TYPE II ANTIFREEZE PROTEINS FROM SMELT (OSMERUS MORDAX) AND ATLANTIC HERRING (CLUPEA HARENGUS HARENGUS): SIMILARITY TO THE C-TYPE LECTIN FAMILY

KATHRYN VANYA EWART

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by

© Kathryn Vanya Ewart

A thesis submitted to the School of Graduate Studies in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Department of Biology
Memorial University of Newfoundland

August 1993

St. John's Newfoundland

ABSTRACT

The structural diversity of fish antifreeze proteins is believed to result from their independent origins in separate fish taxonomic groups. Therefore, antifreeze proteins (AFPs) from two fish species distantly related to all others known to produce AFPs were studied in order to gain a better understanding of the diversity and the distribution of the different AFP types. Results of this study suggest that homologous type II fish AFPs have evolved independently in three separate groups of fish but that they originate from members of a single protein family, the calcium-dependent (C-type) lectins. Moreover, the homology among the three AFPs and the C-type lectins has allowed the recently determined structure of a C-type lectin domain to serve as a prototype for the type II AFPs. This will provide the basis for determining the functional mechanism of this AFP type. These findings also suggest that study of the AFP variants present in diverse fish species may lead to the further insight into the origins, the structures, and the mechanisms of action of the different fish AFPs.

AFPs were isolated from the blood plasma of smelt (Osmerus mordax) and Atlantic herring (Clupea harengus harengus) using gel filtration, ion exchange chromatography, and reverse phase HPLC. Characterization of smelt AFP revealed six isoforms, each with a M_r of 24,000. Amino acid analysis and endoglycosidase digestion revealed that smelt AFP contained N-linked carbohydrate. The herring AFP had two isoforms with a M_r of 14,600 and no carbohydrate. Further analysis of these proteins revealed that, like the type II AFP

of sea raven (Hemitripterus americanus), they were both cystine-rich and sensitive to sulfhydryl reducing agents. Antisera raised against herring and sea raven AFPs cross-reacted with smelt AFP. However, the thermal hysteresis activities of smelt and herring AFPs were lower than that of sea raven AFP.

cDNA libraries were constructed from livers of smelt and herring. Libraries were screened using an oligonucleotide with a sequence derived from tryptic peptides of smelt and herring AFPs. AFP cDNA clones were isolated and sequenced and the primary structures of smelt and herring AFPs were deduced. Protein sequence database searches, sequence alignments, and statistical analyses showed that the AFPs of smelt, herring, and sea raven were homologous to one another and to the carbohydrate-recognition domains (CRDs) present in a family of Ca^{2+}-dependent (C-type) lectins. Furthermore, all but one of the residues known to define the hydrophobic cores of a C-type lectin CRD were present in these AFPs. One CRD Ca^{2+}-binding site appeared conserved in smelt and herring AFPs and these AFPs did require Ca^{2+} for thermal hysteresis activity. Alignment of sea raven AFP with C-type CRDs suggested that it had no functional Ca^{2+}-binding sites and the thermal hysteresis activity of this AFP did not require Ca^{2+}.

Affinity chromatography and agglutination experiments showed that, unlike the C-type lectins, the type II AFPs did not appear to bind strongly to carbohydrates. However, a trace of smelt AFP was retarded on a matrix containing galactose suggesting a slight affinity for this carbohydrate. Two proteins in the C-type lectin family were isolated and tested for thermal hysteresis activity. Neither human pancreatic stone protein nor a C-type lectin from the venom of a snake (Crotalus atrox) showed detectable thermal

hysteresis activity.

Together, these results suggest that the type II AFPs of smelt, herring, and sea raven are related and that all three have evolved from the CRDs of preexisting proteins in the C-type lectin family, the type II AFPs have a fold similar to that of the lectins, and some resemble lectins in requiring Ca^{2+} for activity. However, unlike the lectins, the AFPs appear to interact primarily with ice crystals rather than with carbohydrate.

ACKNOWLEDGEMENTS

I thank Dr. Garth Fletcher for his thoughtful guidance. His optimism and enthusiasm have set an excellent example. I also thank the members of my supervisory committee, Dr. Willie Davidson, Dr. David Idler, and Dr. Alan Vaisius for their encouragement and many helpful discussions.

Members of the lab, including Alice Cadigan, Dr. King-Ming Chan, Juan Gill, Dr. Sally Goddard, Kim Hobbs, Madonna King, and Paul Valerio, have contributed to this project in many ways. I especially thank Dr. Margaret Shears for her generous assistance with the HPLC and helpful suggestions.

The involvement of many other people in this work is gratefully acknowledged. Members of Dr. Davidson's lab, especially Sylvia Bartlett, have shared extensive technical expertise. Discussions with Shashikant Joshi (University of Toronto) were also very helpful. Dr. Ming-Hsiung Kao performed some of the thermal hysteresis measurements and provided patient instruction in the use of the osmometer. Lisa Morgan of the Red Cross and Dr. T. Kent Gartner (University of Tennessee) shared methods for the study and interpretation of erythrocyte agglutination. Dr. Paul Lobel, Dr. Ying So, and Dr. Alan Vaisius allowed my frequent use of their lab equipment and Dr. Deane Renouf provided freezer space. Dr. Choy Hew (University of Toronto) sent antisera to our lab and Dr. Willie Davidson synthesized and donated oligonucleotides on very short notice. Amino acid analyses were performed by Rey Interior (University of Toronto) and Doug

Hall (Memorial University).

Many individuals generously provided samples or assisted in their collection. Pat Carinha, of the Animal Care Facility injected rabbits to raise antibodies and collected many blood samples. Pancreatic stone was donated to our lab by Dr. Gordon Mathieson of the Faculty of Medicine. Herring and smelt samples were obtained with help from Dr. David Cairns, Dr. Gérald Chaput, Dr. Simon Courtenay, Dr. Hélène Dupuis, Dr. Katsuji Haya, Claude LeBlanc, and John Wheeler of the Department of Fisheries and Oceans, Carolyn Gillis, Dr. Vytenis Gotceitas, Madonna King, Dave Methven, Dr. Margaret Shears, Damien Whitten, and Jim Williams of Memorial University, John Watkins of Wesleyville, Nfld., and David Gregan of New Brunswick.

I thank my husband, Stephen Ewart, for his wholehearted support, and his exceptional patience and understanding throughout my studies. I also thank my family for their constant encouragement.

Financial support from Dr. Garth Fletcher's research funds, a Scotiabank Ocean Studies Scholarship, and an NSERC Scholarship is gratefully acknowledged.

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ABBREVIATIONS AND SYMBOLS

AFP	antifreeze protein (includes AFGP)
AFGP	antifreeze glycoprotein
bp	base pair
BS^3	bis(succinimidyl)suberate
BSA	bovine serum albumin
CD	circular dichroism
CRD	carbohydrate recognition domain
Da	Dalton
DEAE	diethylamino ethyl
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis(ß aminoethylether) N,N,N',N'-tetraacetic acid
g	gram
HPLC	high performance liquid chromatography
k	kilo
l	litre
m	meter

μ	micro
m	milli
M	molar
n	nano
PAGE	polyacrylamide gel electrophoresis
PSP	pancreatic stone protein
QAE	quaternary amino ethyl
M_r	relative molecular mass
RNA	ribonucleic acid
RSL	rattlesnake lectin
TBST	tris-buffered saline with tween
TCS	tris-buffered saline with Ca^{2+}
SDS	sodium dodecyl sulphate
UV	ultraviolet

CHAPTER 1:

GENERAL INTRODUCTION

Water is a necessary component of living cells. It is the primary intracellular solvent, it is involved in many of the chemical reactions that define cellular processes, and it is a constituent of the overall structures of most biological macromolecules. Because of the requirement of cells for water, and the physical damage to cells that may result from ice formation, uncontrolled freezing in plants and animals is considered lethal.

It is therefore remarkable that the freezing point of water is almost exactly at the centre of the temperature range which is associated with life on this planet (Franks 1985). Moreover, life is abundant in cold oceans and large numbers of species inhabit polar and north temperate regions. Endothermic animals such as mammals and birds are able to produce heat and most are also insulated so they do not risk freezing in low temperature environments. However, since ectothermic animals and most plants do not generate internal heat, they remain at the temperature of their immediate environment. Study of some of these species has revealed an array of adaptations that reduce the risk of cellular freezing.

The general adaptive strategies that allow ectothermic animals to survive under freezing conditions are freeze tolerance and freeze avoidance. Some adaptations are common to both strategies. Freeze tolerance is achieved through controlled extracellular freezing and it hinges on the production of ice-nucleating proteins that allow freezing to

begin at high subzero temperatures (Storey and Storey 1988). Freeze tolerant animals may also accumulate cryoprotectants, membrane stabilizers, and antifreeze proteins (Zachariassen 1985, Storey and Storey 1988).

Freeze avoidance can be achieved in three ways. One is to colligatively lower the freezing point by accumulation of low molecular weight solutes (Zachariassen 1985). Another is to supercool without freezing by eliminating contact with ice nucleators (Scholander et al. 1957, Zachariassen 1985). The freezing point can also be lowered non-colligatively by inhibiting ice crystal growth through the production of antifreeze proteins (Zachariassen 1985, Davies and Hew 1990).

The production of antifreeze proteins by marine teleost fish is a very interesting freeze avoidance adaptation. The antifreeze proteins (AFPs) appear to be directly responsible for the ability of many fish to survive in icy seawater (Fletcher et al. 1986). In addition, their roles in the freeze avoidance of certain fish species are clearly vital, as evidenced by the maintenance of AFP multigene families in several fish populations (Hew et al. 1988, Scott et al. 1988) and the relationships between AFP levels and freezing risk in separate fish age classes, populations, and species (Scott et al. 1988, Chadwick et al. 1990, Goddard et al. 1992).

The unusual physiological role of AFPs in various fish species, the structures and evolutionary origins of the array of existing AFP forms, and the manner in which they all depress fish freezing points have been the subjects of a diverse assemblage of scientific enquiry. Through these investigations, the AFPs have become a model system in many reseach areas and they now show promise in applications ranging from a diagnostic role in

fisheries oceanography to the production of transgenic crop plants. Presented here are some of the findings that, together, have led to the present understanding of AFPs and current questions regarding these proteins.

1.1 Discovery of "antifreeze" activity and AFPs

The freezing points of teleost fish are normally between -0.5 and -0.9°C (Holmes and Donaldson 1969) whereas the freezing point of seawater is approximately 1°C lower depending on salinity. Thus, when seawater is at its freezing point, the fish in that water are supercooled by about 1°C. Supercooling refers to the maintenance of a liquid phase below the freezing point and it is thermodynamically unstable. In the absence of a nucleation site, supercooled water is unlikely to freeze at -2°C because the probability of homogeneous nucleation is very low. However, in the presence of a nucleating site from which ice can propagate, such as a preexisting ice crystal, supercooled water will rapidly freeze. Consequently, fish in icy seawater are at risk of freezing.

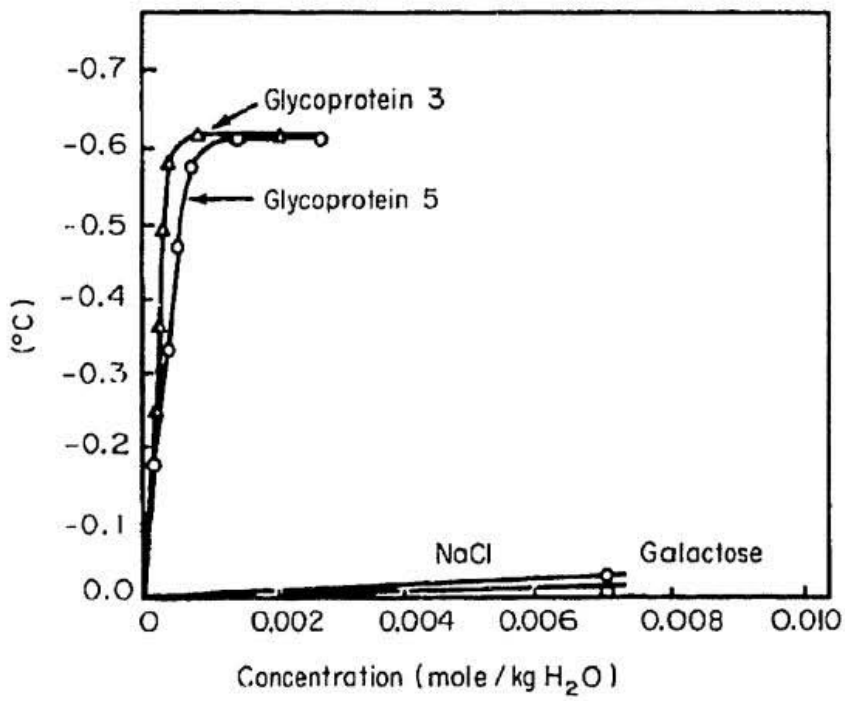
Scholander et al. (1957) observed that certain marine fish species did not freeze in seawater approaching its freezing point. It seemed reasonable to assume that the fish in deep water were able to avoid freezing by avoiding contact with ice crystals. However, the shallow water fish were in direct contact with ice. Clearly, in order to avoid freezing, the shallow water fish had to have unusual freezing characteristics. This led Scholander et al. (1957) to determine the serum freezing points of the shallow water species and they found that these fish had strongly depressed serum freezing points in winter. This was the first

discovery of antifreeze activity in marine fish.

Depressed freezing points were later observed in other fish species from the Barents sea (Eliassen 1960, Raschack 1969) and the northwest Atlantic (Pearcy 1961). The plasma solute responsible for freezing point depression was thought to be a macromolecule because it was non-dialysable. However, it did not appear to be a protein because it was not precipitated by trichloroacetic acid (Gordon et al. 1962). Subsequently, the solutes causing freezing point depression were isolated from the blood of an Antarctic fish and found to be glycoproteins consisting of Ala and Thr (DeVries and Wohlschlag 1969). These proteins were soluble in trichloroacetic acid and they lowered the freezing point non-colligatively (DeVries et al. 1970).

Non-colligative freezing point depression involves a lowering of the apparent freezing point of a solution without affecting the melting point (equilibrium freezing point). Therefore, the supercooled state is stabilized over a certain temperature interval. The resulting difference between the freezing and melting points is called a thermal hysteresis. As shown in Fig. 1, the thermal hysteresis activity (antifreeze activity) of the antifreeze proteins makes them 200-300 times more effective at freezing point depression than solutes that only act colligatively (DeVries 1971a). Consequently, fish with antifreeze proteins can have freezing points equal to that of seawater while maintaining a much lower solute concentration in their body fluids.

Fig. 1. The freezing point depression of solutions containing different molalities of AFGPs and colligative solutes. Taken from DeVries (1971a).


-0.7
-0.6
-0.5
-0.4
-0.3
-0.2
-0.1
0.0
(°C)
Glycoprotein 3
Glycoprotein 5
NaCl
Galactose
0
0.002
0.004
0.006
0.008
0.010
Concentration (mole / kg H_2O)

1.2 Diversity and distribution of AFPs

Since their first discovery, a variety of antifreeze proteins have been found in fish. Antifreeze glycoproteins (AFGP) and three types of antifreeze proteins (AFPs I, II, and III) have been identified. Here, the term AFP applies to all antifreezes, including the AFGPs, because they are all proteins. The distribution, structures and functions, regulation, and gene organization of the different fish AFPs have been widely studied (Davies et al. 1989, Davies and Hew 1990, Cheng and DeVries 1991). In addition, antifreeze proteins or their characteristic hysteresis activity have been found in a number of other animals including invertebrates such as insects (Zachariassen 1985), spiders (Duman 1979), an Antarctic mite (Block and Duman 1989), and bivalves (Theede et al. 1976, Guderley et al. 1985) as well as in plants (Griffith et al. 1992, Urrutia et al. 1992).

1.2.1 AFGPs

The AFGPs were the first AFPs to be discovered. These glycoproteins are found in three families of Antarctic fish in the suborder Notothenioidei: the Nototheniidae, the Channichthydae, and the Bathydraconidae (DeVries and Wohlschlag 1969, Schneppenheim and Theede 1982a, Alghren and DeVries 1984). They have also been identified in four genera in the family Gadidae found in the North Atlantic, North Pacific, and Arctic waters (VanVoorhies et al. 1978, Osuga and Feeney 1978, Raymond et al. 1975, Hew et al. 1981, Fletcher et al. 1982).

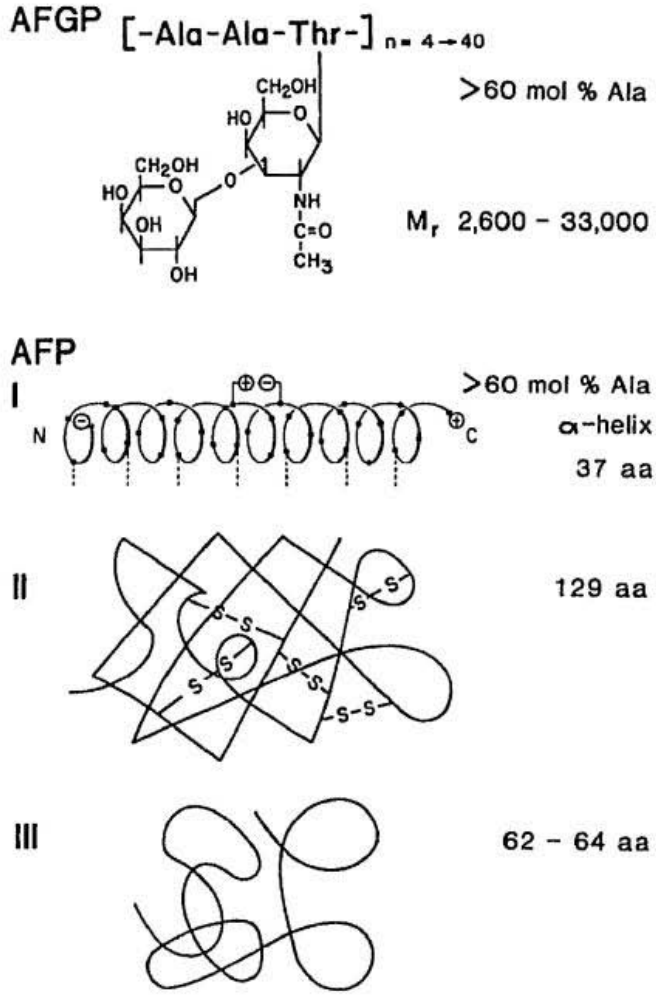
The AFGPs are polymers of the tripeptide Ala-Ala-Thr with a disaccharide of

galactosyl-ß-D(1-3)-α-D-N-acetylgalactosamine attached to each threonyl side chain through a glycosidic bond (Fig. 2a). In all the fish from which AFGPs have been characterized, they consist of a group of polypeptides ranging in M_r from 2500 to 33,000 depending on the number of tripeptide repeats they contain. These peptides are normally numbered, from 1 to 8 for example, beginning with the largest peptide and ending with the smallest. In the smaller polypeptides from some species, Pro occurs in the place of the first Ala of certain tripeptides (Morris et al. 1978, Fletcher et al. 1982) and Arg substitutes for Thr (Fletcher et al. 1982, O'Grady et al. 1982a).

Initial investigations of the AFGP structure by circular dichroism (CD) spectroscopy and viscosity measurements suggested an extended conformation (DeVries et al. 1970, Berman et al. 1980). Evidence suggesting a left-handed ß-structure has also been reported (Franks and Morris 1978). More recently, a structure composed of a threefold left-handed helix was suggested based on the resemblance of AFGP CD spectra to those of other polymers such as polyglutamate, polyproline II, and collagen, that are known to have this structure (Bush et al. 1981). This model allows alignment of the disaccharides on one side of the protein.

The disaccharide moieties of the AFGPs appear to be involved in thermal hysteresis activity. Activity was sharply reduced following the removal of sugar by ß elimination (Ahmed et al. 1973). Modification of hydroxyl groups by periodate oxidation, borate complexation, or acetylation also inhibited activity (Komatsu et al. 1970, DeVries 1971b, Ahmed et al. 1976, Geoghegan et al. 1980). The sugar hydroxyl groups of the AFGP have been suggested to hydrogen bond to the ice lattice (Raymond and DeVries 1977).

Fig. 2. Schematic representation of the 4 AFP types found in marine teleost fish. (A) AFGP, (B) type I AFP, (C) type II AFP, (D) type III AFP. Modified from Davies and Hew (1990).


AFGP
[-Ala-Ala-Thr-] n = 4→40
>60 mol % Ala
Mr 2,600 - 33,000
AFP
I
>60 mol % Ala
α-helix
37 aa
N
C
II
129 aa
III
62 - 64 aa

Alternatively, if the AFGPs are in an extended conformation, the disaccharides may stabilize a structure in which repeated peptide chain carbonyl oxygens on the opposite side of the molecule might interact with ice crystals (DeVries 1988).

1.2.2 Type I AFPs

Type I antifreeze proteins are alanine-rich α-helical proteins with molecular weights ranging from approximately 3000 to 4500 (Fig. 2b). Type I AFPs were first isolated from winter flounder (Pleuronectes americanus) (Duman and DeVries 1984). These AFPs are found in many righteye flounders (family Pleuronectidae) and sculpins (Cottidae) (Duman and DeVries 1974, Hew et al. 1980, Raymond et al. 1975, Scott et al. 1987, Chakrabartty et al. 1988).

The type I AFP of winter flounder has been well characterized. Eight isoforms have been resolved by reverse phase HPLC and they range in molecular weight from 3300 to 4500. The two major forms are HPLC-6 and HPLC-8 (Fourney et al. 1984c). Analysis of their cDNA sequences reveals a repeated eleven amino acid sequence. The consensus sequence is Thr-$(X)_2$-polar amino acid-$(X)_7$ with X being non-polar (Lin and Gross 1981, Davies et al. 1982, Pickett et al. 1983). The AFP of yellowtail flounder (Pleuronectes ferrugineus) largely resembles that of winter flounder. However, it consists of four sequence repeats instead of three and it lacks two of the hydrophilic groups present in winter flounder AFP that have been implicated in ice binding (Scott et al. 1987).

The flounder and sculpin AFPs that have been investigated are primarily α-helical at 0°C (Ananthanaryanan and Hew 1977, Hew et al. 1985, Chakrabartty et al, 1988).

Secondary structure prediction suggests that the yellowtail flounder AFP is also α-helical (Scott et al. 1987). The crystal structure of the major AFP of winter flounder was shown to consist of a single α-helix (Yang et al. 1988). The helices of type I AFPs are mainly amphiphilic. Crystal structure analysis of flounder AFP shows that the hydrophilic side chains all lie on the same side of the helix but this protein is not completely amphiphilic since some Ala residues are on the same side (Yang et al. 1988). Secondary structure predictions suggest that AFPs of yellowtail flounder and grubby and shorthorn sculpins are also amphiphilic (Scott et al. 1987, Chakrabartty et al. 1988). The alignment of polar residues along one side of the helix is reminiscent of the proposed disaccharide alignment in the AFGPs and it is consistent with models predicting that the polar residues of type I AFP hydrogen bond to ice crystals (DeVries and Lin 1977, Yang et al. 1988).

1.2.3 Type II AFPs

The type II AFP of fish was isolated from the sea raven (Hemitripterus americanus), a cottid closely related to the sculpins. The molecular weight of this protein deduced from its predicted sequence is 14,017 Da (Hayes et al. 1989) The sea raven AFP is present in multiple isoforms but in this case they all appear to be the same size (Ng et al. 1986). In contrast to the AFGPs and type I AFPs, the type II AFP contains an average proportion of Ala (14.4%) and it has a relatively high Cys concentration (7.6%) (Slaughter et al. 1981, Ng et al. 1986). In addition, the primary structure of this AFP deduced from its cDNA sequence reveals no repeated sequence elements (Ng et al. 1986). Disulfide bonds appear to be important for the maintenance of an intact structure. The hysteresis

activity of this protein is decreased in the presence of sulfhydryl reducing agents (Slaughter et al. 1981). This characteristic of type II AFP, together with its diverse amino acid composition and its substantial molecular weight, suggests that it adopts a folded structure (Fig. 2c). CD spectra of the AFP and secondary structure prediction for the amino acid sequence obtained from a cDNA clone suggest that little α-helix is present and ß-structure predominates (Slaughter et al. 1981, Ng et al. 1986). Secondary structure prediction also showed a high frequency of reversed turns (Ng et al. 1986).

Similar Cys-rich AFPs have been isolated from insects, including the mealbug (Tenebrio molitor) (Schneppenheim and Theed 1982b), a pyrochroid beetle (Dendroides canadensis) (Wu et al. 1991) and the spruce budworm (Choristoneura fumiferana) (Hew et al. 1983). An AFP with an approximate M_r of 9000 has been isolated from the mealbug and that of the budworm is 13,000. The M_r of the beetle AFP is slightly larger at 24,000 to 26,000. Like the sea raven AFP, the mealbug and beetle proteins appear to be sensitive to reducing agents although the beetle AFP also requires free sulfhydryl groups for activity (Wu et al. 1991). In addition, there is immunological cross-reactivity between sea raven and spruce budworm AFPs while none occurs between sea raven and other fish AFPs. Thus, insect AFPs may also be type II.

The primary structure of sea raven AFP does not allow the prediction of a functional mechanism. However, the high frequency of predicted reverse turns with polar residues such as Asp-Asp in the sea raven AFP may play a role in its hysteresis activity (Ng et al. 1986).

1.2.4 Type III AFPs

The type III AFPs are smaller proteins with molecular weights between 6000 and 7000 Da (Fig. 2d). These proteins show no predominant amino acids, they contain no cysteine, and they have no repeating structure (Hew et al. 1984, Li et al. 1985). The proportions of polar and non-polar residues are approximately 50 mol% (Ananthanaryanan 1989).

Type III AFPs are found in several Zoarcoid families including the Zoarcidae (Hew et al. 1984, Schrag et al. 1987, Cheng and DeVries 1989), Pholididae, Anancharididae, and Stichaedae (Shears et al. 1993). Sequences have been obtained for AFPs of four eelpouts. They are the ocean pout (Macorzoarces americanus) (Li et al. 1985), another northern eel pout, Lycodes polaris, and two Antarctic eel pouts, Rigophila dearborni (Schrag et al. 1987), and Australycicthys brachycephalus (Cheng and DeVries 1989). Sequences have also been obtained from the AFP of wolffish (Anarichas lupus) (Scott et al. 1988). There is substantial identity among all the sequences from different species. Yet within some species, multiple divergent AFPs are found. Twelve AFP isoforms are produced by ocean pout (Li et al. 1985) and they include two immunologically distinct groups (Hew et al. 1984) with divergent amino acid sequences (Hew et al. 1988). Substantial microheterogeneity was also evident among AFPs from L. polaris and R. dearborni (Schrag et al. 1987).

No predominant secondary structure was evident in the AFP of ocean pout (Li et al. 1985). However, the thermal denaturation of AFPs from ocean pout, L. polaris, and A. brachycephalus suggests the presence of secondary and/or tertiary structures

(Ananthanaryanan et al. 1986, Schrag et al. 1987, Cheng and DeVries 1989).

The conservation of amino acid residues among protein isoforms and in proteins from different species is an indication of roles in either the structural integrity or function of a protein. Four of the perfectly conserved residues among ocean pout and wolffish AFPs are Thr or Asn (Hew et al. 1988). Since Thr and Asp residues are thought to be involved in ice crystal binding by winter flounder (type I) AFP (DeVries and Lin 1977), Hew et al. (1988) have suggested the same might be true for conserved Asn and Thr residues in type III AFPs. Furthermore, two Glu residues conserved in many type III AFPs were shown to be involved in ice-binding and hysteresis activity of an ocean pout AFP (Li et al. 1991). Thus, even without knowledge of the folding structure of the type III AFPs, progress has been made in understanding their mechanism of action.

1.3. Physiology of AFPs

The seasonal and ontogenetic regulation of AFP sythesis, the sites of AFP precursor synthesis, proteolytic processing of AFPs, and the distribution of AFPs in fish tissues have been widely studied in order to understand the extent of freeze protection these proteins confer to different fish under various conditions.

1.3.1 Synthesis and tissue distribution of AFPs

The fish AFPs all appear to be extracellular. They are normally purified from blood plasma or serum for ease of collection and purification but they are also present in other

tissues. In a notothenиid, AFGPs are present in interstitial fluids of all tissues except brain (Alghren et al. 1988). They are synthesized in liver, secreted into the blood, and from there they become distributed in the interstitial fluids (Alghren et al. 1988). AFGPs 6, 7, and 8 are also secreted into the intestinal fluids (O'Grady et al. 1982b). The type I AFP of winter flounder is present in approximately equal amounts in skin and blood (Valerio et al. 1992).

The AFPs are synthesized predominantly in liver but in many cases, they are also produced in other tissues. In Antarctic notothenioids, AFGPs are synthesized in liver (O'Grady et al. 1982a, Alghren et al. 1988). However, the possibility of AFGP synthesis in other tissues has not been excluded (Hudson et al. 1978, Haschemeyer and Mathews 1980). Messenger RNAs (mRNAs) for AFP types I, II, and III have been shown in livers from winter flounder, sea raven, and ocean pout, respectively, by Northern blotting (Gong et al. 1992). Type I AFP synthesis in winter flounder liver has also been demonstrated by incorporation studies (Hew et al. 1986). Liver appears to be the only site of type II AFP mRNA accumulation in sea raven. In contrast, the mRNAs for type I AFP in winter flounder and type III AFP in ocean pout are present in many other tissues (Gong et al. 1992). Although liver appears to be a major site of AFP synthesis in winter flounder and ocean pout, high AFP mRNA levels are also present in epithelial tissues (Gong et al. 1992). These results are consistent with some observations of AFP distribution in the bodies of fish. AFP was detected in the skin of European shorthorn sculpin (Myoxocephalus scorpius) and cunner (Tautogolabrus adspersus) but not in the blood of either species (Schneppenheim and Theed 1982a, Valerio et al. 1990). These findings are

consistent with the hypothesis that fish skin is an effective barrier to ice propagation (Valerio et al. 1992).

1.3.2 Processing of AFPs

The synthesis of all the different AFP types involves proteolytic processing with the AFGP representing the most unusual example. The sequence of an AFGP genomic clone from a notothenid, Notothenia coriiceps neglecta, revealed the presence of a polyprotein precursor coding for 44 tandem copies of AFGP-8 and 2 copies of AFGP-7 each separated by 3-amino acid spacers (Hsiao et al. 1990). This polyprotein also appears to have an unusual signal sequence (Hsiao et al. 1990) that is presumably removed following translation. The processing events involved in synthesis of this protein are unclear. However, cleavage at each spacer would be necessary in order to produce the known AFGP products.

Type I AFP of winter flounder is synthesized as a larger preproprotein (Davies et al. 1982). The proprotein has been detected in the blood (Hew et al. 1978) where it is processed to mature AFP (Hew et al. 1986). cDNA and protein sequence analysis of the sea raven type II AFP also suggest that it is synthesized as a preproprotein but the presence of the pro sequence has not been confirmed (Hayes et al. 1989). In contrast, the type III AFP of ocean pout has no apparent pro sequence but some isoforms appear to undergo minor post-translational modifications (Li et al. 1985).

1.3.3 Excretion of AFPs

High concentrations of AFPs are necessary for freeze resistance in fish. Thus, it was suggested that the presence of aglomerular kidneys in 10 Antarctic fish species is an adaptation that allows conservation of circulating AFGPs by avoiding loss through filtration (Dobbs et al. 1974). Consistent with this hypothesis, AFGPs are not found in the urine of Antarctic nototheniids (DeVries 1982).

Northern polar and temperate fish species do have glomerular kidneys but it has been suggested that altered glomerular structures and proportions of functioning glomeruli may result in reduced filtration, thereby minimizing AFP loss (Eastman et al. 1987). However, all four AFP types have been found in the urine of north temperate fish during winter (Fletcher et al. 1989a). The reverse phase HPLC profiles of the AFPs isolated from urine are indistinguishable from those of their counterparts from blood (Fletcher et al. 1989a). In sea raven and winter flounder, urine AFP levels deduced from their hysteresis activity are approximately equal to those in blood plasma, while in ocean pout and cod, urine AFP levels are lower than plasma levels, and no AFP was detected in sculpin urine (Fletcher et al. 1989a). Thus, in general, Antarctic fish may be better able to conserve blood AFP levels by minimizing their excretion while Arctic fish lose AFP to the urine.

1.3.4 Regulation of AFP levels

Differences in AFP levels have been found to occur among species with different distributions, between distinct population within species, and within individuals according to the stage of development and time of the year. It is interesting that, in every case, AFP

levels appear closely coupled to the extent of freezing risk and these findings suggest that the AFPs play a key role in the survival of fish in icy seawater.

The temperature of the Antarctic ocean is always close to -1.9°C and ice is abundant throughout the year in shallow areas (DeVries and Eastman 1982, DeVries 1988) so fish in that environment always require AFPs to survive. In Antarctic fish, AFPs are present permanently (DeVries 1988) and in a nototheniid, Pagothenia borchgrevinki, AFGP synthesis does not respond to changes in environmental factors (O'Grady et al. 1982). Thus, in Antarctic fish, AFP levels do not appear to be environmentally regulated. Fish residing in permanently cold Arctic waters may also have high AFP levels throughout the year. For example, the Arctic shorthorn sculpin has AFP levels in August that are equal to the maximum winter levels of AFP in Newfoundland populations (Fletcher et al. 1982). This suggests that the AFP levels in Arctic shorthorn sculpin are also maintained throughout the year.

In contrast, the AFP levels in fish inhabiting north temperate environments follow seasonal cycles with maximal levels occurring in winter. The plasma levels of all AFPs vary seasonally. In winter flounder, AFP production follows a seasonal cycle in response to photoperiod (Davies et al. 1988) while AFGP production in Atlantic cod (Gadus morhua) is responsive to temperature (Fletcher et al. 1987). There is a seasonal cycle of plasma AFP levels in ocean pout but substantial amounts of AFP are still present in summer (Fletcher et al. 1985). In sea raven, levels are variable; some individuals show seasonal cycles while others have permanently high or low AFP levels (Fletcher et al. 1984a). Regulation of the annual AFP cycle has been most thoroughly studied in winter

flounder. In Newfoundland winter flounder, AFPs appear in the plasma in November, reach maximal levels in January, and disappear in May (Fletcher 1977). Liver mRNA levels follow the same pattern except that mRNA levels fall in April, two months before plasma AFP levels fall to their summer levels (Fourney et al. 1984b).

Initially, it was shown using hypophysectomized flounder that a pituitary factor exerts a negative control on AFP mRNA levels during the summer (Fourney et al. 1984c). The inhibitor produced by the pituitary appears to be regulating AFP gene transcription. During summer, hypophysectomized fish actively transcribe AFP mRNA in liver but intact controls do not (Vaisius et al. 1989). It was also found that if pituitaries were reintroduced into hypophysectomized fish out of contact with the central nervous system, AFP levels remained low throughout the winter. This finding suggests that during winter, the central nervous system of intact flounder blocks the production of an AFP inhibitor by the pituitary, and hence, allows AFP to be produced (Fletcher et al. 1984b).

Growth hormone appears to be the factor responsible for decreased AFP levels in winter flounder during summer (Fletcher et al. 1989b). Injection of growth hormone alone mimicks the inhibition of AFP synthesis that is obtained by using whole pituitary extracts (Idler et al. 1989). Two other lines of lines of evidence support the idea that growth hormone is a key factor in the seasonal AFP cycle. The growth patterns (Chadwick 1986) and the feeding habits (Kennedy and Steele 1971, Fletcher and King 1978) of flounder suggest that it grows only in summer and purified growth hormone has been shown to repress endogenous transcription of the AFP gene (Vaisius 1989).

Although AFP synthesis appears to be regulated primarily by photoperiod through

growth hormone, temperature may also be involved in the variation of AFP levels in flounder. Low temperature alone does not trigger AFP synthesis in autumn (Fletcher 1981) and AFP gene transcription appears unaffected by temperature (Vaisius et al. 1989). However, when fish are kept at high temperatures during winter, plasma AFP levels are lower than normal (Fletcher 1981). This suggests that temperature is affecting AFP accumulation in another way. Northern blotting experiments show that low temperatures are necessary for the accumulation of AFP mRNA (Price et al. 1986, 1990). Thus, low temperatures may influence AFP mRNA stability. Furthermore, translational control mechanisms foster AFP production at low temperature. Winter flounder alanyl-tRNA synthetase shows maximal activity at 0-5°C and tRNA-Ala is more abundant during winter than summer (Pickett et al. 1983).

In addition to the seasonal cycles of AFP expression, in some fish species AFP levels can change over the course of development and the maximal AFP levels and timing of the seasonal cycles can differ between populations. The seasonal cycles of plasma AFP levels differ between geographically distant populations of winter flounder (Fletcher et al. 1985) even when they are kept at the same site under identical conditions (Fletcher and Smith 1980). Timing of AFP production appears to closely precede the beginning of ice accumulation in their natural habitat. Plasma AFP levels appear to change over the course of development in Atlantic cod. AFGP levels in juvenile Atlantic cod are nearly twice the levels found in adults at the same time of time of the year (Kao and Fletcher 1988). Juvenile cod are believed to overwinter near shore (Keats et al. 1987) where ice is prevalent whereas adults are thought to move offshore (Templeman 1979) where less ice is

present. In each case, AFP levels correspond to the apparent magnitude of freezing risk.

1.4 AFP gene organization

Genomic clones for each AFP type have been isolated and sequenced. The ensuing investigations of AFP gene organization revealed that the genes for types I, II, and III AFPs were encoded by high copy number multigene families (Davies et al. 1984, Hew et al. 1988, Scott et al. 1988, Hayes et al. 1989).

The first winter flounder AFP gene sequenced was 1 kb long and it contained a 0.6 kb intron (Davies et al. 1984). Isolation and mapping of different AFP genomic clones and genomic Southern blot analysis revealed the presence of a multigene family with approximately 40 copies (Davies et al. 1984). Further Southern blotting and restriction analysis of genomic clones showed that in Newfoundland winter flounder, the majority of the AFP genes are in tandem clusters of five or more direct repeats (Scott et al. 1985). The tandemly repeated genes code for the two major AFP components and they are more uniform than AFP genes outside the repeated elements (Davies 1992).

In flounders, the extent of tandem repeats differs between species and populations. The Newfoundland yellowtail flounder has an AFP multigene family but it lacks the tandemly repeated elements present in Newfoundland winter flounder (Scott et al. 1985). The tandemly repeated component is also greatly reduced in winter flounder populations from the Bay of Fundy and Brown's bank compared to those in other locations such as Newfoundland (Hayes et al. 1991). In all cases, the presence of high copy number

tandemly repeated elements coincides with cold shallow water habitats where ice is prevalent and high levels of AFP are essential for survival (Scott et al. 1988, Hayes et al. 1991).

The type III AFP is encoded by genes that are approximately 0.7 kb long. Approximately 150 copies are present in the genome of Newfoundland ocean pout (Hew et al. 1988). In contrast, only about one quarter of that number are present in the genome of New Brunswick ocean pout (Hew et al. 1988). This is consistent with the different AFP levels in these populations (Fletcher et al. 1985) and exposure of these different fish groups to icy conditions (Hew et al. 1988). An AFP multigene family has also been documented in wolffish, a related Zoarcoid (Scott et al. 1988). The arrangement of genes in this fish species differs from the others in containing inverted repeats (Scott et al. 1988).

