ISOLATION, CHARACTERIZATION AND STRUCTURAL STUDIES OF THE ANTIFREEZE POLYPEPTIDES FROM OCEAN POUT (MACROZOARCES AMERICANUS)

XIAOMAO LI

ISOLATION, CHARACTERIZATION AND STRUCTURAL STUDIES OF THE ANTIFREEZE POLYPEPTIDES FROM OCEAN POUT

Macrozoarces americanus

BY

© XIAOMAO LI, B.SC.

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ABSTRACT

Antifreeze polypeptides (AFP) were separated successfully from the serum of ocean pout *Macrozoarces americanus* using Sephadex G-75 gel-filtration, QAE-Sephadex and SP-Sephadex ion-exchange chromatography and reverse phase HPLC.

It was found that the ocean pout AFP consists of a multiple family of at least twelve active components of nearly identical size (about 6,000 daltons), which can be classified into two separate groups, named QAE and SP, on the basis of their amino acid compositions and their behaviour on ion-exchange chromatography.

A partial amino acid sequence of SP-1-A, one of the major ocean pout AFP, was established up to 41 residues from its chymotryptic peptides using a protein sequenator. SP-1-A was compared with two other major components, namely SP-1-B and SP-1-C, by tryptic and chymotryptic peptide mapping and amino acid analyses. They showed overall structural similarities with minor differences.

A cDNA coding for the biosynthetic precursor of one of the ocean pout AFP was isolated and purified from the plasmid pBR 322 in *E. Coli* HB101. It was then cloned into phage M13 mp8. The sequence of the cDNA was determined by the dideoxy chain termination method. It had untranslated regions at both 3' and 5', and a coding region for a signal peptide containing 22 amino acid residues and a mature polypeptide containing 65 residues. The sequence of the mature polypeptide matched the protein data from both SP-1-B and SP-1-C. The results

from both protein chemistry and molecular biology showed that SP-1-A, SP-1-B and SP-1-C have very similar amino acid compositions and sequences with only minor differences. For example, SP-1-A contains Ile and Ala at positions 53 and 62, while both SP-1-B and SP-1-C contain respectively Leu and Val at the same positions. SP-1-C has an additional Gly at its C-terminal compared to both SP-1-A and SP-1-B.

The amino acid analyses showed that SP-1-A, SP-1-B and SP-1-C contain fourteen types of amino acids with modest contents of Ala and no half-cystine. There is no obvious repeating structure in ocean pout AFP. These results confirm that ocean pout AFP represent a new type of fish antifreeze proteins.

Ocean pout AFP from individual fish caught in both winter and summer from Newfoundland, and in winter months from New Brunswick were analyzed on reverse phase HPLC. The results did not show any significant populational or seasonal polymorphisms.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AFGP	antifreeze glycoprotein(s)
AFP	antifreeze protein(s)/peptide(s)
Bis	N, N'-methylene-bisacrylamide
BSA	bovine serum albumin
CD	circular dichroism
cDNA	complementary DNA
CM	carboxymethyl
ddNTP	2', 3'-dideoxynucleoside triphosphate
DNAase	deoxyribonuclease
DNS-Cl	dansyl chloride
E. Coli	*Escherichia coli*
HPLC	high performance liquid chromatography
I.D.	internal diameter
IPTG	isopropyl-β-D-thiogalactopyranoside
M.W.	molecular weight
PEG	polyethylene glycol
QAE	diethyl-(2-hydroxy-propyl) aminoethyl
RF	replicative form
RNAase	ribonuclease
RPM	revolutions per minute
SDS	sodium dodecyl sulphate

SP	sulphopropyl
SS	shorthorn sculpin
ss	single-stranded
TBE buffer	0.089M Tris, 0.089M boric acid, 0.002M EDTA buffer
TE buffer	10mM Tris-HCl, pH8, 1mM EDTA buffer
TEAP	triethylamine phosphate
TEMED	N, N, N′, N′-tetramethylethylenediamine
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TLCK	N-α-p-tosyl-L-lysine-chloromethyl ketone
TPCK	L-1-tosylamide-2-phenolethyl-chloromethyl ketone
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Introduction

In the polar and subpolar regions, sea water temperatures can be as low as -1.8°C (1). To survive in such a harsh environment, some marine organisms have developed various types of adaptation. The most intriguing one is the occurrence of a unique class of serum proteins, which specifically lowers the freezing temperature of the plasma to a point that is below that of the surrounding water. These freezing point-depressing proteins are commonly known as antifreeze proteins.

In 1953, Scholander and colleagues first reported a trichloro acetic acid-soluble macromolecular substance in the serum of a northern polar fish which could lower the serum freezing temperature (2, 3). This substance was found later to have a proteinaceous nature. Since then, many species of fish have been found to contain these interesting proteins. Based on their amino acid and carbohydrate compositions, freezing point-depressing proteins can be classified into two distinct groups. They are antifreeze glycoproteins (AFGP) and antifreeze proteins (AFP).

1. PROPERTIES OF MACROMOLECULAR AFGP AND AFP

All antifreeze proteins share the following characteristics.

1. Their effect on freezing point-depression is noncolligative, that is, they lower the freezing point much more than would be expected on the basis of the osmolality of their solutions.

2. They show thermal hysteresis, that is, there is a difference between the apparent freezing and melting temperatures, with the latter being equivalent to what would be expected from the osmolality of the antifreeze solutions.

3. Plots of thermal hysteresis versus antifreeze concentration are convex.

rather than linear, which means that there is a saturation effect at higher protein concentrations.

4. The overall freezing point-depression by antifreeze is the sum of its thermal hysteresis and the depression due to the colligative property of the antifreeze solution, the latter being minimal (4).

2. DISTRIBUTION AND STRUCTURE OF AFGP

AFGP have been found in antarctic fishes, such as ***Trematomus borchgrevinki***, ***Dissostichus mawsoni*** (5), in arctic fishes, like the polar cod ***Boreogadus saida*** (6), saffron cod ***Eleginus gracilis*** (7), and more recently, the tomcod ***Microgadus tomcod*** (8), atlantic cod ***Gadus morhua*** (9), and Labrador cod ***Gadus ogac*** (10) in Newfoundland and Labrador waters. The primary structures of the AFGP from ***T. borchgrevinki***, ***D. mawsoni***, ***B. saida***, ***M. tomcod*** and ***G. morhua*** have been well established (11, 12, 13, 5, 8, 9). They have similar, if not identical, structures. AFGP are a mixture of closely related components which differ structurally in size and the presence of Pro and/or Arg in the smaller components. For example, the AFGP from both ***T. borchgrevinki*** and ***D. mawsoni*** consist of eight polypeptides, made up of a repeating tripeptide unit of Ala-Ala-Thr with the disaccharide galactosyl-N-acetylgalactosamine glycosidically linked to Thr residues. The internal linkage between the sugar monomers is β1-3. The tripeptide unit is repeated seventeen to fifty times, giving the eight components of molecular weights 2,600 to 33,000. The smaller components 6-8 differ from the bigger ones in the replacement of Pro for Ala at certain positions. Similarly, in ***M. tomcod***, Arg replaces Thr in some of the smaller components (8).

Viscosity and circular dichroism studies on the AFGP from ***T. borchgrevinki***

suggested either an extended conformation or a completely flexible random coil secondary structure without any indication of α-helix or β-structure (5). More recently, a careful CD study suggested a 3-fold left-handed helix of the collagen type as the most likely conformation of this AFGP (14).

An interesting observation has been the cooperative function between AFGP (15). The Pro-containing smaller components (AFGP 7 and AFGP 8) exhibited weak antifreeze activities, but if one of them was mixed with any of the active AFGP - the larger components (AFGP 1-5), a very large (2-8 fold) potentiation of antifreeze activity had been observed. It was also reported that structural similarity was necessary for the cooperative function, because the antifreeze activity did not potentiate when the smaller peptide was mixed with flounder AFP (16). However, these observations have recently been disputed by other laboratories (personal communication with Dr. Hew).

3. THEORIES OF THE MECHANISMS OF AFGP ACTIVITY

The so-called "interfacial mechanism" is the most popular one (3). The main idea of this model is that active AFGP function at an interfacial region between the ice or ice-nucleus and the solution to inhibit the growth of the ice. Some experimental data supporting the model include:

1. The a-axis growth of the ice was greatly impaired in the presence of active AFGP (AFGP 1-6); in contrast, the a-axis growth was normal in the presence of inactive AFGP (AFGP 7-8) or a nonantifreeze glycoprotein. This suggested that the active AFGP shield the ice nucleus. There were also observations that active AFGP became "entrapped" as ice growth continued (3).

2. Almost all chemical modifications on the disaccharide groups, such as, graded removal of the sugar side chain by β-elimination, acetylation of

the hydroxyl groups and positioning a negative charge on C-6, inactivated AFGP as antifreeze reagents (17. 12). This suggested that the role of AFGP disaccharide is to disrupt ice surface and that this is indispensible for the antifreeze activity.

Other mechanisms like "liquid phase interaction" or "ice phase interaction" have also been postulated, the former suggesting that certain hydrophilic groups of the AFGP may interact with water molecules to disrupt the local tetrahedral coordination of the liquid water, and the latter suggesting that AFGP can somehow enter into some form of solid solution with water molecules and thus completely alter the structure and properties of the system from those of pure water (3).

4. DISTRIBUTION AND STRUCTURE OF AFP

Research carried out over the past decade has led to a further understanding of AFP. Until now, AFP have been studied in four species of fish (18, 19, 20, 21, 22). They are winter flounder ***Pseudopleuronectes americanus***, shorthorn sculpin ***Myoxocephalus scorpius***, sea raven ***Hemitripterus americanus*** and ocean pout ***Macrozoarces americanus***.

Among these, flounder AFP have been studied in most detail. DeVries and coworkers reported three components of molecular weights 6,000, 8,000 and 12,000 in the AFP from flounder (18). At about the same time, Hew and colleagues reported a major component, separated from a Sephadex G-75 column, of molecular weight 10,000 (19). Later, when the single 10,000 dalton component was analyzed on reverse phase HPLC, it resolved into at least seven components of molecular weights 3,300 to 4,500 (23). The two most abundant components,

No.6 (or A) and No.8 (or B), were 3,300 daltons each. Different from the AFGP discussed above, these two components comprised nine different types of amino acids with Ala accounting for 60 mol % (23, 24, 25). Both of these AFP were 37 residue polypeptides containing three eleven amino acid residue repeats of Thr-Ala-Ala- polar amino acid -Ala-Ala-Ala-Ala-Ala-Ala-Ala. It is clear that the Ala content in AFGP and the AFP from winter flounder are similar inspite of the fact that other amino acid compositions are different and that AFGP have disaccharides. The difference between the A and B components in winter flounder AFP was due to the replacements (from A to B) in position 18 (Lys→Ala), position 22 (Glu→Lys) and position 26 (Ala→Asp). CD studies showed that flounder AFP possessed a large proportion (about 85%) of α-helical conformation in aqueous solution at -1°C. Viscosity studies at -1°C indicated an asymmetric shape (26).