Type II AFPs are encoded by a multigene family in sea raven with about 12-15 gene copies (Hayes et al. 1989). Maps of genomic clones do not show any evidence of tandem linkage over a span of 25 kb but the genes could still be part of a larger repeating unit (Hayes et al. 1989). The relatively low AFP gene copy number in sea raven is consistent with its variable plasma AFP levels and its deeper water habitat where ice is rare.

Although the genomic organization in the AFGPs has not been investigated, Hsiao et al. (1990) have shown tandem iteration in a polyprotein (discussed earlier) in a notothenиid. Their results also suggest the presence of multiple gene copies in this fish.

It is striking that duplication is evident in genes coding for all the distinct AFP types. Developmentally regulated amplification of specific genes occurs in groups ranging from protozoa to amphibians (Stark et al. 1990). In contrast, abnormal (unregulated) gene

amplification occurs only in response to extreme and potentially lethal environmental conditions (Scott et al. 1988, Stark et al. 1990). Sustained selection is thought to be necessary for maintenance of highly repeated or tandemly repeated elements (Stark et al. 1990). Hence, intense environmental selection is probably ongoing in the most freeze-resistant fish populations.

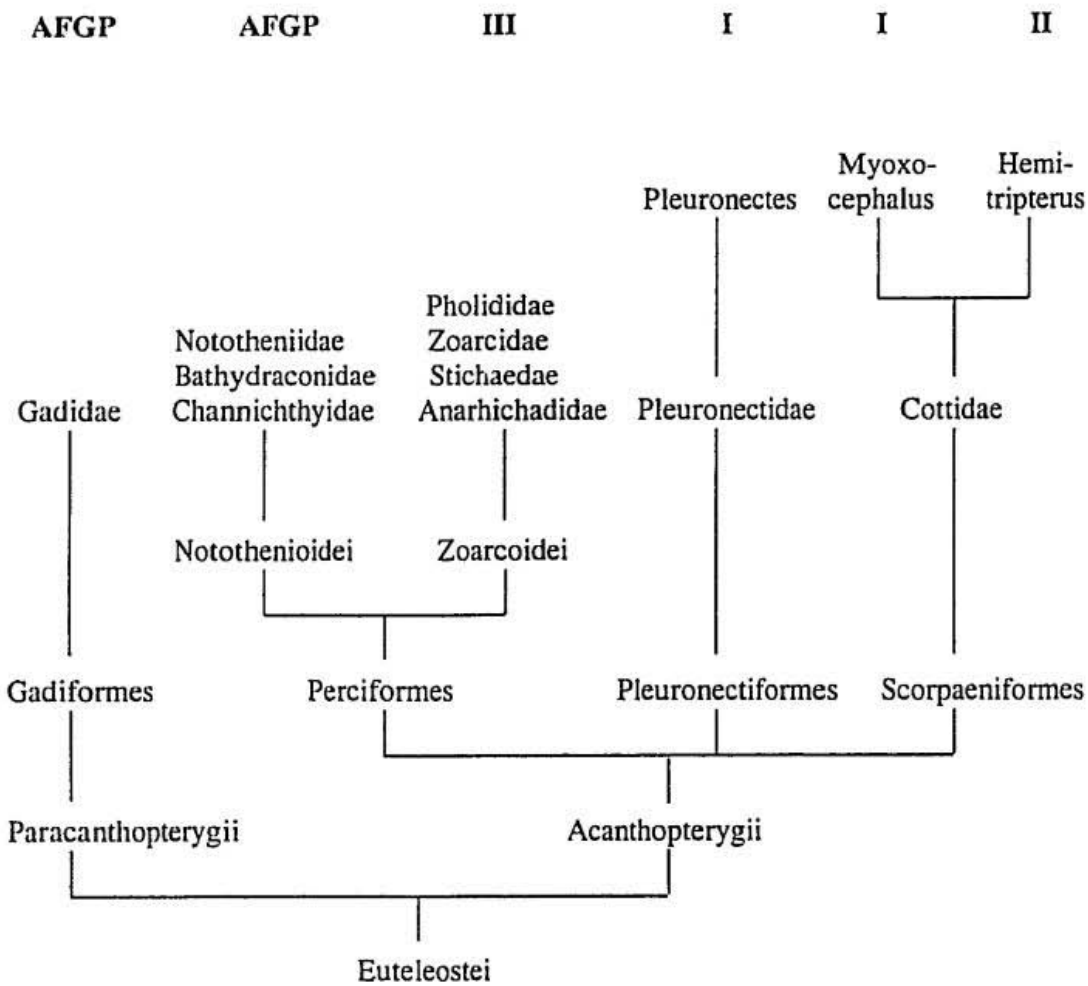
1.5 AFP evolution

It is not unusual for distinct proteins to have similar functions as the different fish AFPs do. However, unlike the AFPs, the majority of proteins are encoded by single genes and they are generally distributed widely across taxonomic groups. Therefore, the presence of four unusual proteins with hysteresis activity each narrowly distributed in specific fish taxonomic groups and encoded by multigene families is unprecedented. The distribution of the different AFP groups is outlined in Fig. 3.

Adaptations of teleost fish to cold and freezing conditions are not likely to have occurred until Cenozoic cooling and glaciation (Keigwin 1980, Kerr 1984) and again more recently during Arctic glaciation (Shakelton et al. 1984). Cenozoic glaciation post-dated much of the teleost radiation. By that time, most present-day fish families were established and they resembled modern assemblages (Greenwood et al. 1966 and reviewed by Scott et al. 1986). Therefore, some of the major adaptations of fish to cold and freezing may have occurred in parallel in separate surviving fish groups.

Based on the timing of large scale cooling events, it has been postulated that

Fig. 3. Distribution of AFP types across fish taxonomic groups. Scale is not linear.


AFGP
AFGP
III
I
I
II
Pleuronectes
Myoxo-
cephalus
Hemi-
tripterus
Pholididae
Zoarcidae
Stichaedae
Anarhichadidae
Nototheniidae
Bathydraconidae
Channichthyidae
Gadidae
Pleuronectidae
Cottidae
Notothenioidei
Zoarcoidei
Gadiformes
Perciformes
Pleuronectiformes
Scorpaeniformes
Paracanthopterygii
Acanthopterygii
Euteleostei

the AFPs evolved recently (Scott et al. 1986). This hypothesis suggests that the different AFP types and their restricted phyletic distributions are the result of independent AFP evolution in fish groups that were already separate when selection for freeze protection first occurred (Scott et al. 1986). In this case, the evolution and amplification of four distinct types of AFPs in different fish groups represents a fascinating example of evolution driven by cooling and suggests that the ability to avoid freezing has played a pivotal role in the survival of many marine fish species.

1.6 Ice-related functions of AFPs

Three known functions of AFPs appear to stem from their interaction with ice crystals. They are thermal hysteresis, inhibition of ice recrystallization, and interaction with ice nucleators. Thermal hysteresis is the freezing point depression that allows fish to survive below their equilibrium freezing points and it is the best known property of fish AFPs.

1.6.1 Interaction of AFPs with ice crystals causing thermal hysteresis

Experiments done *in vivo* and *in vitro* suggest that AFPs lower the freezing point by interacting directly with ice crystals. The lethal freezing point of trout, a fish with no endogenous AFP, is lowered in direct proportion to the amount of pure AFP from winter flounder injected into the fish (Fletcher et al. 1986). This implies that the AFPs lower the freezing point independently of any associated factors. Furthermore, ice crystals acquire

unusual faceted or needle-like morphologies during growth in the presence of AFPs (Scholander and Maggert 1971, Knight et al. 1984, Coger et al. 1992). These results suggest that the AFPs interact directly with ice because a facet will only appear on an ice crystal if growth is inhibited in the direction normal to the facet surface.

Evidence for the adsorption of AFPs onto ice crystals has been gathered from three types of analyses. First, solutions containing ice and AFPs generate surface second harmonics during light spectroscopy (Brown et al. 1985). This signal is not generated by pure ice-water interfaces (Brown et al. 1985). Second, unlike most solutes, AFPs appear to be incorporated into the growing ice crystal during freezing (Raymond and DeVries 1977, Knight et al. 1991). Third, the temperature transition during freezing is sudden for AFP solutions in contrast to the more gradual transition for solutions in which the solute is excluded from ice (Raymond and DeVries 1972).

Drawing on current understanding of the effects of impurities on crystal growth (Raymond 1976), Raymond and DeVries (1977) proposed that AFPs inhibit crystal growth over the temperature interval of hysteresis by adsorbing to ice crystals, and consequently, inhibiting their growth (adsorption-inhibition). These workers suggested that since further ice growth could only occur between adsorbed AFP molecules, a series of curved ice fronts with higher surface free energies would result and subsequent growth would not be favoured.

The model proposed by Raymond and DeVries (1977) also explains the development of faceted ice crystals in the presence of AFP. In solutions with no AFPs, ice crystals usually grow along the a-axes. This causes an increase in size of the basal

plane, which has a surface parallel to the a-axes. However, in solutions containing AFPs, the direction of preferred ice growth is along the c-axis with growth occurring parallel to the c-axis along the prism faces (Raymond and DeVries 1977). This implies that the AFP adsorb preferentially to the prism faces of ice and that their adsorption would allow facets to develop.

Binding patterns of AFPs to ice appear to be more diverse than initially thought, however. Very low concentrations of AFPs cause the development of ice crystal faces that are not clearly aligned with any of the axes (Knight et al. 1984). Preferred planes of adsorption of different type I AFPs are complex and they are not necessarily the prism faces (Knight et al. 1991). Synthetic analogues of winter flounder AFP even inhibit growth along the c-axis at high concentrations, implying binding to the basal plane (Chakrabartty et al. 1989). However, the preferred binding planes of the winter flounder AFP are the pyramidal planes and inhibition of ice growth on every plane appears possible by binding of high concentrations of AFP to a single plane (Knight et al. 1991). In general, AFPs bind to several non-basal planes on ice crystals (Raymond et al. 1989). Even within a single AFP type (I), different preferential planes of binding are observed (Knight et al. 1991).

1.6.1.1 Mechanism of AFP adsorption to ice

The adsorption of the different AFPs onto ice has been suggested to result from optimal spacing between repeated hydrogen bonding groups in the proteins that could interact with atoms at repeated sites along the ice crystal lattice. For the AFGPs, this could

occur in two ways. If the AFGPs are threefold helices, the disaccharides would be aligned along one side of the molecule allowing interaction with ice (Bush et al. 1981, DeVries 1988). Alternatively, if the molecules are extended, alternate main-chain carbonyl groups would align with one side of the ice lattice (DeVries 1988).

In the type I AFPs, the repeated polar residues are predominantly on one side of the helix (Hew et al. 1985, Scott et al. 1987). This also suggests the potential for hydrogen bonding with sites along the ice lattice. The crystal structure of the winter flounder AFP did not reveal any patterns that would lead to preferential binding on non-basal planes (Yang et al. 1988). In view of this, it has been proposed that dipole-dipole interaction between the AFP and vectors on the prism faces of ice might contribute to the interaction of AFP with non-basal planes (Yang et al. 1988). The entire winter flounder AFP appears to be a dipole with a positive charge at the N terminus and a negative charge at the C terminus because it is a single α-helix. However, measurement of dipole strength in the winter flounder suggests that it is not sufficient to induce dipole-dipole interaction between ice and the AFP (Sicheri and Yang 1992). Therefore, the mode of interaction for this AFP is not yet clear.

The manner in which the other AFPs interact with ice crystals is not known. Alignment of repeated hydrogen bonding sites is not yet evident in the type III AFP and the basis for adsorption of type II AFP (Ng et al. 1986) and similar insect AFPs to ice is not clear.

1.6.2 Interactions of AFPs with ice causing recrystallization inhibition

Extremely low concentrations ($\approx 10^{-6}$ M) of AFP are effective in inhibiting ice recrystallization (Knight et al. 1984) though these concentrations are far too low to cause hysteresis. Recrystallization inhibition appears unique to the AFPs because while many other proteins are present in cells or interstitial fluids at concentrations equal to or higher than the range needed for the AFPs' effects, recrystallization is not inhibited in the absence of the AFPs. Inhibition has been shown in the presence of AFGP (Knight et al. 1984), insect AFP (Knight and Duman et al. 1986), and type I AFP (Mueller et al. 1991).

It has been suggested that inhibition of ice recrystallization by AFPs is functionally analogous to their thermal hysteresis activity (Knight and Duman 1986). Since unusual ice crystal faces are expressed in the presence of these very low concentrations of AFP (Knight et al. 1984), it is likely that recrystallization inhibition also stems from their interactions with ice crystals. However, the exact mechanism involved in recrystallization inhibition is not yet understood (Knight et al. 1988).

1.6.3 Interaction of AFGPs with ice nucleators

AFGPs have been shown to inhibit the activity of bacterial ice-nucleating proteins (Parody-Morreale et al. 1988). Ice-nucleating proteins stimulate the nucleation of ice at elevated sub-zero temperatures. They are thought to act by mimicking the structure of an ice crystal surface and, consequently, behaving like a seed ice crystal. Thus, inhibition of nucleation by AFGPs may result from the ability of antifreezes to interact with nucleating proteins as they do with ice cristals. It is not yet clear whether this property of AFGPs

extends to all AFP types or whether it plays a role in freeze tolerance or resistance. In fact, in organisms that possess both ice-nucleating proteins and AFPs, such an interaction could be a disadvantage as both AFPs and nucleating proteins would likely be inactivated if they bound to one another.

1.7 Interaction of AFPs with cell membrane components

AFGPs appear to enhance the survival of mammalian cells during cryopreservation and storage at hypothermic (≈4°C) temperatures. In the presence of AFGPs, 25% of pig and 82% of mouse embryos survived vitrification under conditions that normally do not allow survival (Rubinsky et al. 1992a). In addition, membrane integrity and survival of mammalian oocytes at hypothermic temperatures were increased substantially in the presence of each of the four antifreeze types (Rubinsky et al. 1990, Rubinsky et al. 1991). The effects of AFPs during hypothermia are also evident in more complex structures. Rat livers were better able to maintain functional integrity after hypothermic storage when stored in the presence of ocean pout AFP (Lee et al. 1992).

It is not clear how AFPs make cells cold-tolerant. However, it is interesting that four distinct protein types can each modify ice crystal growth and all four also protect cells at hypothermic temperatures. The two most immediate effects of low temperature on cells are membrane disruption caused by lipid phase transition (Mazur 1984) and membrane depolarization caused by ion pump inefficiency resulting in Ca^{2+} leakage into cells and subsequent unregulated metabolic effects (Hochachka 1986, 1988). It appears that AFPs

may prevent one or both of these problems.

It is possible that the AFPs render cells cold-tolerant by interacting directly with cell membrane surfaces (Rubinsky et al. 1990) and possibly minimizing the effects of temperature on phospholipids. Alterations in membrane composition occur during acclimation of many organisms to low temperatures and phospholipids of cold-adapted species are often less saturated than those of warm-adapted ones. Furthermore, a recent experiment has shown that a cold-sensitive plant can be made cold-tolerant solely by changing its membrane phospholipid acyl chain saturation (Murata et al. 1992) and some membrane cryoprotectants are known to have similar effects. Knight et al. (1991) suggested that crystal surfaces and membrane surfaces are similar in some respects. Thus, it appears plausible that AFPs might interact with ice crystals and membranes in the same manner.

Alternatively, AFPs may protect cells by preventing Ca^{2+} leakage into them. Using standard patch clamping techniques, winter flounder AFP was shown to inhibit Ca^{2+} and K^{+} currents across pig granulosa cell membranes (Rubinsky et al. 1992b). Investigations using an intracellular Ca^{2+}-sensitive dye showed that ocean pout AFP blocks Ca^{2+} entry into rabbit parietal cells (Negulescu et al. 1992) presumably through interaction with certain ion channels. In this manner, AFPs might prevent hypothermia-induced Ca^{2+} accumulation in cells without affecting other cell functions (Negulescu et al. 1992). As mentioned earlier, the interaction of AFGPs with ice-nucleating proteins (Parody-Morreale et al. 1988) is thought to occur because these proteins imitate ice surfaces. It is possible that ion channels susceptible to blockage by AFPs also have a molecular surface that

resembles an ice crystal lattice, thus allowing one or more AFP types to bind and block ion passage.

It is clear that some AFPs interact with Ca^{2+} channels and they may also interact with cell membranes. However, more work will be needed before the mechanism by which they make mammalian cells cold-tolerant is known. The possibility that they play a similar role in fish must also be investigated. Many fish species survive at low (≈4°C) temperatures because of adaptations that make them cold tolerant. Different adaptations may play key roles in the survival of different species. Thus, it is possible that in fish that possess AFPs, these proteins enhance both their cold tolerance and freeze resistance.

1.8 Statement of research problem and objectives

The survival of many teleost fish species in icy seawater depends on their accumulation of high (10-25 mg/ml) concentrations of AFPs. Thus, the study of AFPs provides valuable insight into the cold adaptation, survival and distribution of many fish species in cold oceans. In addition, the AFPs elaborated by fish have become excellent model systems in which to study environmental adaptation, evolution and organization of multigene families, environmental regulation of gene expression, protein evolution, and protein structure/function relationships. In particular, the adsorption of AFPs to ice crystals presents a fascinating example of molecular recognition. Continuing study of the mechanisms by which the different AFPs function will generate knowledge that will be of general significance to many aspects of biorecognition, especially in the interaction of

proteins with crystalline surfaces. Findings in these research areas will, in turn, lead to a more complete understanding of the roles and behaviour of AFPs in fish.

The interaction of AFPs with ice and their roles in generating hysteresis and inhibiting recrystallization of ice are not completely understood. The newly discovered ability of AFPs to render cells cold-tolerant has made the study of their mechanisms of action more complex and the challenge is compounded by the striking diversity of the AFPs. Since the primary and secondary structures of the AFPs are distinct, investigations will need to address whether the different AFPs carry out their known functions in the same way, and determine the functional mechanism of each structural type.

If new types of AFP are discovered, their characterization and study of their mechanisms of action will bring new insights to the different activities of these proteins. Comparative studies employing the four known AFP types (for example, the study of ice crystal morphology by Raymond et al. 1989) have already enhanced our understanding of the interactions of these proteins with ice crystals. Furthermore, as new proteins within the known AFP types are identified, structural comparison of variant proteins will enhance our knowledge of the origins of the AFPs and of their mechanisms of action. Divergence among the variant sequences within an AFP type may reflect constraints on structural or functional integrity. Consequently, structural characteristics involved in hysteresis or other activities are likely to be conserved among protein variants.

The purpose of this study is to gain further insight into the diversity, the distribution, and the structure/function relationships in the AFPs. There are several fish species whose life histories or blood plasma freezing points suggest the presence of AFPs

but from which AFPs have not yet been isolated. Two of these species are smelt (Osmerus mordax) and Atlantic herring (Clupea harengus harengus). These species belong to taxonomic groups very distantly related to those in which fish with known AFP types belong. Therefore, study of their AFPs presented an ideal opportunity to identify new AFP types or new variants within an existing AFP type.

The specific objectives of this study are as follows:

(1) Isolation of the AFPs using chromatography and characterization by chromatography, activity measurements, gel electrophoresis, and immunoblotting.

(2) Construction of a smelt cDNA library and sequencing of an AFP clone in order to deduce the smelt AFP primary structure.

(3) Construction of a herring cDNA library and sequencing of an AFP clone in order to deduce the herring AFP primary structure.

(4) Functional characterization of the two new AFPs based on their sequence characteristics and their relationships to known AFPs or to other proteins.

Achievement of these objectives will provide two new AFPs, fully characterized, cloned and sequenced. This information, together with the AFP clones, can be used in the study of AFP structure/function relationships if they are expressed in heterologous systems. In addition, this work will generate the molecular tools necessary to seek an understanding of the roles and the regulation of AFPs in smelt and herring.

CHAPTER 2:

ISOLATION AND CHARACTERIZATION OF ANTIFREEZE PROTEINS FROM SMELT AND ATLANTIC HERRING

2.1 Introduction

Each of the four known AFP types is thought to be narrowly distributed among taxonomic groups (Scott et al. 1986). However, several fish species outside the groups in which the known AFPs occur are also likely to produce AFPs. Two such species are the smelt (O. mordax) and the Atlantic herring (C. harengus harengus). The smelt belong to the family Osmeridae (order Salmoniformes) and the herring belong to the family Clupeidae (order Clupeiformes) (Nelson 1984). Both groups are distantly related to those in which AFPs have previously been found.

In the western Atlantic ocean, the smelt ranges from Lake Melville, Labrador to Delaware River, Pennsylvania (Scott and Scott 1988). Smelt overwinter in nearshore locations, where they encounter subzero seawater temperatures (McKenzie 1964, Belyanina 1969), and antifreeze activity has been detected in smelt blood (DeVries 1974, Duman and DeVries 1975). The Atlantic herring is found from Greenland to North Carolina in the northwest Atlantic (Svetovidov 1963, Blaxter et al. 1985). Atlantic herring have been observed to survive at temperatures below -1.1°C in the presence of ice (Brawn 1960).

A preliminary investigation of the blood plasma of smelt and Atlantic herring revealed the presence of antifreeze activity. Thus, the aim of this work was to isolate and characterize the AFPs from these fish species.

2.2 Materials and methods

2.2.1 Sample collection

Atlantic herring (C. harengus hargenus) were caught using an otter trawl in Chedabucto Bay, N.S. in January 1986 and on Brown's Bank, N.S. in February 1986. They were caught using gill nets in Placentia Bay, Nfld. in March 1986 and in Conception Bay, Nfld. during March and April, 1987. Smelt (O. mordax) were captured using box nets in Trinity Bay, Nfld. in December 1987 and near Hardwick, Miramichi Bay, N.B. in February 1988. Blood samples were collected from freshly caught fish either by severing the caudal peduncle or by syringe and needle. The blood was allowed to clot and the serum collected following centrifugation (4000 x g) and stored at -20°C prior to analysis.

2.2.2 Measurement of antifreeze activity

Thermal hysteresis, a direct measure of antifreeze activity, is defined as the difference between melting and freezing temperatures of a small seed ice crystal (Kao et al. 1986). Thermal hysteresis measurements were made using a nanolitre osmometer (Clifton Technical Physics, Hartford, NY). Drops of serum were pipetted into mineral or immersion oil suspended in the holes of a metal disk on a cooling platform and the platform

was covered with a glass coverslip to minimize condensation. Observations were made using a Zeiss stereomicroscope. Nucleation was induced in the drops by rapid freezing to -40°C. Then, the samples were thawed in order to obtain a single crystal in each. The temperature at which the crystal grew was taken as the freezing point and the temperature at which the crystal got smaller was the melting point. The difference between these two values is the thermal hysteresis. Each measurement was done in triplicate.

2.2.3. Purification of antifreeze proteins

Atlantic herring serum collected from Conception Bay, Nfld. (8 ml) and smelt serum (10 ml) from Miramichi Bay, N.B. were each applied directly on a Sephadex G150 column (2.5 x 90 cm) in 0.1 M NH_4HCO_3 buffer. The column used to fractionate smelt proteins was calibrated for estimating M_r using BSA, ovalbumin, chymotrypsinogen A, and cytochrome C. The one used to fractionate herring proteins was calibrated using only the last three proteins. Proteins were detected in individual fractions by UV spectrophotometry. Optical density readings were made at 230 and at 280 nm. Following lyophilization, individual fractions were redissolved in 300 µl of 0.01 M NH_4HCO_3 and antifreeze activity was measured as described above. Fractions containing antifreeze activity were pooled and lyophilized again.

The samples were redissolved in no more than 10 ml of 5 mM Tris-HCl buffer pH 9.5 containing 0.1 M NaCl. They were then chromatographed on a QAE-Sephadex (A25) column (1.6 x 36 cm) equilibrated in the same buffer using a 0.1-0.4 M NaCl gradient. Absorbance peaks at 230 nm were each pooled and desalted either by using a Sephadex

G25 column (1.5 x 30 cm) or by dialyzing against 0.1 M NH_4HCO_3 (Spectrapore membrane tubing, M.W. cut-off 3500). They were then lyophilized and tested for antifreeze activity. The active peaks were relyophilized and designated as QAE AFP.

The AFP from herring and smelt were further fractionated using high performance liquid chromatography (HPLC) (Waters Associates). AFP were dissolved (10 mg/ml) in 0.2 M $HCOONH_4$ and 25 µl samples were applied to a Waters µBondapak C_{18} column (7.8 mm x 30 cm). They were eluted using a 0.1% trifluoroacetic acid-acetonitrile gradient at a flow rate of 0.75 ml/min. After lyophilization, individual peaks were reapplied to the column and eluted under the same conditions. Following repeated lyophilization to remove TFA, protein from each peak was dissolved in a minimal amount of 0.01 M NH_4HCO_3 and antifreeze activity was measured.

Winter flounder and ocean pout antifreeze polypeptides were purified by Sephadex G75 column chromatography (Fourney et al. 1984a, Hew et al. 1984). Sea raven antifreeze proteins were purified on Sephadex G75 followed by HPLC (Ng et al. 1986).

2.2.4 Amino acid and hexosamine analysis

The major herring and smelt AFP collected from the HPLC C_{18} column were hydrolysed in 6 N HCl at 110°C for 24 h for amino acid analysis. The samples were also hydrolysed in 4 N HCl at 110°C for hexosamine analysis. Methionine and cysteine were determined as methionine sulphone and cysteic acid, respectively, following performic acid oxidation. Amino acids and hexosamines were determined on a Waters Picotag amino acid analysis system.

2.2.5 Antifreeze activity studies

Smelt and herring QAE AFPs were used to plot thermal hysteresis curves. Two separate samples of lyophilized protein were weighed and dissolved in 0.01 M NH_4HCO_3. The protein concentrations of these solutions were verified using a BCA protein assay (Pierce Chemical Company, Rockford, Il). Identical dilutions were made from each starting solution and the thermal hysteresis of each solution was measured. Hystersis values obtained using the buffer alone were used as blanks and subtracted from each measurement.

The effect of dithiothreitol (DTT) on the antifreeze activities of smelt AFP, herring AFP, and flounder AFP (cystine-free) were determined. Proteins were dissolved in 0.01 M NH_4HCO_3. Aliquots of these solutions were combined in a 9:1 ratio with 0.5 M DTT or water. Final smelt and herring AFP concentrations were 15 mg/ml and the final winter flounder AFP concentration was 2.4 mg/ml. Solutions were kept at 50°C for 1 hour and thermal hysteresis was measured. For these hysteresis measurements, samples were suspended directly in the holes of the metal disk on the cooling platform of the osmometer and no oil was used. Blanks were subtracted from each measurement.

2.2.6 Determination of relative molecular mass

The M_rs of smelt and herring QAE antifreeze proteins were determined using SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). Gels were stained for protein with 0.1% Coomassie blue R250 in a solution of isopropanol-acetic acid-water (25:10:65) containing 0.1% cupric acetate. They were destained in 2 changes of methanol-

acetic acid-water (30:10:60) followed by several changes of methanol-acetic acid-water (10:5:85).

2.2.7 Immunoblotting

Rabbit polyclonal antibodies were raised previously against SR2, the major sea raven AFP and against a major ocean pout AFP SP-1-A as described by Ng et al. (1986). Herring and smelt QAE AFPs were run on 15% polyacrylamide SDS-PAGE and electrophoretically transferred to a Hybond-N nylon membrane (Amersham). Transfer was carried out using either a TE 52 transfer electrophoresis unit (Hoefer Scientific Instruments, San Fransisco CA) for 3 hours or a Mini Protean II transfer unit (Bio-Rad) overnight. Prior to transfer, gels were equilibrated in transfer buffer (25 mM Tris, 150 mM glycine, pH 8.3). Following transfer, membranes were notched for alignment with the gel, allowed to dry, and kept at 4°C until use.

Detection was carried out using the Protoblot Western Blot AP System (Promega, Madison WI) essentially following the directions provided. Blocking was carried out in order to cover any non-specific binding sites on the membrane. This step was carried out overnight at 42°C in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 10% BSA and 1% gelatin. The membrane was removed from the blocking solution and placed directly in the first antibody solution for 1 hour. This solution was TBST containing 0.33 ml anti-sea raven AFP antiserum or 0.2 ml anti-ocean pout antiserum per 10 ml. Three washes were carried out in 100 ml each TBST. The membrane was then placed in the second antibody solution (goat anti-rabbit IgG alkaline

phosphatase conjugate diluted 1:7500 in TBST) for 1 hour followed by 3 more washes in TBST. The membrane was incubated in a colour development solution (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate for several minutes until colour development was complete and then rinsed in several changes of water.

2.2.8 Preparation of antisera

Rabbit polyclonal antibodies were raised against smelt HPLC isoforms S2 and S3 and herring isoforms H1 and H2. Antibodies were raised in New Zealand white rabbits held at the Memorial Univeristy Animal Care facility. Protocols conformed to guidelines from the Canadian Council on Animal Care.

All procedures used to raise antibodies and to collect and store antisera were as described by Harlow and Lane (1988). One rabbit was used for each protein isoform. For initial injections, 200 µl solutions of 0.9% NaCl containing 80 µg smelt or 40 µg herring protein were prepared. To each, a further 300 µl 0.9% NaCl was added, followed by 500 µl Freund's complete adjuvant (ICN Biomedical). Each mixture was vortexed for at least 10 minutes or until homogeneous. Two 500 µl aliquots were taken from each mixture. One was injected into the thigh muscle of each hind leg. After 3-4 weeks, boosts were done. Half the original amount of protein was dissolved in saline as above, mixed with 500 µl of incomplete Freund's adjuvant, vortexed, divided into 2 aliquots, and injected as before. Further boosts were done 2 months and 5 months later. Antiserum was collected from each rabbit 9 days after each boost by bleeding from a vein in the ear. Blood was

allowed to clot at 4°C or at room temperature overnight. Serum was collected and frozen at -20°C or at -70°C.

Antisera were tested for avidity and specificity by immunoblotting. Serial tenfold dilutions of the respective QAE AFPs were dot blotted onto positively-charged nylon (Gelman Sciences). The blots were then processed as described above for the Western blots using 0.33 ml antiserum in a total volume of 10 ml for the first antibody incubation. Specificity of the antisera obtained after the second boost was also evaluated by immunoblotting. One µg smelt or herring blood plasma were electrophoresed (SDS-PAGE) in 4-20% acrylamide gradient gels (Bio-Rad), transferred to nylon membranes, and then incubated in the appropriate antibody solutions as described earlier. Immunoblotting was repeated using the anti-herring and anti-smelt AFP antisera in order to determine the cross-reactivities of each for the different cystine-rich AFPs.

2.3 Results

2.3.1 Isolation and identification of smelt and herring AFPs

Analysis of smelt and herring serum from different locations showed different levels of thermal hysteresis (Table 1). AFP were present in most populations sampled. Temperatures obtained off Logy Bay, Nfld. (Walker et al. 1986) were used for Conception Bay, April 1985. Since water temperature on the northeast Newfoundland shelf appears to vary in a coherent fashion (Petrie et al. 1988), these temperatures are

Table 1. Plasma hysteresis values in fish from different locations.

	Thermal hysteresis (°C)	Water Temperature[a] (°C)
Atlantic herring		
Brown's Bank, N.S. 16 February, 1986	Absent	7.0
Conception Bay, Newfoundland 15 April, 1985	0.23 (pool n>35)	-1.5
Chedabucto Bay, Nova Scotia 30 January, 1986	0.12 (pool n=8)	0.6
Placentia Bay, Newfoundland 22 March, 1986	0.318 ± 0.011 (n=55)	-1.0
Smelt		
Miramichi Bay, New Brunswick 8 February, 1988	0.318 ± 0.011 (n=22)	-1.8 - 0
Trinity Bay, Newfoundland 10 December, 1987	0.17 ± 0.01 (n=7)	-0.5

[a]Sources: Conception Bay, Walker et al. 1986; Chedabucto Bay, Placentia Bay, Walker, et al. 1987.

expected to be very similiar to one another. Although temperatures were not available for Miramichi Bay, N.B., the temperature was estimated as 0 to -1.8°C since the fish were caught in shallow water under ice.

Sephadex G150 chromatography of smelt and herring serum produced peaks of antifreeze activity (Fig. 4a, 4b). The herring AFP eluted at a volume corresponding to an approximate M_r of 25,000 whereas the elution volume of smelt AFP proteins suggested a M_r of approximately 50,000. QAE-Sephadex chromatography of the smelt G150 peak of activity resulted in 6 absorbance peaks at 230 nm (Fig. 5a). When they were tested for thermal hysteresis, only peak 4 showed activity. An identical ion exchange chromatography of the herring G150 active peak gave 4 absorbance peaks (Fig. 5b) of which only the third was active.

The QAE AFP were further purified using HPLC. The herring AFP resolved into 2 active isoforms, H1 and H2, present in nearly equal amounts, (Fig. 6a). Both isoforms showed antifreeze activity. The smelt AFP was fractionated into 6 active components of which S2 and S3 were the major ones (Fig. 6b).

2.3.2 Relative molecular masses of smelt and herring AFPs

On SDS-PAGE in 12% and 15% polyacrylamide gels, the M_r of the smelt AFP was 24 000 and that of herring was 14 600 (Fig. 7a,b). However, Sephadex G150 produced higher M_r estimates than did electrophoresis. Such differences between estimates of M_r from gel filtration and from SDS-PAGE also occur for

Fig. 4. Sephadex G150 fractionations of (A) 8 ml smelt and (B) 6 ml Atlantic herring serum. The column size was 2.5 x 90 cm, the fraction size collected was 3.8 ml, and the elution buffer was 0.1 M NH_4HCO_3, pH 8.2. Individual fractions were lyophilized and redissolved in 300 µl of 0.01 M NH_4HCO_3 and their antifreeze activity determined. Fractions 46-64 from (A) and fractions 67-79 from (B) were pooled for subsequent analysis.

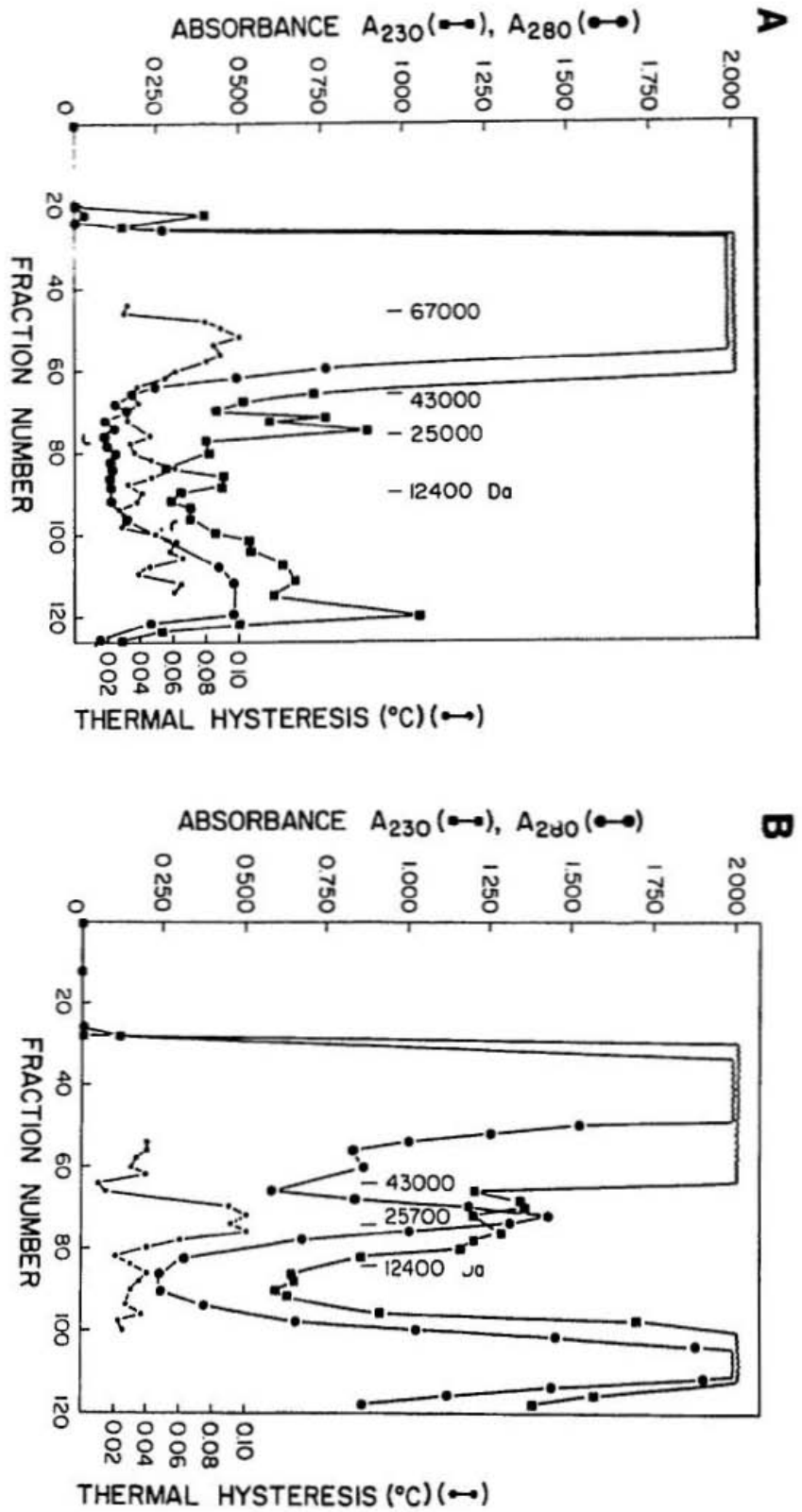

A
ABSORBANCE A_{230}(•—•), A_{280}(•—•)
0
0.250
0.500
0.750
1.000
1.250
1.500
1.750
2.000
FRACTION NUMBER
20
40
60
80
100
120
– 67000
– 43000
– 25000
– 12400 Da
0.02
0.04
0.06
0.08
0.10
THERMAL HYSTERESIS (°C)(•—•)
B
ABSORBANCE A_{230}(•—•), A_{280}(•—•)
0
0.250
0.500
0.750
1.000
1.250
1.500
1.750
2.000
FRACTION NUMBER
20
40
60
80
100
120
– 43000
– 25700
– 12400 Da
0.02
0.04
0.06
0.08
0.10
THERMAL HYSTERESIS (°C)(•—•)

Fig. 5. QAE Sephadex ion exchange chromatographic profiles of G150 AFP peaks from (A) smelt and (B) Atlantic herring. The column size was 1.6 x 36 cm, the fraction size was 3.6 ml and the elution buffer was 5 mM Tris-HCl, pH 9.5 with a 0.1-0.4 M NaCl gradient. The fractions containing optical density peaks at 230 nm were pooled, lyophilised, redissolved in the smallest possible volumes of 0.01 M NH_4HCO_3 and monitored for antifreeze activity. Fractions 113-130 from (A) and fractions 105-124 from (B) were pooled for subsequent analysis.

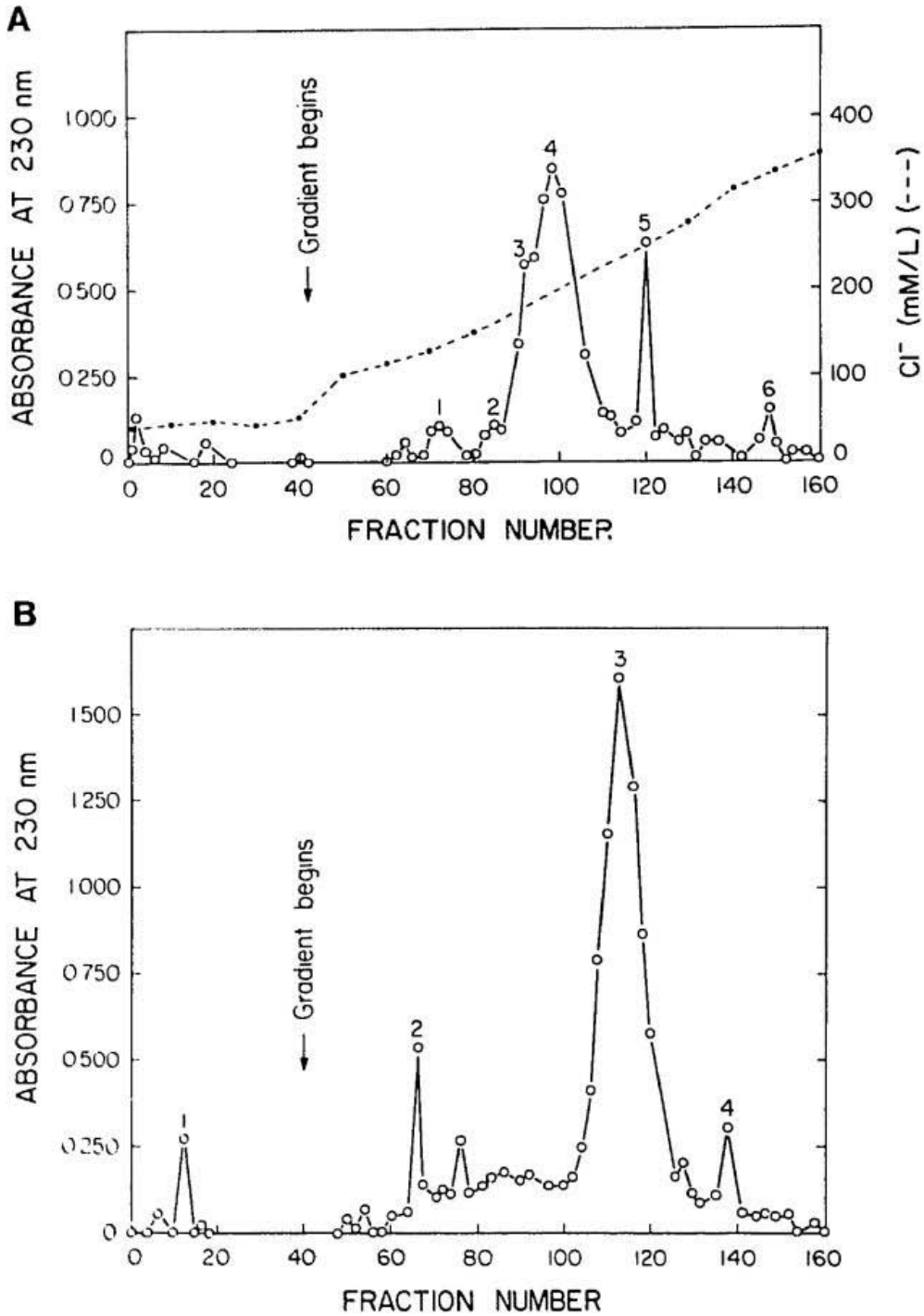

A
ABSORBANCE AT 230 nm
1000
0750
0500
0250
0
Gradient begins
400
300
200
100
0
Cl⁻ (mM/L) (---)
1
2
3
4
5
6
0
20
40
60
80
100
120
140
160
FRACTION NUMBER
B
ABSORBANCE AT 230 nm
1500
1250
1000
0750
0500
0250
0
Gradient begins
1
2
3
4
0
20
40
60
80
100
120
140
160
FRACTION NUMBER

Fig. 6. Reverse phase HPLC of (A) smelt and (B) Atlantic herring AFP QAE peaks using a Waters μBondapak C_{18} column (7.8 mm x 30 cm). Proteins were eluted using a 0.1% TFA-acetonitrile gradient with a flow rate of 0.75 ml/min. Optical density peaks were pooled, rechromatographed separately, redissolved in the smallest possible volumes of 0.01 M NH_4HCO_3 and monitored for activity.

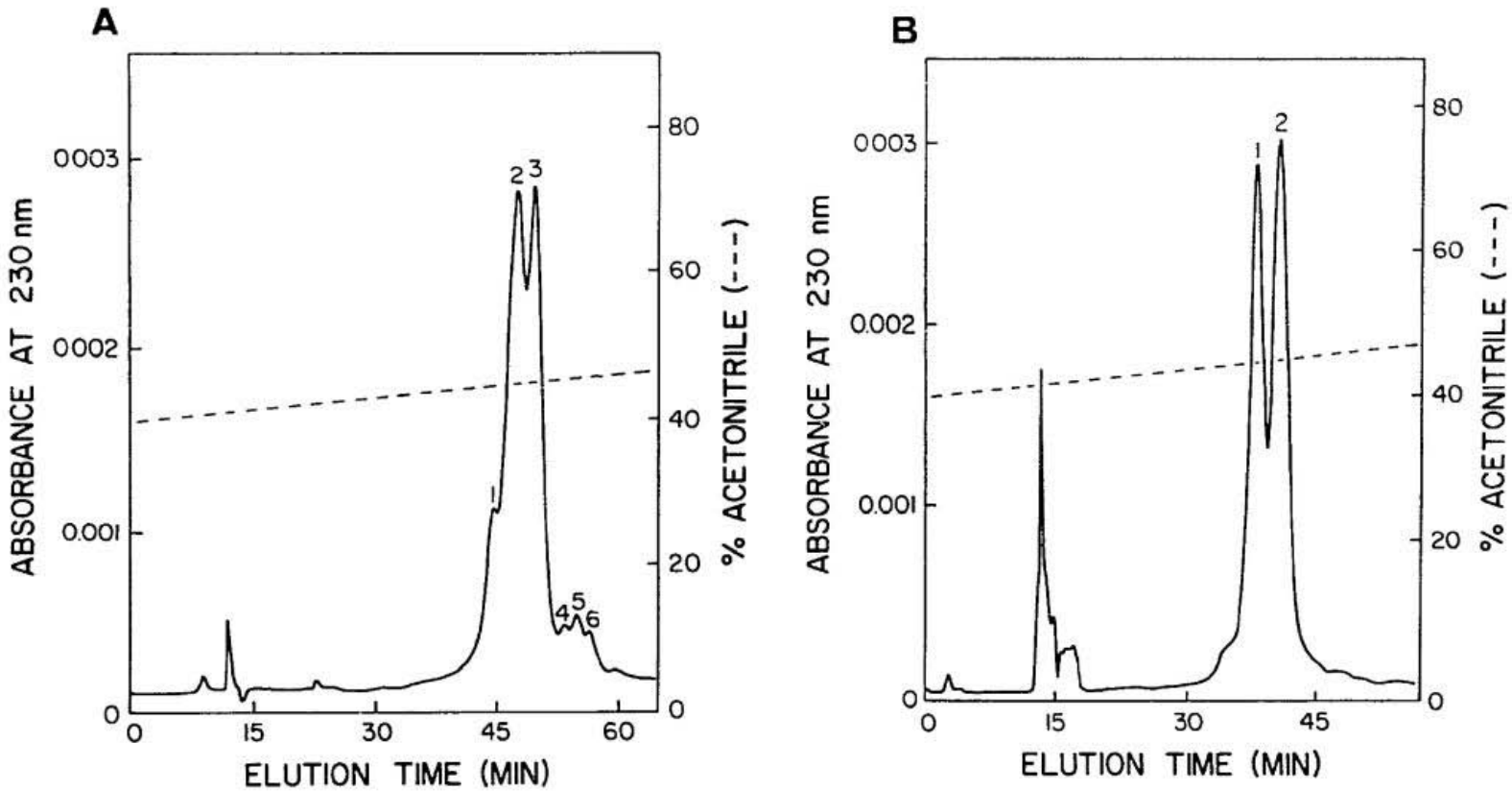
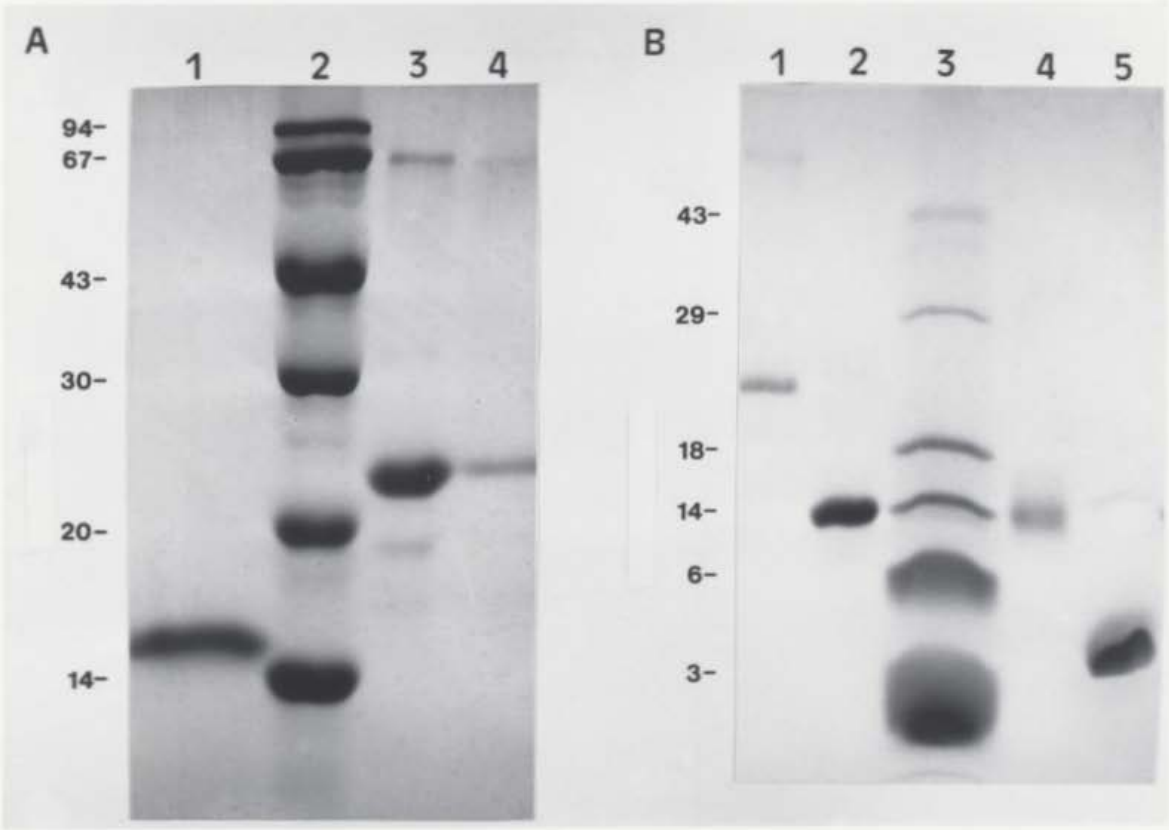

A
ABSORBANCE AT 230 nm
0.003
0.002
0.001
0
0
15
30
45
60
ELUTION TIME (MIN)
80
60
40
20
0
% ACETONITRILE (---)
1
2
3
4
5
6
B
ABSORBANCE AT 230 nm
0.003
0.002
0.001
0
0
15
30
45
ELUTION TIME (MIN)
80
60
40
20
0
% ACETONITRILE (---)
1
2

Fig. 7. SDS-PAGE of smelt and herring AFPs: (A) SDS-PAGE in 12% polyacrylamide. Lanes: (1) herring AFP from QAE; (2) molecular mass protein markers; (3) smelt AFP from G150; (4) smelt QAE AFP. (B) SDS-PAGE in 15% polyacrylamide. Lanes: (1) smelt AFP; (2) herring AFP; (3) low molecular mass protein markers; (4) sea raven AFP; (5) ocean pout AFP. Numbers on the left represent the M_r of the markers ($x10^{-3}$).


A
1
2
3
4
94-
67-
43-
30-
20-
14-
B
1
2
3
4
5
43-
29-
18-
14-
6-
3-

winter flounder, sculpin and ocean pout AFP (Hew et al. 1980, Fourney et al. 1984a, Hew et al. 1984).

When the smelt and herring AFPs were electrophoresed along with AFP from sea raven and ocean pout, the sea raven and herring AFP comigrated, indicating that their M_r values were similar (Fig. 7b). The mobilities of sea raven and ocean pout AFP were consistent with previous results for these AFP under the same conditions (Hew et al. 1984, Ng et al. 1986).

In some instances, smelt QAE AFP contained a minor contaminating protein evident on SDS-PAGE. However, it appeared to be a small percentage of total protein.

2.3.3 Amino acid and hexosamine compositions

The amino acid compositions and the hexosamine contents of the 2 herring AFP isoforms and the 2 major smelt AFP isoforms are shown in Table 2. Repeated analyses gave results consistent with those shown here. Ocean pout and sea raven AFPs were included for comparison.

2.3.4 Antifreeze activity of smelt and herring AFPs

The antifreeze activities of smelt and herring QAE AFPs as a function of mass and molarity are shown in Fig. 8a and 8b respectively. The activities were lower than those of most other fish AFPs. Unlike other AFPs, the antifreeze activity of smelt and herring did not saturate at 20 mg/ml (Fig. 8a), but continued to increase with concentration.

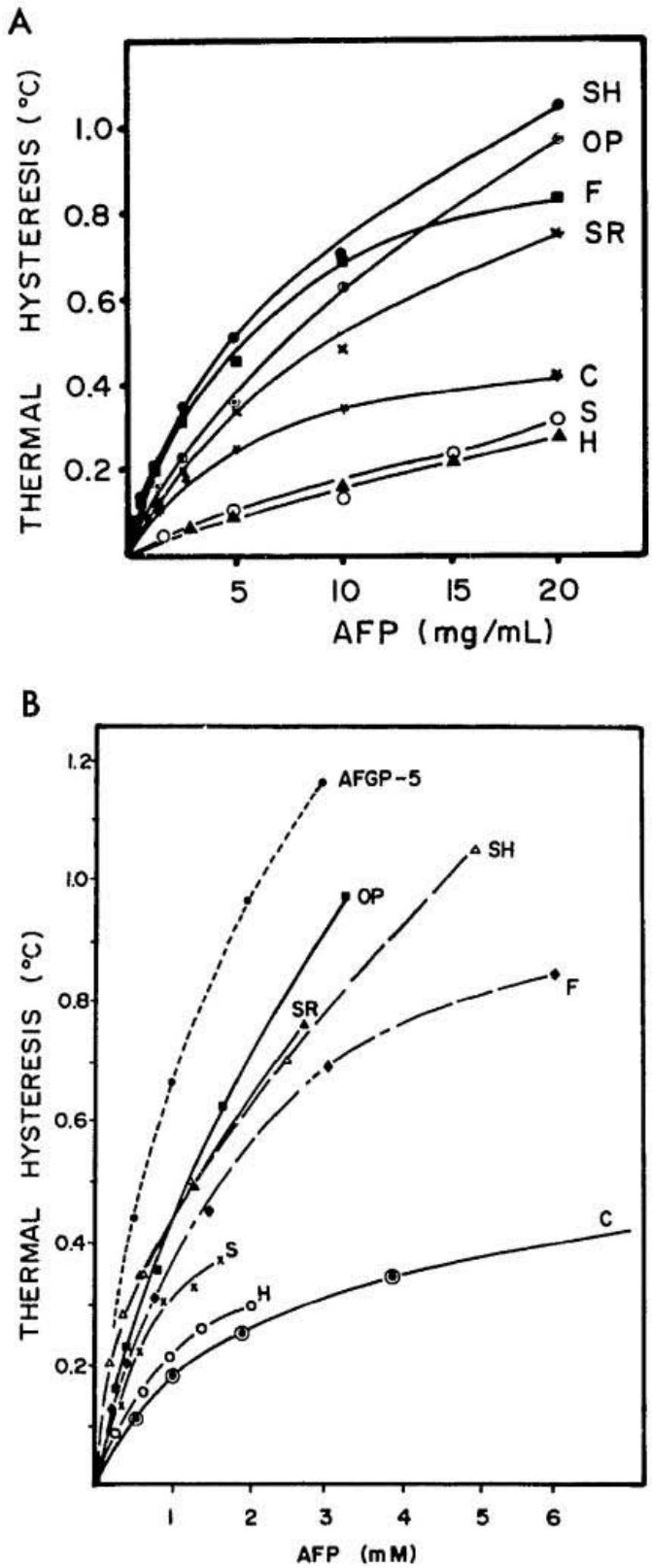
Since smelt and herring AFPs had a relatively high percentage of half-cystine,

Table 2. The compositions (mol %) of AFPs.

	Sea Raven SR2[a]	Ocean Pout SP1-A[a]	Herring		Smelt	
			H1	H2	S2	S3
Asx	9.28	6.32	13.30	12.85	12.54	11.92
Glx	9.61	12.08	10.15	10.28	8.01	8.19
Ser	7.75	3.87	8.49	9.18	7.88	8.92
Gly	7.83	7.85	6.12	6.10	8.68	8.66
His	3.27	0.00	2.91	2.51	1.95	2.40
Arg	2.59	0.00	1.81	2.21	1.26	1.95
Thr	8.11	9.21	8.47	8.16	9.77	9.90
Ala	14.45	10.38	8.22	8.31	10.11	9.51
Pro	6.64	10.44	4.56	4.77	4.22	4.74
Tyr	1.62	1.49	0.00	0.15	0.49	0.00
Val	1.89	11.50	2.98	2.25	5.52	5.49
Met	4.79	8.21	4.74	5.28	4.04	3.69
½Cys	8.29	0.00	9.13	8.88	6.81	6.49
Ile	2.64	7.21	5.01	5.40	4.37	4.38
Leu	6.49	3.37	7.70	7.74	6.48	6.50
Phe	2.59	1.69	3.38	3.12	4.39	4.54
Lys	2.16	6.38	3.02	2.81	3.48	2.72
glucosamine	0	0	0	0	~3%	~3%
galactosamine	0	0	0	0	0	0

[a]Sources: sea raven, Ng et al. 1986; ocean pout, Li et al. 1985.

Fig. 8. Comparison of thermal hysteresis curves on (A) a mass basis and (B) a molar basis of AFP and AFGP obtained from shorthorn sculpin (SH), ocean pout (OP), winter flounder (F), sea raven (SR), Atlantic cod (C), smelt (S) and Atlantic herring (H). Curves for AFP from all species except smelt and herring are taken from Kao et al. (1986).


A
THERMAL HYSTERESIS (°C)
1.0
0.8
0.6
0.4
0.2
SH
OP
F
SR
C
S
H
5
10
15
20
AFP (mg/mL)
B
THERMAL HYSTERESIS (°C)
1.2
1.0
0.8
0.6
0.4
0.2
AFGP-5
SH
OP
SR
F
C
S
H
1
2
3
4
5
6
AFP (mM)

the effect of disulfide bond reduction on AFP activity was determined. Thermal hysteresis of smelt AFP was 72% inhibited and that of herring AFP was 57% inhibited in the presence of DTT, a sulfhydryl reducing agent. DTT alone in solution did not affect thermal hysteresis, and winter flounder antifreeze activity remained unchanged under the same conditions (Table 3).

2.3.5 Evaluation of anti-smelt and anti-herring AFP antisera

The antisera raised against smelt AFP isoforms had a high affinity for their antigens. Antisera collected after the second boost could be used to detect as little as 50 ng of smelt QAE AFP (Fig. 9). In contrast, the antisera against herring AFPs only produced a detectable signal when 5µg of antigen was used (Fig. 9).

The antisera appear to be specific for the AFPs. The only herring or smelt blood plasma proteins recognized by the antibodies were the AFPs (Figs. 10, 11). However, this does not eliminate the possibility that proteins the same size as the AFPs might be recognized by the antisera and still result in a single band on the blot.

2.3.6 Cross-reactivity of the AFPs with antisera against sea raven, smelt, and herring AFPs

Antiserum previously produced against the major sea raven AFP component SR2 by Ng et al. (1986) cross-reacted with smelt AFP but not with herring AFP (Fig. 12). Thus, smelt AFP is immunologically similar to that of sea raven whereas herring AFP is not. Further immunoblotting showed that the anti-herring AFP antiserum cross-reacted

Table 3. The effects of dithiothreitol (DTT) on AFP activity.

	Thermal hysteresis (°C)		% Inhibition
Protein	- DTT	+DTT	
Smelt AFP 15 mg/ml	0.205 ± 0.018	0.057 ± 0.015	72%
Atlantic herring AFP 15 mg/ml	0.215 (n=2)	0.093 (n=2)	57%
Winter flounder AFP 2.4 mg/ml	0.345 ± 0.028	0.337 ± 0.020	-
No protein	- (n=1)	- (n=1)	-

Note: n = 3 except where indicated.

Fig. 9. Immunoblots by dot blotting of a serial dilution of smelt and herring QAE AFPs using anti-smelt and anti-herring AFP antisera. Membranes strips 1 and 2 were prepared using smelt QAE AFP and strips 3 and 4 using herring AFP. For all blots, the first, second, third, and fourth rows contain 5, 0.5, 0.05, and 0.005 μg of antigen respectively. Anti-smelt AFP antiserum was used to detect antigen in lanes 1 and 2 and anti-herring AFP antiserum was used in strips 3 and 4.

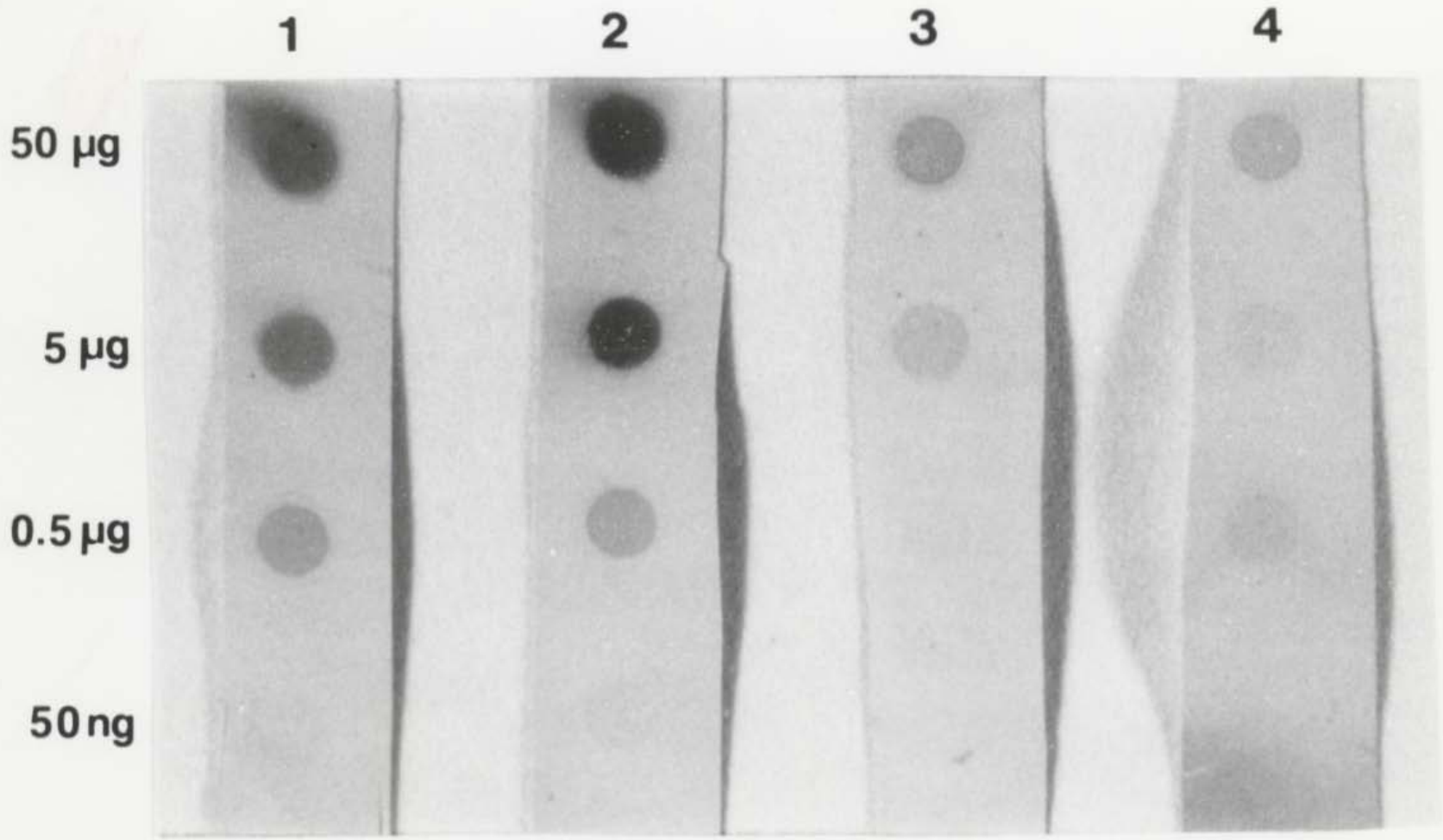

1
2
3
4
50 µg
5 µg
0.5 µg
50 ng

Fig. 10. Immunoblots by Western blotting of smelt blood plasma using anti-smelt AFP antisera. All strips are blots of 2 µl of smelt plasma. Antisera used in each lane: (1-3) anti-S2 antisera collected after 1st, 2nd, and 3rd boosts respectively; (4-6) anti-S3 antisera collected after 1st, 2nd, and 3rd boosts respectively.

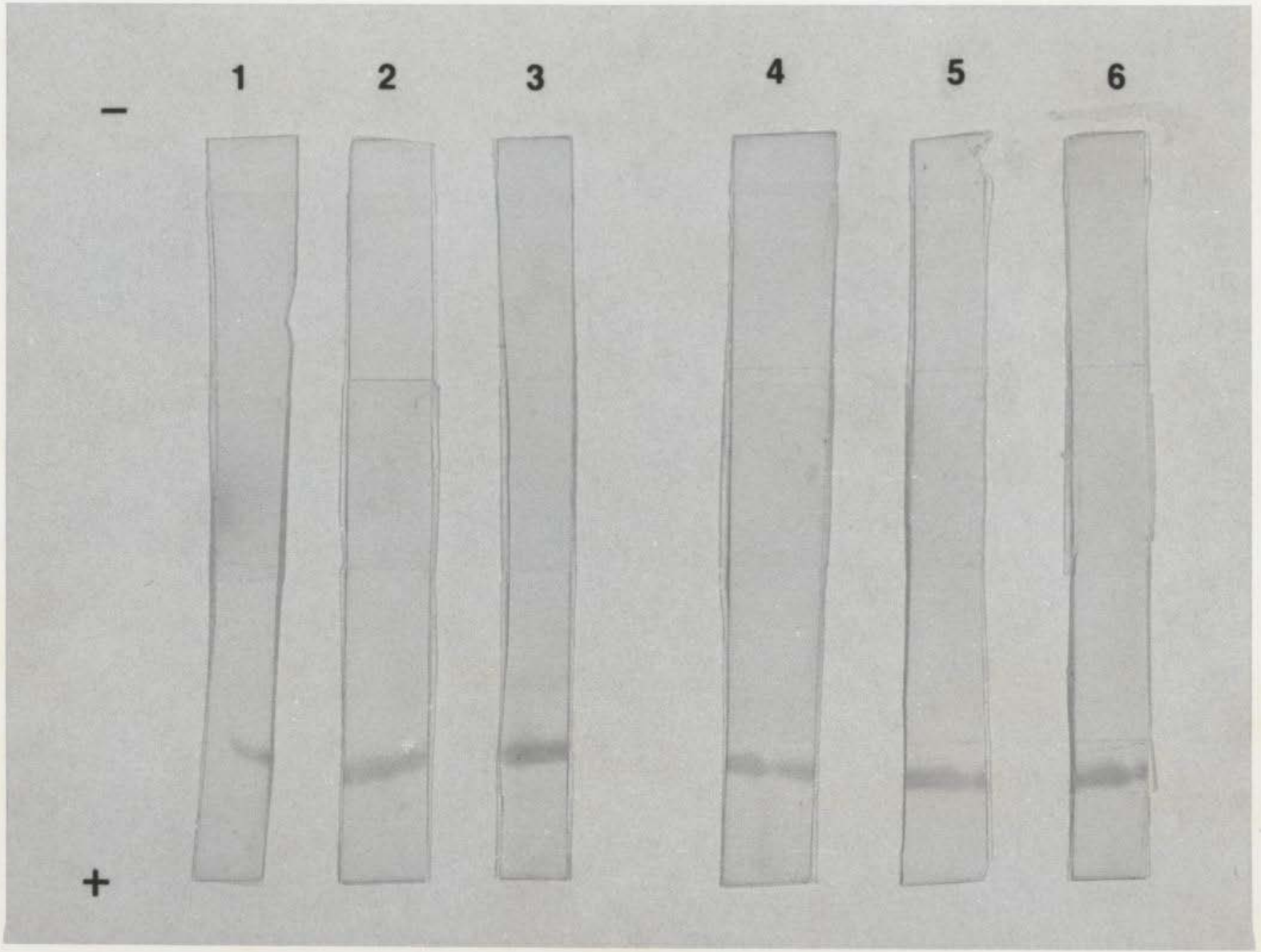

1
2
3
4
5
6
–
+

Fig. 11. Immunoblots by Western blotting of herring blood plasma using anti-herring AFP antisera. All strips are blots of 1 µl of herring plasma. Anti-herring AFP antisera used in each lane: (1-2) anti-H1 collected after 1st and 3rd boosts; (3-5) anti-H2 collected after 1st, 2nd, and 3rd boosts, respectively. Numbers on the left represent the M_r of the markers ($x10^{-3}$).

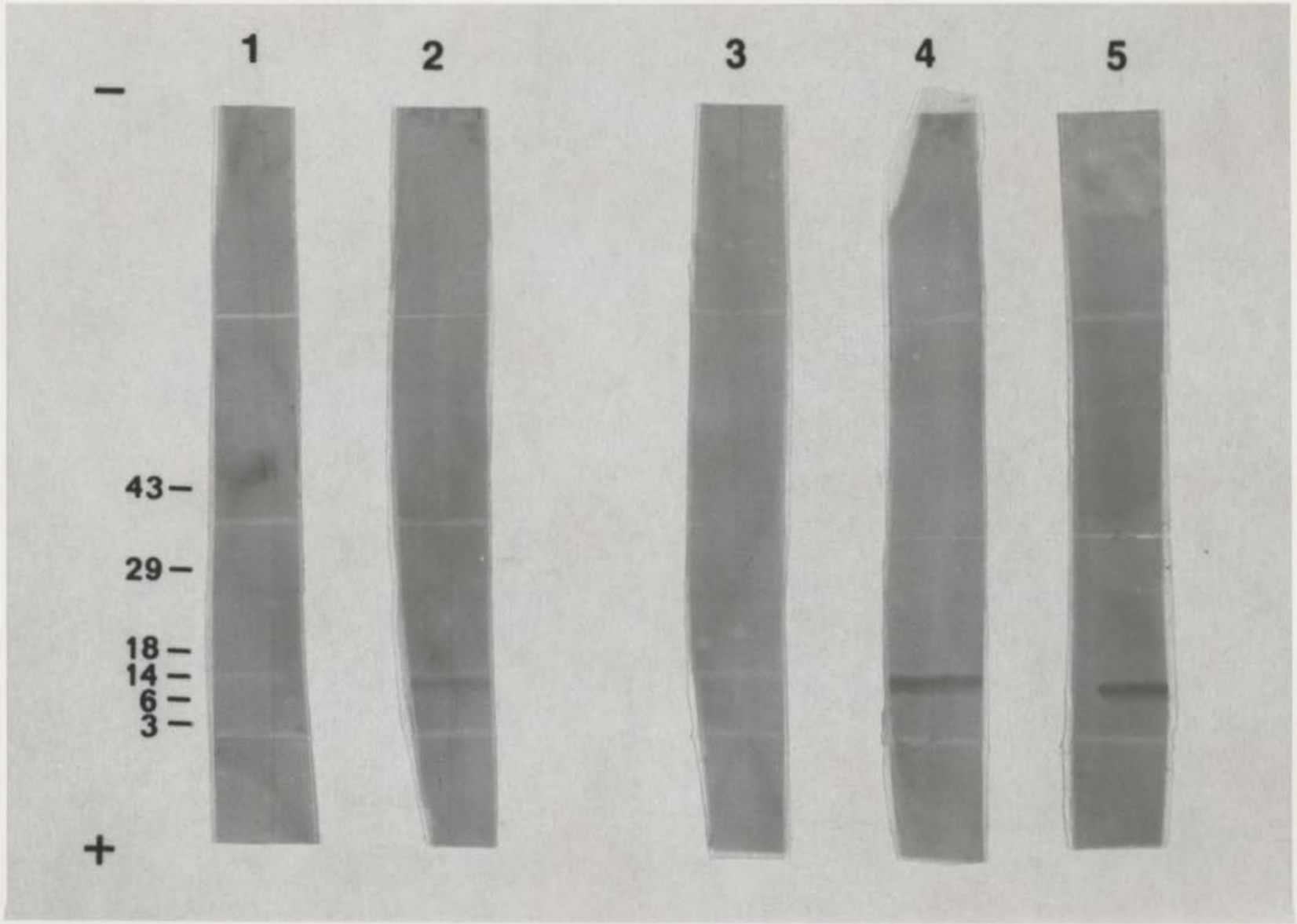
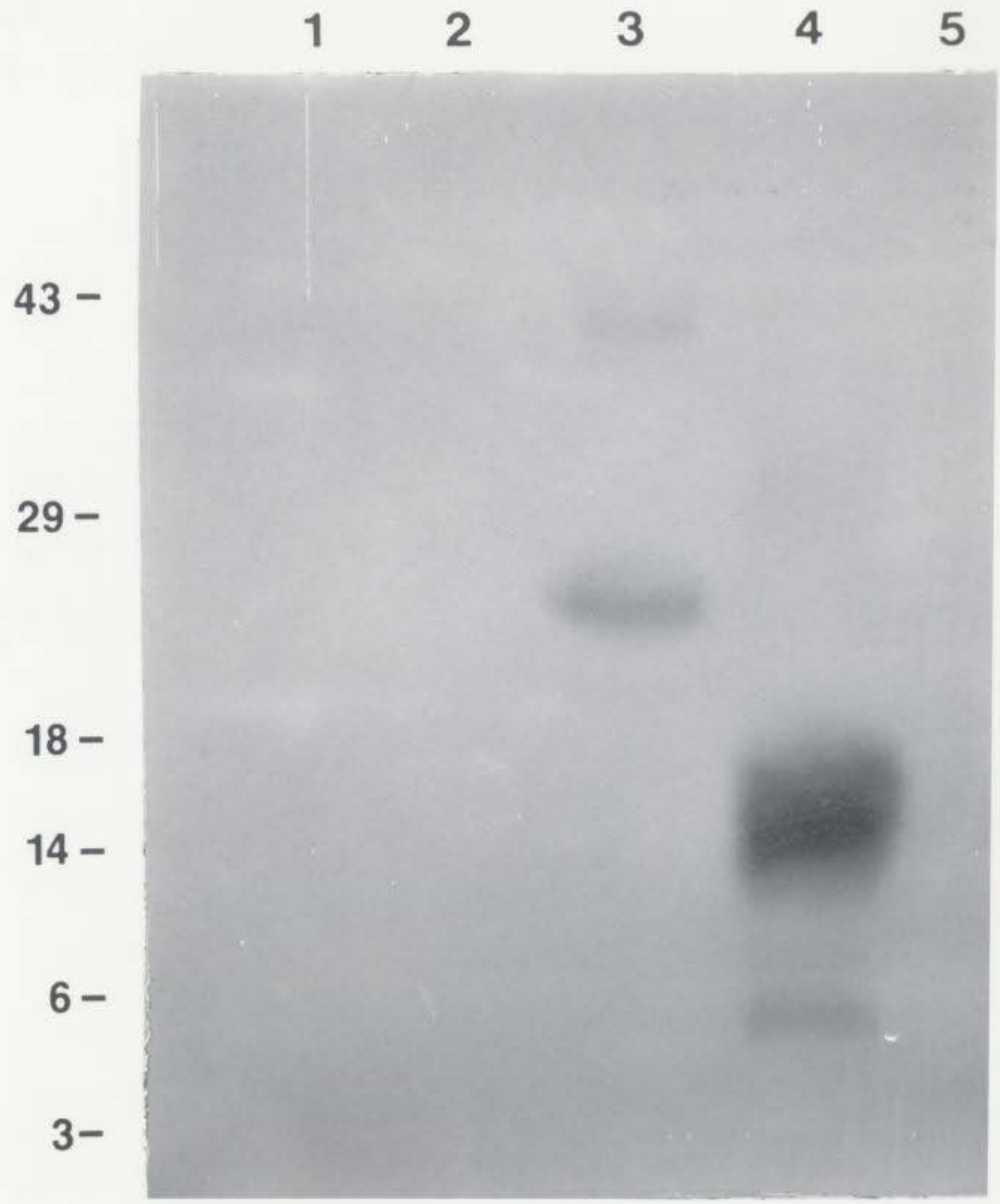

1
2
3
4
5
−
43
29
18
14
6
3
+

Fig. 12. Immunoblotting of AFPs by Western blotting showing cross-reactivity to anti-SR2 antiserum. Lanes: (1) blank; (2) herring AFP; (3) smelt AFP; (4) sea raven AFP; (5) low molecular mass protein markers. Numbers on the left represent the M_r of the markers ($x10^{-3}$).


1
2
3
4
5
43 –
29 –
18 –
14 –
6 –
3 –

only with smelt AFP and anti-smelt AFP antiserum did not cross-react with the AFPs of herring or sea raven (Fig. 13).

Antiserum previously produced against ocean pout AFP, SP-1-A, did not cross-react with either smelt or herring AFP (Fig. 14). This antiserum appeared specific for ocean pout AFP. It did not cross-react with an AFP from Atlantic snailfish, Liparis atlanticus, (Fig. 14) and it was previously shown not to cross-react with the sea raven AFP (Ng et al. 1986).

2.4 Discussion

Herring, smelt, and sea raven belong to three distinct orders of fish (Clupeiformes, Salmoniformes, and Scorpaeniformes respectively), yet their AFPs have considerable similarities. On the basis of amino acid composition, particularly half cystine residues, the smelt and herring AFP more closely resemble type II AFP from sea raven than they do AFPs from ocean pout (type III) or flounder and sculpin (type I). This similarity between smelt, herring and sea raven AFPs is further illustrated by the importance of disulfide bonds (oxidized half-cystine) to their activity (Slaughter et al. 1981).

The cross-reactivity between anti-sea raven AFP and anti-herring AFP antisera and smelt AFP indicate that the AFPs from these species have at least one immunological or structural feature in common. Yet, the absence of cross-reactivity between herring and sea raven AFPs suggests that they are, in some ways, immunologically distinct. These results are difficult to interpret because the anti-smelt antiserum does not recognize either of the

Fig. 13. Immunoblotting of cystine-rich AFPs by Western blotting showing cross-reactivity to anti-SR2 (sea raven), anti-S3 (smelt), and anti-H2 (herring) AFP antisera. Lanes for all blots: (1) herring QAE AFP; (2) smelt QAE AFP; (3) sea raven AFP. Blots A, B, and C were incubated with antisera against smelt, herring, and sea raven AFPs, respectively. Numbers on the left represent the M_r of the markers ($x10^{-3}$).