Flounder AFP are synthesized individually via larger precursors (24, 25). Complementary DNA made to the mRNA of the A and B component precursors were cloned in the plasmid pBR322 and their sequences were determined by the method of Maxam and Gilbert (24, 25). The preproprotein sequences of both components are 82 residues long. The 'prepro' parts of the two precursors are identical. The mature sequences of both A and B components contain 37 amino acid residues. The difference between the two is at three positions as mentioned above. Also there are several silent changes in the nucleotide sequences encoding the mature proteins (24, 25).

With shorthorn sculpin AFP, two components were detected using Sephadex

G-75 gel-filtration and QAE-Sephadex (A25) ion-exchange columns with the major one (B) accounting for 76% of the material and the minor one (A) for 24% of the material (21). The component B had many characteristics in common with flounder AFP, such as, size (as estimated on Sephadex G-75 column, about 10,000-11,000 daltons), amino acid composition, the abundance of Ala (about 60 mol %), thermolysin peptide mapping and high α-helical content (about 58% at -1°C), but it had twelve types of amino acids instead of nine (21, 27). Recently, the two most abundant components (SS-8, SS-3) have been isolated by Sephadex G-75 column and reverse phase HPLC. SS-8 and SS-3 are polypeptides containing 45 and 33 amino acid residues respectively. Both of these have blocked N-terminals (28). The presence of three repeating structures of eleven amino acid residues in sculpin AFP was consistent with that of flounder AFP and supported the idea that they belong to the same group.

The AFP from sea raven, on the other hand, have different characteristics from those of the AFP discussed above. One major component was isolated using Sephadex G-75 and QAE-Sephadex (A25) columns. The molecular weight of the component was larger (approximately 14,000-16,000). It contained eighteen types of amino acids with a high content of half-cystine (about 8 mol %) and only an average amount of Ala (about 14.4 mol %). Furthermore, it was sensitive to sulphhydryl reagents. Circular dichroism studies indicated the absence of a significant amount of α-helix and a possible presence of β-structure. Immunological studies also showed that sea raven AFP does not cross-react with antibodies raised to either flounder or sculpin AFP (20).

AFP from winter flounder and shorthorn sculpin appear to belong to the same group, while the AFP from sea raven represent another type of fish antifreeze polypeptides.

5. SEASONAL VARIATIONS OF AFP

With most of the fishes studied, the overall freezing point-depression, thermal hysteresis and level of AFP in the blood plasma show annual cycling with the maxima in winter and the minima in summer. In Newfoundland winter flounder, the maxima appeared from January to April and the minima from July to October when the water temperatures were -1.1 to -1.4°C and 10 to 14°C, respectively. The AFP began to appear in the serum in November and disappeared during May when the water temperatures were 4 to 6°C and -1.0 to 3.0°C respectively. There was very little or no AFP from May to November. The above cycle of the AFP correlated closely with that of the Newfoundland water temperature (29). With another population of the same species, the Nova Scotian flounder, the AFP level increased approximately one month later and declined two months earlier than it did in its counterpart Newfoundland flounder. This cycle also correlated closely with that of Nova Scotian water temperature (30). In shorthorn sculpin, the appearance of AFP in the plasma was at essentially the same time of the year as that of Newfoundland flounder, but the AFP disappeared later than in Newfoundland flounder. Significant amounts of AFP were still present in Newfoundland shorthorn sculpin during July when no AFP could be detected in the plasma of the Newfoundland flounder (21). Another population of sculpin, the arctic sculpin, however, showed that the concentrations of AFP in the plasma, even during summer, were comparable to those observed in the plasma of Newfoundland sculpin during winter (27).

From the above observations, it seems that the annual cycle of the AFP level is a common feature among the subpolar fishes, but there is a tendency for fishes inhabiting more polar oceans to maintain higher concentrations of AFP even in summer and their AFP levels decline later than those of less polar fishes. This is an example of how biological organisms adapt to the environment.

6. CONTROL OF THE AFP BIOSYNTHESIS

The annual cycles of the AFP indicate that environmental factors influence the appearance and the disappearance of the AFP from the plasma. It has been found that photoperiod plays a major role in the timing of the appearance of the AFP (31). Long day-lengths (>12 h) delayed the onset of the appearance of AFP. Water temperature affected only the rate of the disappearance of the AFP (4). Winter flounder maintained under long day-length had both a delayed appearance and a decreased accumulation of the AFP mRNA (32). It was found that the pituitary gland regulates the level of AFP via a negative mechanism. Hypophysectomized winter flounder continued to synthesize the AFP in summer or under the conditions of warm water (6 to 12 °C) and long day-length (18 h), while the sham-operated control group did not (33). Another experiment revealed that hypophysectomy of winter flounder resulted in the increase in liver weights, total liver poly A+ mRNA and the AFP mRNA (34). It was also found that the level of AFP mRNA in winter flounder showed an annual cycle, similar to but preceeding slightly that of AFP (35). All of these results suggested that photoperiod and water temperature may act as the initial cues for the control of the AFP biosynthesis, while a pituitary hormone may be the mediator and that the hormone may regulate the AFP biosynthesis by changing the level of the AFP mRNA using a negative transcriptional control mechanism.

Evidence has been found that translational control mechanisms operate to optimize the production of AFP in the winter flounder (36). Total tRNA from the liver of the winter fish showed an approximately 40% increase in Ala acceptor capacity over the tRNA from the summer fish; in contrast, the acceptor capacities for other amino acids showed no seasonal differences. Biosynthetic studies suggested that Alanyl-tRNA synthetase functioned best between 0 and 5°C which is the seawater temperature when AFP synthesis occurs, while Prolyl and Valyl-tRNA synthetases were most active between 20 and 30°C. So the Ala tRNA acceptor and the Alanyl-tRNA synthetase both function especially in winter to increase the translational efficiency of the AFP mRNA.

Recently, in winter flounder, three nonoverlapping chromosomal regions coding for the AFP have been reported (37). Each region contains two AFP genes spaced from 3 to 7 kilobase pairs apart, but all of the three regions represent only 10-20% of the complement of this multigene family. One of the six genes has been sequenced and identified as a variant of the gene coding for the most abundant AFP, component A. It is 1.0 kilobase pairs long and contains an intervening sequence of 0.6 kilobase pairs between the coding regions for the bulk of the signal sequence (presequence) and for the proprotein.

7. POSSIBLE MECHANISMS OF AFP ACTIVITY

Concerning the mechanisms of the AFP function, no direct evidence is available. DeVries suggested a model in which the α-helical antifreeze polypeptide oriented in such a way that all the polar residues, like Thr and Asp, were located on one side, while the nonpolar residues, like Ala, were located on the other side.

The polar residues could form hydrogen bonds with the water molecules in the ice lattice. In other words, the water molecules in the ice crystals adsorbed the AFP (38). The adsorption of the antifreeze to ice crystals could conceivably lead to an increase in the surface area with only a small increase in the volume, which could cause an increase in the surface free energy. In order for freezing to occur, the energy must be removed from the system and it would be done by lowering the freezing temperature. This model explained the noncolligative lowering of freezing temperature by AFP.

The model described above could not explain the high content of Ala in flounder and sculpin AFP. The conserved eleven amino acid residue repeat, present in both flounder and sculpin AFP which has a high content of Ala, implies that it may play a crucial role in the antifreeze function. A synthetic polypeptide containing 65 mol % Ala and 35 mol % Asp randomly distributed showed antifreeze activity (39). This result strongly emphasized the role of Ala. On the other hand, the sea raven AFP contains an average amount of Ala (14 mol %), but a high half-cystine content (8 mol %). This suggests that half-cystine may be involved in the function. Therefore, sea raven AFP may represent another type of mechanism. Coincidently, another half-cystine containing AFP was isolated recently from the second instar larvae of the spruce budworm ***Choristoneura fumiferana***. Like sea raven AFP, it only contained a modest amount of Ala (8 mol %) (40). It was interesting to observe that there was an immunological cross-reactivity between this AFP and the antibodies raised to sea raven AFP (40).

8. RATIONALE FOR THE PRESENT INVESTIGATION

Ocean pout (*Macrozoarces americanus*) AFP have been found earlier by our laboratory to differ from all of the three AFP types mentioned above. Ocean pout AFP have at least eight active components which fall into two distinct groups based on their behaviour on ion-exchange chromatography: that is, the CM-binding group and the QAE-binding group (22). Amino acid analyses showed that ocean pout AFP contain most, if not all, types of amino acids with modest Ala contents and no half-cystine at all. However, the amino acid compositions of the peptides in one group were quite similar. The circular dichroism spectra of ocean pout AFP at 0°C resembled the spectra from AFGP in the far UV region. Unlike the latter, however, they exhibited aromatic CD bands in the 250-300 nm region. Under different denaturing conditions, the CD spectra of ocean pout AFP were different, e.g. a sigmoidal conformational transition when heated to 60°C, a typical spectrum of denatured protein with 6M guanidine hydrochloride treatment and a change-over to α-helical spectrum when treated with SDS. Taken together, these results indicate that ocean pout AFP has a well defined secondary and tertiary structure, which differs from those of the other known AFP (41).

The level of ocean pout AFP also showed an annual cycle. However, unlike the three species discussed above, ocean pout contained a significant amount of AFP even in summer (22).

From the information above, it can be seen that ocean pout AFP have many unique features and it has been suggested that they represent a new type of fish AFP.

As a further step to understand the structure and mechanism of action of these AFP, a project was planned with the following objectives:

1. further characterization of the multiple ocean pout AFP components with ion-exchange chromatography and reverse phase HPLC;

2. elucidation of the primary structure of one of the major ocean pout AFP components using techniques in protein chemistry;

3. elucidation of the structural relationship of the members of the AFP family by peptide mapping and amino acid analyses;

4. investigation of the structure of the biosynthetic precursor(s) of the AFP;

5. investigation of the seasonal and populational variations of ocean pout AFP.

Chapter 1

Isolation And Characterization Of Ocean Pout AFP

Previous work carried out in our laboratory has shown that ocean pout AFP can be isolated and separated successfully by Sephadex G-75 gel-filtration chromatography, QAE, CM, SP-Sephadex ion-exchange chromatography and analytical reverse phase HPLC (22). Ocean pout AFP can be divided into at least eight active components of approximately identical size (about 6,000 daltons) by the above procedures (22). In the present study, Sephadex G-75 gel-filtration chromatography, QAE, SP-Sephadex ion-exchange chromatography and preparative reverse phase HPLC were used with some modifications and improvements. The purity as well as the seasonal and geographic variations of ocean pout AFP were examined.

1.1. MATERIALS AND METHODS

Ocean pout were collected by divers from waters around the Avalon Peninsula, Newfoundland and were kept at ambient seawater temperatures and photoperiods. Serum samples were prepared by low speed centrifugation (4,000 g x 15 min) of clotted blood and stored at -20°C.

The procedure used for the purification of the ocean pout AFP is described below. Although this is essentially the same as used before (22), modifications in the buffer systems and gradients were made. In addition, the CM-Sephadex ion-exchange chromatography step was omitted and the HPLC was used on both analytical and preparative scales.

1.1.1. SEPHADEX G-75 GEL-FILTRATION CHROMATOGRAPHY

Ocean pout serum (5 ml) was loaded on a Sephadex G-75 column (2.5 cm I.D. x 59 cm) in 0.1 M NH_4HCO_3 buffer. Fractions of 3 ml (or 50 drops) each were collected and pooled according to their absorbance at 230 nm. Two peaks were resolved. The first one corresponded to most of the serum proteins and the second peak contained antifreeze polypeptides as demonstrated earlier in our laboratory (22).

Fractions corresponding to the AFP peak were pooled, lyophilized and rechromatographed on the same column once. The material was designated G-75 AFP.

1.1.2. QAE-SEPHADEX (A25) ION-EXCHANGE CHROMATOGRAPHY

50 mg of G-75 AFP in 2 ml of starting buffer (5 mM Tris-HCl, pH 9.4) was dialysed against a litre of the starting buffer at 4°C overnight using dialysis tubing (M.W. cut-off 3,000). After application of the sample, elution was carried out with the starting buffer. Fifty fractions were collected (40 drops or 2.4 ml per tube), then a linear gradient of 0 to 0.2 M NaCl in the starting buffer was applied. Two peaks were obtained; the first one was the unbound peak and the second one was named QAEA.

The two samples were desalted on a Sephadex G-25 gel-filtration column (2.0 cm I.D. x 25 cm) equilibrated with 0.1 M NH_4HCO_3 buffer and lyophilized

1.1.3. SP-SEPHADEX (C50) ION-EXCHANGE. CHROMATOGRAPHY

The unbound peak from QAE-Sephadex (A25) chromatography (50 mg in 2 ml) was dialysed against 1 litre of starting solution (0.01 M NaCl, pH 3) at 4°C overnight. After sample loading, the column was washed with 90 ml of the starting solution. A linear gradient of 200 ml 0.01 M NaCl, pH 3 and 200 ml 0.2 M NaCl, pH 3 was employed afterwards. Fractions were collected in 3.0 ml sizes. Based on absorbance at 230 nm, 4 peaks were obtained and designated SP-1, SP-2, SP-3 and SP-4 respectively. They were lyophilized and desalted as in section 1.2.

1.1.4. REVERSE PHASE HPLC

Separation of peptides by reverse phase HPLC is based on the difference in their hydrophobicities. With a solvent gradient of increasing hydrophobicity, peptides elute from the column in order of increasing hydrophobicity. The gradient can be formed by increasing the percentage of the organic phase B (such as CH_3CN), while decreasing that of the water phase A.

By Sephadex G-75 gel-filtration and QAE, SP ion-exchange chromatography, ocean pout AFP had been separated into 5 groups: QAEA, SP-1, SP-2, SP-3 and SP-4. They were analyzed by reverse phase HPLC respectively. SP-1 was further purified by reverse phase HPLC.

1.1.5. MOLECULAR WEIGHT ESTIMATION BY GEL-FILTRATION HPLC AND SDS POLYACRYLAMIDE SLAB GEL ELECTROPHORESIS

It has been reported that, on gel-filtration by HPLC with a TSK-G3000-PW column and an eluant containing 0.1 % TFA and 45% acetonitrile, the elution volumes of proteins or peptides were linearly related to the logarithm of their molecular weights. This offers a rapid and sensitive method for the molecular weight estimation of polypeptides or proteins (42).

In the present study, Altex Spherogel TSK-3000-SW columns were used. Two columns (7.5 mm I.D. x 30 cm) were connected in series. The eluant used was 0.1 % TFA in 45 % CH_3CN with a flow rate of 0.5 ml per min.

A SDS polyacrylamide slab gel of gradient 9-25% acrylamide was employed (43). A 3 % stacking gel was layered on the top of the running gel. Samples were mixed with 2 % SDS, 5.7% β-mercaptoethanol, 10% glycerol and bromophenol blue, boiled for 3 min and then loaded on the gel.

Initially the electrophoresis was carried out at 60 V in 0.6 % Tris, 2.88 % glycine and 0.2 % SDS buffer. After the samples had entered the running gel, the voltage was increased to 120 V. Following electrophoresis, the gel was stained with brilliant blue (Coomassie R-250) and destained with methanol (5 % v/v) and acetic acid (7.5 % v/v).

1.1.6. AMINO ACID AND N-TERMINAL ANALYSES

50 μg of sample were hydrolyzed in 200 μl of 6 M HCl at 110°C for 24 h. The hydrolysis was carried out in a closed ampule which was initially evacuated. Amino acid analysis was performed on a Durum Amino Acid Analyzer by the Amino Acid Facility, The Hospital for Sick Children, Toronto.

For N-terminal analysis, 10 μg of sample were dissolved in 20 μl of 0.2 M $NaHCO_3$ and 20 μl of DNS-Cl (2 mg/ml in acetone). After incubation at 37°C for 1-2 h, the sample was dried and hydrolyzed with 6 M HCl at 110°C for 12-16 h. Polyamide TLC (5 cm x 5 cm) was used to identify the fluorescent N-terminal amino acid by comparing the position of the sample spot on one side of the plate with those of the amino acid standards on the other side after two dimensional running (44).

The solvents used in TLC were 1.5 % formic acid for the 1st dimension, benzene: acetic acid 9:1 (v/v) for the 2nd dimension (1st solvent), and ethylacetate:methanol:acetic acid 20:1:1 (v/v/v) for the 2nd dimension (2nd solvent).

1.1.7. MEASUREMENTS OF THERMAL HYSTERESIS OF OCEAN POUT AFP

Thermal hysteresis was measured by [redacted]. M. H. Kao using a nanolitre osmometer (Clifton Technical Physics, Hartford, CT, USA). Crystal growth or dissolution was observed under a light microscope. The osmometer was first calibrated with NaCl solutions of known osmolalities. The osmolalities of the

AFP solutions were then obtained and converted into thermal hystereses (T.H.) in °C by the equation, T.H. (°C) = 1.856 x 10^3 x mosmol/kg.

1.1.8. PURITY OF REAGENTS

Sephadex G-75, Sephadex G-25, QAE-Sephadex A25 and SP-Sephadex C50 were purchased from Pharmacia (Dorval, Montreal). Reagents for SDS polyacrylamide gel electrophoresis, such as, SDS, acrylamide, ammonium persulphate, TEMED, bromophenol blue and Coomassie brilliant blue were "Electrophoresis Purity Reagents" and purchased from Bio-Rad (Richmond, CA. USA). Acetonitrile was "High Purity Solvent" and purchased from Burdick & Jackson Laboratories, Inc. (Muskegon, MI. USA). All other chemicals were of reagent grade.

1.2. RESULTS

1.2.1. PRESENCE OF A MULTIPLE COMPONENT FAMILY OF OCEAN POUT AFP

Earlier research from our laboratory had shown that the slower running peak from Sephadex G-75 chromatography had antifreeze activity and that when this AFP (designated as G-75 AFP) was further fractionated by ion-exchange chromatography and reverse phase HPLC, it was resolved into at least eight active components (22).

In the present study, the G-75 AFP (Figure 1) was resolved into five groups, i.e. QAEA, SP-1, SP-2, SP-3 and SP-4 on QAE and SP-Sephadex ion-exchange chromatography (Figure 2). The G-75 AFP could be resolved analytically into at

Figure 1. Chromatography of the ocean pout winter serum on a Sephadex G-75 gel-filtration column in 0.1 M NH_4HCO_3 buffer at 4°C. The sample volume was 5 ml. The first peak had most of the serum proteins and the second one contained the antifreeze polypeptides (designated as G-75 AFP). (first run: o——o ; rechromatography •——•).