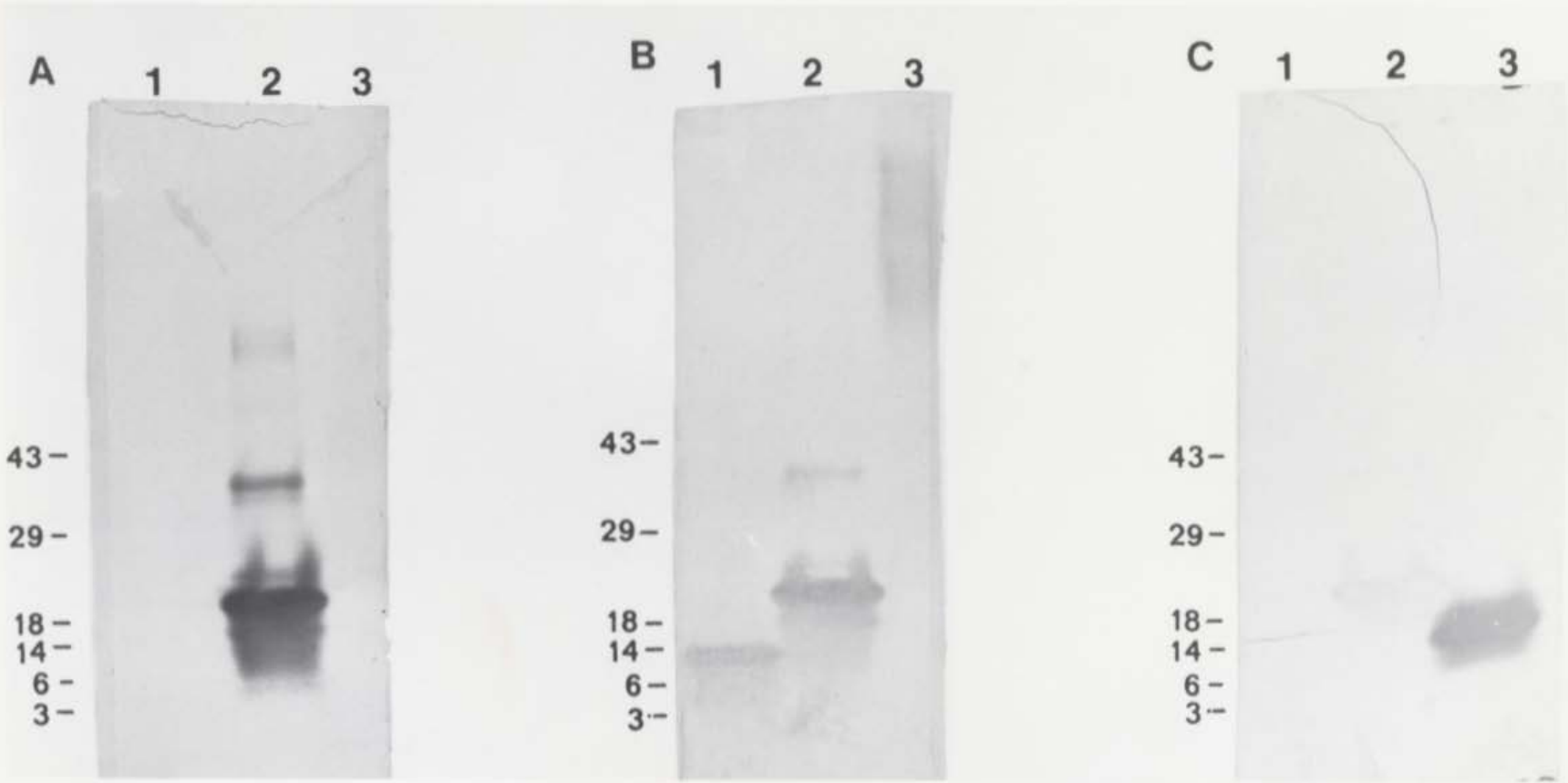
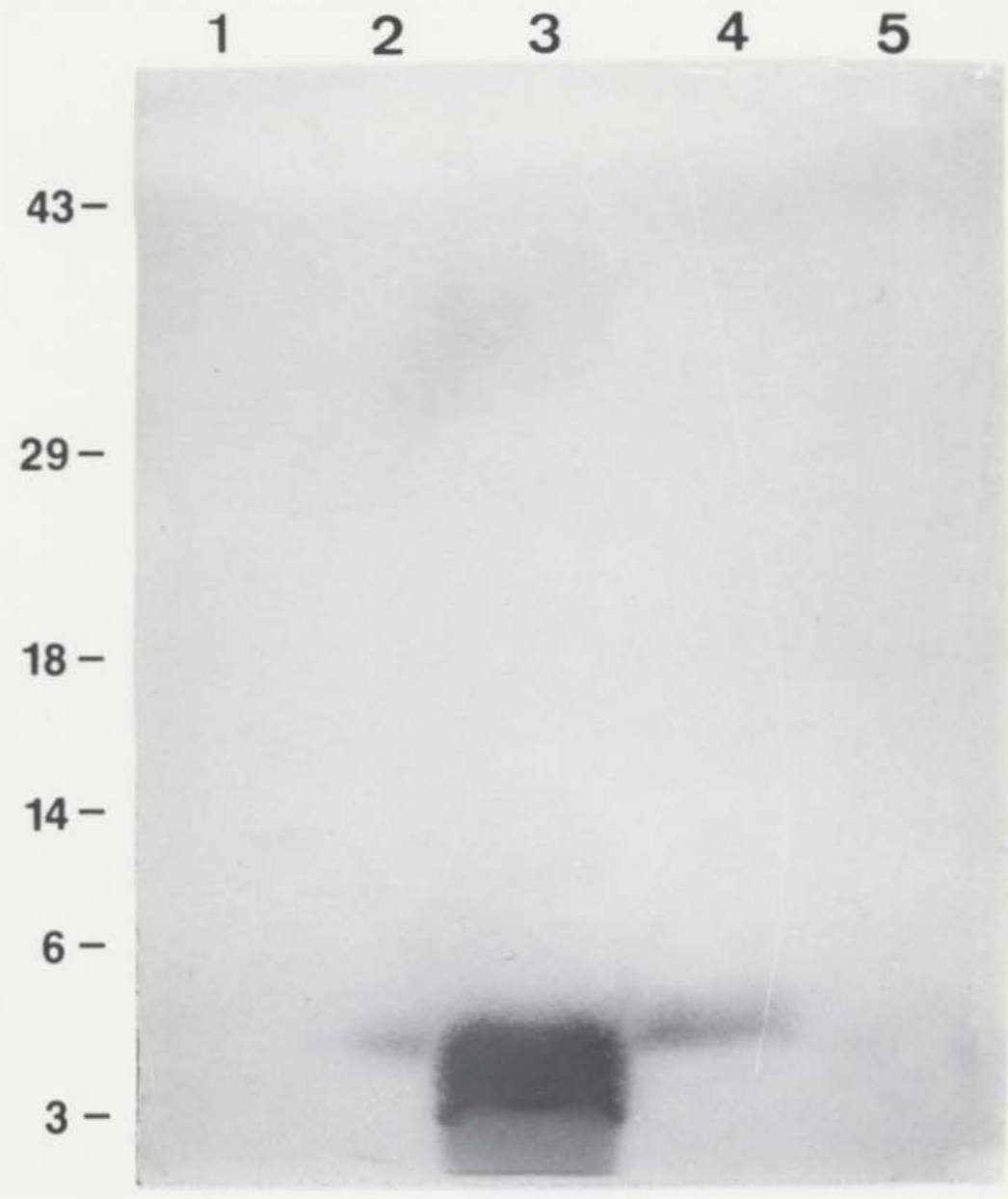

A
1
2
3
43
29
18
14
6
3
B
1
2
3
43
29
18
14
6
3
C
1
2
3
43
29
18
14
6
3

Fig. 14. Immunoblotting of AFPs by Western blotting, using anti-ocean pout AFP antiserum. Lanes: (1) smelt QAE AFP; (2) herring QAE AFP; (3) ocean pout AFP; (4) low molecular mass markers; (5) liparis AFP. Numbers on the left represent the M_r of the markers ($x10^{-3}$).


1
2
3
4
5
43
29
18
14
6
3

other 2 AFPs. However, since the smelt AFP is glycosylated, anti-smelt AFP antiserum may recognize primarily the carbohydrate on this AFP rather than the protein epitopes it shares with the other AFPs.

The resemblance among the three cystine-rich fish AFPs may reflect a common structure resulting from convergent evolution. This appears to be the case for other AFPs. Although the flounder and sculpin AFPs are both alanine-rich α-helical AFPs with similar amino acid compositions, current evidence suggests that they are the products of different genes (Scott et al. 1986). The spruce budworm (Choristoneura fumiferana) also contains a cystine-rich AFP with an amino acid composition, an M_r, and immunological properties all similar to that of sea raven AFP (Hew et al. 1983). Given the isolated findings of cystine-rich AFPs, it will be useful to investigate their occurence among the divergent fish groups where they are found. Interestingly, among Clupeoid fish, antifreeze activity has also been detected in the closely related gaspereau, Alosa pseudoharengus, (Duman and DeVries 1974) and Pacific herring, Clupea harengus pallasi, (Eastman et al. 1987, Raymond 1989).

The presence of glucosamine in smelt AFP suggests that it has an N-linked carbohydrate. The similar antifreeze activities of smelt and herring AFPs suggest that the carbohydrate of smelt AFP has little or no functional significance. To date, carbohydrate has only been found associated with the AFGP where it contributes approximately 60% of the molecular mass (DeVries et al. 1970, Hew et al. 1981).

The activities of AFPs isolated from smelt and herring are the lowest, on a milligram basis, of all antifreeze studied to date (Fig. 5a). However, when the M_r of the proteins are taken into consideration, their ability to depress the freezing temperature of

aqueous solutions comes into line with those of the other AFPs (Fig. 5b). These observations are consistent with the suggestion that as the molecular mass of the AFP increases above 6000 it becomes increasingly less efficient (lower activity per unit weight) at depressing the freezing temperatures (Kao et al. 1986). Furthermore, the smelt and herring AFPs have activities in the range observed for other AFPs (Kao et al. 1986). Yet, on a milligram or a molar basis, their activities appear lower than that of sea raven AFP. This suggests that smelt and herring AFPs are distinct in some way from the sea raven AFP.

The growth of ice crystals in solutions of herring and smelt AFPs was unique among the AFPs studied to date. The ice crystals were bipyramidal in the presence of all concentrations of smelt AFP and high concentrations of herring AFP. However, in solutions containing lower concentrations (1-10 mg/ml) of herring AFP, the crystals were hexagonal or irregularly shaped. In solutions of smelt and herring AFPs, ice crystal growth also occured in small increments at temperatures above the apparent freezing point which was taken as the point where rapid growth occurred. A similar incremental habit of ice growth was noted by Raymond (1989) in the blood plasma of Pacific herring (Clupea harengus pallasi). Since physiological concentrations of antifreezes normally halt the growth of ice crystals at temperatures above the freezing point (Raymond et al., 1989), smelt and herring AFPs appear to be preventing ice growth differently from other AFPs.

The presence of AFP in Atlantic herring appears related to their enhanced ability to survive in icy seawater. Herring from Newfoundland residing in subzero seawater appear to be quite freeze tolerant judging by their substantial plasma hysteresis. In contrast,

herring from the relatively warm (7°C) waters of Brown's Bank, N.S. showed no evidence of AFP. Gulf of St. Lawrence herring are believed to avoid cold waters by migrating offshore in winter (M. Chadwick, pers. comm.). However, Hodder et al. (1972) hypothesized that juvenile herring may overwinter in coastal areas within the Gulf rather than migrating with the adults. Consistent with this hypothesis are recent findings that juvenile herring overwintering in coastal areas have plasma hysteresis levels nearly three times higher than those of adults in offshore areas (Chadwick et al. 1990).

Considerable interpopulation and intrapopulation differences in AFP levels, in AFP gene copy numbers, and in the regulation of AFP production have been observed in winter flounder, ocean pout and cod along the Northwest Atlantic coast (Davies et al. 1988, Kao and Fletcher 1988, Hew et al. 1988). It will be interesting to determine whether similar differences exist among groups of herring.

CHAPTER 3:

STRUCTURE AND FUNCTION OF SMELT AND HERRING AFPs: SIMILARITY TO THE SEA RAVEN AFP AND TO THE CALCIUM-DEPENDENT LECTINS

3.1 Introduction

The AFPs of smelt and herring were shown to resemble the type II AFP of sea raven. Their similar amino acid compositions and sensitivity to reducing agents suggest that they are all globular proteins whose hysteresis activity relies on an intact folded structure stabilized by disulfide bonds. The close molecular weights of herring and sea raven AFPs and the immunological cross reactivity between smelt and sea raven AFPs and smelt and herring AFPs further illustrate their similarity and suggest that they might be homologous.

However, through convergent evolution, separate protein families may give rise to very similar proteins. Interesting examples can be found among the fish AFPs. The type I AFPs of flounder and sculpin appear to be the products of different genes that do not cross-hybridize (Scott et al. 1986). Furthermore, the AFGP and the type I AFP, which both differ from most proteins in their unusually high Ala contents, appear to be unrelated.

If the different type II AFPs have evolved from distinct protein families, their study will widen the scope of distinct AFPs available for the investigation of AFP functional

mechanisms. Alternatively, if these proteins are related, sequence comparison among them will be useful in probing the structure-function relationship of type II AFPs. To determine whether or not two proteins are related, it is necessary to compare their amino acid sequences (primary structures) (Doolittle 1990) or their predicted or experimentally determined tertiary structures (Bowie et al. 1991). The amino acid sequence of a protein can be obtained by direct sequencing or it can be inferred from the protein's corresponding cloned cDNA.

The amino acid sequence of the sea raven AFP has been deduced previously from its cDNA and genomic sequences (Ng et al. 1986, Hayes et al. 1989). Therefore, obtaining the sequences of the other type II AFPs will allow comparisons to be made among all three proteins. The goal of the present study was to begin this comparison by determining the amino acid sequences of the smelt and herring AFPs from their cDNAs and comparing them with the known sequence of the sea raven AFP (Ng et al. 1986, Hayes et al. 1989) and those of other proteins.

3.2 Materials and methods

3.2.1 Partial protein sequencing and preparation of an oligonucleotide probe

Pure smelt and herring AFPs were obtained as described in Chapter 2. Smelt HPLC fraction S2 and herring HPLC fraction H1 were reduced and alkylated following the method of Henschen (1986) in order to prepare them for direct sequencing. In each case, approximately 500 µg of protein was denatured in 6 M guanidine HCl containing 0.1 M

Tris-HCl pH 8.5, and flushed with N_2. Disulfide bonds were cleaved using ß-mercaptoethanol and resulting free sulphydryl groups were alkylated by iodoacetic acid. The protein was separated from these reagents by gel filtration on a PD-10 column (Pharmacia) in 0.1 M NH_4HCO_3 and freeze dried. Initial sequencing revealed that the N-terminus of each protein was blocked.

In order to obtain peptides that could be sequenced, smelt and herring AFPs were digested using trypsin. Each protein was suspended in 500 µl 0.1 M NH_4HCO_3. A gel slurry (300 µl) of immobilized TPCK-treated trypsin (Pierce) was washed in the same buffer and added to the protein solution. These mixtures were incubated at 37°C for 3 to 4 hours with shaking. The trypsin was removed by centrifugation and the supernatant containing peptides was applied to a Waters µ Bondapack C_{18} column (7.8 mm x 30 cm). Peptides were eluted using a 0.1% trifluoroacetic acid-acetonitrile gradient with a flow rate of 0.5 ml/min. The smelt and herring AFP tryptic peptides were sequenced using a Porton Gas-Phase Microsequencer. Sequencing was done by the Biotechnology Service Centre, Hospital for Sick Children, Toronto, Canada.

The amino acid sequence from one smelt peptide, which was identical to a peptide from herring, was used to predict the sequence of a corresponding degenerate oligonucleotide. Sense and antisense oligonucleotides corresponding to this sequence were synthesized by the Nucleic Acids Analysis Facility, Memorial Univeristy of Newfoundland. The antisense oligonucleotide was used as a probe for Northern blotting and library screening. The probe was ^{32}P labeled on its 5' end by γ-^{32}P-ATP, >5000 Ci/mmol (Amersham) using T4 polynucleotide kinase (Bio/Can Scientific). The labelling

reaction was carried out as described by Davis et al. (1986). The probe was isolated from reagents and excess label by chromatography on a NAP column (Pharmacia) following the method of Davis et al. (1986).

3.2.2 RNA isolation and Northern blotting

3.2.2.1 Isolation and evaluation of smelt liver mRNA

Smelt were collected through the ice in the Northumberland Strait near Grande-Digue, N.B., Canada in February. Smelt livers were frozen in liquid nitrogen and stored at -70°C until use. Total RNA was isolated from smelt livers using the protocol of Chomczynski and Sacchi (1987) that relies on extraction in acid guanidinium isothiocyanate phenol-chloroform. This protocol was followed as described except volumes were scaled up 10 fold in order to accommodate 1 g of tissue and centrifugation times were increased to 30 minutes or more because a clinical centrifuge was used. Approximately 1g of frozen liver was crushed and homogenized in the extraction buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.75% v/v ß-mercaptoethanol), extracted twice with phenol-chloroform and precipitated twice with ethanol. The resulting pellet dissolved very slowly in water. It was suspended in water for storage at -70°C.

Poly(A)$^+$ RNA was prepared from total RNA following the method of Aviv and Leder (1972) as described by Davis et al. (1986). Oligo (dT) cellulose was purchased from Pharmacia and others and chromatography was carried out in 10 ml autoclavable disposable plastic columns (Bio-Rad). Oligo (dT) cellulose was washed in a NaOH

solution, rinsed several times in a modified high salt buffer, and poured into the column. RNA in water was denatured by heating, combined with an equal volume of high salt buffer (40 mM Tris-HCl pH 7.4, 0.1 M NaCl, 1 mM EDTA, 0.1% SDS), and poured into a plastic column. The column was washed in modified high salt buffer to elute poly(A)$^-$ RNA. The poly(A)$^+$ RNA was eluted in 1-1.5 ml low salt buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.05% SDS), pooled, and aliquoted. Aliquots were precipitated in 2.5 volumes of ethanol and 0.1 volume of 3 M sodium acetate, pH 6, and then they were resuspended in water containing an RNAase inhibitor, RNAade (Bio/Can Scientific).

Poly(A)$^+$ RNA was also isolated directly from homogenized livers by direct oligo (dT) hybridization using a kit (Invitrogen). One g of tissue was homogenized in a buffer containing 0.2 M NaCl, a mixture of proteases, and SDS and incubated at 45°C for 2 hours. NaCl was added to the solution to bring the concentration to 0.5 M and oligo (dT) cellulose was added. The solution was placed on a rotary shaker for 1 hr and then oligo (dT) cellulose was pelleted by centrifugation. The pellet was resuspended and pelleted by centrifugation repeatedly in several changes of high-salt buffer. It was then poured into a disposable column as above. Poly(A)$^+$ RNA was eluted in low salt buffer, pooled, precipitated, and resuspended as above. All RNA was stored at -70°C. Poly(A)$^+$ was used no later than three weeks after isolation.

The integrity of the RNA was verified before cloning by formaldehyde gel electrophoresis following the method of Davis et al. (1986) except that ethidium bromide was added to the sample buffer prior to denaturing the RNA instead of staining the gel following electrophoresis and RNA was denatured by keeping it at 65°C for 10 minutes.

The ability of the oligonucleotide probe to recognize a discrete transcript was also evaluated. Poly(A)$^+$ RNA obtained using a kit (Invitrogen) was electrophoresed in a 1.2% agarose gel containing formaldehyde and transferred to a positively-charged nylon membrane (Gelman Sciences) by capillary blotting in 10X SSC (1.5 M NaCl, 0.15 M Na_3citrate•$2H_2O$, pH 7.0) using quick-blot papers (Sigma) following the procedure of Davis et al. (1986). RNA was then UV-crosslinked to the membrane on a shortwave transilluminator for approximately 20 seconds. Hybridization and washing were carried out according to the method of DiLella and Woo (1987). The membrane underwent prehybridization for 12 hrs in the absence of labelled probe in a solution of 0.9 M NaCl, 6 mM EDTA, 0.5% SDS, 0.89 M Tris-HCl pH 7.5 containing 0.2 mg/ml calf thymus DNA as a blocking agent. In most hybridization protocols, herring or salmon DNA are used as blocking agents. Since fish nucleic acids were being studied in this case, mammalian DNA was used instead. Following prehybridization, the membrane was hybridized for 24 hours in the same solution in the presence of the labeled oligonucleotide. Four membrane washes were performed in TMAC buffer (3 M tetramethylammonium chloride, 2 mM EDTA, 50 mM Tris-HCl pH 8.0, 0.2% SDS) with shaking. The membrane was washed twice at 4°C for 30 minutes, once at room temperature for 30 minutes, and once at 52°C for 1 hour. It was then rinsed briefly at room temperature in two changes of the same buffer with no SDS. The membrane was exposed to Kodak XAR-5 film for approximately 14 hours at -70°C with an intensifying screen.

3.2.2.2 Isolation and evaluation of herring liver mRNA

Herring were collected between February and April in the Bay of Chaleur, N.B., Canada. Blood and liver samples were taken. Livers were frozen in liquid nitrogen and stored at -70°C for 8 to 10 months before use. Livers from individuals with the highest blood AFP levels were used to isolate poly(A)$^+$ RNA. Poly(A)$^+$ RNA was isolated directly from a herring liver tissue homogenate using a kit (Invitrogen) as described above. The integrity of this RNA was also assessed by formaldehyde gel electrophoresis following the method of Davis et al. (1986). Northern blotting, oligonucleotide hybridization, and X Ray film exposure were also performed as for smelt mRNA except herring mRNA was UV-crosslinked to the nylon membrane using a Stratalinker (Stratagene).

3.2.3 Preparation, evaluation, and screening of a smelt liver cDNA library

3.2.3.1 Preparation of a library from smelt

cDNA was prepared from smelt liver poly(A)$^+$ RNA according to the method of Gubler and Hoffman (1983) using a cDNA synthesis system (Invitrogen). The first complimentary DNA strand was obtained through a series of steps that involved denaturing the RNA using methylmercury hydroxide, annealing an oligo (dT) primer to the poly(A) tails of the RNA, and then synthesizing the DNA strand using reverse transcriptase. To synthesize the second DNA strand, the RNA strand of the resulting duplex was nicked using RNAase H, RNA-primed DNA synthesis was carried out using E. coli DNA polymerase I, and the resulting fragments were attached using E. coli DNA ligase. The

cDNAs were blunt-ended by extending the duplex using the E. coli DNA polymerase 1 Klenow fragment (Bio/Can Scientific). Adaptors which were blunt at one end and compatible with BstX1 at the other end were ligated onto the blunt ends of the cDNA using T4 DNA ligase. cDNA was then size-selected and separated from excess adaptors by electrophoresis in a 1% agarose gel. Total cDNA was cut out and electroeluted from the gel using a Geneluter (Invitrogen). This cDNA was electrophoresed again and fragments ranging in size from 400 to 2000 bps were cut out and electroeluted as before. Electroelution was carried out as recommended by the supplier except that Spectrapore 3000 dialysis membrane was used instead of the wider pore-size membrane supplied with the Geneluters in order to avoid losing cDNAs shorter than 1000 bp.

A smelt cDNA library was constructed using a series of commercially prepared reagents, cells, and protocols (The librarian II, Invitrogen). cDNA was ligated into the BstX1-cut vector, pcDNAII (Invitrogen) using T4 DNA ligase. Competent cells (E. coli strain Inv1aF') were transformed with the resulting plasmids using the heat shock method. Fifty µl ß-mercaptoethanol, 1 ml of competent cells and the full-scale ligation products were combined, mixed gently, and incubated on ice for 30 minutes. The mixture was transferred to a 42°C water bath and incubated for exactly 1 minute and 30 seconds and then returned to ice for 2 minutes. Nine ml of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 20 mM glucose, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$) were added to the transformed bacteria. This mixture was incubated at 37°C on a gyratory shaker to allow the bacterial cells to express the plasmid-born ampicillin resistance gene. Aliquots were plated on LB-agar plates (1% bacto tryptone, 0.5% bacto yeast

extract, 1% NaCl, pH 7.4, with 0.5% agar) containing 100 µg/µl ampicillin (Boehringer Mannheim) and colonies were allowed to grow overnight at room temperature. The library consisted of approximately 2000 clones.

3.2.3.2 Evaluation and screening of the smelt library

Twelve colonies were picked at random and cultures were grown overnight at 37°C in 2 ml of LB broth (as above but without agar). Plasmids were isolated from each culture following the procedure of Zhou et al. (1990) and then digested following the method described by Davis et al. (1986). HindIII and XbaI (Bio/Can Scientific) were used because they cut at single sites in the plasmid at opposite sides of the cDNA insert and allow release of the cDNA. Digestions were carried out following the method described by Davis et al. (1986) except they were scaled up to a total volume of 20 µl and 1 µl DNAase-free RNAase (Boehringer Mannheim) was included in each reaction. Products were electrophoresed in a 1% agarose gel, stained with ethidium bromide (0.4 mg/l) for 15 minutes, and visualized by UV transillumination. Since all twelve clones contained cDNA, the library was considered good enough to screen.

Duplicate colony lifts were made onto positively-charged and uncharged nylon membranes (Amersham) and baked in a microwave oven for 3 minutes in 2X SSC with 5% SDS to lyse bacteria following the procedure of Buluwela et al. (1989) as described in the Invitrogen Librarian II manual. DNA was then UV-cross linked to the membranes using a Stratalinker (Stratagene). Hybridization and autoradiography were performed according to the procedures used for the Northern blot. Cultures were grown from each

putative positive clone. Plasmids were isolated from them, digested as described above, and electrophoresed on a 1% agarose gel. Southern blotting was performed following the method of Ausubel et al. (1989) for transfer onto nylon membranes. Membranes were hybridized as described earlier using the labelled oligonucleotide in order to determine which clones were true positives.

3.2.4 Preparation, evaluation, and screening of a herring liver cDNA library

3.2.4.1 Preparation of a library from herring

cDNA was prepared from herring liver poly(A)$^+$ RNA following the protocol supplied by Invitrogen except reagents remaining from 2 cDNA synthesis systems (BRL and Invitrogen) were used and the reaction included only 80% of the recommended concentration of reverse transcriptase. BstX1-cor·patible adaptors (Invitrogen) were ligated onto the cDNAs using T4 DNA ligase and cDNAs were size selected and separated from excess adaptors by electrophoresis in a 1.2% agarose gel prepared using low melting point agarose (Bio-Rad). cDNA ranging in size from 450 to 2000 bp was cut out of the gel and electroeluted using a Geneluter (Invitrogen) as described above.

The cDNA was ligated into the vector pcDNAII and the resulting chimeric plasmids were used to transform competent bacterial cells (E. coli strain Inv1aF'). Approximately 9000 clones were obtained.

3.2.4.2 Evaluation and screening of the herring library

Ten bacterial colonies were picked at random and cultures were grown overnight at 37°C in LB Broth. Plasmid isolation, digestion, and electrophoresis were performed. A single colony lift was made from each plate onto positively-charged nylon (Amersham). Membranes were microwave-treated, hybridized and autoradiographed as described above.

Since only single lifts were done in library screening and cDNA inserts were not identified by Southern blotting, the identity of the positive clones had to be verified prior to full-length sequencing. Twenty positive clones were chosen for analysis. They were grown in culture, their plasmids were isolated and digested, and digestion products were separated by electrophoresis. Preliminary sequencing reactions were carried out on plasmid clones with the 6 longest cDNAs. Clone 20 was shown to contain the nucleotide sequence corresponding to the oligonucleotide and to the corresponding herring peptide. Sequencing at the 5' end revealed the beginning of an open reading frame preceded by a putative untranslated region. Since this clone also had a complete 3' region including a poly(A) sequence, it appeared full length. This clone was chosen for further sequencing.

3.2.5 Sequencing of the AFP cDNA clones

For sequencing of the smelt AFP cDNA, plasmids were prepared using a miniprep protocol from the Protocols and Applications Guide (Promega). The sequences of both strands of a positive clone were obtained by dideoxynucleotide sequencing on double stranded DNA using modified T7 polymerase (Bio/Can Scientific) according to protocols provided by the supplier which were based on the method of Tabor and Richardson

(1987). Sequencing was carried out on the intact clone, on a XbaI-PstI cut and religated deletion clone, and on XbaI-AvaII and AvaII-PstI fragments subcloned into pGEM-7Zf(+) (Promega).

For herring AFP cDNA sequencing, plasmids were isolated using the Magic Minipreps DNA isolation system (Promega). The sequences of both strands of a positive clone were obtained by dideoxynucleotide sequencing of double stranded DNA using modified T7 polymerase (Pharmacia) using the accompanying protocols which also followed the method of Tabor and Richardson (1987). Sequences were determined from the intact clone, and also from HindIII-AccI and AccI-XbaI deletion clones.

For all sequencing reactions, the label used was ^{35}S. Primers used for sequencing were oligonucleotides corresponding to the plasmid SP6 and T7 promoter sequences (Promega) and the sense and antisense oligonucleotides corresponding to smelt AFP peptide S2-P2. Sequencing reaction products were resolved on 6% or 8% polyacrylamide gels prepared according to the procedure outlined in the Promega Protocols and Applications Guide (Promega) except catalyst concentrations were reduced to avoid rapid polymerization. Electrophoresis was carried out using the Seqi/Gen sequencing cell (Bio/Rad) following the methods of Davis et al. (1986). Urea was removed from the gels by soaking them in 10% methanol and 10% acetic acid for 15 minutes. The gels were dried and exposed to ß-Max film (Amersham) overnight or to Xomat RP film (Kodak) for 2-3 days.

3.2.6 Sequence analysis

Using PC/Gene software, the reading frames of the cDNAs and the encoded amino acid sequences were obtained and sequence characteristics were predicted. Using the FASTA programme (Pearson and Lipman 1988), the Swiss-Prot database (release 16) was searched for proteins similar to the smelt and sea raven AFP precursors and release 20 was searched for proteins similar to the the herring AFP precursor. Sequences with the highest similarity scores on FASTA were chosen for further analysis. Since most high scoring sequences were calcium-dependent (C-type) lectins, the recently published snake venom lectin (Hirabayashi et al. 1991) was also chosen. These sequences were compared to herring, smelt, and sea raven AFP sequences using the method of Needleman and Wunsch (1970) with a scoring matrix from the Dayhoff mutation data matrix (Dayhoff et al. 1979) using the default parameters on PC/Gene. A jumble test (Monte Carlo simulation) was used to determine whether each sequence was significantly homologous to the AFPs of herring, smelt, or sea raven. A similarity score greater than 5 standard deviations above the mean in a 100-run jumble test was considered as evidence of significant homology.

3.2.7 Antifreeze activity measurements

Thermal hysteresis was measured as described (Chapter 2) in solutions of antifreeze proteins in the presence and absence of Ca^{2+} ions. Antifreeze proteins were dissolved in Tris-HCl, pH 8.0. Aliquots of these solutions were brought to 2.5 mM ethylene glycol-bis(ß-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA) or to 10 mM $CaCl_2$ with a final Tris concentration of 15 mM in both groups.

3.2.8 Detection of Ca^{2+}-binding using ruthenium red

Binding of Ca^{2+} to AFPs was evaluated qualitatively using ruthenium red. This dye appears to binds specifically to Ca^{2+}-binding proteins (Charuk et al. 1990). The procedure followed was as described by Charuk et al. (1990). Various proteins (10-12 µg each) were run on SDS-PAGE under non-reducing conditions. To achieve this, SDS-PAGE was run as described in Chapter 1 except ß-mercaptoethanol was omitted from the sample buffer. The proteins were transferred to a reinforced nitrocellulose membrane with a 0.22 µ pore size (Schleicher and Schueli). Transfer was as described in Chapter 2 except the buffer contained 20% methanol and transfer was completed overnight at 15 V in a Mini-Protean II transfer unit (Bio-Rad). After transfer, the membrane was placed in binding solution (60 mM KCl, 3 mM $MgCl_2$, 10 mM Tris-HCl pH 7.5) containing 25 g/l ruthenium red for 1 hour with slow shaking and it was dried and photographed. The membrane was then placed in binding solution containing 50 mM $CaCl_2$ for 1 hour with slow shaking to displace ruthenium red, dried, and photographed again.

3.3 Results

3.3.1 Tryptic peptide sequences and corresponding oligonucleotides

Tryptic peptides obtained from the smelt AFP isoform in HPLC peak S2 (Chapter 2) were sequenced. Their sequences are shown in Fig. 15. Sequences were also obtained for peptides of the herring AFP isoform in HPLC peak H1 and they are shown in Fig. 16. Some peptide sequences from smelt and herring were similar to one another and also to the

Fig. 15. Tryptic peptides from smelt AFP S2. (A) Amino acid sequences of peptides obtained by tryptic digest of smelt AFP S2. (B) First 6 amino acids of peptide S2-P2 with nucleotide sequences of corresponding degenerate oligonucleotide probe.

(A) Tryptic peptides from smelt AFP S2:

S2-P2 Cys-Trp-Asn-Asp-Thr-Pro-Cys-Thr-His-Leu

S2-P3 Glu-Leu-His-Trp-Ala-His-Ala-Gln-Ile-Ser

S2-P5 Pro-Ala-Thr-Val-Ile-Pro-Glu-Val-Thr-Pro-Pro-Ser

S2-P6 Asp-Gly-Ala-Asn-Leu-Ala-Ser-Ile-His-Ser

S2-P7 Cys-Phe-Leu-Phe-Asn-Pro-Leu-Gln-Leu-His

(B) S2-P2 and corresponding nucleotide sequences:

```
S2-P2                Cys-Trp-Asn-Asp-Thr-Pro-Cys-...

oligo-          5'-TGT-TGG-AAT-GAT-ACT-CCT-TG-3'
nucleotide        C       C   C   C   C
                                  A   A
                                  G   G
```

Fig. 16. Tryptic peptides from herring AFP H1. (A) amino acid sequences of peptides obtained by tryptic digest of herring AFP H1. (B) First 6 amino acid residues of peptide H1-P2 with nucleotide sequences of corresponding degenerate oligonucleotide probe.

(A) Tryptic peptides from herring AFP H1:

H1-P1 Trp-Ile-Gly-Gly-Thr-Asp-Cys-Gln-Val-Ser

H1-P2
major Cys-Trp-Asn-Asp-Thr-Pro-Cys-Thr-His-Leu
minor Gln-Leu-His-Trp-Ala-Asp-Ala-Gln-Glu-Ser

H1-P3 Glu-Gly-Ala-Asn-Leu-Ala-Leu-Ala-Ser-Ile-His-Ser-Leu-Glu-Glu-Ser

H1-P4 Glu-Leu-Thr-Ser-Ala-Asp-Leu-Ile-Pro-Ser

H1-P5 Trp-Phe-Trp-Met-Asp-Ser-Thr

H1-P7 Trp-Phe-Trp-Met-Asp-Ser

(B) H1-P2 (major) and corresponding nucleotide sequences:

```
H1-P2                Cys-Trp-Asn-Asp-Thr-Pro-Cys-...

oligo-          5'-TGT-TGG-AAT-GAT-ACT-CCT-TG-3'
nucleotide        C       C   C   C   C
                                  A   A
                                  G   G
```

sea raven AFP sequence (Ng et al. 1986) (results not shown). The codons corresponding to the amino acid sequences of the peptides were deduced. The codons coding for the first 7 amino acids of peptide S2-P2 were the least degenerate (Figs. 15, 16). This degenerate sequence also corresponds to peptide H1-P2. Therefore, this DNA sequence was used to synthesize mixed oligonucleotides. Both sense and antisense oligonucleotides were prepared.

3.3.2 Cloning and sequencing of a smelt AFP cDNA

3.3.2.1 Analysis of smelt liver mRNA

Formaldehyde gel electrophoresis of smelt liver poly $(A)^+$ RNA showed that the isolation methods yielded intact RNA (Fig. 17). Northern blotting of poly$(A)^+$ RNA revealed a single band with an approximate length of 0.8 kb (Fig. 18). The presence of a single well-defined band showed that the AFP was not degraded in this preparation and suggested that the oligonucleotide probe is specific for AFP. In order to rule out the possibility that the probe recognizes other transcripts that are the same size as that of the AFP, total RNA was isolated from winter flounder liver and was included in the blot. Winter flounder has no type II AFP and no hybridization to winter flounder liver RNA was detected on the blot. Thus, the oligonucleotide did appear to recognize the smelt AFP exclusively and not more widely distributed related proteins.

Fig. 17. Formaldehyde gel electrophoresis of RNAs from smelt and flounder livers. Lanes: (1) RNA standards, 9.5 and 7.1 kbs; (2) 20 μg flounder liver total RNA; (3-5) 1,5, and 10 μg smelt liver poly $(A)^+$ RNA, respectively. The positions of the 28S, 18S, and 5S RNAs are indicated.

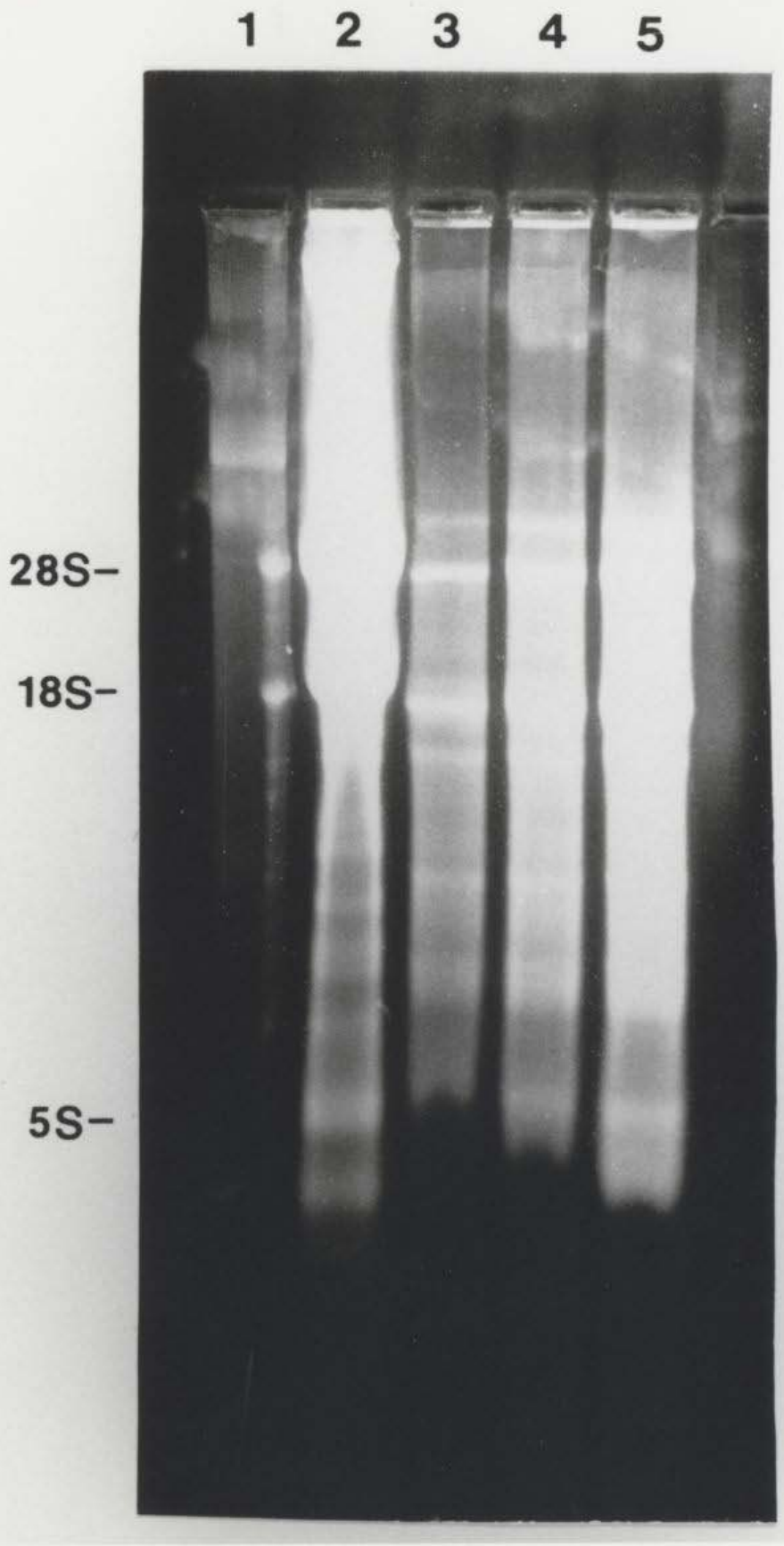
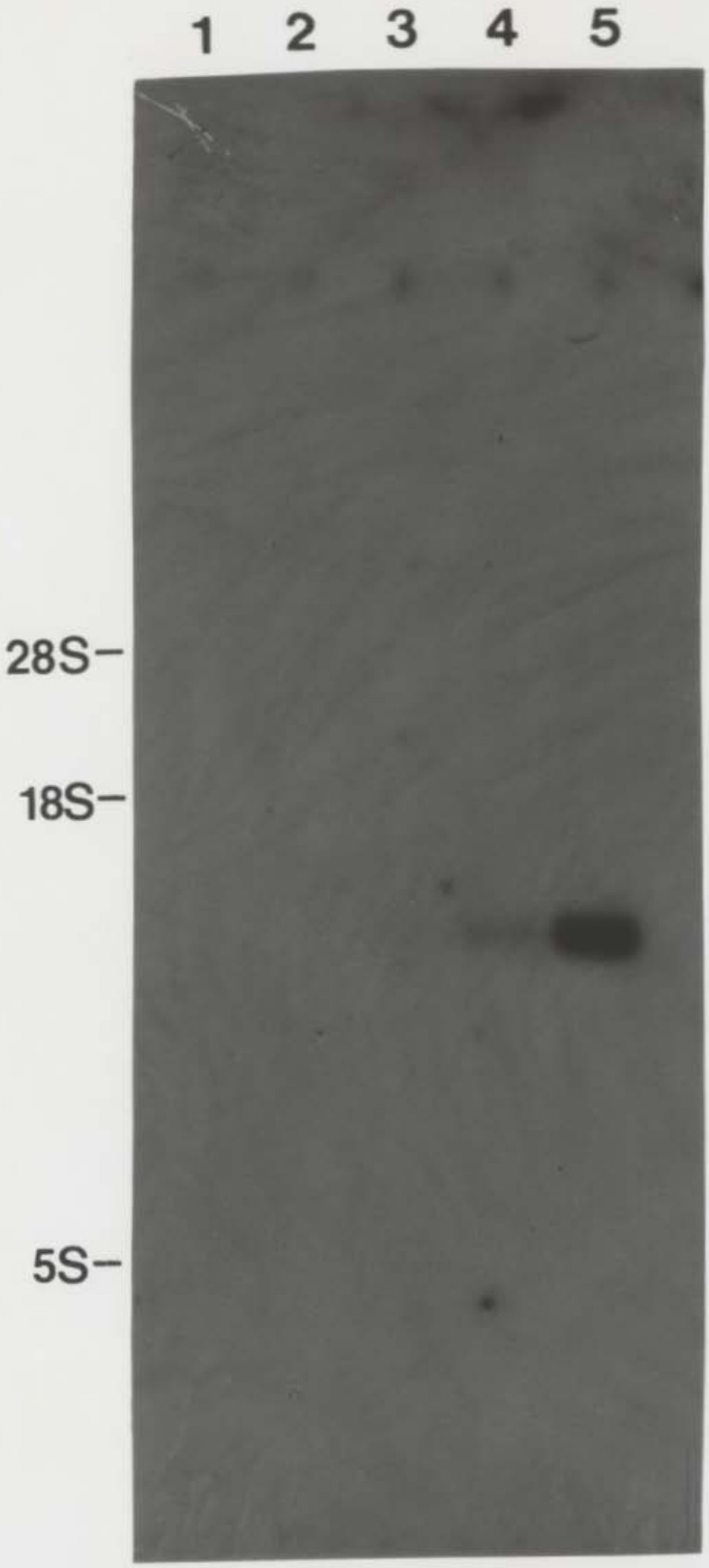

1
2
3
4
5
28S–
18S–
5S–

Fig. 18. Northern Blot showing specificity of the oligonucleotide probe for a smelt liver transcript. Lanes: (1) RNA standards, 9.5 and 7.1 kbs; (2) 20 μg winter flounder total RNA; (3-5) 1,5,and 10 μg respectively of smelt liver poly(A)$^+$ RNA. The positions of the 28S, 18S, and 5S RNAs are indicated.


1
2
3
4
5
28S
18S
5S

3.3.2.2 Characterization of a smelt AFP cDNA

Evaluation of the library showed that all 12 of the clones examined contained cDNA. Therefore, library screening was undertaken. Eighteen potential positive clones were detected in a duplicate screening of the smelt liver cDNA library. All contained cDNA inserts (Fig. 19). Seventeen positive clones were confirmed by Southern blot analysis (Fig. 20). Their sizes ranged from approximately 600 to 800 bps. Clone 17 (lane 18) was sequenced. It was 611 bps long (Fig. 21). A 525 bp open reading frame was present in the clone. It also contained a 63 bp 5'-untranslated region and a 24 bp 3'-untranslated region. No polyadenylation site was present. However, the short length of the 3'-untranslated region suggests that the clone was truncated. Poly(A)$^+$ RNA was used to construct the library and cDNA synthesis was primed using oligo (dT). Therefore, in order to be represented in this library, this smelt AFP message would need to have been polyadenylated.

Sequence analysis showed a 525 bp open reading frame coding for a 175 residue AFP precursor (Fig. 21). The amino acid sequence corresponded exactly to the sequences of all tryptic peptides from smelt AFP. The predicted molecular mass of the primary translation product was 19,054. This approximates the 20,000 M_r of deglycosylated smelt AFP on SDS-PAGE (see Chapter 5). The predicted signal sequence cleavage site lies between residues Ala 16 and Ala 17 and a potential N-glycosylation site was located on Asn 34. Potential phosphorylation sites were also present.

Fig. 19. Digestion products from plasmids of putative positive smelt AFP cDNA clones resolved on an agarose gel. Lanes: (1-8) clones 1-8; (9) DNA size markers; (10-19) clones 9-18; (20) DNA size markers.

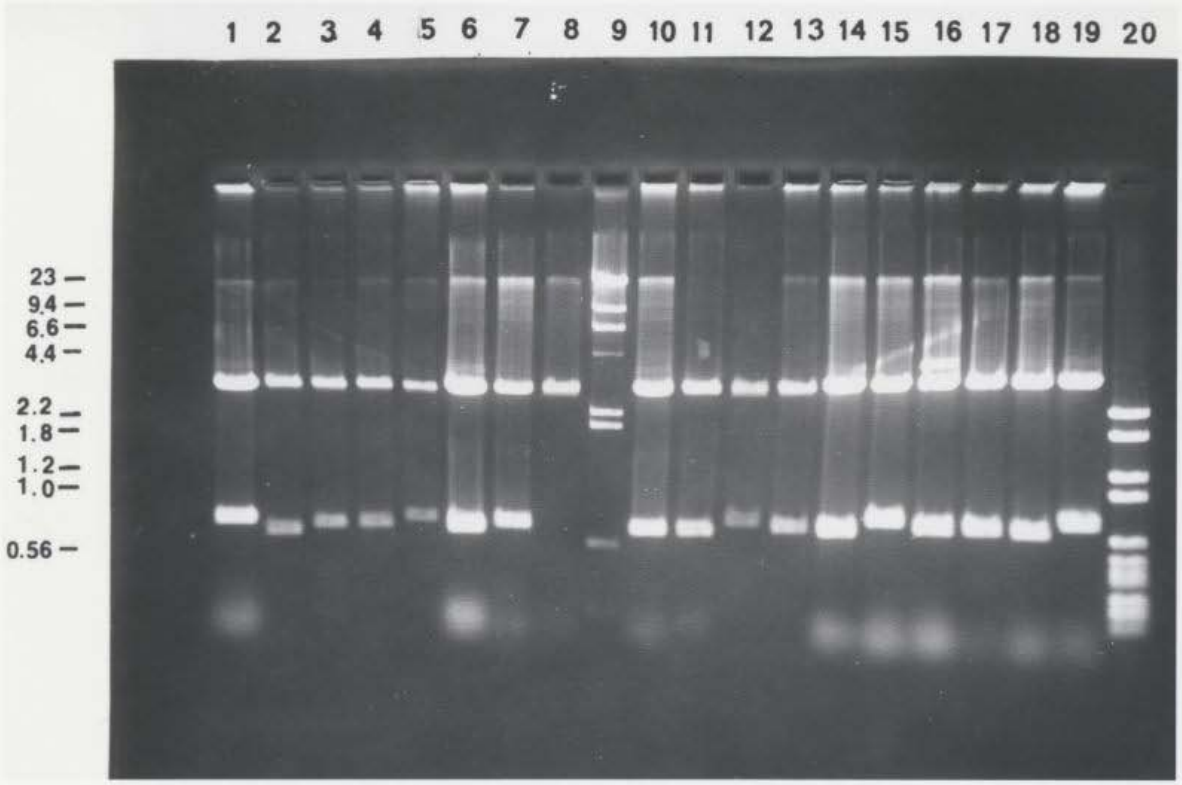

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
23
9.4
6.6
4.4
2.2
1.8
1.2
1.0
0.56

Fig. 20. Southern blot of DNA from gel in Fig. 19. The oligonucleotide used to screen the smelt cDNA library was also used for this hybridization. Lanes: (1-8) clones 1-8; (9) DNA size markers; (10-19) clones 9-18; (20) DNA size markers.

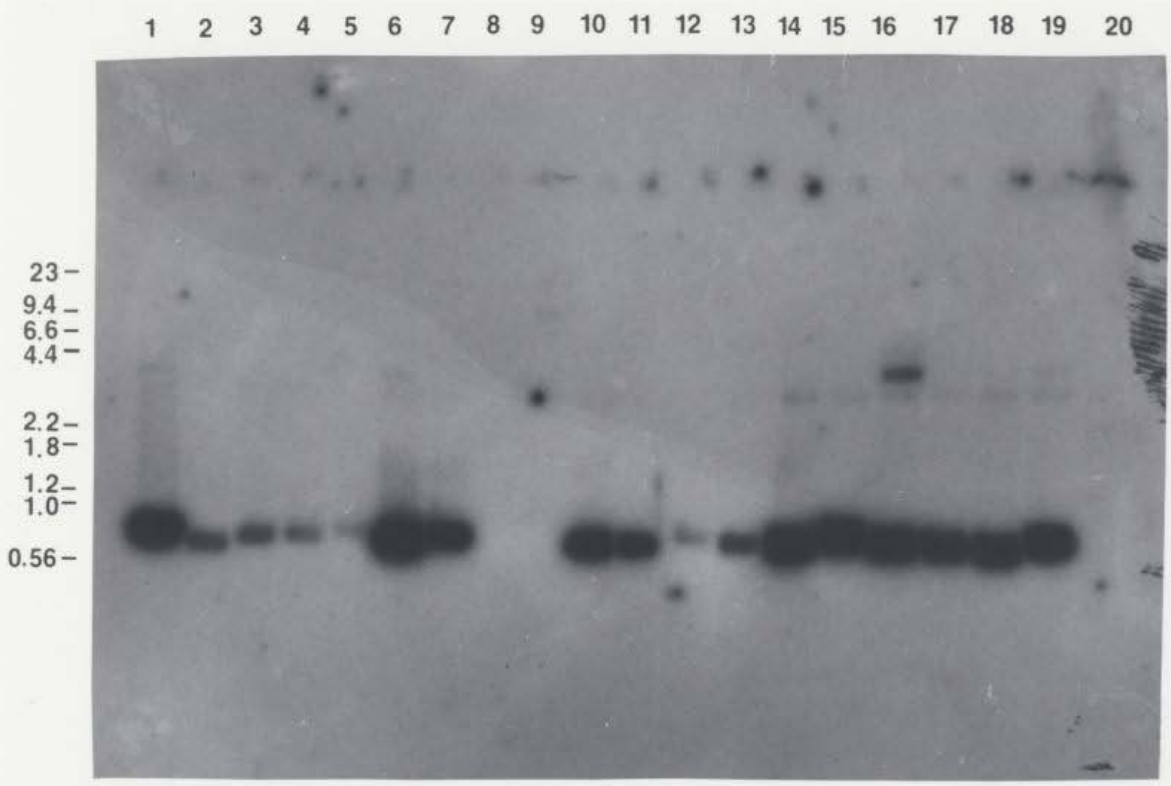

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
23
9.4
6.6
4.4
2.2
1.8
1.2
1.0
0.56

Fig. 21. The smelt cDNA nucleotide sequence and predicted primary translation product. The first nucleotide of the initiating codon in numbered as +1. Amino acid sequences also obtained by Edman degradation of tryptic peptides from smelt AFP are underlined. The predicted signal sequence cleavage site is indicated by an arrow. The putative N-linked glycosylation site is boxed.

```
                                                                AGA   -60

CAGAAGAGAATATATTTTTCCAACAGGTCATCAACGTCAAAGTGATCTCCACCACCAGG    -1

ATG CTG GCT GCT CTA CTT GTT TGT GCC ATG GTG GCT CTG ACC AGG   +45
Met Leu Ala Ala Leu Leu Val Cys Ala Met Val Ala Leu Thr Arg    15

GCT GCA AAT GGT GAC ACG GGG AAA GAG GCT GTG ATG ACA GGG TCC   +90
Ala Ala Asn Gly Asp Thr Gly Lys Glu Ala Val Met Thr Gly Ser    30
    ↑
AGT GGA AAG AAT TTG ACA GAA TGT CCC ACT GAT TGG AAG ATG TTC   +135
Ser Gly Lys Asn Leu Thr Glu Cys Pro Thr Asp Trp Lys Met Phe    45

AAT GGT CGC TGT TTC CTT TTT AAT CCA TTA CAA TTG CAT TGG GCT   +180
Asn Gly Arg Cys Phe Leu Phe Asn Pro Leu Gln Leu His Trp Ala    60

CAC GCT CAG ATA AGC TGC ATG AAG GAT GGG GCA AAC CTT GCA TCC   +225
His Ala Gln Ile Ser Cys Met Lys Asp Gly Ala Asn Leu Ala Ser    75

ATA CAC AGC CTT GAA GAG TAT GCG TTT GTT AAG GAG CTG ACA ACT   +270
Ile His Ser Leu Glu Glu Tyr Ala Phe Val Lys Glu Leu Thr Thr    90

GCA GGC TTA ATA CCA GCA TGG ATT GGA GGC TCA GAT TGC CAT GTG   +315
Ala Gly Leu Ile Pro Ala Trp Ile Gly Gly Ser Asp Cys His Val   105

TCA ACA TAT TGG TTT TGG ATG GAT AGT ACA AGT ATG GAT TTT ACT   +360
Ser Thr Tyr Trp Phe Trp Met Asp Ser Thr Ser Met Asp Phe Thr   120

GAC TGG TGC GCT GCA CAA CCT GAT TTT ACC TTA ACT GAG TGC TGC   +405
Asp Trp Cys Ala Ala Gln Pro Asp Phe Thr Leu Thr Glu Cys Cys   135

ATA CAG ATA AAT GTT GGA GTT GGA AAA TGC TGG AAT GAC ACA CCT   +450
Ile Gln Ile Asn Val Gly Val Gly Lys Cys Trp Asn Asp Thr Pro   150

TGT ACC CAT CTT CAT GCA TCA GTC TGC GCC AAG CCT GCC ACC GTG   +495
Cys Thr His Leu His Ala Ser Val Cys Ala Lys Pro Ala Thr Val   165

AAT CCC GAG GTG ACA CCA CCA TCA ATC ATG TGATTGTGTGTATGTTTGC   +544
Ile Pro Glu Val Thr Pro Pro Ser Ile Met

ATACT
```

3.3.3 Cloning and sequencing of a herring AFP cDNA

3.3.3.1 Analysis of herring liver mRNA

Formaldehyde gel electrophoresis of herring liver poly(A)$^+$ RNA showed that the isolation method yielded intact RNA (Fig. 22). Although a large size range is evident, a transcript approximately 650 bp long appeared to be the most abundant in herring liver. Northern blotting revealed that the oligonucleotide recognizes the very abundant transcript that was evident on the formaldehyde gel (Fig. 23).

3.3.3.2 Characterization of a herring AFP cDNA

Library evaluation showed that 8 of the 10 clones examined contained cDNA so library screening was undertaken. A large number of putative positive clones were identified in the herring liver cDNA library. Twenty were chosen for further analysis. Ninteen clones were shown to contain cDNA and one was incompletely digested (Fig. 24). One positive clone was sequenced on both strands. The cDNA insert was 654 bp long and it contained a 441 bp open reading frame (Fig. 25). The nucleotide sequence of the Atlantic herring AFP cDNA was extremely similar to that of the smelt AFP cDNA, and also similar to the sea raven AFP cDNA (not shown). A putative polyadenylation signal (AATAAA) began 17 bp upstream of the poly(A) extension. The herring AFP cDNA encoded a 147 residue AFP precursor (Fig. 25). The predicted amino acid sequence corresponded exactly to the sequences of all tryptic peptides from herring AFP. The calculated molecular mass of the primary translation product was 16,374. The predicted signal sequence cleavage site was between residues Ala 17 and Ala 18 and

Fig. 22. Formaldehyde gel electrophoresis of herring liver poly(A)$^+$ RNA. Lanes: (1) 5 μg RNA; (2) 10 μg RNA. The positions of 28S, 18S and 5S RNAs are indicated.

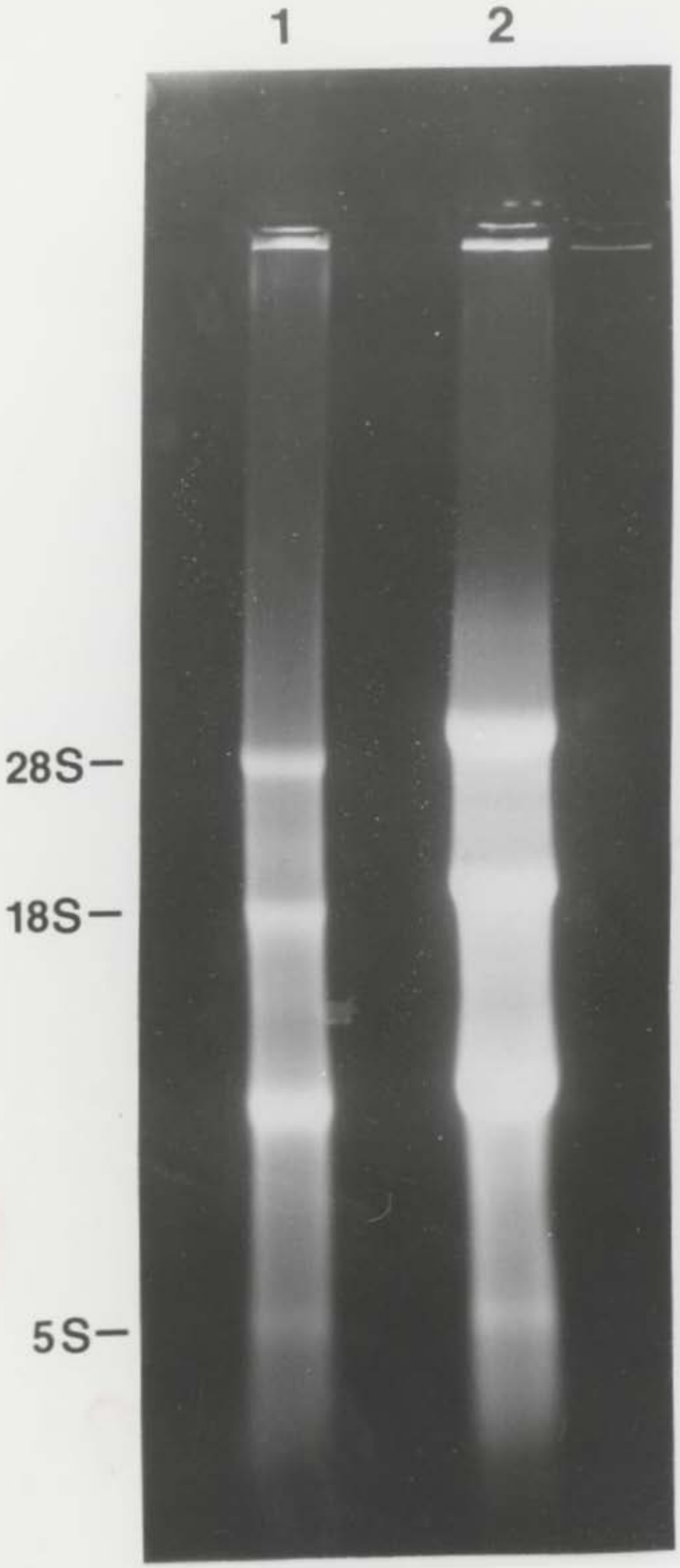

1
2
28S
18S
5S

Fig. 23. Northern blot showing specificity of the oligonucleotide probe for a herring liver transcript. Lanes: (1) 5 μg and (2) 10 μg of herring liver poly(A)$^+$ RNA. The positions of the 28S, 18S, and 5S RNAs are indicated.

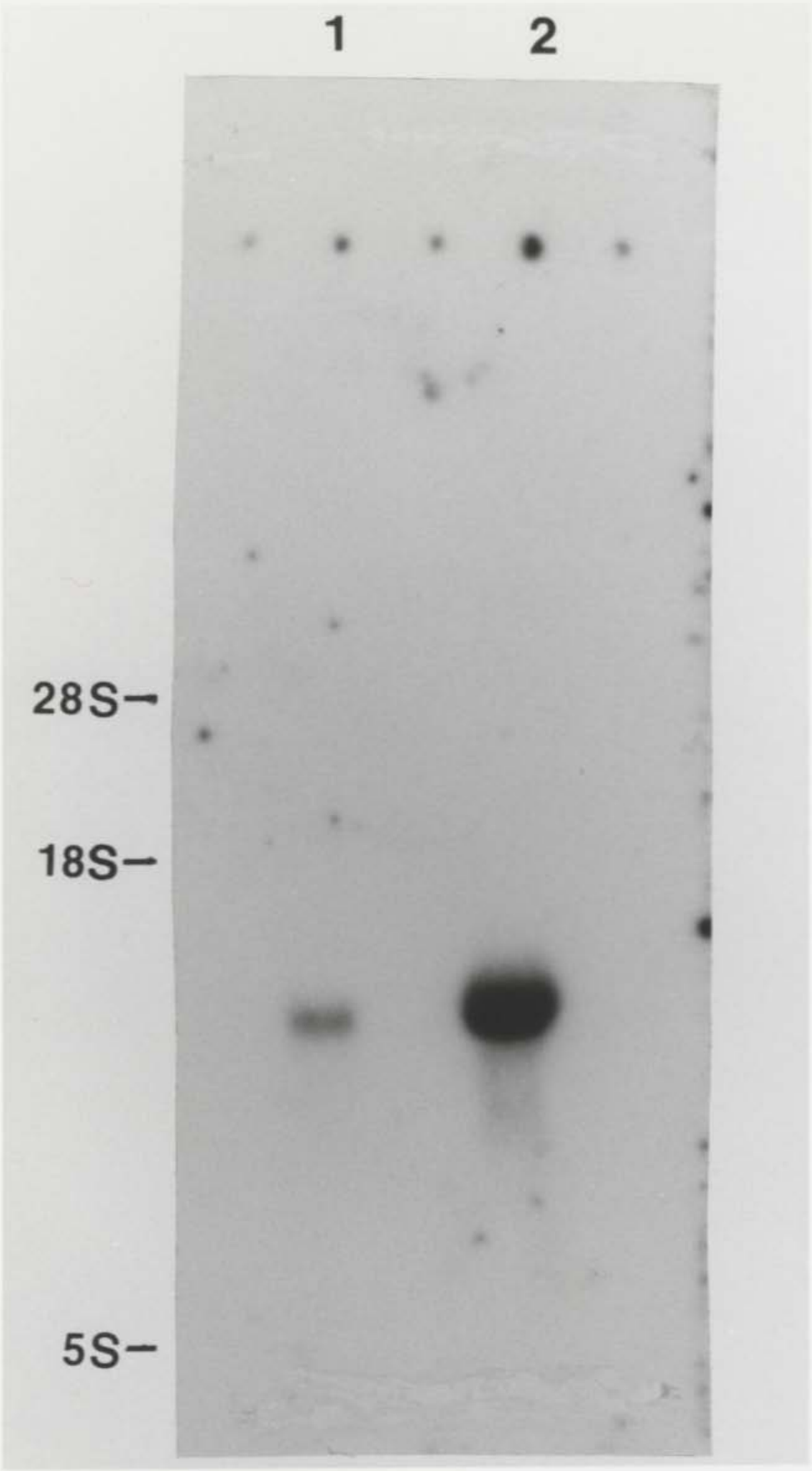

1
2
28S
18S
5S

Fig. 24. Digestion products from plasmids of putative positive herring AFP cDNA clones resolved on an agarose gel. Lanes: (1) DNA size markers; (2-21) plasmids from clones 1 to 20 respectively.

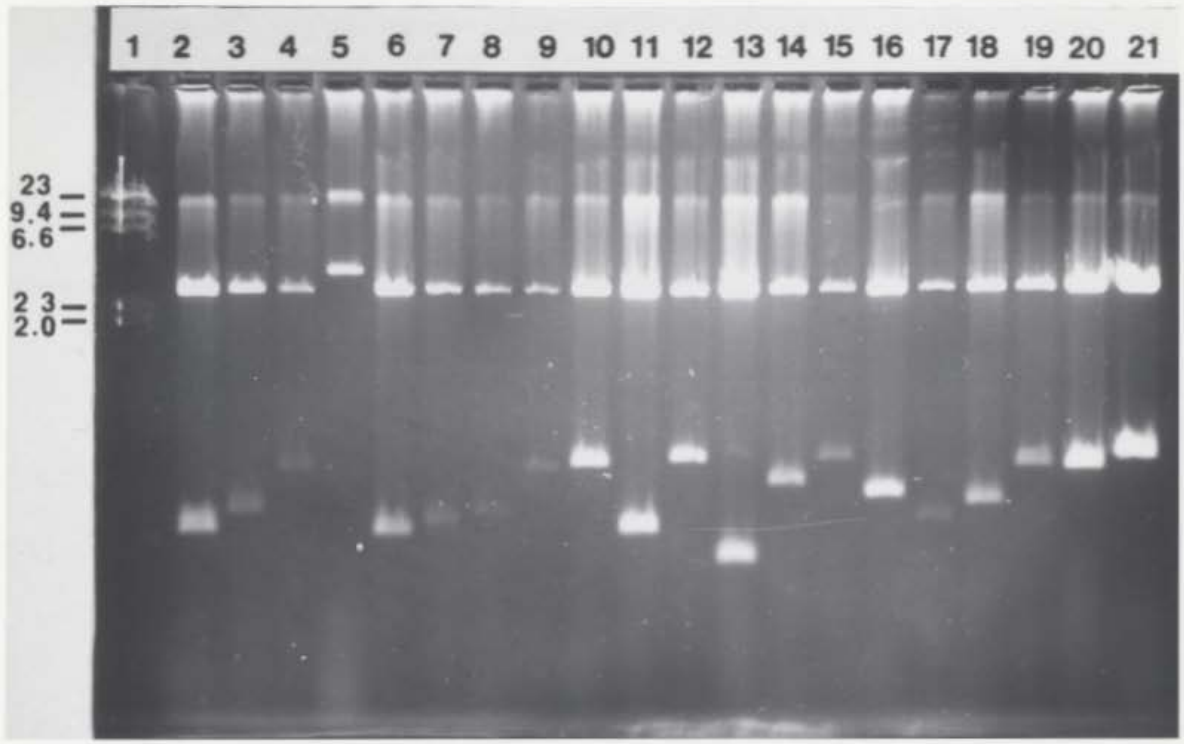

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
23
9.4
6.6
2.3
2.0

Fig. 25. The herring cDNA nucleotide sequence and predicted primary translation product. The first nucleotide of the initiating codon is numbered as +1. The polyadenylation signal is in bold letters. Amino acid sequences that were also obtained by Edman degradation of tryptic peptides from herring AFP are underlined and the predicted signal sequence cleavage site is indicated by an arrow.

```
                                CTCTAAAGGGAAGACAGAGGCAACAGGCTGAAATTGTGCAGA   -60

CAGAAGAGAAGATATTTCTCCAGCAGGTCATCAAGGTCAAAGTCATCTCCACTACCAGG                  -1

ATG CTG ACT GTG TCT CTA CTC GTT TGT GCC ATC GTG GCT CTG ACT                  +45
Met Leu Thr Val Ser Leu Leu Val Cys Ala Ile Val Ala Leu Thr                   15

AGG GCT GCT GAT GAA TGT CCC ACT GAT TGG AAG ATG TTC AAT GGT                  +90
Arg Ala Ala Asp Glu Cys Pro Thr Asp Trp Lys Met Phe Asn Gly                   30
    ↑
CGC TGT TTC CTT TTT AAT CCA TTA CAA TTG CAT TGG GCT GAC GCT                  +135
Arg Cys Phe Leu Phe Asn Pro Leu Gln Leu His Trp Ala Asp Ala                   45

CAG GAA AGC TGT ATG AAG GAG GGG GCA AAC CTT GCA TCC ATT CAC                  +180
Gln Glu Ser Cys Met Lys Glu Gly Ala Asn Leu Ala Ser Ile His                   60

AGC CTT GAA GAG TCT ACG TTT GTT AAG GAG CTG ACA AGT GCA GAC                  +225
Ser Leu Glu Glu Ser Thr Phe Val Lys Glu Leu Thr Ser Ala Asp                   75

TTA ATC CCA TCA TGG ATT GGA GGC ACA GAT TGC CAA GTG TCA ACC                  +270
Leu Ile Pro Ser Trp Ile Gly Gly Thr Asp Cys Gln Val Ser Thr                   90

CGT TGG TTT TGG ATG GAT AGC ACA AGT ATG GAT TAT GCT GAC TGG                  +315
Arg Trp Phe Trp Met Asp Ser Thr Ser Met Asp Tyr Ala Asp Trp                   105

TGC GCT GCA CAA CCT GAT ACT ACC TTA ACT GAG TGC TGC ATA CAG                  +360
Cys Ala Ala Gln Pro Asp Thr Thr Leu Thr Glu Cys Cys Ile Gln                   120

ATG AAT GTT GGA ATT GGA AAA TGC TGG AAT GAC ACA CCT TGT ACG                  +405
Met Asn Val Gly Ile Gly Lys Cys Trp Asn Asp Thr Pro Cys Thr                   135

CAT CTT CAT TCA TCA ATC TGC GCC AAG CCA CTG AAA TGATTCCTGAG                  +452
His Leu His Ser Ser Ile Cys Ala Lys Pro Leu Lys

CTGACACAGAGGCATCCACCACCATCAATCACGTGATTGTGTGTATGTTTGCATACTCT                  +511

TCTGTGTTTTTCATGTATCAAGTCAAATAAAAGGCTGAAAC(A)n
```

potential phosphorylation sites were present. No N-glycosylation site was found in the deduced herring AFP sequence. This was consistent with the lack of detectable hexosamine in herring AFP.

3.3.4 Comparison of the three type II AFP sequences

A search for similarity to the smelt AFP precursor among sequences in the Swiss-Prot database (release 16) revealed that the smelt AFP most strongly resembled the sea raven AFP. Smelt and sea raven AFP precursor sequences share 40.5% identity when aligned using PC/Gene software. The herring AFP precursor was 83% identical to that of smelt and it was 44.2% identical to the sea raven AFP precursor (Ng et al. 1986, Hayes et al. 1989) when gaps were introduced for optimal alignment using PC/Gene. The 3 type II AFP sequences are aligned by eye and the regions common to all three proteins are highlighted in Fig. 26.

All cysteine residues were completely conserved between herring, smelt, and sea raven AFPs. The number of cysteines involved in disulfide bonds in herring and smelt AFPs and their positions were not assigned experimentally. However, the 10 cysteines in sea raven AFP make 5 disulfide bonds (Ng and Hew, 1992). Thus, it appears that the cysteine residues in smelt, herring, and sea raven AFPs form disulfide bonds (cystines) in the same positions.

The herring AFP precursor sequence differed from the others in having a gap corresponding to residues 20-37 of the smelt AFP precursor and residues 21-40 of the sea

Fig. 26. Comparison of the amino acid sequences of herring, smelt, and sea raven AFP precursors. Residues identical in all 3 proteins are shaded. Numbering is according to positions in AFP precursor sequences. Gaps (---) have been introduced for optimal alignment by eye.

```
hafp      MLTVSLLVCAIVALTRAADE--------------------CPTDW  (1-25)
smafp     ML-A_LVCAMVALTRAANGDTGKEAVMTGS--SGKNLTECPTDW  (1-42)
srafp     MLTVSLLVCAMMALTQANDDKILKGTATEAGPVSQRAPPNCPAGW  (1-45)

hafp      KMFNGRCFLFNPLQLHWADAQESCMKEGANLASIHSLEESTFVKE  (26-70)
smafp     KMFNGRCFLFNPLQLHWAHAQISCMKDGANLASIHSLEEYAFVKE  (43-87)
srafp     QPLGDRCIYYETTAMTWALAETNCMKLGGHLASIHSQEEHSFIQT  (46-90)

hafp      LTSADLIPSWIGGTDCQVSTRWFWMDSTSMDYADWCAAQPDTTLT  (71-115)
smafp     LTTAGLIPAWIGGSDCHVSTYWFWMDSTSMDFTDWCAAQPDFTLT  (88-132)
srafp     LN-AGV--VWIGGSACLQAGAWTWSDGTPMNFRSWCSTKPDDVLA  (91-132)

hafp      ECCIQMNVGIGKCWNDTPCTHLHSSICAKPLK                (116-147)
smafp     ECCIQINVGVGKCWNDTPCTHLHASVCAKPATVIPEVTPPSIM     (133-175)
srafp     ACCMQMTAAADQCWDDLPCPASHKSVCAMTF                 (133-163)
```

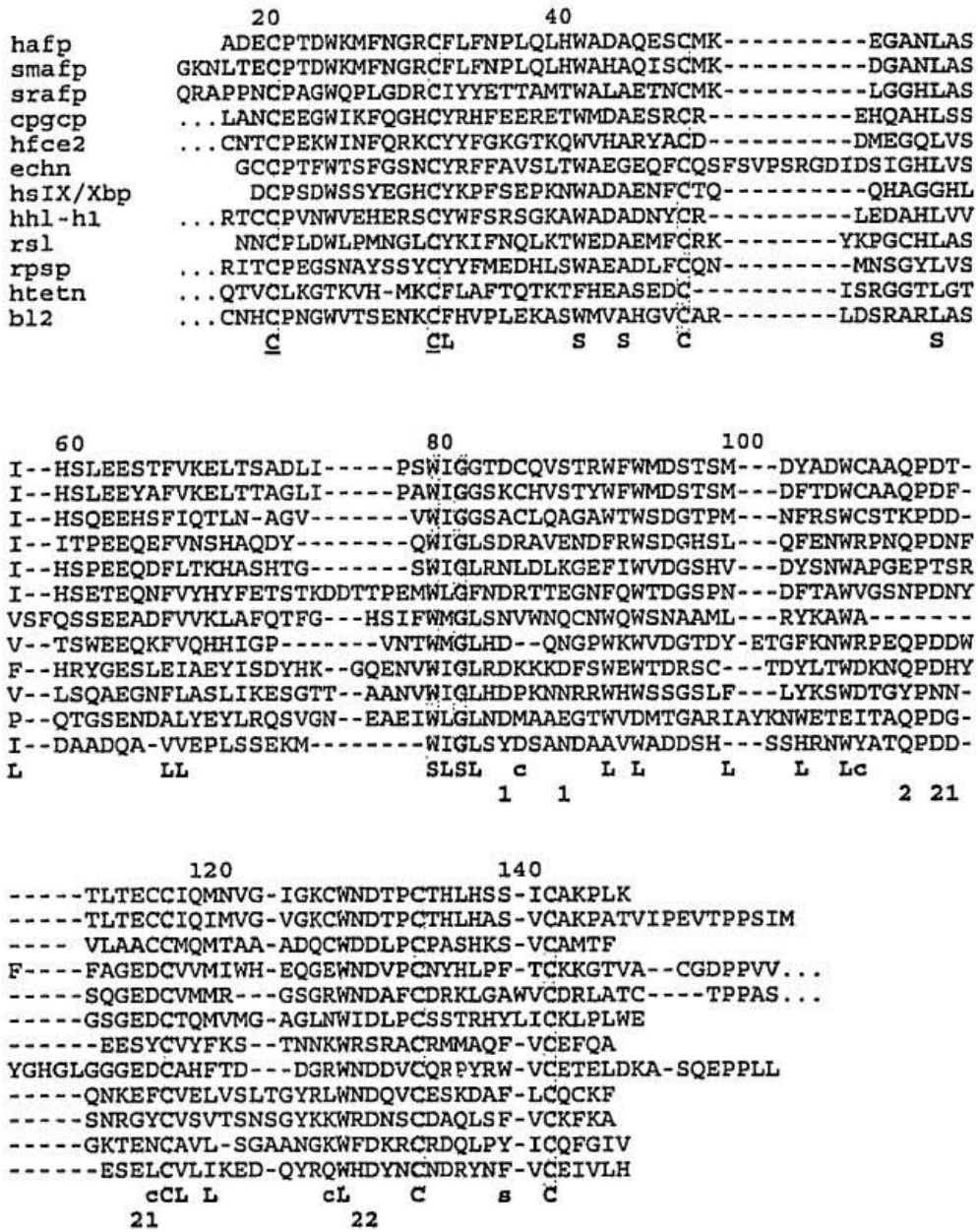
raven precursor (Fig. 26). This portion constitutes the putative prosequence and 6 residues of the mature sea raven AFP (Hayes et al. 1989).

3.3.5 Comparison of the type II AFPs to the C-type lectin family

A search of the Swiss-Prot data base using FASTA revealed that the AFP precursors of herring, smelt, and sea raven were similar to members of the C-type lectin family. Statistical analysis showed that fifteen proteins containing C-type carbohydrate recognition domains (CRDs) or related domains were homologous to all three type II fish AFPs. They are the human (Terazono et al. 1988), rat (Rouquier et al. 1991) and bovine (de la Monte et al. 1990) pancreatic stone proteins, the human (Ikuta et al. 1987) and mouse (Bettler et al. 1989) FcER2 receptors, Habu snake venom coagulation factor IX/X-binding protein B chain (Atoda et al. 1991), chicken cartilage proteoglycan core protein (Sai et al. 1986), human tetranectin (Fuhlendorff et al. 1987), human hepatic lectins H1 (Spiess et al. 1985) and H2 (Spiess and Lodish 1985), chicken hepatic lectin (Mellow et al. 1988), western diamondback rattlesnake venom lectin (Hirabayashi et al. 1991), barnacle lectins 2 (Muramoto and Kamiya 1990) and 3 (Muramoto and Kamiya 1986), and the sea urchin lectin, echinoidin (Giga et al. 1987).