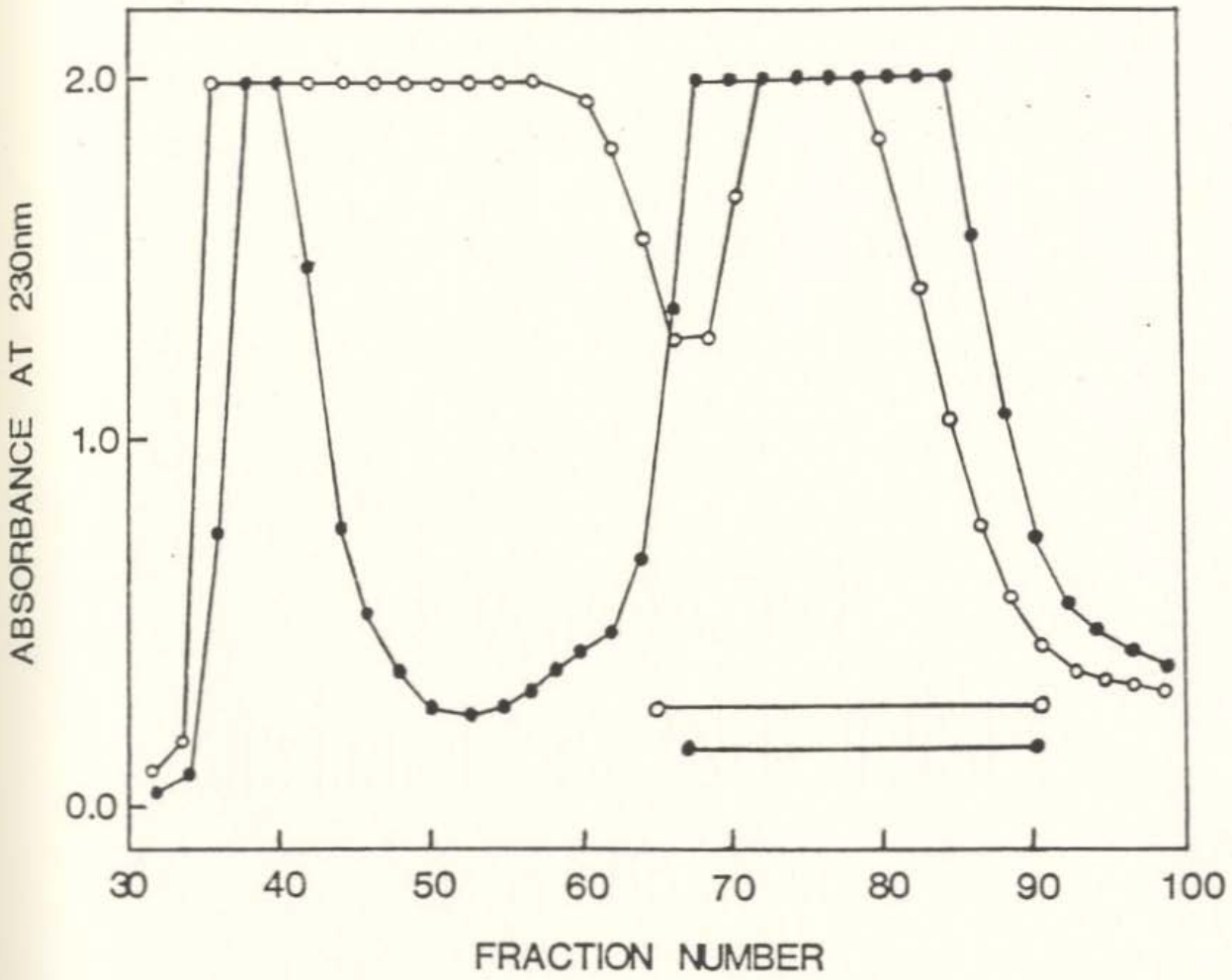
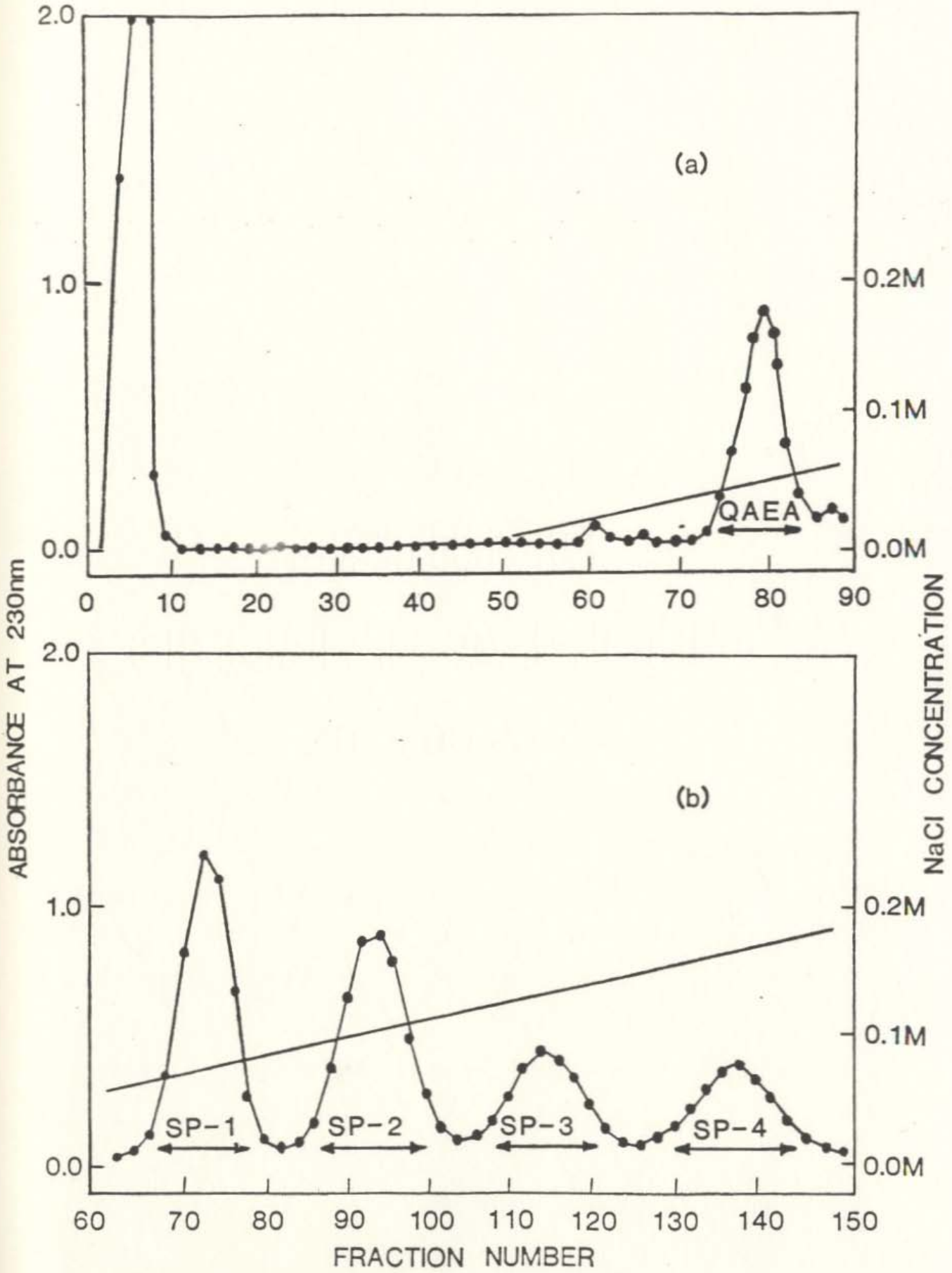

ABSORBANCE AT 230nm
2.0
1.0
0.0
30
40
50
60
70
80
90
100
FRACTION NUMBER

Figure 2. (a): Chromatography of G-75 AFP on a QAE-Sephadex (A25) column in 5 mM Tris-HCl buffer, pH 9.4 at room temperature. The column was eluted with a 0.0 to 0.2 M NaCl gradient in the same buffer. The first peak was QAE-unbound peak, and the second one was designated QAEA. (b): Chromatography of QAE-unbound component on SP-Sephadex (C50) column in 0.01 M NaCl, pH 3 at room temperature. The column was eluted with a 0.01 to 0.2 M NaCl gradient, pH 3. The four peaks obtained were designated SP-1, SP-2, SP-3 and SP-4 respectively in order of elution time.


(a)
QAEA
(b)
SP-1
SP-2
SP-3
SP-4
ABSORBANCE AT 230nm
NaCl CONCENTRATION
FRACTION NUMBER
2.0
1.0
0.0
0.2M
0.1M
0.0M
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150

least twelve components on reverse phase HPLC (Figure 3). These results confirm an earlier suggestion that ocean pout AFP is a multiple family of closely related components (22).

1.2.2. CORRELATION OF G-75 AFP WITH INDIVIDUAL COMPONENTS FROM ION-EXCHANGE CHROMATOGRAPHY USING REVERSE PHASE HPLC

As shown in Figure 4, there was a good correlation between G-75 AFP and peaks QAEA, SP-1, SP-2, SP-3 and SP-4. These five fractions from ion-exchange chromatography could account for all the components from the G-75 AFP on analytical HPLC, indicating that the fractionation procedures were successful. Reverse phase HPLC alone can fractionate all the components separated by QAE and SP ion-exchange chromatography. It appears to be the best way to prepare pure materials.

1.2.3. CHARACTERISTICS OF OCEAN POUT AFP

1.2.3.1. MOLECULAR WEIGHT ESTIMATION

On gel-filtration by HPLC with two TSK-3000-SW columns, the molecular weights of SP-2, SP-3 and G-75 AFP were all nearly the same, ranging from around 5,700 to 6,500 (Figure 5).

It was also found that the molecular weight estimation using TSK-3000-SW columns was quite reliable. A mixture of insulin, SP-1 and aprotinine was resolved into three peaks with aprotinine eluting first, SP-1 second and insulin the last.

Figure 3. Chromatography of G-75 AFP on reverse phase HPLC in 0.05 % TFA at room temperature. Samples were eluted with an acetonitrile gradient as indicated and numbered 1 to 12 respectively in order of elution time. The column used was μBondapak™ (7.8 mm I.D. x 30 cm) (Waters). The flow rate was 1 ml/min.

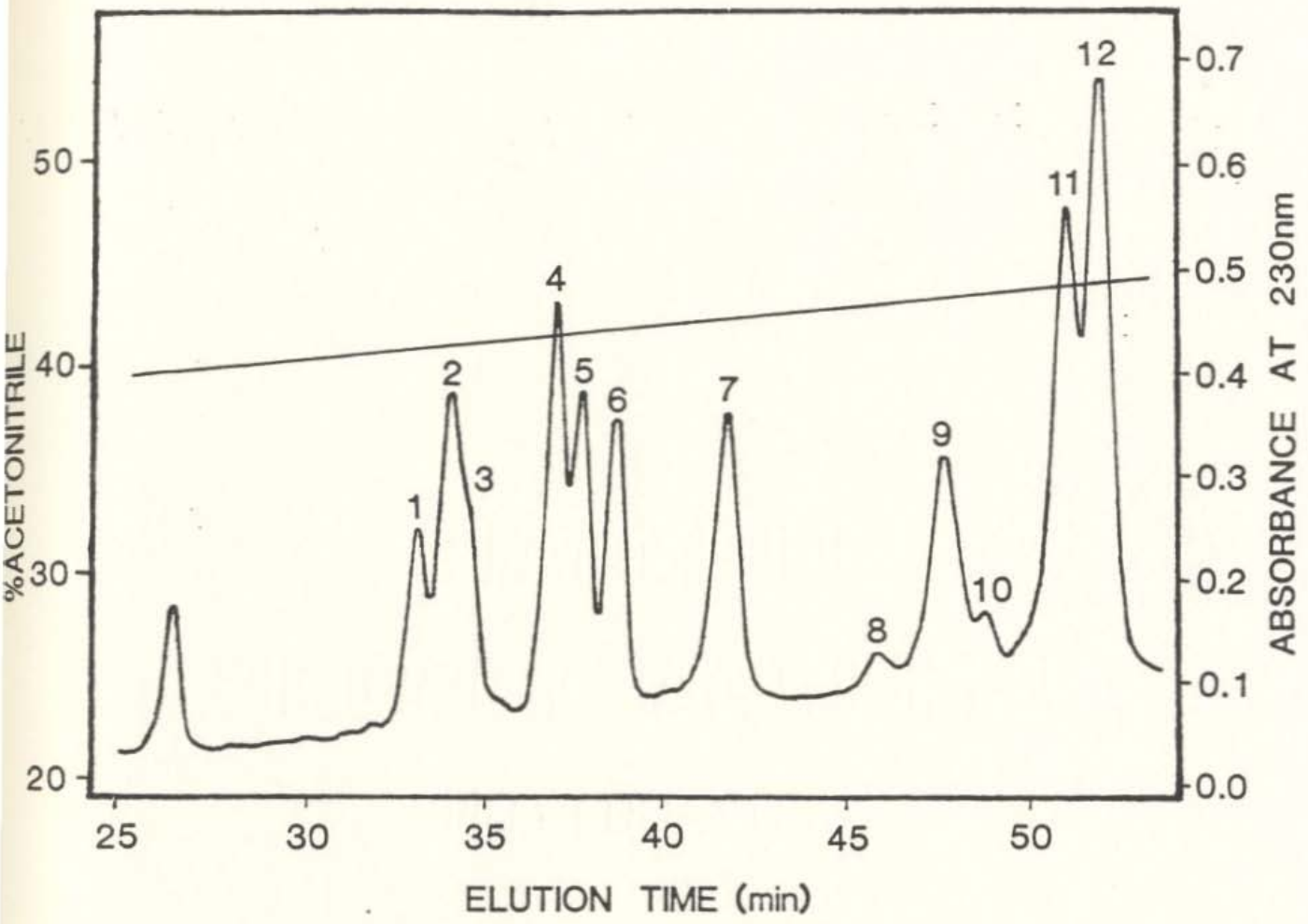
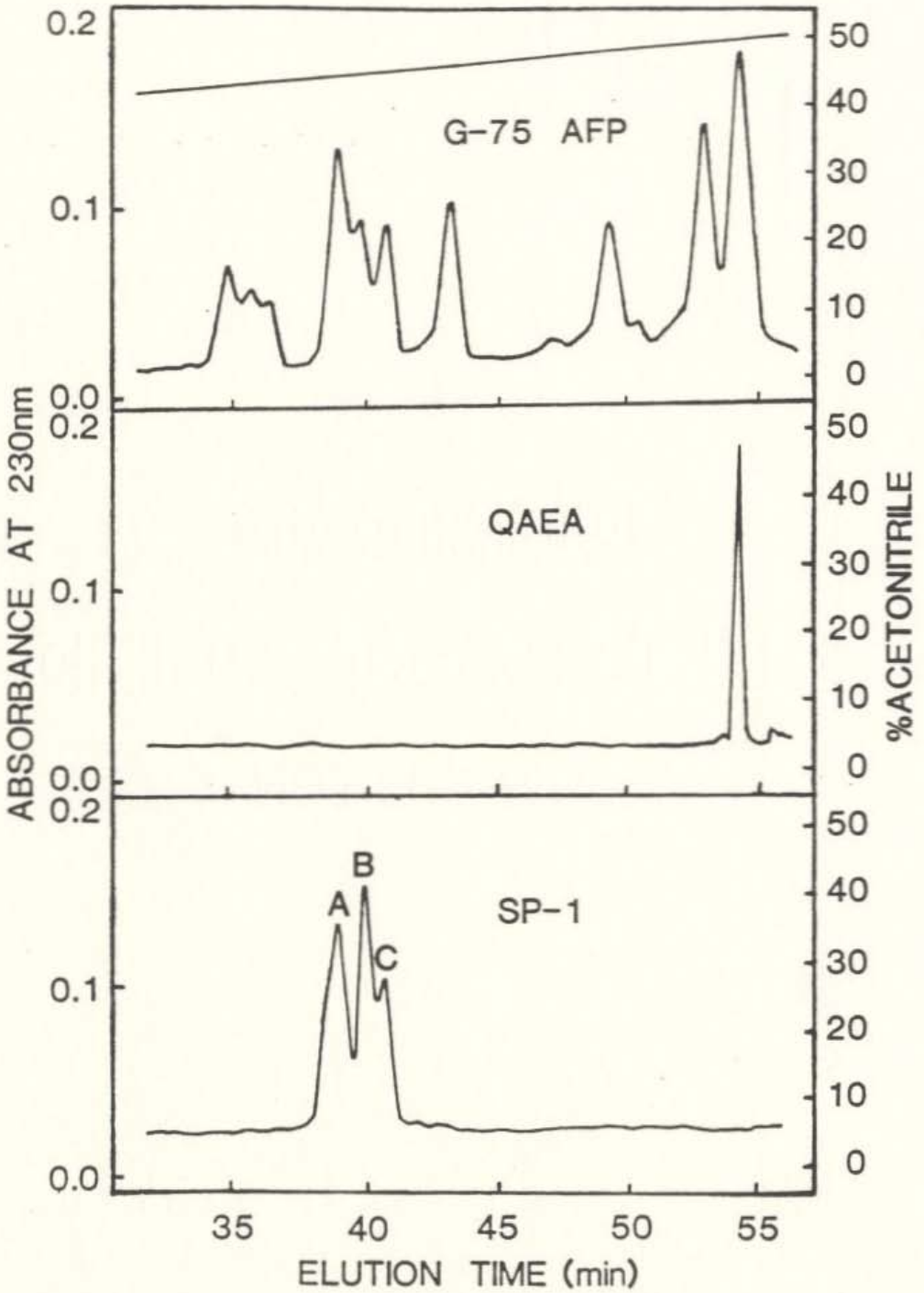