The CRDs or CRD-like regions of representative proteins from this group are aligned with the type II AFPs in Fig. 27. In this figure, the sequence of the mature sea raven AFP was used (Hayes et al. 1989) and the predicted pre and pro sequences of the herring and smelt AFPs were removed where applicable to show only the mature sequences.

Fig. 27. Alignment of type II AFPs with proteins homologous to all 3 type II AFPs. All proteins are C-type lectins or lectin-like proteins. Only the CRDs or CRD-like regions of these proteins are shown. Numbering is according to position in the herring AFP precursor. Residues identical in all proteins are shaded. Some sequences have been truncated at the N or C terminal ends (...) and gaps (---) have been introduced to optimize alignment. Sequences are: hafp, herring AFP; smafp, smelt AFP; srafp, sea raven AFP; cpgcp, chicken proteoglycan core protein; hfce2, human FcER2 receptor; echn, echinoidin; hsIX/Xbp, Habu snake factor IX/X binding protein; hhl-h1, human asialoglycoprotein receptor H1; rsl, rattlesnake lectin; rpsp, rat pancreatic stone protein; htetn, human tetranectin; bl2, barnacle lectin 2. Residues forming the large hydrophobic core of MBP-A and conserved among C-type lectins (Weis et al. 1991a), are denoted by **L**. Residues of the small hydrophobic core of MBP-A and conserved among C-type lectins that are present in the type II AFPs are denoted by **S** and those absent in the type II AFPs by **s**. Cysteines conserved among all groups of C-type lectins are denoted by **C** and those present only in galactose-binding lectins (Drickamer 1989) are denoted by **C**. Cysteine residues that appear unique to the AFPs are represented by **c**. Residues of the MBP-A Ca^{2+}-binding site 1 are denoted by **1** and those of Ca^{2+}-binding site 2 are denoted by **2** (Weis et al. 1991a).


20 40
hafp ADECPTDWKMFNGRCFLFNPLQLHWADAQESCMK----------EGANLAS
smafp GKNLTECPTDWKMFNGRCFLFNPLQLHWAHAQISCMK----------DGANLAS
srafp QRAPPNCPAGWQPLGDRCIYYETTAMTWALAETNCMK----------LGGHLAS
cpgcp ...LANCEEGWIKFQGHCYRHFEERETWMDAESRCR----------EHQAHLSS
hfce2 ...CNTCPEKWINFQRKCYYFGKGTKQWVHARYACD----------DMEGQLVS
echn GCCPTFWTSFGSNCYRFFAVSLTWAEGEQFCQSFSVPSRGDIDSIGHLVS
hsIX/Xbp DCPSDWSSYEGHCYKPFSEPKNWADAENFCTQ----------QHAGGHL
hhl-h1 ...RTCCPVNWVEHERSCYWFSRSGKAWADADNYCR----------LEDAHLVV
rsl NNCPLDWLPMNGLCYKIFNQLKTWEDAEMFCRK--------YKPGCHLAS
rpsp ...RITCPEGSNAYSSYCYYFMEDHLSWAEADLFCQN---------MNSGYLVS
htetn ...QTVCLKGTKVH-MKCFLAFTQTKTFHEASEDC----------ISRGGTLGT
bl2 ...CNHCPNGWVTSENKCFHVPLEKASWMVAHGVCAR--------LDSRARLAS
C CL S S C S
60 80 100
I--HSLEESTFVKELTSADLI-----PSWIGGTDCQVSTRWFWMDSTSM---DYADWCAAQPDT-
I--HSLEEYAFVKELTTAGLI-----PAWIGGSKCHVSTYWFWMDSTSM---DFTDWCAAQPDF-
I--HSQEEHSFIQTLN-AGV-------VWIGGSACLQAGAWTWSDGTPM---NFRSWCSTKPDD-
I--ITPEEQEFVNSHAQDY--------QWIGLSDRAVENDFRWSDGHSL---QFENWRPNQPDNF
I--HSPEEQDFLTKHASHTG-------SWIGLRNLDLKGEFIWVDGSHV---DYSNWAPGEPTSR
I--HSETEQNFVYHYFETSTKDDTTPEMWLGFNDRTTEGNFQWTDGSPN---DFTAWVGSNPDNY
VSFQSSEEADFVVKLAFQTFG---HSIFWMGLSNVWNQCNWQWSNAAML---RYKAWA-------
V--TSWEEQKFVQHHIGP-------VNTWMGLHD--QNGPWKWVDGTDY-ETGFKNWRPEQPDDW
F--HRYGESLEIAEYISDYHK--GQENVWIGLRDKKKDFSWEWTDRSC---TDYLTWDKNQPDHY
V--LSQAEGNFLASLIKESGTT--AANVWIGLHDPKNNRRWHWSSGSLF---LYKSWDTGYPNN-
P--QTGSENDALYEYLRQSVGN--EAEIWLGLNDMAAEGTWVDMTGARIAYKNWETEITAQPDG-
I--DAADQA-VVEPLSSEKM--------WIGLSYDSANDAAVWADDSH---SSHRNWYATQPDD-
L LL SLSL c L L L L Lc
1 1 2 21
120 140
-----TLTECCIQMNVG-IGKCWNDTPCTHLHSS-ICAKPLK
-----TLTECCIQIMVG-VGKCWNDTPCTHLHAS-VCAKPATVIPEVTPPSIM
---- VLAACCMQMTAA-ADQCWDDLPCPASHKS-VCAMTF
F----FAGEDCVVMIWH-EQGEWNDVPCNYHLPF-TCKKGTVA--CGDPPVV...
-----SQGEDCVMMR---GSGRWNDAFCDRKLGAWVCDRLATC----TPPAS...
-----GSGEDCTQMVMG-AGLNWIDLPCSSTRHYLICKLPLWE
------EESYCVYFKS--TNNKWRSRACRMMAQF-VCEFQA
YGHGLGGGEDCAHFTD---DGRWNDDVCQRPYRW-VCETELDKA-SQEPPLL
-----QNKEFCVELVSLTGYRLWNDQVCESKDAF-LCQCKF
-----SNRGYCVSVTSNSGYKKWRDNSCDAQLSF-VCKFKA
-----GKTENCAVL-SGAANGKWFDKRCRDQLPY-ICQFGIV
------ESELCVLIKED-QYRQWHDYNCNDRYNF-VCEIVLH
cCL L cL C s C
21 22

3.3.6 Comparison of the type II AFP sequences with CRD sequences and structure

The crystal structure of the CRD from rat serum mannose binding protein (MBP-A) was determined and revealed the roles of sequence elements conserved among C-type CRDs (Weis et al. 1991a). Sequence alignment of the type II AFPs with the CRDs allowed the same elements to be identified in the antifreezes. All 14 residues that form the large hydrophobic core of the CRD in the rat serum-type mannose binding protein (MBP-A) (Weis et al. 1991a) are conserved in the AFPs (Fig. 27). Five of 6 conserved residues in the small hydrophobic core of the CRDs are also found in the AFPs.

The AFPs contain the 4 cysteines conserved in MBP-A and other lectins and the 2 other cysteines found only in galactose-binding lectins (Fig. 27). However, the disulfide bonding pattern in the AFPs appears more complex than in C-type lectin CRDs. Cysteine residues occur in AFP sequences corresponding to loops 1, 3, and 4 in the MBP-A crystal structure (Weis et al. 1991a). A cysteine residue was also present in a sequence corresponding to a ß-sheet region (ß4) of MBP-A (Weis et al. 1991a). No cysteines occur at these positions in known C-type lectins.

Residues shown to form the first Ca^{2+}-binding site in C-type lectins (Weis et al. 1991a) are not conserved in smelt, herring, and sea raven AFPs (Fig. 27). Non-conservative amino acid substitutions at positions corresponding to Ca^{2+}-binding site 1 in the AFPs suggests that this site may not be functional. The second Ca^{2+}-binding site also appears non-functional in sea raven because of non-conservative amino acid substitutions. However, all of the amino acid residues conserved in the second Ca^{2+} binding site of C-type lectins are present in smelt and herring AFPs (Fig. 27).

3.3.7 Antifreeze activity of type II AFPs in the presence and absence of Ca^{2+}

Thermal hysteresis activities of AFPs from smelt, herring, sea raven, and winter flounder were measured in the presence and absence of free divalent cations. Winter flounder AFP (type I) and sea raven AFP activities were unaffected by Ca^{2+}. However, activities of smelt and herring AFPs were sharply reduced in the absence of Ca^{2+} (Table 4). Addition of excess $CaCl_2$ to EGTA-inactivated samples restored activity of smelt and herring proteins 74% and 87%, respectively, compared to active controls.

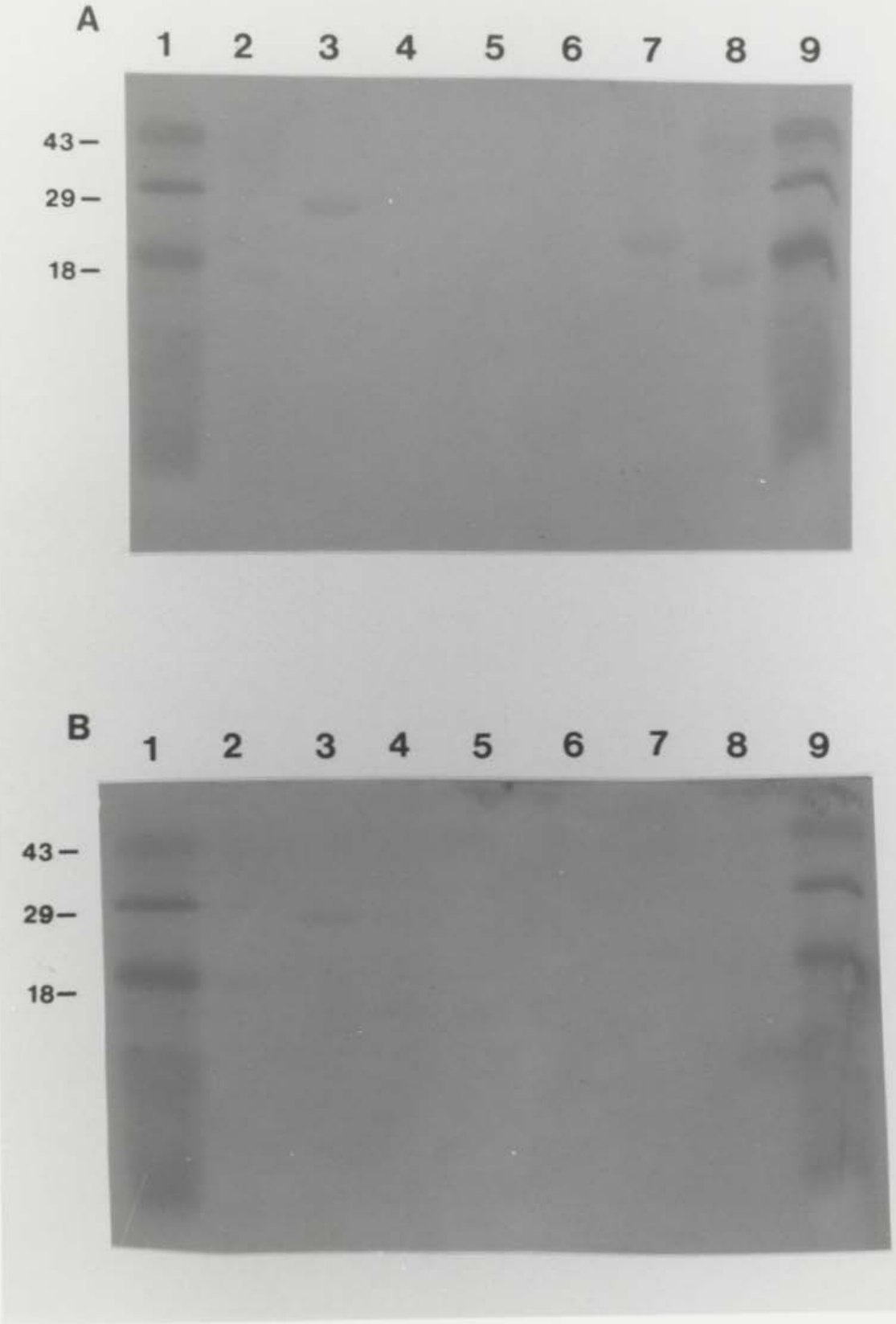
3.3.8 Binding of Ca^{2+} to sea raven AFP

A qualitative dye-binding assay did not reveal any Ca^{2+}-binding activity in sea raven AFP. Ruthenium red bound to three immobilized proteins (Fig. 28a). Two were proteins previously known to bind Ca^{2+}. These were rattlesnake lectin (Hirabayashi et al. 1991) and the 18 kDa molecular weight marker, ß-lactoglobulin (Pappas and Rothwell 1991). The other was the smelt AFP. It appears that the dye was binding at Ca^{2+}-binding sites on the proteins because washing in $CaCl_2$ completely displaced ruthenium red from smelt AFP and displaced it partially from the other 2 proteins (Fig. 28b). Ruthenium red did not bind detectably to sea raven, ocean pout, or winter flounder AFPs, nor did it bind to any of the molecular weight markers that are not known to bind Ca^{2+}. The absence of ruthenium red on sea raven AFP is consistent with results of sequence analysis and activity measurements.

Table 4. Activities of AFPs in the presence and absence of free Ca^{2+} ions. Values are means ± SEM from 5 determinations.

AFP		Thermal hysteresis (°C)	
Fish	mg/ml	10 mM $CaCl_2$	2.5 mM EGTA
Herring	15	0.201 ± 0.027	-0.005 ± 0.004
Smelt	15	0.192 ± 0.012	0.006 ± 0.002
Sea raven	5	0.369 ± 0.008	0.373 ± 0.032
Winter flounder	3	0.255 ± 0.022	0.256 ± 0.016

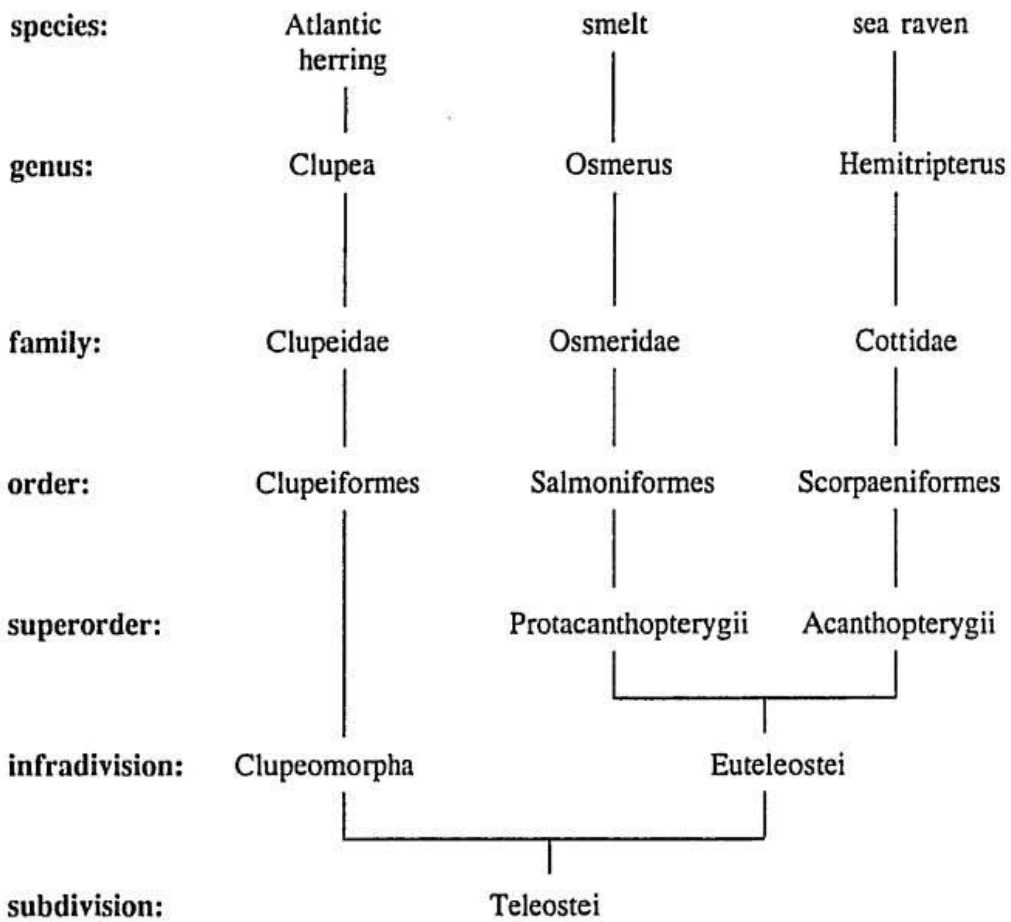
Fig. 28. Ruthenium red staining of AFPs. Lanes for both blots: (1,9) prestained molecular mass protein markers; (2,8) regular molecular mass protein markers; (3) rattlesnake lectin (RSL); (4) winter flounder AFP; (5) ocean pout AFP; (6) sea raven AFP; (7) smelt AFP. Numbers on the left represent the M_r of the markers ($x10^{-3}$).


A
1 2 3 4 5 6 7 8 9
43–
29–
18–
B
1 2 3 4 5 6 7 8 9
43–
29–
18–

3.4 Discussion

In teleost fish, freeze resistance is presumed to have evolved recently during Cenozoic cooling and glaciation (Scott et al. 1986). Yet, homologous type II AFPs are found in three distantly related species. The distribution of type II fish AFPs is outlined in Fig. 29. Sea raven are in the family Cottidae (order Scorpaeniformes, superorder Acanthopterygii), a group very distantly related to the order Salmoniformes (superorder Protacanthopterygii) to which smelt belong. These two superorders (Protacanthopterygii and Acanthopterygii) are believed to have diverged over 40 million years ago (Greenwood et al. 1966) prior to the onset of glaciation (Kennet and Shakelton 1976, Keigwin 1980, Kerr 1984). Hence, the cooling events that appear to have led to selection for antifreeze function are thought to have occurred after these fish groups had parted (Scott et al. 1986). Furthermore, according to the classification of Nelson (1984), herring belong to the infradivision Clupeomorpha, a group separate from the infradivision Euteleostei, which encompasses all other AFP-producing fish species. Thus, the homology among the three type II AFPs was surprising. It is even more remarkable if the distributions of other AFPs are considered. For example, the shorthorn sculpin, a close relative of the sea raven, produces a type I AFP (Hew et al. 1985). To reconcile the narrow distribution of type II AFPs among cottids with their appearance in widely divergent species, it is reasonable to suggest that they evolved recently in three separate fish groups from a single preexisting protein family.

Fig. 29. Outline of the phylogenetic relationships among fish producing type II AFPs. The scale used here is not linear.


species:
Atlantic herring
smelt
sea raven
genus:
Clupea
Osmerus
Hemitripterus
family:
Clupeidae
Osmeridae
Cottidae
order:
Clupeiformes
Salmoniformes
Scorpaeniformes
superorder:
Protacanthopterygii
Acanthopterygii
infradivision:
Clupeomorpha
Euteleostei
subdivision:
Teleostei

A similarity search in a protein sequence database revealed that a large number of sequences were similar to the type II AFPs. All of these sequences were CRDs or CRD-like sequences in proteins belonging to the C-type lectin family (Drickamer 1988). Similarities between sea raven and some proteins in the C-type lectin family have also been reported by others (Ng and Hew 1992). To determine whether the observed similarities reflected an evolutionary relationship, a statistical comparison between the sequences of the high scoring CRD-containing proteins and each of the type II AFPs was undertaken. A large subset of the CRD-containing proteins showed scores above the cutoff point indicating homology. The AFPs appear most closely related to these proteins. However, C-type CRDs whose scores were below the minimum required for statistical evidence of homology must also share a common origin with the AFPs. All proteins containing CRDs belong to a single family. Hence, they all are related to each other and, consequently, to the AFPs as well. In addition, the statistical tests normally employed for this purpose may reject actual homologies if they are discriminating enough to eliminate non-homologous sequences (Feng et al. 1985).

The homology between the herring AFP and the C-type lectins and lectin-like proteins is illustrated by their alignment in Fig. 27. Bezouska et al. (1991) classified C-type lectin CRDs from higher vertebrates into four groups according to amino acid sequence similarity. This grouping is also consistent with CRD-associated flanking domain differences and the presence and positions of introns in lectin genes (Bezouska et al. 1991). The vertebrate C-type lectins that are significantly homologous to all three type II AFPs belong to group I (proteoglycans) and group II (type II receptors). Most of these

proteins bind galactose and its derivatives (Bezouska et al. 1991) as do the rattlesnake and invertebrate lectins that are homologous to the type II AFPs (Hirabayashi et al. 1991, Muramoto and Kamiya 1990, Giga et al. 1987).

The C-type lectin family includes a large number of proteins with diverse functions but whose common feature is the presence of a C-type CRD. The CRD was originally identified as a domain that binds carbohydrates in a calcium-dependent manner (Drickamer 1988). This domain spans approximately 130 residues and contains several residues that are identical or conserved among CRDs from different proteins (Drickamer 1988). The type II AFPs appear to adopt a fold similar to that of MBP-A, considered to be a prototype CRD structure. Many of the residues forming the large and small hydrophobic cores of the C-type CRDs have been located in the crystal structure of the rat MBP-A and are conserved among 22 vertebrate lectins (Weis et al. 1991a). Conserved Ca^{2+}-binding residues have been similarly identified (Weis et al. 1991a). Herring, smelt, and sea raven AFPs contain all but one of the residues conserved among the C-type lectins that form the large and small hydrophobic cores of MBP-A. In addition, smelt and herring AFPs each appear to contain one intact Ca^{2+}-binding site.

There are, however, some interesting differences between the type II AFPs and the C-type lectins. Two extra disulfide bonds are present in the type II AFPs (Ng and Hew 1992) and the results of Ng and Hew (1992) indicate that some of the conserved cysteine residues do not form the same disulfides in the AFPs and the lectins. These differences may cause the CRD-like tertiary structure of these AFPs to be distinct from the CRDs of lectins and possibly cause them to be more tightly folded. In addition, the presence of a

non-conserved residue in the AFPs at a position corresponding to one of the small hydrophobic core-forming residues in MBP-A may cause the AFPs to have different properties from the lectins. An MBP-A mutant containing the non-conservative substitution Ala → Ser at this position had a lower Ca^{2+} affinity than its wild-type counterpart (Quesenberry and Drickamer 1992). Since the AFPs all contain Ser at this position, their Ca^{2+} affinity or other properties may differ from those of MBP-A or C-type lectins in general.

The lectins in the C-type lectin family all require Ca^{2+} in order to function (Drickamer 1988, 1989). Using the CRD from chicken hepatic lectin, it was also shown that upon binding Ca^{2+}, the C-type CRD undergoes a conformational change in which the structure becomes more compact and more resistant to proteolysis (Loeb and Drickamer 1988). The sea urchin C-type lectin, echinoidin, appears to undergo a similar change in response to Ca^{2+} (Giga et al. 1985). Therefore, as a member of the C-type lectin family, the sea raven AFP is unusual because sequence analysis revealed no functional Ca^{2+}-binding site and its hysteresis activity is not responsive to Ca^{2+}. To determine whether sea raven AFP might bind Ca^{2+} but not require it for activity, the ruthenium red staining was carried out. Results of this procedure suggested that the sea raven AFP did not bind Ca^{2+}. In contrast, smelt AFP and a C-type lectin from rattlesnake (Crotalus atrox) did appear to bind Ca^{2+} in the same assay. Furthermore, the inactivity of smelt and herring AFPs in solutions devoid of free Ca^{2+} suggests that, like the lectins, these proteins bind ligand only when in a Ca^{2+}-dependent active conformation. Although the C-type lectins and smelt and herring AFPs may have different affinities for Ca^{2+}, they clearly respond to it in the same

way. The difference here is that the ligand of the AFPs is ice. Thus, the interaction between type II AFPs and ice and that between lectins and sugars appear functionally similar.

The homology between the C-type lectins and type II AFPs does not necessarily mean that the AFPs are also able to bind carbohydrate. A number of lectin-like proteins are homologous to all three type II AFPs and contain CRD-like domains but bind to ligands other than carbohydrate. The human pancreatic stone protein appears to inhibit $CaCO_3$ crystal growth through interaction with the crystal lattice (Multigner et al. 1983) and the human lymphocyte Fc receptor for IgE (FcER2) recognizes deglycosylated IgE, presumably through protein recognition (Vercelli et al. 1989). Tetranectin binds to the kringle 4 part of plasminogen (Clemmensen et al. 1986) as well as to sulphated polysaccharides (Clemmensen 1989). However, it is not clear whether both types of ligand bind to the same site or to distinct sites on tetranectin.

Some aspects of the smelt and herring AFP primary structures remain unresolved. The exact lengths of the mature smelt AFP sequences are not known because the N termini of the proteins are blocked. The sea raven AFP precursor contains 34 residues preceding the mature N terminus. This is thought to include a 17 residue presequence (predicted signal sequence) followed by a 17 residue prosequence (Hayes et al. 1989). The predicted signal sequences of smelt and sea raven AFP are very similar (Fig. 26). However, smelt and herring AFPs may not have a corresponding prosequence cleaved. The M_r of smelt AFP is much greater than that of sea raven AFP (Chapter 2). Thus, the possibility that smelt AFP retains the residues corresponding to the predicted sea raven AFP prosequence

cannot be ruled out. The herring AFP precursor appears to be simply a preprotein because it does not contain the residues corresponding to the prosequence of the sea raven AFP. In addition, the presence of an N-linked glycosylation template in the sequence of smelt AFP and its absence in those of herring and sea raven is consistent with amino acid analysis results. Neither herring nor sea raven AFPs contain detectable hexosamine (Slaughter et al. 1981, Ng et al. 1986) yet analysis of smelt AFP (Chapter 1) has revealed the presence of N-acetylglucosamine, which forms the linkage with Asn in N-linked carbohydrates.

Further understanding of type II AFP structure and function may result from comparisons of the three type II AFP primary structures. Although the type II AFPs are homologous proteins, some differences exist among them. The herring and smelt AFPs appear the most similar. Their precursor primary structures are very much alike (83% identity) and they both require Ca^{2+} ions for activity. In contrast, the sea raven AFP has a more distinct primary structure (Ng et al. 1986) and it does not require Ca^{2+} for activity. Sea raven AFP also shows the highest thermal hysteresis activity of the type II AFPs measured over a range of millimolar concentrations (Chapter 2 and Kao et al. 1986). It is unlikely that the lower activities of the smelt and herring AFPs are due to suboptimal Ca^{2+} concentrations. Although no Ca^{2+} was added to the buffers in earlier experiments (Chapter 2), the hysteresis values were comparable to those obtained in the presence of 10 mM added $CaCl_2$. Thus, the differences between the type II AFP primary structures appear to correspond to their Ca^{2+}-binding characteristics and activity levels. This knowledge may allow the eventual identification of some of the AFP residues that interact

with ice. Additional comparison with the MBP-A prototype CRD structure might reveal the location of the ice-binding surface of the type II AFPs.

The homology between the type II AFPs and members of the C-type lectin family suggests that the CRDs of C-type lectins or related proteins might share properties with the AFPs. AFPs lower solution freezing points by binding to the non-basal planes of ice crystals and thereby inhibiting ice growth (Davies and Hew 1990). Thus, for an antifreeze function to have evolved, the progenitor C-type lectin must have been able to interact with ice crystals. Lee et al. (1991) investigated the carbohydrate recognition mechanisms of three C-type lectins and found that the hydroxyl groups at postions 3 and 4 of the target sugars were indispensible for interaction with the lectins. These authors suggested that the oxygen and hydrogen of the 3-OH hydrogen binds with the lectins. Therefore, it seems reasonable to suggest that the CRDs of certain lectins may have the ability to bind to water molecules organized in an ice crystal lattice as do the AFPs.

CHAPTER 4:

FUNCTIONAL COMPARISON OF TYPE II AFPS AND OTHER PROTEINS IN THE C-TYPE LECTIN FAMILY

4.1 Introduction

The majority of proteins in the C-type lectin family function as lectins. Some C-type lectins consist of CRDs with no distinct associated domains. Examples are the lectins of an acorn barnacle, a sea urchin, and a rattlesnake (Muramoto and Kamiya 1986, 1990, Giga et al. 1987, Hirabayashi et al. 1991). These lectins all bind galactose. However, most lectins in this family are composed of CRDs coupled to a number of other domains. The carbohydrate specificities of the CRDs in the multidomain lectins are more diverse as well. These lectins include several different groups. Endocytic receptors of mammalian and avian liver, also called hepatic lectins, clear desialated glycoproteins from circulation (Spiess 1990). MBPs of mammalian liver and blood plasma bind the mannose-containing oligosaccharides of several pathogens (Ikeda et al. 1987, Ezekowitz et al. 1990, Ezekowitz 1991). Selectins are cell adhesion molecules that target leucocytes to inflammation sites and to lymphoid tissues (Johnston et al. 1989, Bevilaqua et al. 1989, Lasky et al. 1989). Mammalian and avian proteoglycan core proteins also contain CRD sequences (Krusius et al. 1987, Halberg et al. 1988) and a proteoglycan CRD expressed in bacteria can function

as a lectin (Halberg et al. 1988). However, the proteoglycans are not likely to function as lectins in vivo because their CRDs are heavily glycosylated and processed.

Other proteins in the C-type lectin family bind to different ligands. An example is the pancreatic stone proteins which bind to $CaCO_3$ crystals (Multigner et al. 1983). In this protein, and others such as the IgE Fc receptor (Ikuta et al. 1987), lectin activity has not yet been reported.

Since the type II AFPs and the C-type lectin CRDs are clearly related structures and their known biological activities are for the most part Ca^{2+}-dependent, it seems reasonable to suggest that they might work in a similar way. Binding to carbohydrates and binding to ice both require the Ca^{2+}-induced "active" conformation of the CRD. In addition, carbohydrate recognition occurs through hydrogen bonding to specific hydroxyl groups on target sugars (discussed in chapter 4). These findings suggest a relationship between carbohydrate and ice recognition and raise the possibility that they might occur at the same site on the domain.

As a first step towards exploring the functional mechanism of the type II AFPs, the possibility that the AFPs may have ligands in common with the C-type CRDs or related domains must be investigated. Thus, the goal of this study was to determine whether the C-type lectin CRDs and the type II AFP can bind the same ligands. The ability of type II AFPs to bind carbohydrate and the interaction of other proteins in the C-type lectin family with ice were both evaluated.

4.2 Materials and methods

4.2.1 Agglutination tests using type II AFPs

4.2.1.1 Initial screening for agglutination

Agglutination of erythrocytes from Atlantic cod, winter flounder, rat, and rabbit was assayed in the presence of type II AFPs. Procedures were based on previously described methods (Komano et al. 1980, Gartner and Ogilvie 1984, Hirabayashi et al. 1991). Erythrocytes were collected in the presence of sodium heparin and washed three times in TCS (10mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.4) by resuspension and centrifugation. They were then brought to 4% v/v in TCS and stored for a maximum of 1 week at 4°C. AFPs partially purified by gel filtration of herring, smelt, and sea raven plasma were used. Single concentrations and twofold serial dilutions of the AFPs in TCS were prepared in duplicate in microtiter plates with a volume of 25 µl in each well. For each assay, a positive control (rattlesnake lectin), a negative control (ovalbumin), and a well containing only TCS were included. To each well, 25 µl of erythrocyte suspensions were added and well contents were mixed by pipetting. Plates were covered, kept at room temperature for 1 hr, and evaluated by direct observation as described previously (Komano et al. 1980, Sharon and Lis 1975, MacLaren 1986). Agglutinated cells formed a diffuse mat across the well whereas non-agglutinated cells formed a distinct spot at the bottom of the well.

4.2.1.2 Evaluation of erythrocyte agglutination by pure sea raven AFP

Sea raven AFP purified by gel filtration on Sephadex G75 was applied to a gel filtration column (1.5 x 90 cm) containing Sephacryl S100-HR (Sigma) in 0.1 M NH_4HCO_3 buffer. Absorbances at 230 nm were measured for each fraction and 25 µl aliquots were taken from fractions spanning the optical density peak and were resolved by SDS-PAGE as described in Chapter 2 to locate those containing the AFP. AFP-containing fractions were pooled, freeze-dried, and applied to a Bio-Gel DEAE Sepharose column (1 x 30 cm) in 0.1-0.25 M gradient. Fractions spanning the optical density peaks were evaluated by SDS-PAGE as above. Fractions containing pure AFP were pooled and freeze-dried. Agglutination of rabbit erythrocytes by sea raven AFP was evaluated as above.

4.2.2 Determination of subunit structure of a type II AFP

An NH_3-specific monobifunctional cross-linker, bis-(sulfosuccinimidyl) suberate (BS^3) (Pierce), was used to cross-link protein following the method described by Weis et al. (1991b). A 50 mM solution of BS^3 was perpared in 0.1 M Hepes pH 7.4 containing 10 mM $CaCl_2$ and it was used to prepare a series of twofold serial dilutions on a microtiter plate. Sea raven AFP purified by ion exchange chromatography was brought to 0.625 mg/ml in the same buffer. Twenty µl aliquots of this solution were pipetted into 8 wells. Then 5 µl of each BS^3 dilution was added to a corresponding well containing sea raven AFP. The samples were covered and kept at room temperature for 1 hour. To each well, 25 µl of 1:1 SDS-PAGE reducing sample buffer was added. These samples were boiled

for 5 minutes and loaded on a 15% SDS-PAGE gel. This experiment was repeated and products were resolved by non-reducing SDS-PAGE.

4.2.3 Carbohydrate affinity chromatography of type II AFPs

A 1 mg/ml solution of G150 purified smelt AFP was made in 10 mM Tris-HCl pH 8.0 containing 2.5 mM $CaCl_2$ and 2.5 mM $MgCl_2$ and aliquots were frozen. Herring and sea raven AFPs purified by gel filtration were also used. However, because of the limited supply of these proteins, they were only applied to galactose columns.

One to three ml of immobilized D-mannose, L-fucose, ß-D-glucose, D-galactose, N-acetylglucosamine, N-acetylgalactosamine, and heparin (all from Sigma or Pierce) were poured into disposable 10 ml chromatography columns (Bio-Rad) and these were equilibrated in 10 mM Tris-HCl pH 8.0 containing 2.5 mM $CaCl_2$ and 2.5 mM $MgCl_2$. Chromatography was carried out at 4°C. For each, 200 µg of AFP was applied to the column followed by approximately 10 ml of buffer. Ten 50-drop fractions (approximately 1 ml each) were collected to wash off unbound protein and then excess buffer was removed from above the gel surface. To elute any Ca^{2+}-dependent lectins, 10 ml of 10 mM Tris-HCl pH 8.0 containing 10 mM EDTA were added to the column, and 10 more 50-drop fractions were then collected. Flow speed was kept at or below 15 ml/hr. To ensure that this procedure would allow lectins to bind to the columns in the Ca^{2+}/Mg^{2+} buffer and elute in the EDTA buffer, the assay was also done using rattlesnake lectin with a galactose gel, Sepharose 4B (Sigma). All fractions were lyophilized and resuspended in sample buffer. For each chromatography, fractions 1-18 were resolved by SDS-PAGE.

To accomodate the fractions, 2 identical 10-well gels were run at the same time in a single unit (Mini-Protean II, Bio-Rad) and they were stained and destained together.

4.2.4 Removal of carbohydrate from smelt AFP

Smelt AFP was partially purified by gel filtration of smelt blood plasma on Sephadex G150. The protein pooled from active fractions was evaluated by SDS-PAGE and lyophilized. A 1 mg/ml solution of smelt AFP was made in N-glycosidase F reaction buffer (100 mM NaH_2PO_4, 50 mM EDTA, 0.2% SDS, pH 7.8). Two 500 µl aliquots were taken from this solution. Five µl of ß-mercaptoethanol were added to each and they were boiled for 3 minutes and cooled on ice for 8 to 10 minutes. To each tube, 450 µl of N-glycosidase buffer and 16 µl of Nonidet P40 were added. Fifty µl of water were added to the control tube and 50 µl (10 units) of N-glycosidase F (Boehringer Mannheim) were added to the reaction tube. Both tubes were incubated at 37°C overnight. Equal volumes of protein from the reaction and control tube were analysed by SDS-PAGE.

4.2.5 Isolation and antifreeze activity measurement of PSP

A human pancreatic stone was obtained from an autopsy at the Health Sciences Centre, St. John's, Nfld. The stone protein was purified following the methods described by DeCaro et al. (1979) and Multigner et al. (1983). The pancreatic stone was enclosed in gauze and washed for 24 hours in 10 mM Tris-HCl pH 7.8 containing 150 mM NaCl. It was then placed in 0.5 mM EDTA pH 8.0, vortexed, and shaken overnight to dissolve the $CaCO_3$ in the stone. The resulting solution was dialysed extensively against 0.1 M

NH_4HCO_3 and lyophilized. The protein was evaluated by SDS-PAGE and its hysteresis activity was measured as described in Chapter 1.

4.2.6 Isolation and evaluation of RSL

Rattlesnake venom from the western diamondback rattlesnake, Crotalus atrox (Sigma), was purified using affinity chromatography essentially as described by Gartner and Ogilvie (1984). Venom was suspended in starting buffer (10 mM Tris-HCl pH 8.0 containing 10 mM $CaCl_2$) and applied to a column (2.5 x 40 cm) containing Sepharose CL-4B (Pharmacia and Sigma) in the same buffer. In the presence of Ca^{2+}, the lectin will bind galactose present on the column while other proteins will flow through. Unbound protein was washed off the column in starting buffer. Then, to elute the lectin, the column buffer was changed to 10 mM Tris-HCl pH 8.0, containing 10 mM EDTA. Proteins were detected in individual fractions by optical density measurement at 280 nm. Fractions containing protein eluted in EDTA buffer were pooled and dialysed against 10 mM Tris-HCl pH 8.0, and then extensively against 0.1 M NH_4HCO_3, and lyophilized.

In order to verify that the lectin was active after lyophilization, the carbohydrate-binding ability of the lyophilized protein was evaluated by affinity chromatography as described in section 4.2.3 and by agglutination on microtiter plates in TCS as described in section 4.2.1.1. The specific activity of this lectin against rabbit erythrocytes was evaluated using the microtiter plate assay. The concentration of lectin at which the agglutination endpoint occurred was determined in duplicate twofold serial dilutions by

finding the maximum dilution of a known concentration of RSL at which agglutination occurs.

4.2.7 Measurement of RSL antifreeze activity

Thermal hysteresis activity of RSL was measured as described in Chapter 2. RSL, ovalbumin, and herring AFP were each dissolved in Tris-HCl pH 8.0. Aliquots of each protein were brought to 2.5 mM EGTA or to 10 mM $CaCl_2$. The final Tris concentration was 15 mM in each sample.

4.3 Results

4.3.1 Agglutination activity of type II AFPs

Herring and smelt AFPs did not agglutinate fresh erythrocytes from cod, winter flounder, rat, or rabbit at concentrations up to 0.5 mg/ml under standard conditions used to study agglutination (Figs. 30, 31). Wells containing all concentrations of these proteins were identical to controls containing ovalbumin or without added protein. In addition, no agglutination was detected in wells containing 10 mg/ml of these AFPs. They also appeared identical to non-lectin controls. Under the same conditions, a C-type lectin from rattlesnake caused full agglutination.

The AFP preparation from sea raven did show agglutination of rat and rabbit erythrocytes at concentrations of 0.5, 0.256, and 0.125 mg/ml (Fig. 32). However, this appeared to be caused by a contaminant in the crude protein preparation. Purified sea raven

Fig. 30. Evaluation of agglutination of erythrocytes from different species in the presence of herring AFP. A, B, C, and D represent identical experiments using erythrocytes from rabbit, rat, cod, and flounder, respectively. In each, microtiter wells 1-24 show a twofold serial dilution of AFP. Wells 1, 12, 13, and 24 are numbered to show orientation. Control wells contain: (R) RSL; (O) ovalbumin; and (C) no added protein.

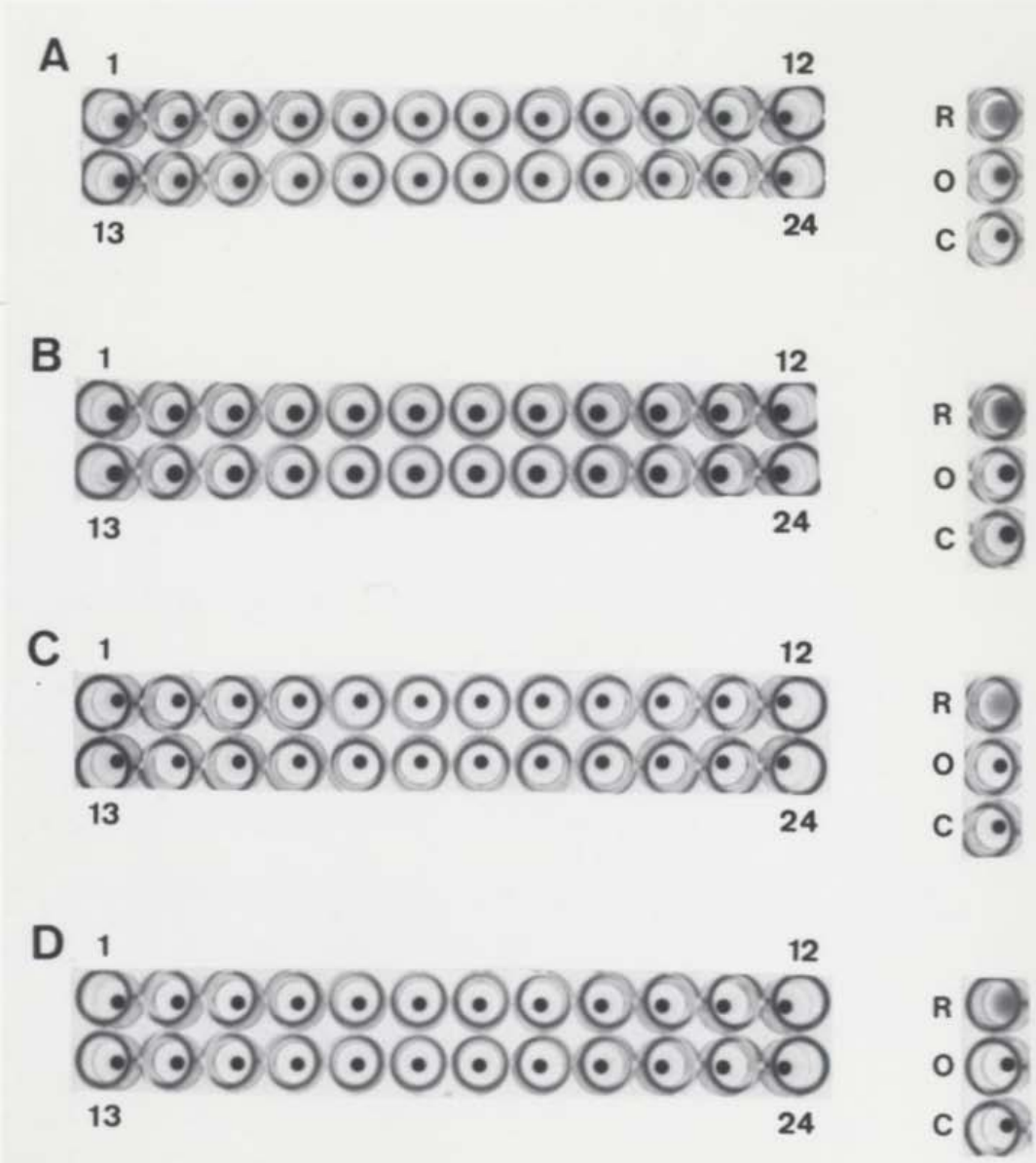

A
1
12
13
24
R
O
C
B
1
12
13
24
R
O
C
C
1
12
13
24
R
O
C
D
1
12
13
24
R
O
C

Fig. 31. Evaluation of agglutination of erythrocytes from different species in the presence of smelt AFP. A, B, C, and D represent identical experiments using erythrocytes from rabbit, rat, cod, and flounder, respectively. In each, microtiter wells 1-24 show a twofold serial dilution of AFP. Wells 1, 12, 13, and 24 are numbered to show orientation. Control wells contain: (R) RSL; (O) ovalbumin; and (C) no added protein.

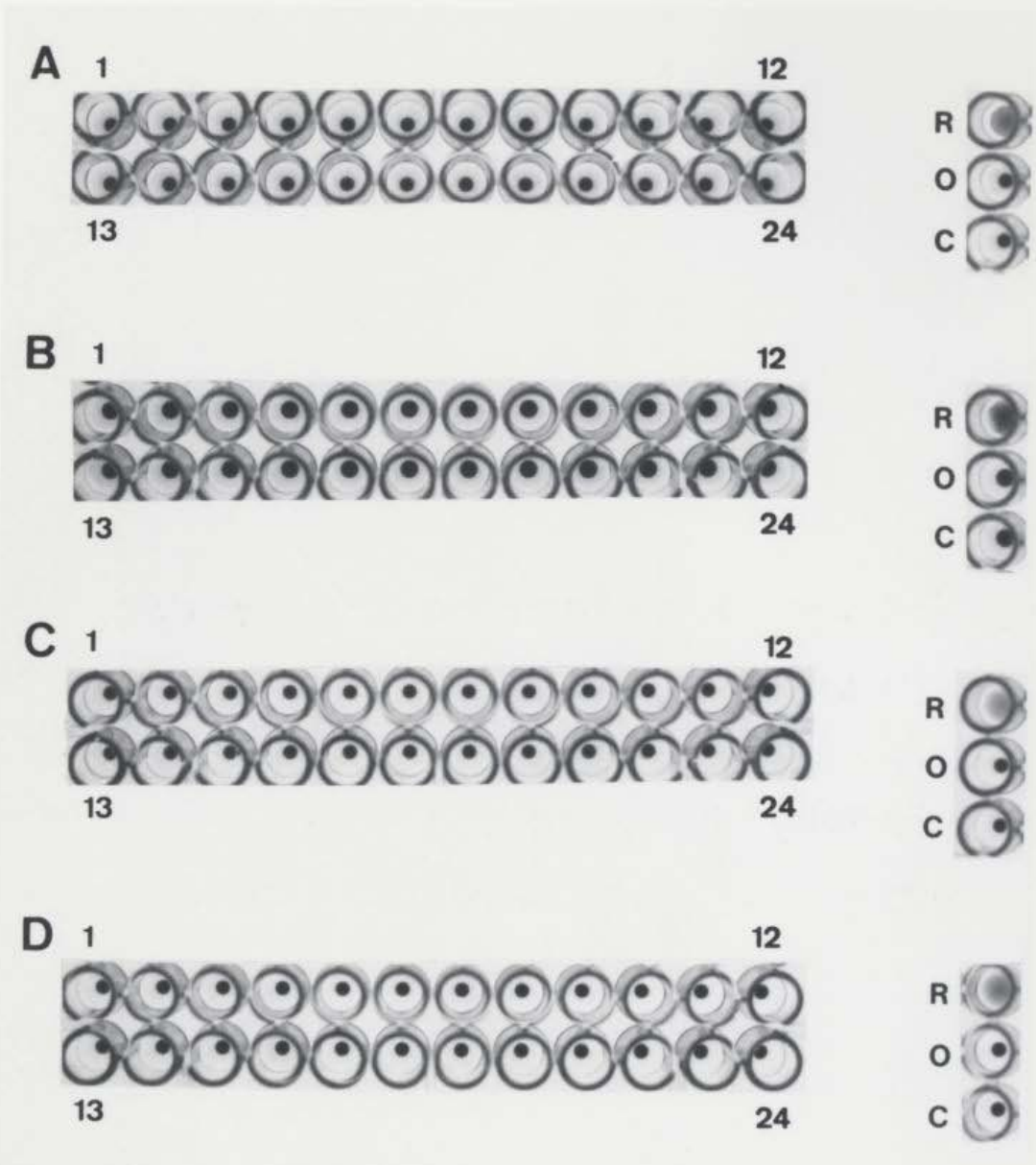
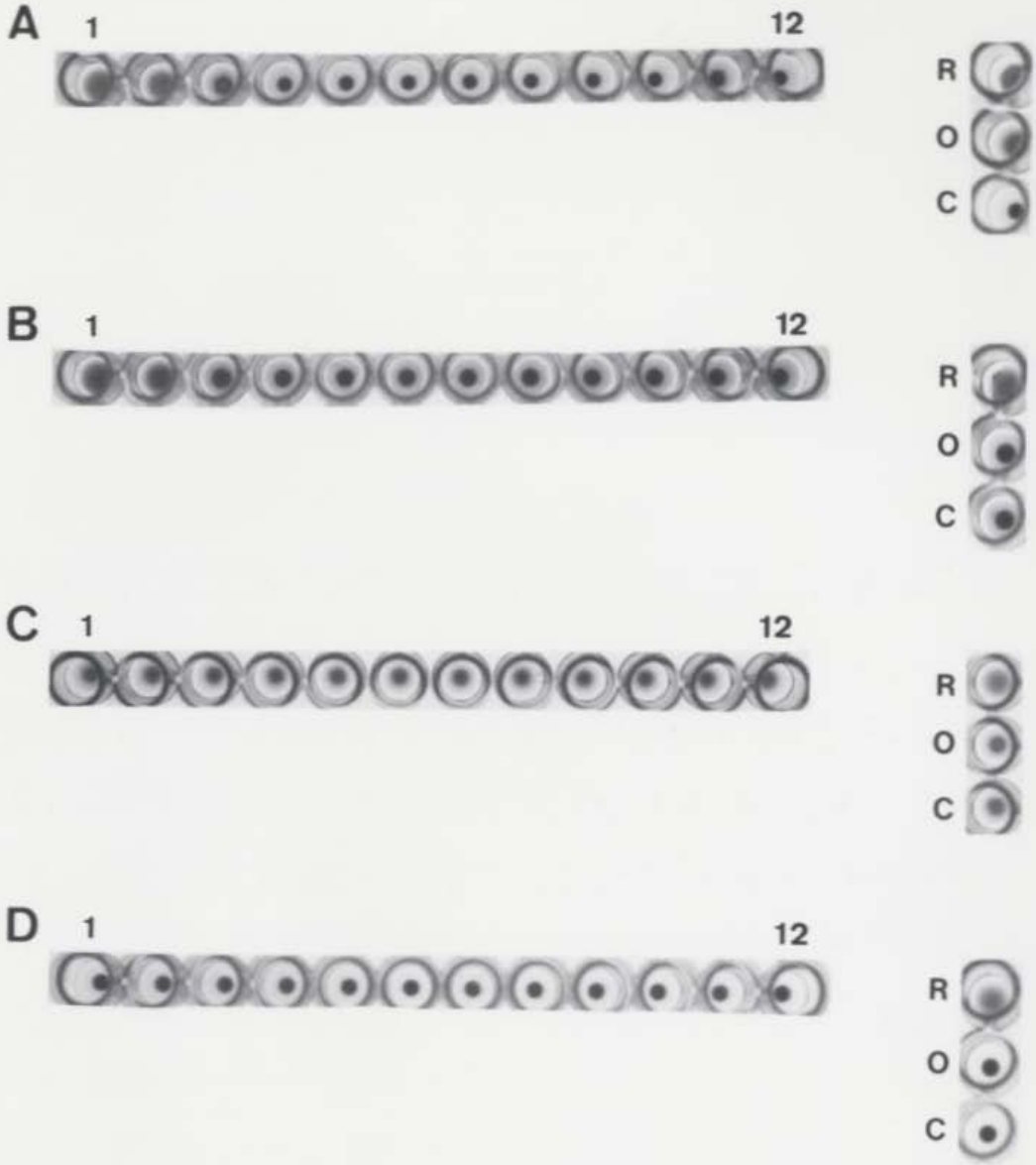

A
1
12
13
24
R
O
C
B
1
12
13
24
R
O
C
C
1
12
13
24
R
O
C
D
1
12
13
24
R
O
C

Fig. 32. Evaluation of agglutination of erythrocytes from different species in the presence of sea raven AFP. A, B, C, and D represent identical experiments using erythrocytes from rabbit, rat, cod, and flounder, respectively. In each, microtiter wells 1-12 show a twofold serial dilution of AFP. Wells 1 and 12 are numbered to show orientation. Control wells contain: (R) RSL; (O) ovalbumin; and (C) no added protein.


A
1
12
R
O
C
B
1
12
R
O
C
C
1
12
R
O
C
D
1
12
R
O
C

AFP did not agglutinate rabbit erythrocytes under the same conditions, even at a tenfold higher concentration (Fig. 33).

4.3.2 Subunit structure of a type II AFP

Sea raven AFP was treated with a cross-linking agent in order to determine whether it forms oligomers in solution. In the presence of an appropriate cross-linker, oligomers should be covalently joined and these products are detected by SDS-PAGE as bands with lower mobilities that the monomer. In the presence of a range of cross-linker concentrations, sea raven AFP appeared to remain in its monomeric form (Fig. 34).

4.3.3 Carbohydrate-affinity chromatography of AFPs

For the affinity chromatography experiments, to ensure that any protein bound to a column was indeed AFP and in order to detect low levels of bound protein, fractions were analysed by SDS-PAGE. Analysis of fractions 1 to 18 from most chromatographies by SDS-PAGE gave nearly identical results. The protein eluted in the Ca^{2+}-containing initial buffer, mainly in fractions 3 to 6, in every case. This indicated that the AFP did not interact with the immobilized carbohydrates. A typical result, that from N-acetylglucosamine affinity chromatography, is shown in Fig. 35. However, a trace of smelt AFP was retarded on immobilized galactose (Sepharose 4B) and was eluted in the EDTA-containing fractions 12 and 13 (Fig. 36). Herring and sea raven AFP did not bind to immobilized galactose. These proteins eluted completely in fractions 3-6. A known C-type lectin, RSL, was used as a positive control. It did bind to immobilized ß-D-galactose

Fig. 33. Evaluation of agglutination of rabbit erythrocytes in the presence of sea raven AFP prepared by gel filtration (G75) or further purified by repeated gel filtration and ion exchange chromatography (DEAE). In each, microtiter wells 1-6 show a twofold serial dilution of AFP beginning with the indicated protein concentrations. Wells 1 and 6 are numbered to show orientation.

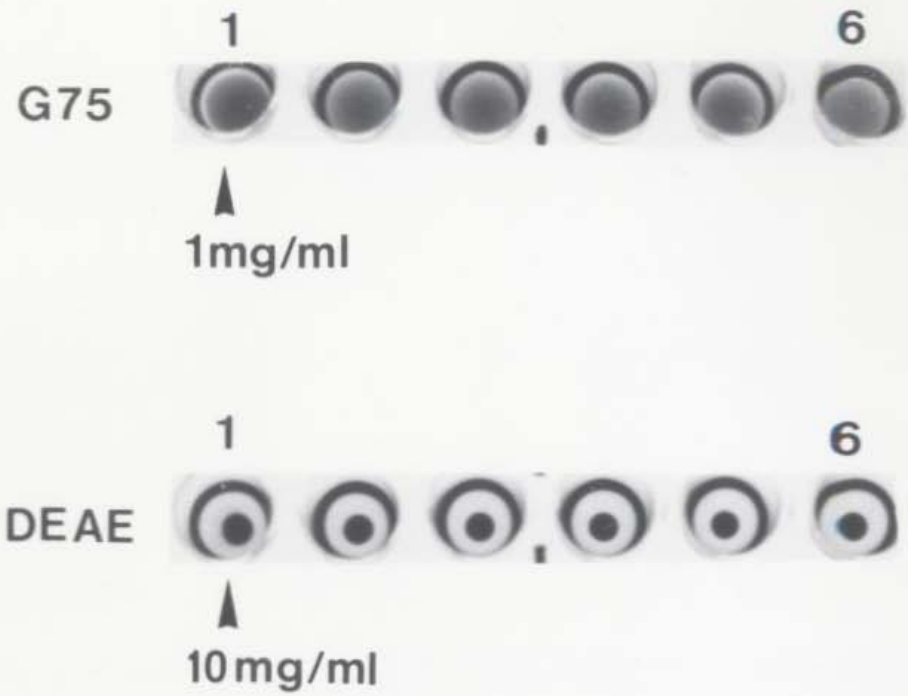

1
6
G75
1mg/ml
1
6
DEAE
10mg/ml
1
6
DEAE
1mg/ml

Fig. 34. Evaluation of oligomeric structure of sea raven AFP. Lanes: (1) molecular mass protein markers; (2-10) sea raven AFP treated with 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.08, and 0 mM BS^3, respectively.

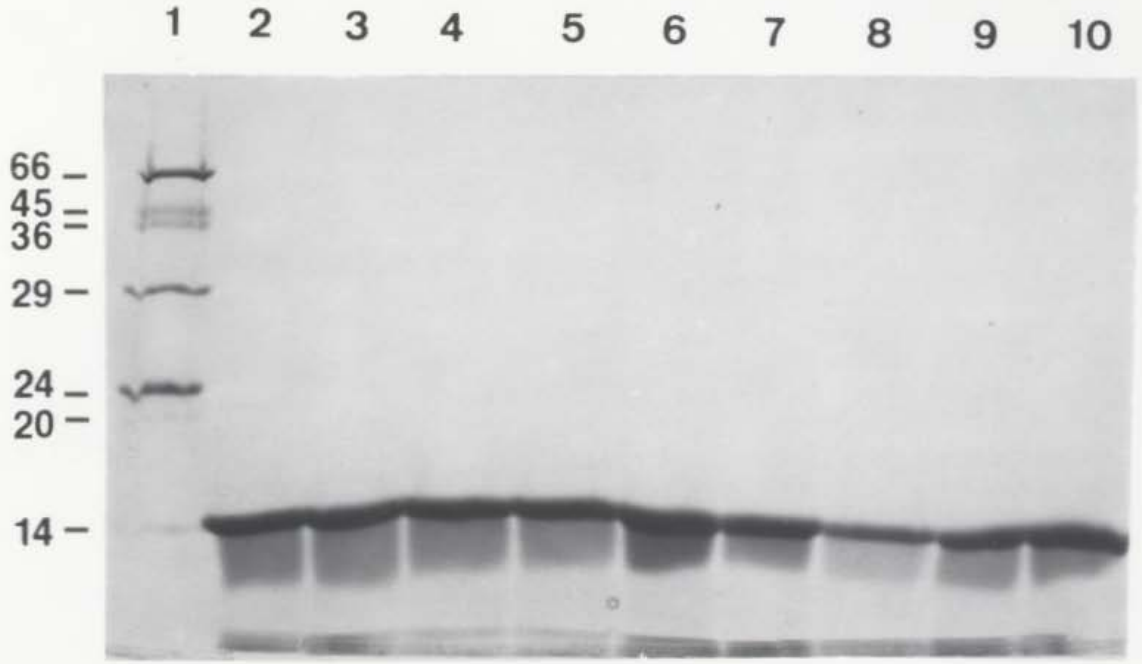

1
2
3
4
5
6
7
8
9
10
66
45
36
29
24
20
14

Fig. 35. SDS-PAGE of fractions from N-acetylglucosamine affinity chromatography of smelt AFP. Lanes: (1) molecular mass protein markers; (2-10) chromatography fractions 1-9; (11) ovalbumin; (12-20) chromatography fractions 11-18. Numbers on the left represent the M_r of the markers ($x10^{-3}$).

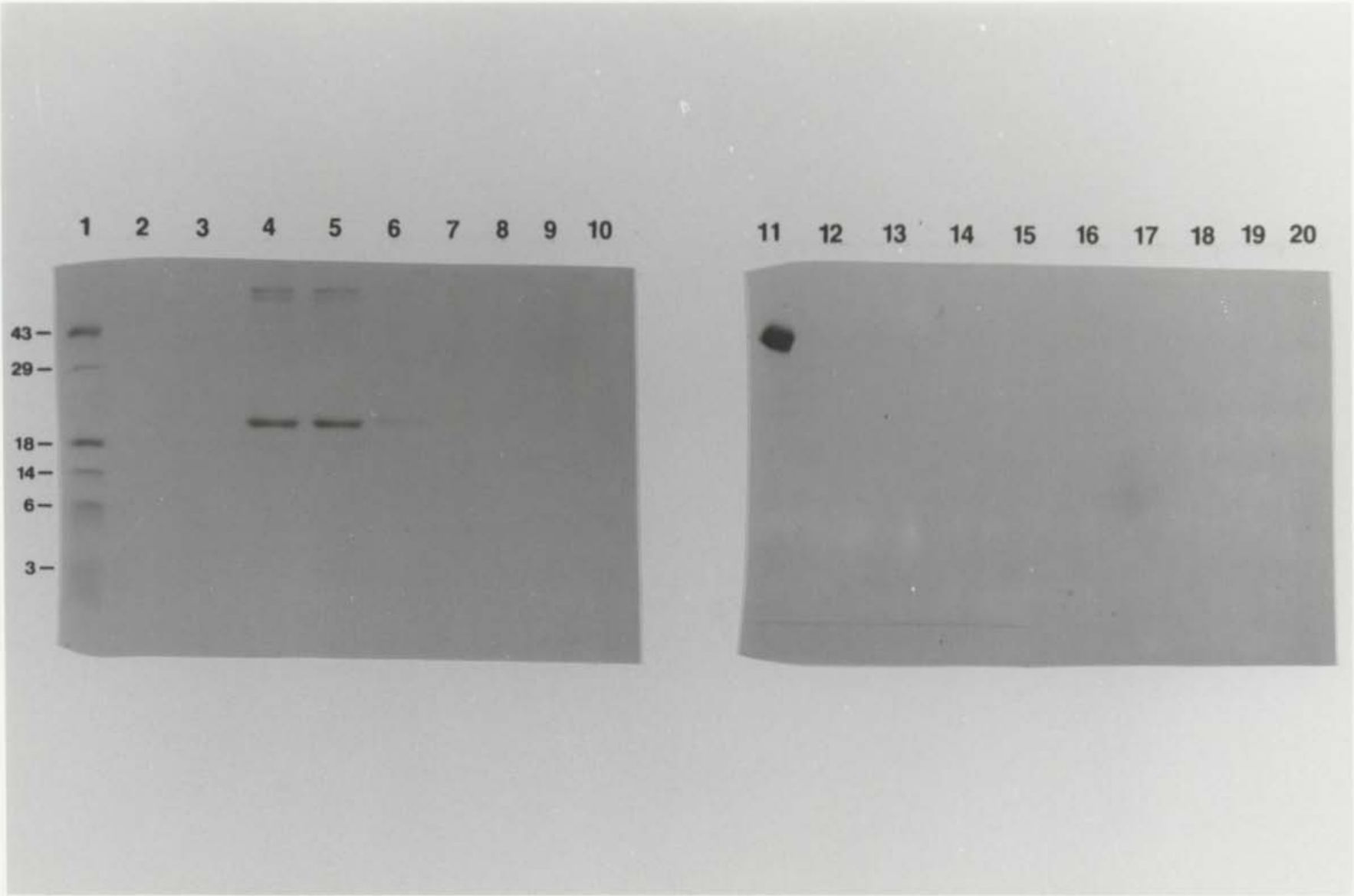
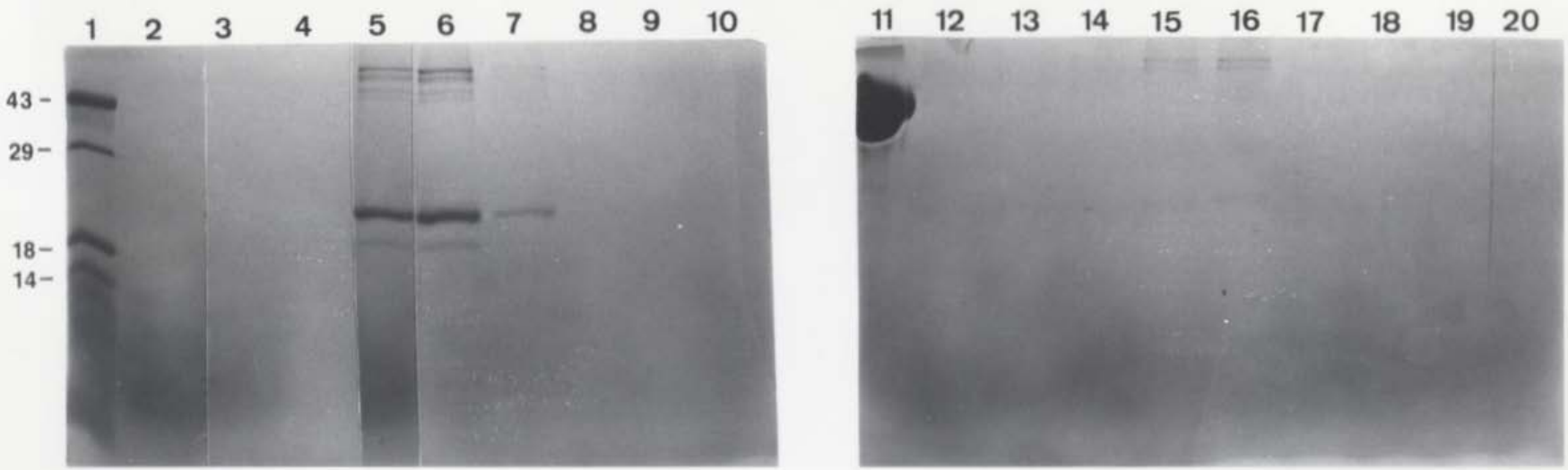

1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20
43
29
18
14
6
3

Fig. 36. SDS-PAGE of fractions from galactose affinity chromatography of smelt AFP on Sepharose 4B. Lanes: (1) molecular mass protein markers; (2-10) fractions 1-9; (11) ovalbumin; (12-20) fractions 10-18. Numbers on the left represent the M_r of the markers ($x10^{-3}$).


1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20
43 –
29 –
18 –
14 –

under the same conditions and it was eluted in the EDTA-containing buffer mainly in fractions 14 to 16 (Fig. 37).

4.3.4 The carbohydrate of smelt AFP

The smelt AFP treated with N-glycosidase F and the untreated AFP control had different mobilities when evaluated by SDS-PAGE (Fig. 38). The smelt AFP had an M_r of 23,000 as measured from this gel, while the deglycosylated smelt AFP had an M_r of 20,000. These results indicate that the smelt AFP contains covalently-bound carbohydrate that accounts for approximately 13% of its apparent M_r on SDS-PAGE.

4.3.5 Isolation and antifreeze activity measurement of human PSP

A small amount of protein was obtained from the pancreatic stone. On SDS-PAGE, a single protein with an M_r of approximately 14,000 was detected (Fig. 39) consistent with previous results (DeCaro et al. 1979). The protein was difficult to dissolve at high concentrations. It was considered dissolved when the solution appeared clear in bright light but, in an NH_4HCO_3 buffer, a precipitate formed over a period of several hours. The hysteresis activity of the PSP was measured as described in Chapter 2. Hysteresis was not detected in a 30 mg/ml solution of PSP prepared in 0.1 M NH_4HCO_3 or in 15 mM Tris-HCl pH 8.0 containing 25 mM $CaCl_2$. Thus, this protein appears unable to function as an antifreeze.

Fig. 37. SDS-PAGE of fractions of galactose affinity chromatography of RSL on Sepharose 4B. Lanes: (1) molecular mass protein markers; (2-10) fractions 1-9; (11) ovalbumin; (12-20) fractions 10-18. Numbers on the left represent the M_r of the markers ($x10^{-3}$).

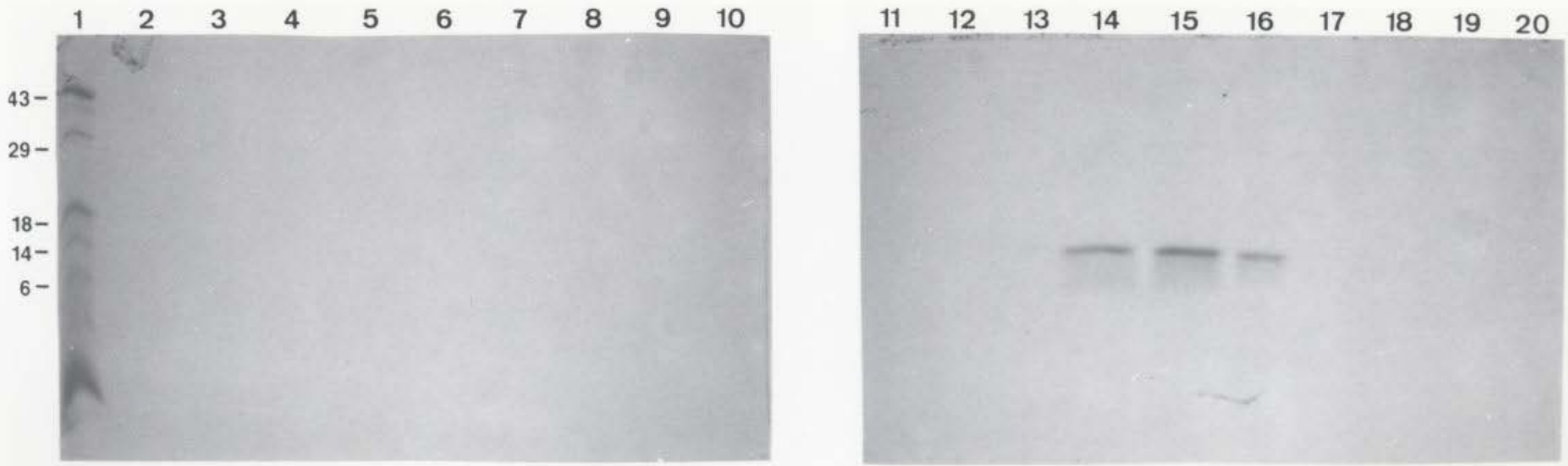

1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20
43
29
18
14
6

Fig. 38. SDS-PAGE of control and N-glycosidase F-treated smelt AFP. Lanes: (1) molecular mass markers; (2) deglycosylated smelt AFP; (3) native smelt AFP. Numbers on the left represent the M_r of the markers ($x10^{-3}$).

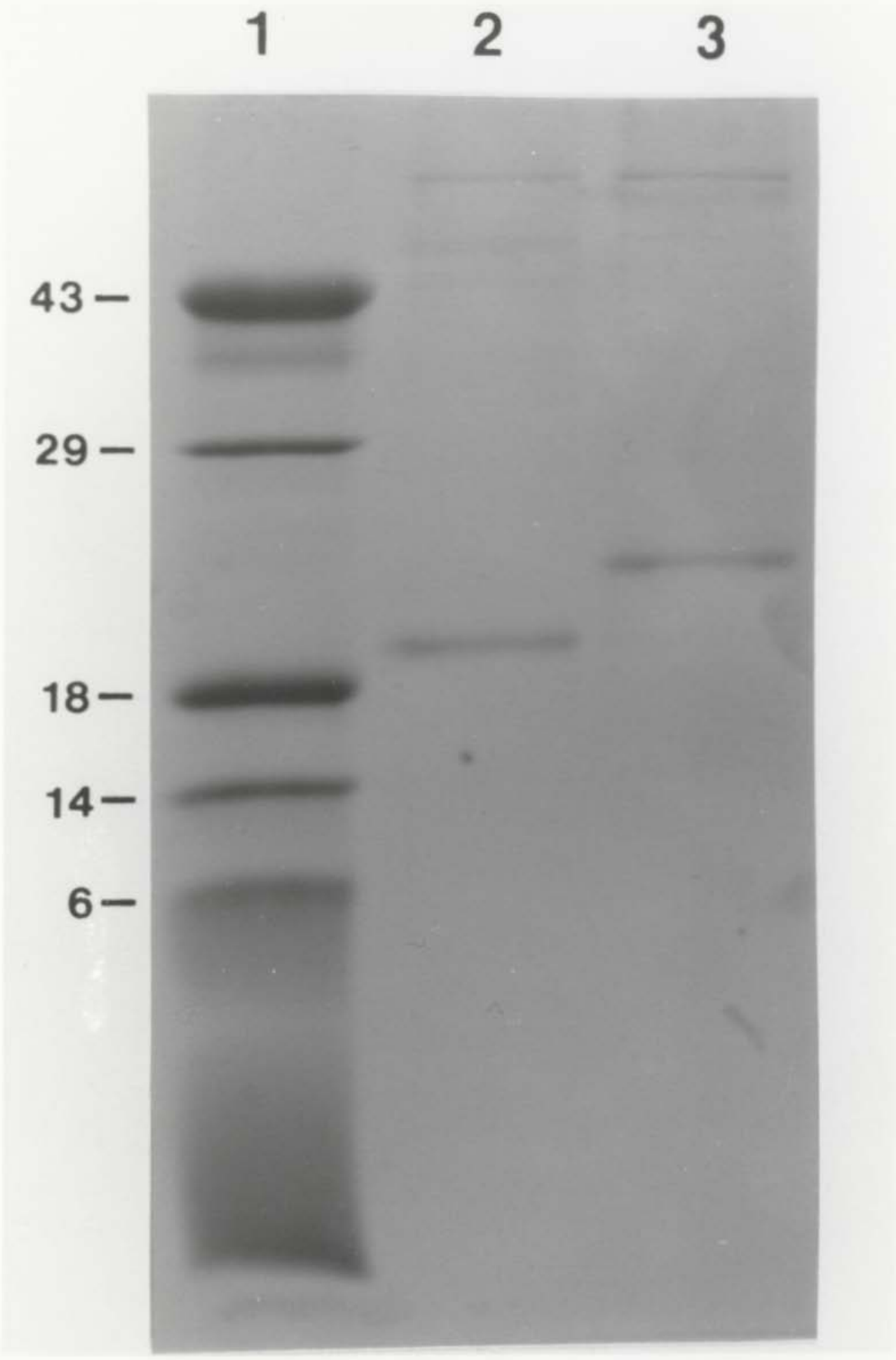
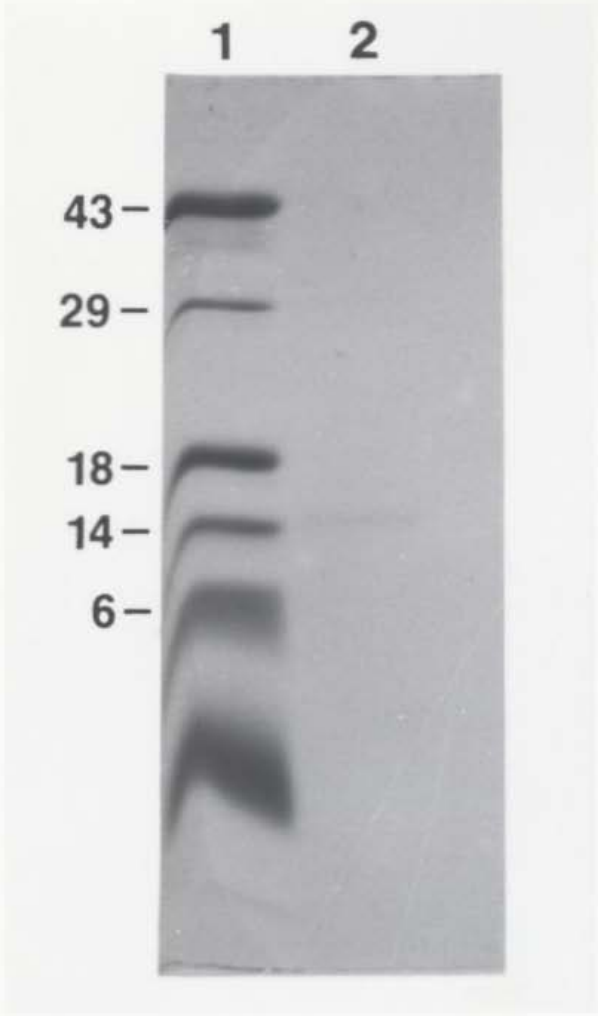

1
2
3
43
29
18
14
6

Fig. 39. SDS-PAGE of human PSP. Lanes: (1) molecular mass protein markers; (2) human pancreatic stone protein. Numbers on the left represent the M_r of the markers ($x10^{-3}$).


1
2
43
29
18
14
6

4.3.6 Isolation and carbohydrate-binding activities of RSL

The galactose-binding lectin from the venom of western diamondback rattlesnake was adsorbed onto Sepharose CL-4B and eluted using EDTA. A typical chromatogram is shown in Fig. 40. SDS-PAGE under reducing and non-reducing conditions revealed a dimer with an approximate M_r of 28,000, which could be separated into monomers with an apparent M_r of 15,000 (Fig. 41). However, the molecular weight of the monomer calculated from its protein sequence is 16,317 (Hirabayashi et al. 1991). A single protein band was visible on SDS-PAGE under reducing conditions suggesting that the protein was pure (Fig. 41).

The purified, lyophilized rattlesnake lectin showed normal carbohydrate-binding activity. Using carbohydrate-affinity chromatography, rattlesnake lectin was shown to bind galactose (Sepharose 4B). Elution of adsorbed RSL could be achieved using the usual buffer containing EDTA (Fig. 37) or the same buffer containing 100 mM lactose (Fig. 42). Agglutination of rabbit erythrocytes was also evaluated using the microtiter plate assay. The lowest concentration in a twofold serial dilution of RSL showing agglutination of intact rabbit erythrocytes was 15 ng/ml (Fig. 43). This is in line with the previously reported end-point concentration of 10 ng/ml found to agglutinate intact rabbit erythrocytes (Hirabayashi et al. 1991).

4.3.7 Investigation of RSL antifreeze activity

No thermal hysteresis was detected in 15 mg/ml solutions of RSL using the osmometer as described in Chapter 2 (Table 5). This high concentration of RSL was

Fig. 40. Sepharose CL-4B affinity chromatography of rattlesnake venom. The column size was 2.5 x 40 cm and the fraction size was approximately 4 ml. The starting buffer was 10 mM Tris-HCl pH 8.0 containing 10 mM $CaCl_2$. This buffer was replaced by the elution buffer (10 mM Tris-HCl pH 8.0 containing 10 mM EDTA) at tube 181. Fractions 229-238, comprising the optical density peak eluted using EDTA, were pooled and lyophilized.