%ACETONITRILE
50
40
30
20
ABSORBANCE AT 230nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1
2
3
4
5
6
7
8
9
10
11
12
25
30
35
40
45
50
ELUTION TIME (min)

Figure 4. Chromatography of G-75 AFP, QAEA, SP-1, SP-2, SP-3 and SP-4 on reverse phase HPLC in 0.05 % TFA at room temperature. The column was μBondapak™ C18 (7.8 mm I.D. x 30 cm) (Waters). The other conditions were the same as in Figure 3.


G-75 AFP
QAEA
SP-1
A
B
C
ABSORBANCE AT 230nm
%ACETONITRILE
ELUTION TIME (min)
0.2
0.1
0.0
50
40
30
20
10
0
35
40
45
50
55

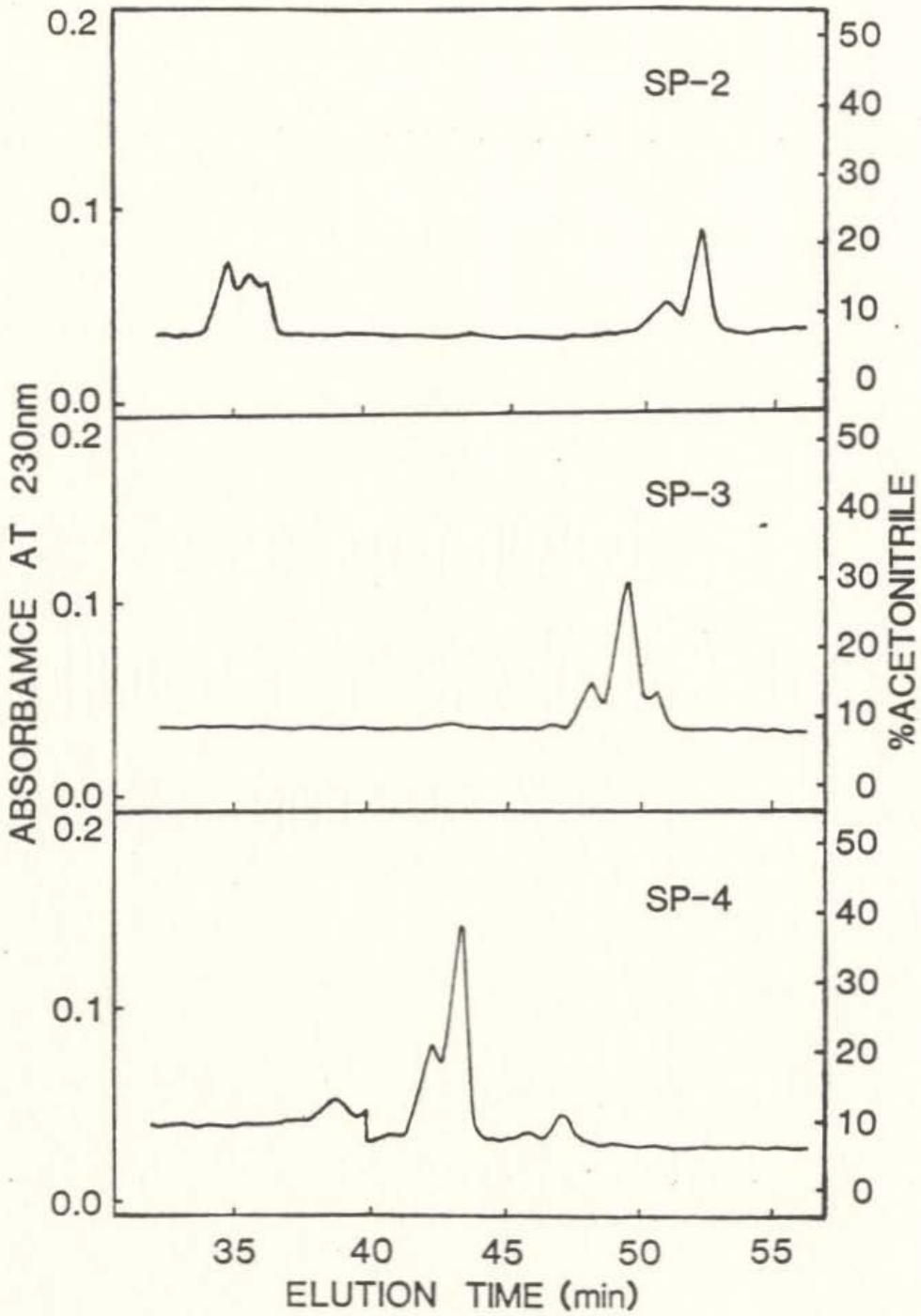
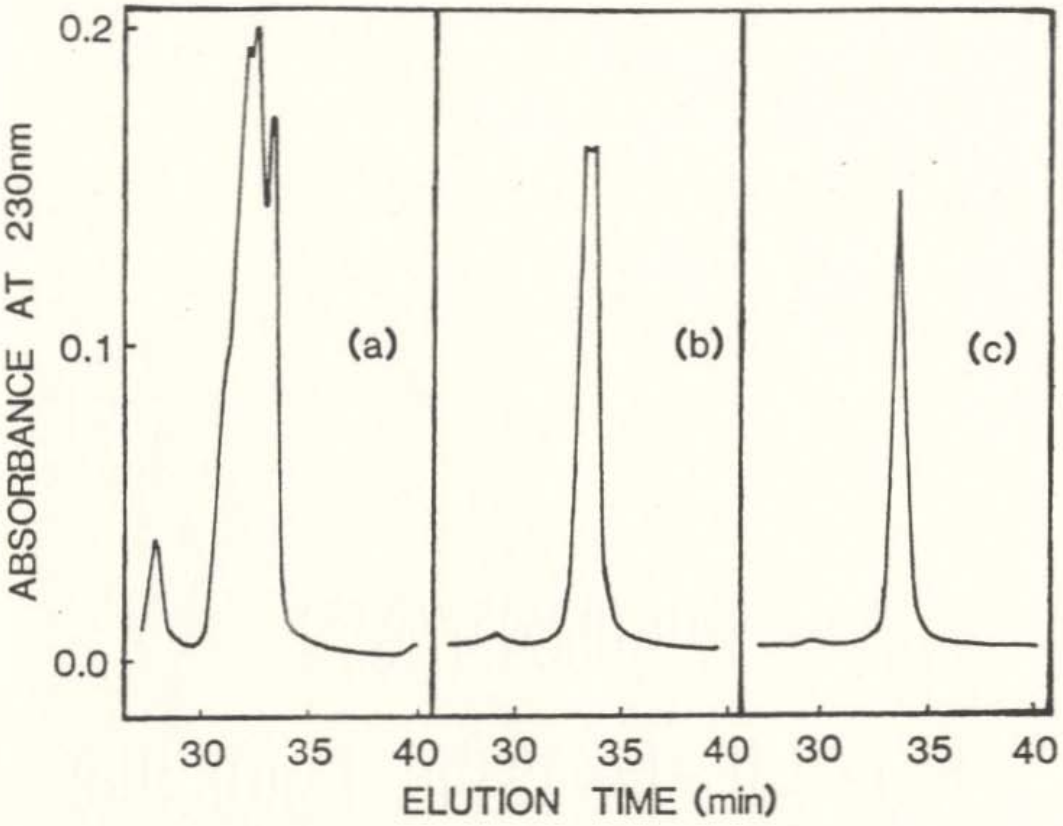
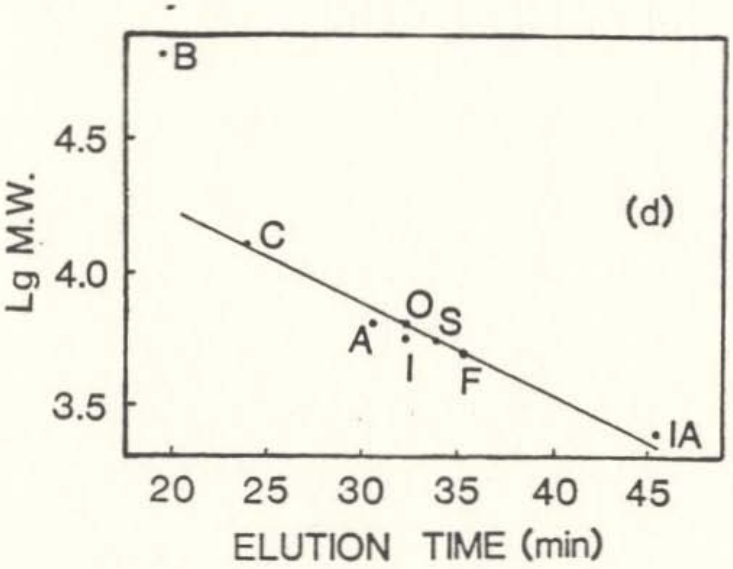

SP-2
SP-3
SP-4
ABSORBAMCE AT 230nm
%ACETONITRILE
ELUTION TIME (min)
0.0
0.1
0.2
0
10
20
30
40
50
35
40
45
50
55

Figure 5. Estimation of the molecular weights of ocean pout AFP on gel-filtration HPLC. Figures (a), (b) and (c) are the profiles of G-75 AFP, SP-2 and SP-3 in 45 % acetonitrile (containing 0.1 % TFA) on two Spherogel TSK-3000-SW (2 x 7.5 mm I.D. x 30 cm) (Beckman) columns. The flow rate was 0.5 ml/min. Figure (d) is the plot of elution time versus Lg molecular weight. The standards were B (BSA, 68 kD.), C (cytochrome C, 13 kD), A (aprotinine, 6.5 kD.), I (insulin, 5.7 kD), S (shorthorn sculpin AFP, 4.3 kD.), F (flounder AFP 3.3 kD.), IA (insulin A chain, 2.5 kD.). O stands for ocean pout AFP.


0.2
0.1
0.0
ABSORBANCE AT 230nm
(a)
(b)
(c)
30
35
40
30
35
40
30
35
40
ELUTION TIME (min)


B
4.5
4.0
3.5
Lg M.W.
(d)
C
O
S
A
I
F
IA
20
25
30
35
40
45
ELUTION TIME (min)

By SDS polyacrylamide slab gel electrophoresis, the estimated molecular weights of the G-75 AFP, QAEA, SP-1, SP-2, SP-3 and SP-4 were all under 6,500 (Figure 6).

Based on these determinations, the molecular weights of G-75 AFP, QAEA, SP-1, SP-2, SP-3 and SP-4 are all similar. They are between 5,700 and 6,500. These values were in good agreement with the molecular weights estimated using an analytical ultracentrifuge (22).

1.2.3.2. AMINO ACID ANALYSES

There are at least three components in SP-1, namely SP-1-A, SP-1-B and SP-1-C (see Figure 4). They were collected from SP-1 and rechromatographed individually on HPLC with a less steep gradient. The results of the amino acid analyses of QAEA, SP-1-A, SP-1-B and SP-1-C are shown in Table 1. QAEA, SP-1-A, SP-1-B and SP-1-C all contained fourteen to fifteen types of different amino acids. Arg was present in QAEA, but not in any of the SP-1 components (SP-1-A, SP-1-B and SP-1-C). The amino acid compositions of SP-1-A, SP-1-B and SP-1-C were very similar to each other. The fact that the Ala content is very modest and that there is no half-cystine at all would suggest that unlike flounder AFP which have a very high content of Ala (60 mol %), or sea raven AFP which have an extraordinarily high content of half-cystine (8 mol %), ocean pout AFP represent a new type of fish AFP. These results were in good agreement with the previous observations made in our laboratory (22).

It should be pointed out that the calculation of the number of amino acid residues for a given sample was made in a rather arbitrary way. The average

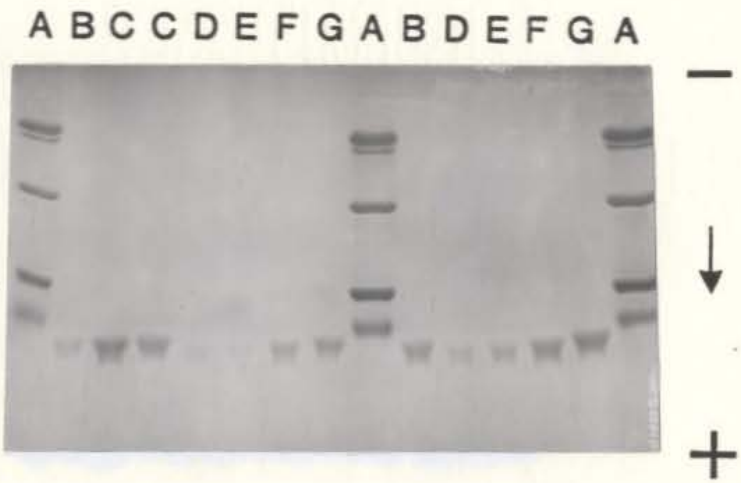


Figure 6. Polyacrylamide gel electrophoresis of ocean pout AFP. Lanes A, B, C, D, E, F and G corresponded to standards, G-75 AFP, QAEA, SP-1, SP-2, SP-3 and SP-4 respectively. The standards consisted of ovalbumin (43 kD.), chymotrypsinogen A (25.7 kD.), lysozyme (14.3 kD.), aprotinine (6.5 kD.) and insulin A chain (2.5 kD.) (not stained).

Table 1. Amino Acid Analyses of the Ocean Pout AFP
(yield in nmol)

	SP-1-A	SP-1-B	SP-1-C	QAEA
Asp	3.17(4)	1.81(4)	4.60(4)	15.76
Thr	4.62(6)	3.28(7)	9.84(8)	14.17
Ser	1.94(2)	0.80(2)	2.41(2)	7.04
Glu	6.06(7)	2.42(6)	9.64(8)	14.82
Pro	5.24(6)	2.56(6)	7.10(6)	21.44
Gly	3.94(5)	2.05(5)	5.58(5)	9.40
Ala	5.21(6)	3.04(7)	7.3[redacted]	16.96
Val	5.77(7)	3.13(7)	6.76(6)	22.01
Met	4.12(5)	2.28(5)	6.06(5)	13.71
Ile	3.62(4)	2.00(5)	5.46(5)	10.58
Leu	1.69(2)	1.47(3)	3.60(3)	16.12
Tyr	0.75(1)	0.62(1)	0.94(1)	2.23
Phe	0.85(1)	0.30(1)	0.94(1)	0.18
Lys	3.20(4)	1.75(4)	4.47(4)	3.84
Arg				5.60
Total	(60)	(63)	(64)	
M.W.	6302	6571	6761	
nmol/res.#	0.85	0.45	1.20	

* The figures in ( ) indicate the calculated numbers of residues.
# This is the assumed average value of nmol corresponding to one residue in the calculations, which was chosen to match the M.W. obtained by chromatography and electrophoresis (see 1.2.3.1.).

value of the nanomole/residue (shown in the last line of Table 1) was chosen and the integral number of all the residues calculated therefrom in such a way as to obtain maximum agreement with the molecular weight data obtained by gel-filtration HPLC and SDS polyacrylamide gel electrophoresis (see 1.2.3.1. for detail).

1.2.3.3. N-TERMINAL AMINO ACID ANALYSES

Dansylation tests were carried out on SP-1-A, SP-1-B and SP-1-C individually. No fluorescent α-N-dansyl amino acids were detected on polyamide plates after two dimensional chromatography using two or three solvents, suggesting that the N-terminals of all these AFP are blocked.

The above observation was confirmed by the fact that the intact SP-1-A component failed to be sequenced by the protein sequenator.

The same phenomenon was found with shorthorn sculpin AFP whose N-terminal amino acid Met was modified (28).

1.2.3.4. ANTIFREEZE ACTIVITY MEASUREMENTS

As shown in Table 2, all the twelve components from C18 column chromatography (μBondapak^{TM}) of the G-75 AFP showed thermal hysteresis. The most active ones were No.4 (SP-1-A), No.5 (SP-1-B), No.11 (one component of SP-2) and No.12 (QAEA).

Table 2. Antifreeze Activities of the Ocean Pout AFP Components

	Component No.					
Activity	1	2	3	4	5	6
mosmol/kg	48.3	43.3	26.6	106.6	153.3	53.0
T. H.	0.090	0.080	0.049	0.200	0.285	0.098

	Component No.					
Activity	7	8	9	10	11	12
mosmol/kg	31.6	38.3	46.6	36.6	76.6	103.3
T. H.	0.059	0.071	0.087	0.068	0.142	0.192

T. H. (thermal hysteresis, °C) = 1.856×10^{-3} x mosmol/kg

The component numbers 1 to 12 correspond to the 12 peaks of the G-75 AFP resolved on reverse phase HPLC C18 column (Figure 3).

The above measurements were made by Dr. M. H. Kao (see 1.1.7.).

1.2.4. COMPARISON OF OCEAN POUT AFP FROM NEWFOUNDLAND AND FROM NEW BRUNSWICK

AFP isolated from individual ocean pout caught in Newfoundland and New Brunswick were analysed on reverse phase HPLC. The sera were obtained during winter months.

No significant difference could be seen between the G-75 profiles of ocean pout AFP from these two locations (Figure 7). The HPLC profiles of both samples were found, by visual inspection, to be similar, if not identical, except for the small peak eluting at around 25 min (Figure 8). This peak is highly unstable or unreproducible. Due to its low yield, it is impossible to determine whether it has antifreeze activity or not. Therefore, there does not seem to be any significant populational polymorphism. All the twelve individual components are present in both Newfoundland and New Brunswick ocean pout and they occur in somewhat similar ratios. Recently, AFP from winter flounder inhabiting different waters have been studied (45). The results obtained from ocean pout in the present investigation are similar to those from that study.

1.2.5. COMPARISON OF NEWFOUNDLAND OCEAN POUT AFP IN SUMMER AND IN WINTER

As mentioned in the introduction, ocean pout contains a significant amount of AFP even in summer. One question is whether the AFP produced in summer is the same as that in winter.