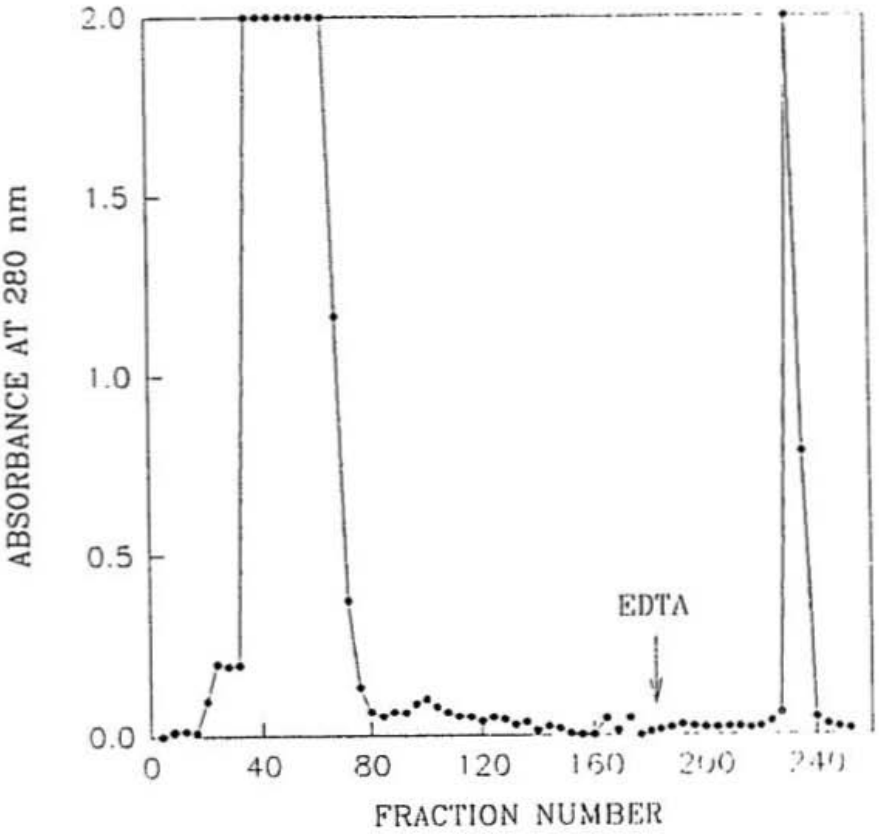

2.0
1.5
1.0
0.5
0.0
ABSORBANCE AT 280 nm
EDTA
0
40
80
120
160
200
240
FRACTION NUMBER

Fig. 41. SDS-PAGE of RSL. Lanes: (1,2) RSL, not reduced; (3,4) reduced RSL; (5) low molecular mass protein markers. Numbers on the left represent the M_r of the markers ($x10^{-3}$).

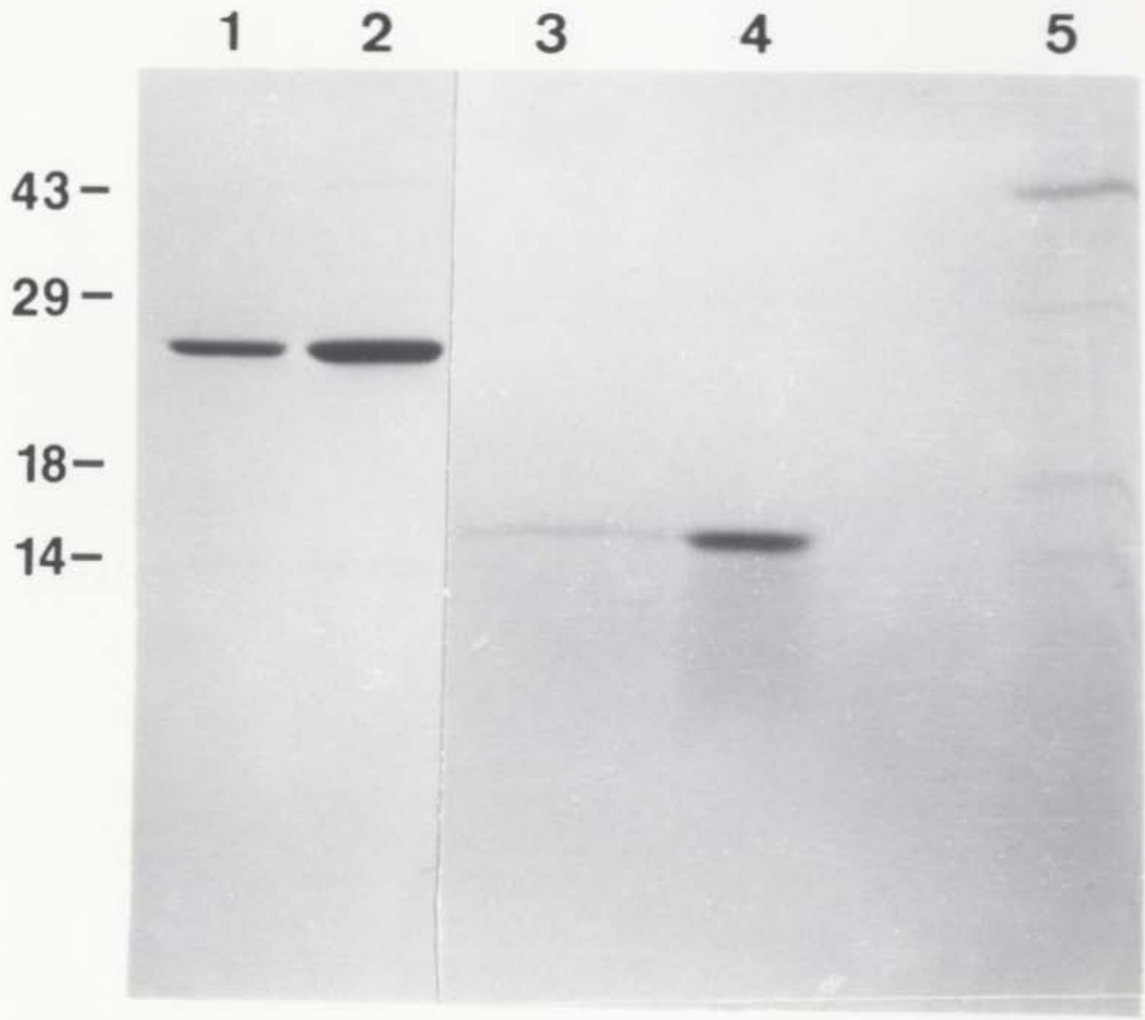

1
2
3
4
5
43–
29–
18–
14–

Fig. 42. SDS-PAGE of fractions from galactose affinity chromatography of RSL on Sepharose 4B. Bound protein was eluted using 10 mM Tris-HCl pH 8.0 containing 100 mM lactose. Lanes: (1) molecular mass markers, (2-10) fractions 1-9; (11) no protein; (12-20) fractions 10-18. Numbers on the left represent the M_r of the markers ($x10^{-3}$).

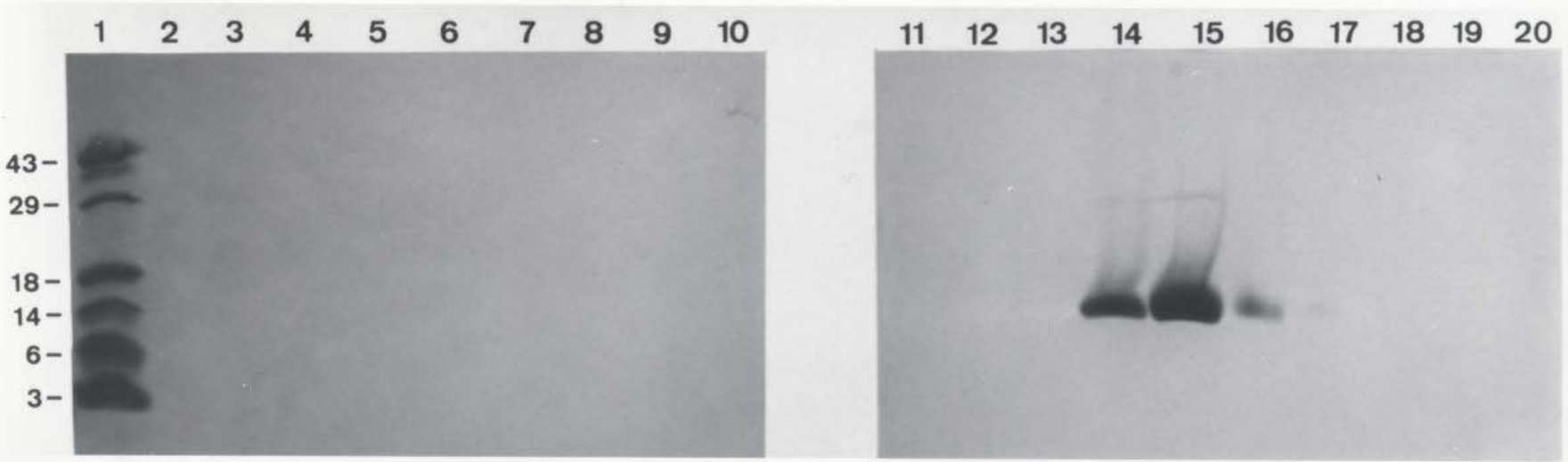

1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20
43–
29–
18–
14–
6–
3–

Fig. 43. Determination of endpoint of agglutination of fresh rabbit erythrocytes by RSL. Microtiter wells 1-24 show a twofold serial dilution of RSL. Wells 1, 12, 13, and 24 are numbered to show orientation. The well containing the minimum RSL concentration causing agglutination is indicated by an arrow and the concentration of RSL in this well is indicated.

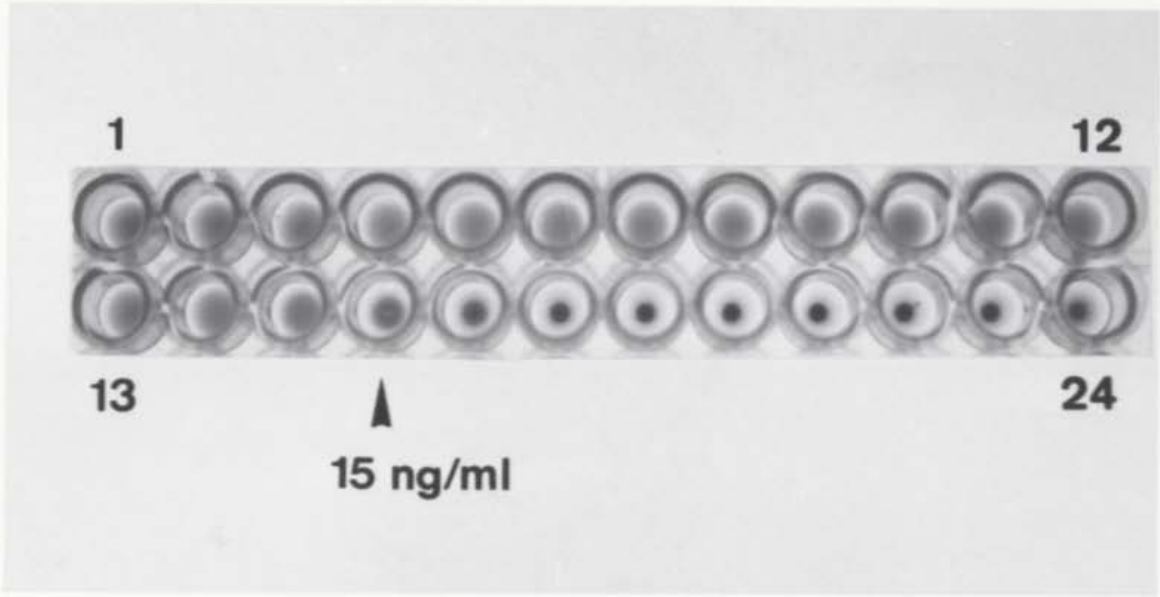

1
12
13
24
15 ng/ml

Table 5. Antifreeze activities of RSL in the presence and absence of free Ca^{2+} ions. Values are means ± SEM from 3 determinations except where indicated.

Protein		Thermal hysteresis (°C)	
Protein	mg/ml	10 mM $CaCl_2$	2.5 mM EGTA
RSL	15	-0.006 ± 0.003	-0.003 ± 0.006
Ovalbumin	15	-0.007 ± 0.004	-0.006 ± 0.003
Herring AFP	15	0.204 (n=1)	0.023 (n=1)

chosen in order to maximize the likelihood of detecting thermal hysteresis. Herring AFP showed normal activity when measured under the same conditions.

4.4 Discussion

The C-type lectin family includes a large number of proteins with C-type CRDs or related domains. The CRDs of different lectins in this family bind at least 5 different carbohydrates (Drickamer 1989). In addition, there are related proteins with CRD-like sequences that bind to other ligands including other proteins (Clemmensen 1989, Vercelli et al. 1989), bacterial cell walls (Jomori and Natori 1991), and $CaCO_3$ (Multigner et al. 1983). Thus, it appears that the CRD is a structure that, in different contexts, has evolved the ability to bind a variety of different target molecules. Carbohydrate binding has recently been shown to involve the residues of Ca^{2+}-binding site 2 (Drickamer et al. 1992). However, it is not clear whether CRD-related structures such as the AFPs bind their ligands at that site or on another surface of the molecule that is formed when the Ca^{2+}-dependent conformation is adopted.

Smelt and herring AFPs contain the conserved carbohydrate-binding residues in the second Ca^{2+}-binding site of galactose-binding CRDs (Weis et al. 1992, Drickamer et al. 1992). In contrast, sea raven AFP does not contain the conserved residues of Ca^{2+}-binding site 2. Therefore, it was anticipated that smelt and herring AFPs might bind galactose or other carbohydrates and that sea raven AFP would not. However, no carbohydrate-binding activity was detected by agglutination in any of the type II AFPs.

Agglutination of cells relies on divalent or multivalent carbohydrate binding. No oligomer formation was detected when the sea raven AFP was treated with a cross-linking agent. However, since the pattern of cross-linking is sensitive to many factors including the specificity of the cross-linker and the distribution of functional groups in the protein, the failure of to demonstrate an oligomer cannot be considered as proof that oligomers do not form (Das and Fox 1979). If the AFPs are monomeric, they would be unable to agglutinate cells even if they did recognize cell surface carbohydrates. Therefore, the initial agglutination experiments were followed up by affinity chromatography to determine whether monovalent carbohydrate binding might occur. Only a trace of carbohydrate binding was evident between smelt AFP and galactose. No detectable binding to this sugar was observed using herring or sea raven AFPs.

N-acetylglucosamine was detected in conjunction with the amino acid analysis of smelt AFP (Chapter 2). This was taken as evidence that the smelt AFP was a glycoprotein. The presence of an N-linked glycosylation motif in the sequence of the smelt AFP also supported this conclusion. However, these findings did not eliminate the possibility that the smelt AFP was behaving like a lectin and had bound the N-acetylglucosamine as a ligand. A more definitive answer was obtained by digesting the smelt AFP with N-glycosidase F. This enzyme cleaves the N-glycan linkage between Asn and N-acetylglucosamine without affecting other bonds within the protein or the carbohydrate moieties (Tarentino et al. 1985). The marked increase in mobility of the smelt AFP on SDS-PAGE following digestion with the enzyme indicated that an N-linked carbohydrate had been removed. Thus, it appears that N-acetylglucosamine is part of the covalent

structure of smelt AFP and not a ligand. This is consistent with the results of N-acetylglucosamine affinity chromatography which revealed no interaction between this carbohydrate and the smelt AFP.

The CRD-like structure of the type II AFPs may differ from the lectin CRDs in a way that impairs carbohydrate-binding. Drickamer (1992) noted that the discrimination against mannose in galactose-specific lectin CRDs and subtle differences in sugar specificities among lectins within the mannose and galactose binding groups cannot be accounted for strictly by differences at their carbohydrate-binding sites. Furthermore, the Ala → Ser substitution in the AFPs at the position corresponding to the 6th small hydrophobic core-forming residue of the MBP-A CRD may impair binding to carbohydrate as it does in MBP-A (Quesenberry and Drickamer 1992). The AFPs also contain two disulfide bonds not present in the lectins (Ng and Hew 1992 and Chapters 3 and 4). Thus, it is possible that the Ca^{2+}-binding site 2 of the AFPs is not fully accessible to carbohydrate. Alternatively, if Ca^{2+}-binding site 2 is the ice-binding site of the AFPs, it might adopt a conformation that allows recognition of a specific arrangement of hydrogen bonding sites in an ice crystal plane instead of the expected recognition of carbohydrate.

To determine whether C-type CRDs or related structures might have hysteresis-related activities, PSP and RSL were used. These proteins were ideal for this investigation because they consist of CRDs or CRD-like structures with no associated domains. Consequently, the presence or absence of hysteresis activity could be directly attributable to the CRD.

PSP appeared to be an interesing protein in which to search for hysteresis because it binds to an inorganic crystal, $CaCO_3$ (Multigner et al. 1983). Yet, no hysteresis was detected in a solution of PSP. Since pancreatic stones are extremely rare, it was not possible to obtain enough protein to optimize conditions or to use more sensitive assays. Thus, an AFP-like activity may have gone undetected. However, considering that some AFPs appear to rely on repeated hydrophilic amino acids for lattice matching to ice and that the preferred orientations of certain AFPs on ice crystals appear distinct and specific (Davies and Hew 1990, Knight et al. 1991), the suggestion that human PSP might act like an AFP, based simply on its ability to bind a different crystal, may not be well founded.

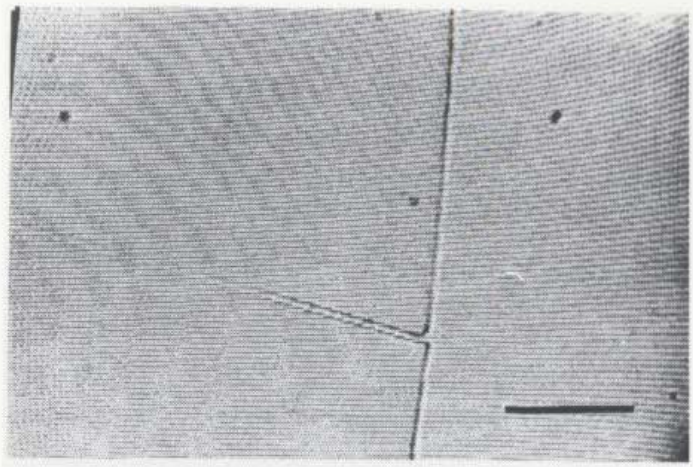
The absence of measurable hysteresis in a solution of PSP does not rule out the possibility that the CRDs of other proteins in the C-type lectin family might possess this property. To continue the search for thermal hysteresis activity in the C-type lectin CRDs, the RSL was used. This protein shares a higher percentage of identical amino acids with the AFPs than do the rat and human PSPs. Furthermore, C-type lectins that bind galactose, including RSL, appear the most closely related to the AFPs (see Chapter 4). In addition, the closer evolutionary relationship between reptiles and fish suggested that RSL might be less divergent from fish AFPs than are those found in invertebrates or in higher vertebrates.

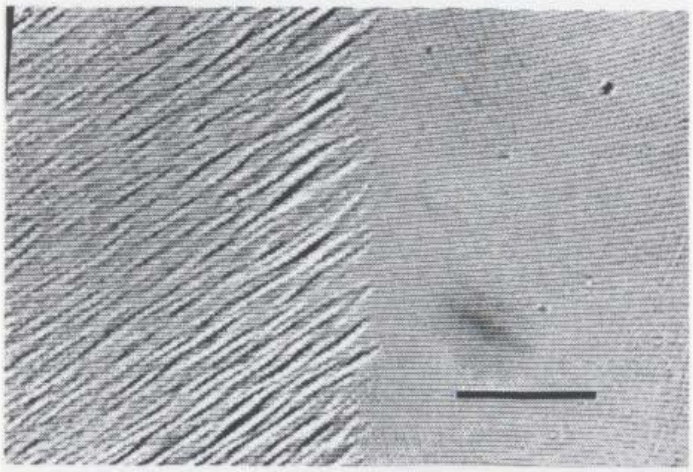
No thermal hysteresis was detected in solutions containing high concentrations of RSL. Thus, RSL does not appear to be an AFP. However, a study using RSL purified exactly as described here showed that this protein alters the morphology of the ice-water interface in a solution undergoing directional solidification in a manner similar to the AFPs

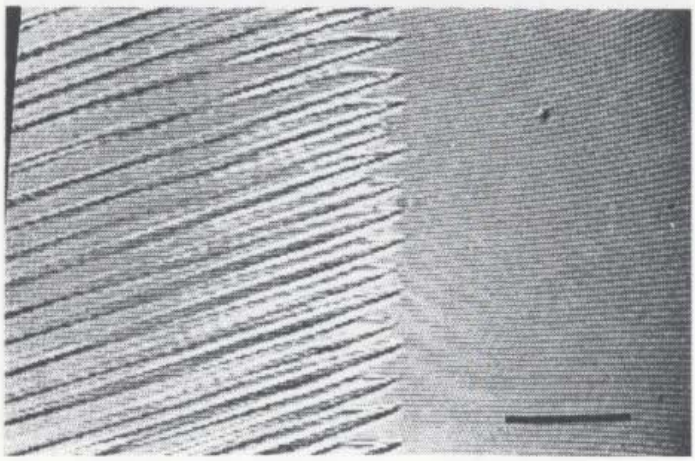
(Rubinsky et al. 1992c) (Fig. 44, centre and lower panels). Modification of the ice-water interface in the presence of RSL was evaluated using a directional solidification stage (Rubinsky and Ikeda 1985). The development of faceted ice crystals in RSL solutions was not caused by the colligative properties of this protein. Inorganic solutes, thought to have strictly colligative effects, resulted in flat ice-water interfaces even when present at much higher concentrations than RSL or the AFPs (Rubinsky et al. 1992c) (Fig. 44, upper panel). Furthermore, the presence of faceted ice crystals in RSL solutions implies that ice growth in the direction normal to the facet plane has been inhibited. Thus, it appears that RSL is adsorbing specifically to one crystallographic plane. This result is similar to the unique binding plane specificities of different type I AFPs reported by Knight et al. (1991).

If RSL binds to a specific ice crystal plane and produces facets as the AFPs do, why does it not cause hysteresis in the same concentration range as the AFPs? Crystal growth rates in the presence of a synthetic type I AFP and a number of sequence variants suggest that ice growth is a 2 step process (Wen and Laursen 1992). First, at low concentrations, AFPs bind to specific ice crystal planes causing faceted bipyramidal crystals to develop. Second, it is at higher AFP concentrations that ice crystal growth inhibition occurs. The weaker the affinity of an AFP for ice, the higher its concentration must be in order to reach the threshold at which ice growth is inhibited (Wen and Laursen 1992). Thus, RSL may have a relatively weak affinity for ice such that high concentrations are sufficient to cause facets to develop but not sufficient to stop ice crystal growth. This is consistent with the properties of AFPs at extremely low concentrations which include the

Fig. 44. Water-ice interface morphologies observed on a directional solidification stage. Interfaces formed in the presence of the following solutions: 5 mM NaCl (upper panel); 0.71 mM sea raven AFP (centre panel) ; and 0.71 mM RSL (lower panel) . The line represents 50 µm. Taken from Rubinsky et al. (1992c).







ability to cause ice facets to develop and to inhibit recrystallization (Knight et al. 1984, Knight and Duman 1986) but not the ability to generate hysteresis.

Since the RSL CRD can bind to an ice crystal plane, it is reasonable to suggest that type II AFPs evolved from one or more members of the C-type lectin family simply through selection for CRDs with an enhanced affinity for ice. The AFPs do not appear to bind appreciably to carbohydrates. Therefore, the characteristics that enable the CRD-like structure of the type II AFP to bind ice effectively enough to cause hysteresis may also restrict carbohydrate binding. The conserved residues of Ca^{2+}-binding site 2 in galactose-binding lectins (Drickamer 1992) are all present in the herring and smelt AFPs. Thus, it appears that in the AFPs, these sites are altered in some way. This site appears even further altered in the sea raven AFP since this AFP does not bind Ca^{2+}. The sea raven AFP is also a more effective AFP than those of smelt and herring (Chapter 2). Thus, improved hysteresis activity appears to correspond with the degree of divergence from C-type lectin CRDs. These results raise the possibility that a modified Ca^{2+} and carbohydrate-binding site may be the ice-binding site of the type II AFPs. This would be an interesting starting point for structure-function studies of the type II AFPs.

CHAPTER 5:

GENERAL DISCUSSION

5.1 Review of primary objectives

The present study was undertaken in order to gain a better understanding of the structures and functions of AFPs and the occurrence of different AFPs among teleost fish. The strategy used was to investigate the AFPs of fish species divergent from those in which AFPs had previously been identified. Specifically, the objectives of this study were to (1) isolate and characterize the AFPs of smelt and herring, (2) clone and sequence cDNAs for these AFPs in order to deduce their protein sequences, and (3) use knowledge derived from the new AFP sequences in order to further characterize these proteins. These goals were achieved and, in doing so, interesting discoveries were made concerning the type II AFP that was originally only known to occur in sea raven. Insights were gained into the origins of the type II AFPs, aspects of their main chain folding patterns were made evident, and study of the structure/function relationship in this AFP type was begun.

5.2 The presence of AFPs in smelt and herring

In a first approach, the AFPs of both fish species were isolated using gel filtration, ion exchange chromatography, and reverse phase high performance liquid

chromatography. The AFPs of smelt appeared to consist of 6 active isoforms and those of herring of two active isoforms.

Isolation of AFPs from smelt and herring was necessary in order to study these new AFPs. However, the identification of AFPs in smelt and herring also presents many opportunities to gain further knowledge of the biology of these 2 species. For example, in Atlantic cod, patterns of AFP production appear to influence habitat selection and overwintering behaviour (Goddard et al. 1992). In herring, like cod, juvenile fish have higher blood plasma AFP levels that the adults do (Chadwick et al. 1990). Since the overwintering behaviour of some herring populations is unclear at present, investigation into the onset of AFP production and levels attained may help to elucidate their migration potential. Similarly, the overwintering behaviour of smelt is not completely understood, although they are widely believed to overwinter in shallow nearshore locations (MacKenzie 1964, Scott and Scott 1988), so study of their AFP production may be informative.

Now that AFPs are known to occur in smelt and herring, the synthesis and the regulation of these AFPs and their roles in the freeze resistance and possibly in the cold tolerance of these fish can be investigated. Herring and smelt collected during winter months appear to resist freezing over extended time intervals in icy seawater (Brawn 1960, and personal observations). Thermal hysteresis appears to contribute significantly to the freezing point depression of smelt and herring blood. However, other adaptations may also be involved. In smelt, AFPs may not be the only means of protection from freezing. In a Pacific subspecies of smelt (Osmerus mordax dentex), high concentrations of glycerol are found and this compound appears to act as a colligative antifreeze (Raymond 1992).

Since elevated glycerol levels have also been detected in Q. mordax (Raymond 1992), it may further lower their freezing points. In winter flounder and in salmonids, it has been shown that the epidermis may act as a barrier to ice crystal propagation (Fletcher et al. 1988, Valerio et al. 1992). Similar mechanisms may operate in smelt and herring. In addition, since herring are euryhaline and smelt are anadromous (MacKenzie 1964, Brawn 1960, Scott and Scott 1988), they may avoid freezing by moving into waters with lower salinities, and consequently, higher freezing points.

The isolation of AFPs from smelt and herring and the availability of antisera and cDNA probes for both will make it possible to investigate the contribution of AFPs to the winter survival of these fish species.

5.3 Smelt and herring AFPs identified as type II

Several features of smelt and herring AFPs were generally consistent with those of other known AFPs. The antifreeze activities of the smelt and herring AFPs on a concentration basis were in line with those previously found (Kao et al. 1986). The AFPs of smelt and herring also displayed microheterogeneity as the other AFPs do. The presence of multiple isoforms of AFPs appears to result, at least in part, from their being encoded by multigene families. This appears to be the case for ocean pout, in which the AFP variants appear to be separate gene products (Hew et al 1988) and in the sea raven, which has 12-15 copies of the AFP gene and at least 2 sequence isoforms (Hayes et al. 1989). Thus, it will be interesting to determine whether smelt and herring also have AFP multigene families.

The smelt AFP differed from that of herring in that it contained a small amount of glucosamine. The presence of carbohydrate in smelt AFP showed that the AFGP are not the only glycoprotein antifreezes. However, there are differences between the AFGPs of cods and notothenioids and the smelt AFP. The carbohydrate of smelt appears to be a relatively minor component of this protein. It is an N-linked sugar and it appears to contribute a small proportion of the molecular mass. To date, there is no evidence suggesting a role for this carbohydrate in antifreeze activity. In contrast, the carbohydrate moieties of the AFGP are O-linked disaccharides present on every third residue (Thr) and they contribute close to half of the total mass. They are also necessary for antifreeze activity of the AFGPs (Ahmed et al. 1973, 1976, Geoghegan et al. 1980).

Characteristics that so far had distinguished sea raven AFP from the AFPs of other fish were shown to be shared by those of smelt and herring. The M_rs of smelt and herring AFPs were 24,000 and 14,600, respectively. Amino acid analysis showed both proteins to be cystine-rich, like that of sea raven and the activity levels of smelt and herring AFPs were reduced 72 and 57% in the presence of DTT, indicating the importance of their disulfide bonds. In addition, smelt AFP was found to be immunologically similar to those of sea raven and herring. The apparent similarity between the AFPs of smelt, herring, and sea raven prompted a determination of the primary structures of the two new AFPs.

5.4 Homology among smelt, herring, and sea raven AFPs

A cDNA library was prepared from smelt liver and an AFP cDNA was isolated from it. The protein sequence derived from this cDNA was homologous to that of sea raven. Cloning was repeated to construct a herring liver cDNA library. Analysis of a herring AFP cDNA revealed that it too encoded an AFP precursor homologous to those of both smelt and sea raven.

Smelt, herring, and sea raven belong to taxonomic groups believed to have diverged prior to Cenozoic glaciation. Yet, antifreeze function is believed to have evolved initially in response to this cooling event (Scott et al. 1986). Thus, it appears that the type II AFPs evolved from a single protein or related proteins that were widely distributed across fish taxonomic groups.

All 3 AFPs were homologous to members of the C-type lectin family. Sequence alignment revealed that the type II AFPs all form structures related to the CRDs that occur alone or joined to other domains in all C-type lectins. It appears that C-type lectins and related proteins exist in fish and that during recent cooling events, AFPs evolved from one or more proteins in the C-type lectin family.

It is not clear whether the three type II fish AFPs have evolved from the same protein or different ones. However, the high percentage of identity (83%) shared by the sequences of smelt and herring AFPs argues for a common direct ancestor. Presumably, the evolutionary precursors of type II AFPs were soluble proteins present in blood and extracellular fluids where they could interact with ice crystals. In addition, sequence

alignment shows that the AFPs are most closely related to the galactose-binding lectins and related non-lectin proteins. Therefore, the proteins that gave rise to these AFPs are more likely to have been one of these proteins than a mannose- or fucose-binding lectin. In this regard, it is interesting that a soluble Ca^{2+}-dependent lectin has been purified from the plasma of a freshwater fish, Channa punctatus, and it binds Gal(ß1-3)GalNac (Manihar and Das 1990). Although no sequence has been reported for this lectin, its properties are consistent with those of C-type lectins. If a lectin like this one is present in the plasma of marine fish species, it could be a direct ancestor of the AFPs.

5.5 Structral and functional similarity between AFPs and proteins of the C-type lectin family

Alignment of vertebrate C-type lectins has revealed the presence of many identical and conserved residues (Drickamer 1989). Determination of the crystal structure of a C-type lectin CRD from rat MBP-A revealed that most of these conserved residues were cysteines involved in disulfide bonds, residues forming the hydrophobic cores, and the oxygen-containing residues of the two Ca^{2+}-binding sites in that CRD (Weis et al. 1991a). This suggested that the fold of rat MBP-A determined from its crystal structure is shared by the different C-type lectins. Analysis of smelt, herring, and sea raven AFP sequences revealed that they contained 19 of the 20 conserved residues that constitute the C-type CRD hydrophobic cores. In addition, smelt and herring AFPs had the residues conserved in one

Ca^{2+}-binding site (site 2) while sea raven AFP did not appear to contain conserved residues corresponding to either CRD Ca^{2+} binding site.

These results suggested a structure for the type II AFPs. They also indicated that the type II AFPs might share some functional characteristics with the C-type lectins. The detection of an intact Ca^{2+}-binding site in smelt and herring AFPs led to an investigation of the effects of Ca^{2+} on antifreeze activity. Both proteins require Ca^{2+} for thermal hysteresis. In contrast, sea raven AFP does not require Ca^{2+} in order to function. Thus, Ca^{2+}-binding site prediction by sequence alignment was consistent with the Ca^{2+} requirements of these proteins for activity.

Further investigation of functional similarity between the type II AFPs and other members of the C-type lectin family revealed a slight overlap in the repertoire of ligands recognized by these proteins. A trace of smelt AFP bound to a gel composed of galactose. It is not clear from these experiments whether the AFP isoforms of smelt have a uniform but weak affinity for galactose or a single minor AFP isoform binds galactose while the others do not. This question could be addressed by isolating the different isoforms of smelt AFP and measuring their affinities for carbohydrates separately.

All residues of Ca^{2+}-binding site 2 in galactose-binding lectins (Drickamer 1992) are present in the smelt and herring AFPs. However, the AFPs have a more complex disulfide bonding pattern (Ng and Hew 1992) than do the known C-type lectins. Furthermore, a non-conserved residue in the small CRD-like hydrophobic core that is known to weaken Ca^{2+} and carbohydrate binding in MBP-A (Quesenberry and Drickamer

1992) is present in the AFPs. These differences may explain the very low affinity of the AFPs for carbohydrate.

5.6 Future directions

5.6.1 Structure and function of type II AFPs

In AFGPs and AFP type I, short repeating units and small overall sizes have facilitated relatively direct approaches to the study of their structure/function relationships (Davies and Hew 1990). In addition, the limited number of candidate residues for interaction with ice in the type III AFP has made site-directed mutagenesis a feasible approach to identifying key residues in ice interaction (Li and Hew 1991). In contrast, the larger sizes and apparently folded structures of the type II AFPs have made it impossible to predict from their sequences which residues, or even which surfaces, might bind to ice. It appears likely that the type II AFPs function as antifreezes in a similar manner to types I, III, and the AFGPs. Surface second harmonics measurements showed that AFGPs adsorb to ice (Brown et al. 1985) and all AFP types generate similar crystal growth modifications (Raymond et al. 1989). However, the interactions between type II AFPs and ice are not understood.

The structural and functional similarity between the type II fish AFPs and members of the C-type lectin family has allowed a direct jump from primary structures to a tertiary model. Since the crystal structure of MBP-A is known, alignment of the AFPs with the lectins can be used to predict which amino acids are on the surface of these AFPs. This

will narrow down considerably the number of potential residues for investigation in structure/function studies.

Continuing refinement of the understanding of C-type lectin structure and function is also likely to generate new information pertinent to the functional mechanism of the type II AFPs. The recent discovery that MBP-A binds carbohydrate at its second Ca^{2+}-binding site (Weis et al. 1992) raises the possibility that ice may also be recognized at that site. There are, however, two caveats to this idea. The first is that AFGPs and AFP type I both appear to interact with ice through a repeated linear array of contact points. In the AFGPs, the longer polymers are the more effective antifreezes (Raymond and DeVries 1977, Burcham et al. 1986). Similarly, type I AFP analogues with 4 or 5 internal repeats are more effective than a 3-repeat AFP like that of winter flounder (Mueller et al.1991). The Ca^{2+} and carbohydrate-binding site of C-type CRDs (Weis et al. 1992) appears rather limited in comparison. The second is that the residues corresponding to the Ca^{2+} and carbohydrate binding site of the C-type lectins are identical in the smelt and herring AFPs and the galactose-specific C-type lectins (Drickamer 1992). Hence, it is difficult to explain why one of these lectins, RSL, does not show antifreeze activity. The subtle structural differences between the type II AFPs and the C-type lectins will have to be investigated in order to better understand their different activities.

Structure/function studies involving the generation of AFP mutants altered at the Ca^{2+}-binding sites, at other surface residues, at some of the cysteine residues, and in the hydrophobic cores will be useful in determining whether there is a relationship between carbohydrate binding and interaction with ice. To begin with, changing the non-conserved

Ser in the AFP small hydrophobic core to a residue normally found in lectins at that position would be a very interesting approach.

5.6.2 Search for homologous insect AFPs

One or more of the cystine-rich AFPs of insects may be related to the fish type II AFPs. The independent evolution of type II AFPs in separate fish groups from proteins in the C-type lectin family implies that homologous type II AFPs could have evolved in insects as well. No cDNA or protein sequence from an insect AFP has been reported to date. However, immunological cross-reactivity between the AFPs of sea raven and spruce budworm (Hew et al. 1983) suggest a similarity. Proteins in the C-type lectin family have been found throughout the animal kingdom. Examples in insects are the lectin of fleshfly, (Sarcophaga peregrina) (Takahashi et al. 1985) and a similar protein found in the cockroach (Periplaneta americana) (Jomori and Natori 1991). If a homologous AFP is found in an insect, comparison to the fish AFP might also further our understanding of the structure/function relationship of type II AFPs.

5.6.3 Investigation of other AFP functions

In addition to their antifreeze activity, the type II AFP of sea raven and AFPs of the three other types are able to protect mammalian cells from hypothermic damage (Rubinsky et al. 1990, 1991). Since type I AFP from winter flounder and type III AFP from ocean pout have been shown to block Ca^{2+} currents (Rubinsky et al. 1992b, Negulescu et al. 1992), it is possible that all four types protect cells from hypothermia by blocking flux

through specific ion channels. Since herring and smelt AFPs resemble all four AFP types in their antifreeze activity and they are homologous to the sea raven AFP, they are also likely to protect cells from hypothermic damage.

The discovery of new properties shared by the AFPs raises several questions. Do all AFPs protect cells from hypothermic damage? If so, do they all achieve this by interacting with Ca^{2+} channels? Is this function related to adsorption to ice crystals? One way to begin investigating the relationship between these two seemingly disparate functions of AFP is to determine whether AFP antifreeze activity levels correspond to their effectiveness in hypothermic protection. The three type II AFPs are homologous and they appear structurally similar. Since their antifreeze activities are different, it would be useful to determine whether their abilities to protect cells at low temperatures correspond to their activity levels. Furthermore, if mutants are generated for structure-function studies that have altered antifreeze activity, it would be interesting to determine whether they also differ from authentic AFP in hypothermic protection.

5.6.4 Exploration of possible C-type lectin functions

C-type lectins occur in a variety of animals. The functions of some of these lectins, such as the mammalian asialoglycoprotein receptors (hepatic lectins), appear to be well defined (Spiess 1990). In contrast, the roles of many other C-type lectins, such as the invertebrate lectins, are not as clearly understood. Roles in host defense against pathogens and in developmental processes have been postulated (Takahashi et al. 1985, Muramoto

and Kamiya 1990, Suzuki et al. 1990). However, the homology of these C-type lectins with the type II AFPs suggests a possible functional relationship.

It is unlikely that C-type lectins protect these animals from freezing. In most environments, freeze avoidance is not necessary. In addition, RSL showed no antifreeze activity and lectins are not normally present in the concentration range at which most AFPs are effective. However, all four fish AFP types can protect mammalian cells from hypothermic damage (Rubinsky et al. 1990, 1991) by a mechanism that may be related to their ability to block specific ion currents. It will therefore be useful to investigate whether any C-type lectins or related proteins can have a role in hypothermic protection or interact with ion channels. If such a function were attributed to a C-type lectin, it could be considered as host defense, but in a more general sense.

Bryozoan lectins that are Ca^{2+}-dependent show seasonal patterns of expression that are distinct from one another (Colon-Urban and Oppenheim 1990). The relationship between lectin levels and seasonal parameters has not been investigated and the role of these lectins is not yet known. Yet, the possibility of AFP-like properties in proteins other than AFPs in the C-type lectin family could become an interesting area for future research.

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