To answer the question, Newfoundland ocean pout AFP in summer and

Figure 7. Chromatography of the ocean pout serum on a Sephadex G-75 column in 0.1 M NH_4HCO_3 buffer at 4°C. The sample volume was 5 ml. Figures (a) and (b) were the profiles of winter serum obtained from Newfoundland and New Brunswick fish respectively. (first run: o——o ; rechromatography: •——•)

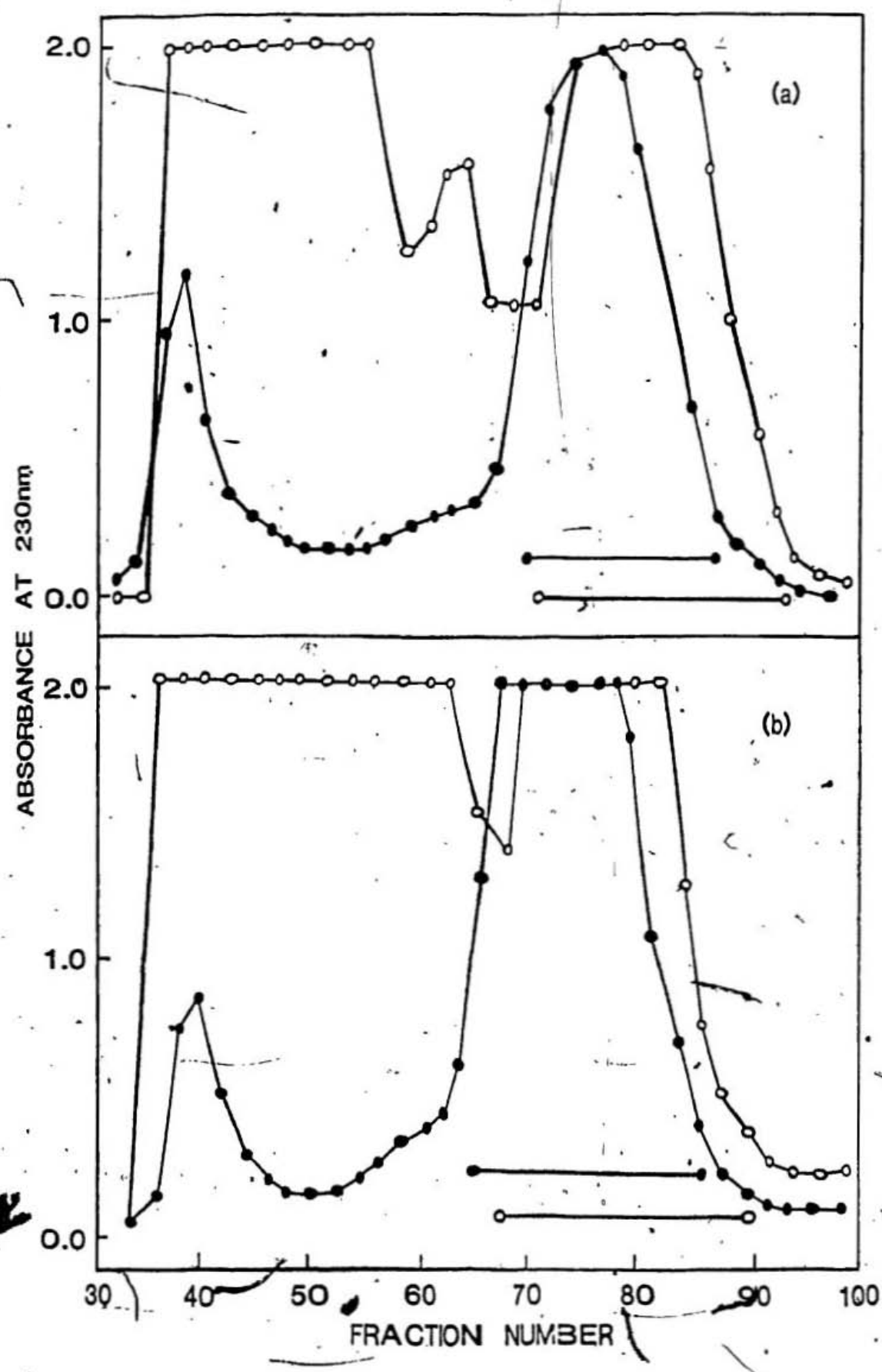
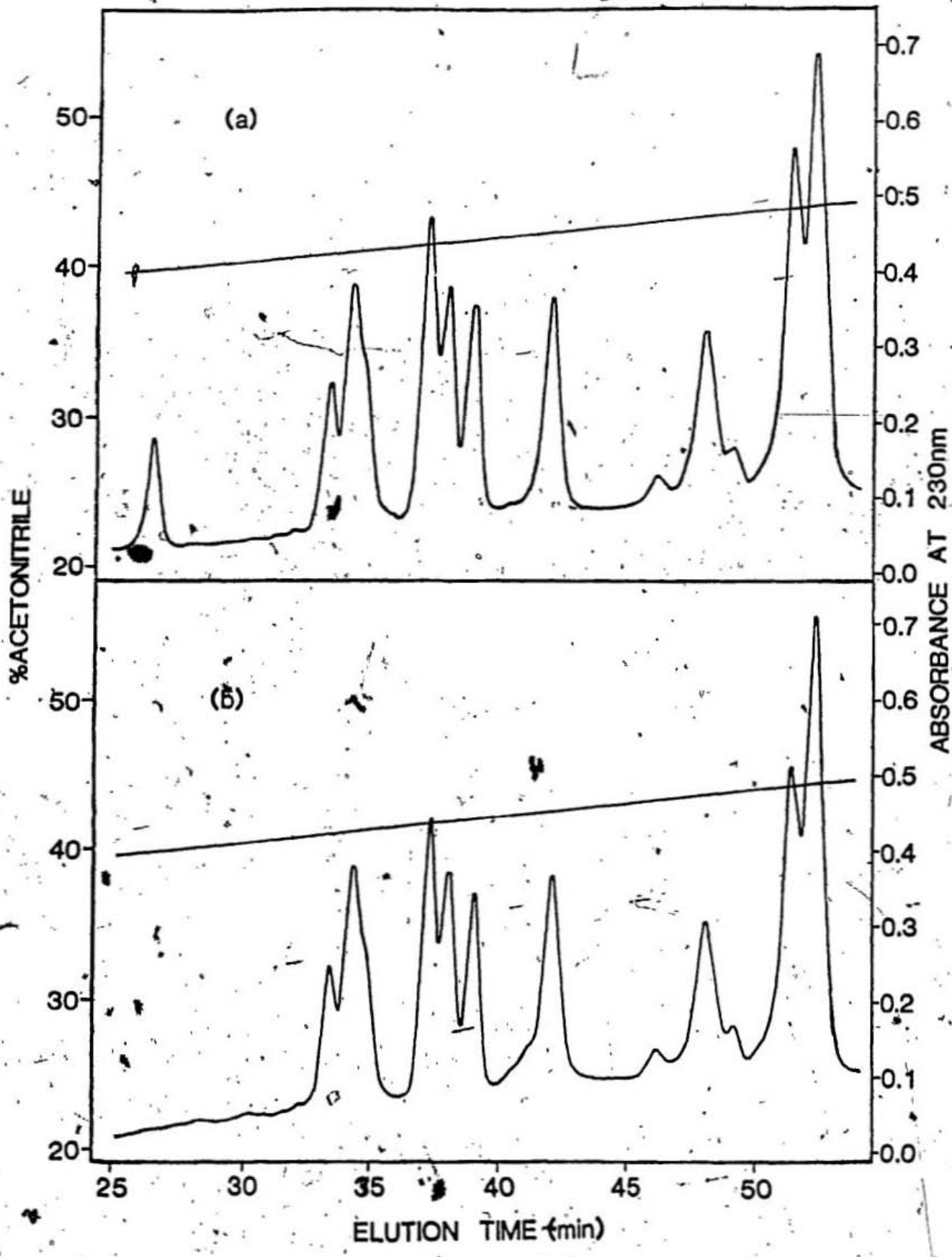

2.0
1.0
0.0
(a)
ABSORBANCE AT 230nm
2.0
1.0
0.0
(b)
30
40
50
60
70
80
90
100
FRACTION NUMBER

Figure 8. Chromatography of ocean pout G-75 AFP from Newfoundland (Figure (a)) and New Brunswick (Figure (b)). The AFP were purified from winter sera on μBondapak™ C18 (7.8 mm I.D. x 30 cm) column (Waters). The buffer system and the gradient were the same as in Figure 3.


(a)
(b)
%ACETONITRILE
ABSORBANCE AT 230nm
ELUTION TIME (min)
50
40
30
20
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
25
30
35
40
45
50

winter were isolated and analysed on reverse phase HPLC individually. Figure 9 shows that in both cases, the Sephadex G-75 patterns were very similar. While on reverse phase HPLC (C18 or, C3 column), one or two components out of the twelve had higher relative amounts in summer than in winter (Figure 10 and Figure 11), as deduced by visual inspection. It is interesting to observe that a small peak eluting at about 17 min on the C3 column in summer corresponds to the highly unstable component appearing on the C18 column in winter. It appears that the contents of ocean pout AFP in both winter and summer are very similar, even though the relative amounts of one or two components are higher in summer than in winter. Thus the variation is quantitative rather than qualitative.

1.3. SUMMARY

The present chapter has established that:

1. Ocean pout AFP can be isolated and separated successfully by Sephadex G-75 gel-filtration chromatography, QAE-Sephadex and SP-Sephadex ion-exchange chromatography and reverse phase HPLC.

2. Ocean pout AFP comprise a family of at least twelve active components of nearly identical size (about 6,000 daltons).

3. The amino acid compositions of some of the major components of ocean pout AFP suggest that the group may represent a new type of fish AFP.

4. Ocean pout AFP have no obvious populational or seasonal polymorphisms as analyzed on HPLC.

Figure 9. Chromatography of the winter (Figure (a)) and summer (Figure (b)) sera from Newfoundland ocean pout on a Sephadex G-75 column. The other conditions were the same as in Figure 1. (first run: ——; rechromatography: ——)

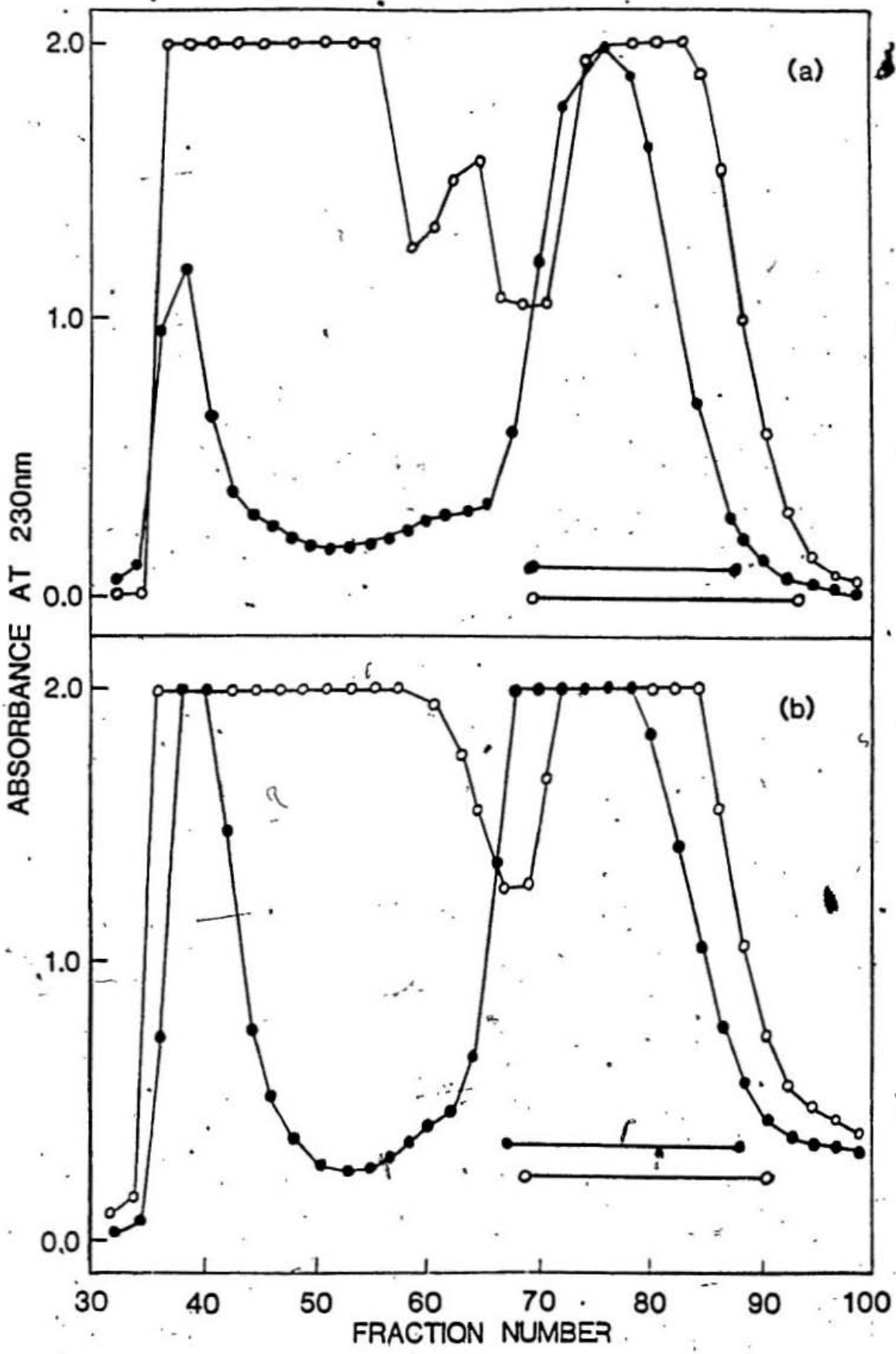

(a)
(b)
2.0
1.0
0.0
ABSORBANCE AT 230nm
30
40
50
60
70
80
90
100
FRACTION NUMBER

Figure 10. Chromatography of the G-75 AFP of the Newfoundland ocean pout from winter (Figure (a)) and summer months (Figure (b)) on μBondapak^{TM} C18 (Waters) column. The experimental conditions were the same as in Figure 3.

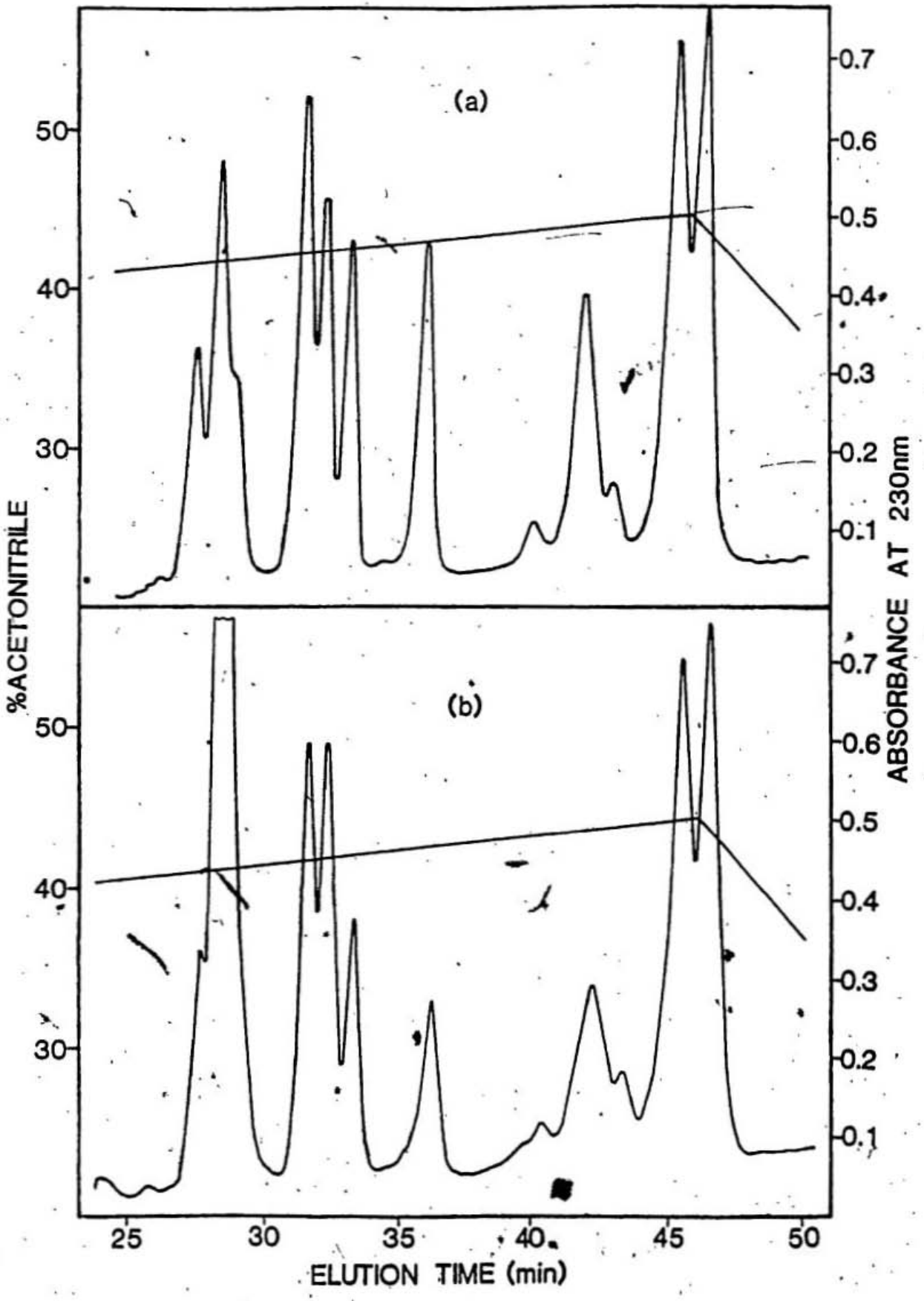
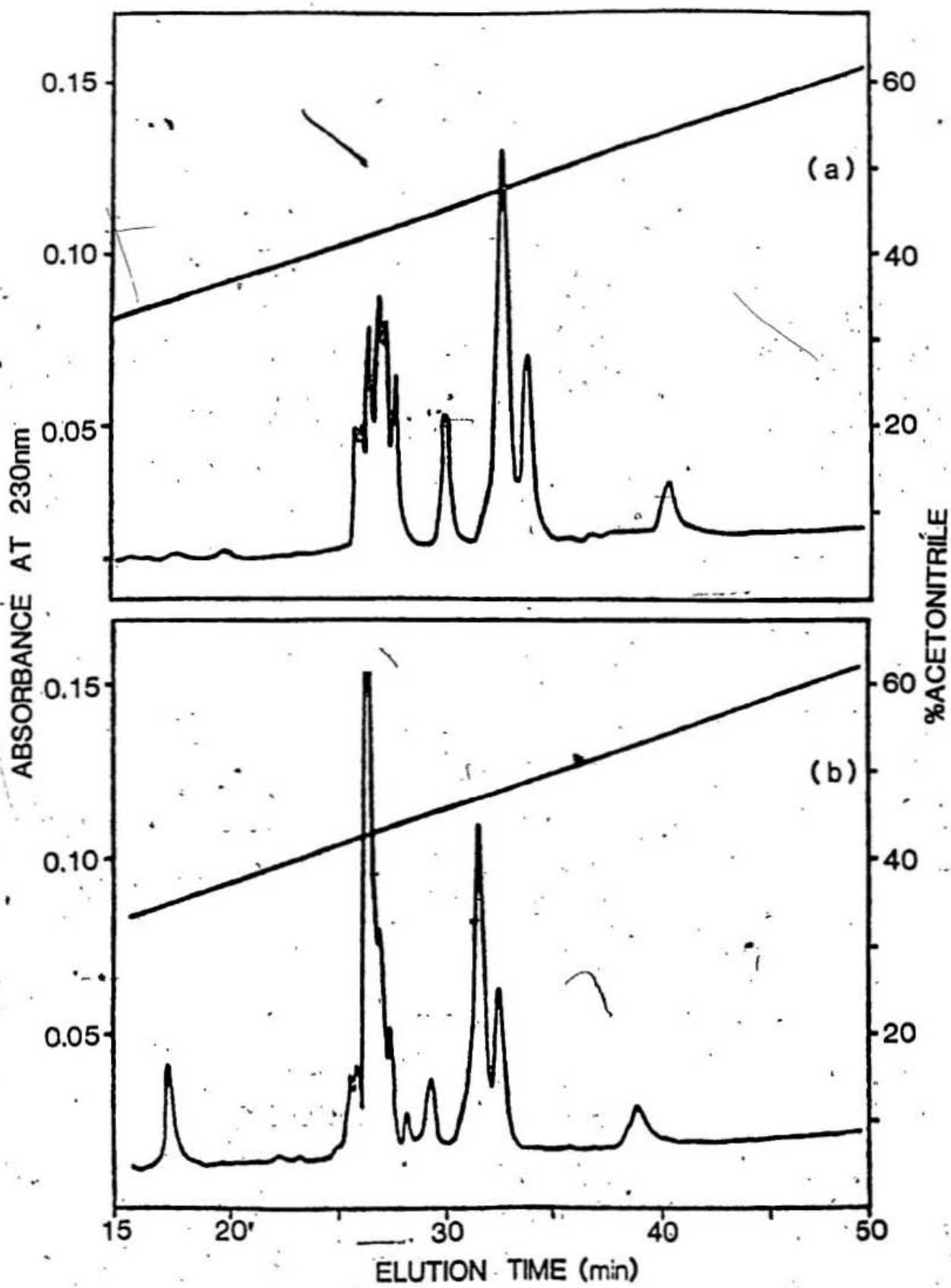

(a)
(b)
%ACETONITRILE
50
40
30
ABSORBANCE AT 230nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
25
30
35
40
45
50
ELUTION TIME (min)

Figure 11. Chromatography of the G-75 AFP of the Newfoundland ocean pout from winter (Figure (a)) and summer months (Figure (b)) on reverse phase HPLC in 0.05 % TFA. Samples were eluted with acetonitrile gradient as indicated. The column was Ultrapore RPSC C3 (4.6 mm I.D. x 7.5 cm) (Beckman). The flow rate was 0.5 ml/min.


0.15
0.10
0.05
ABSORBANCE AT 230nm
(a)
(b)
60
40
20
%ACETONITRILE
15
20'
30
40
50
ELUTION TIME (min)

Chapter 2

Structural Studies On SP-1-A, SP-1-B And SP-1-C

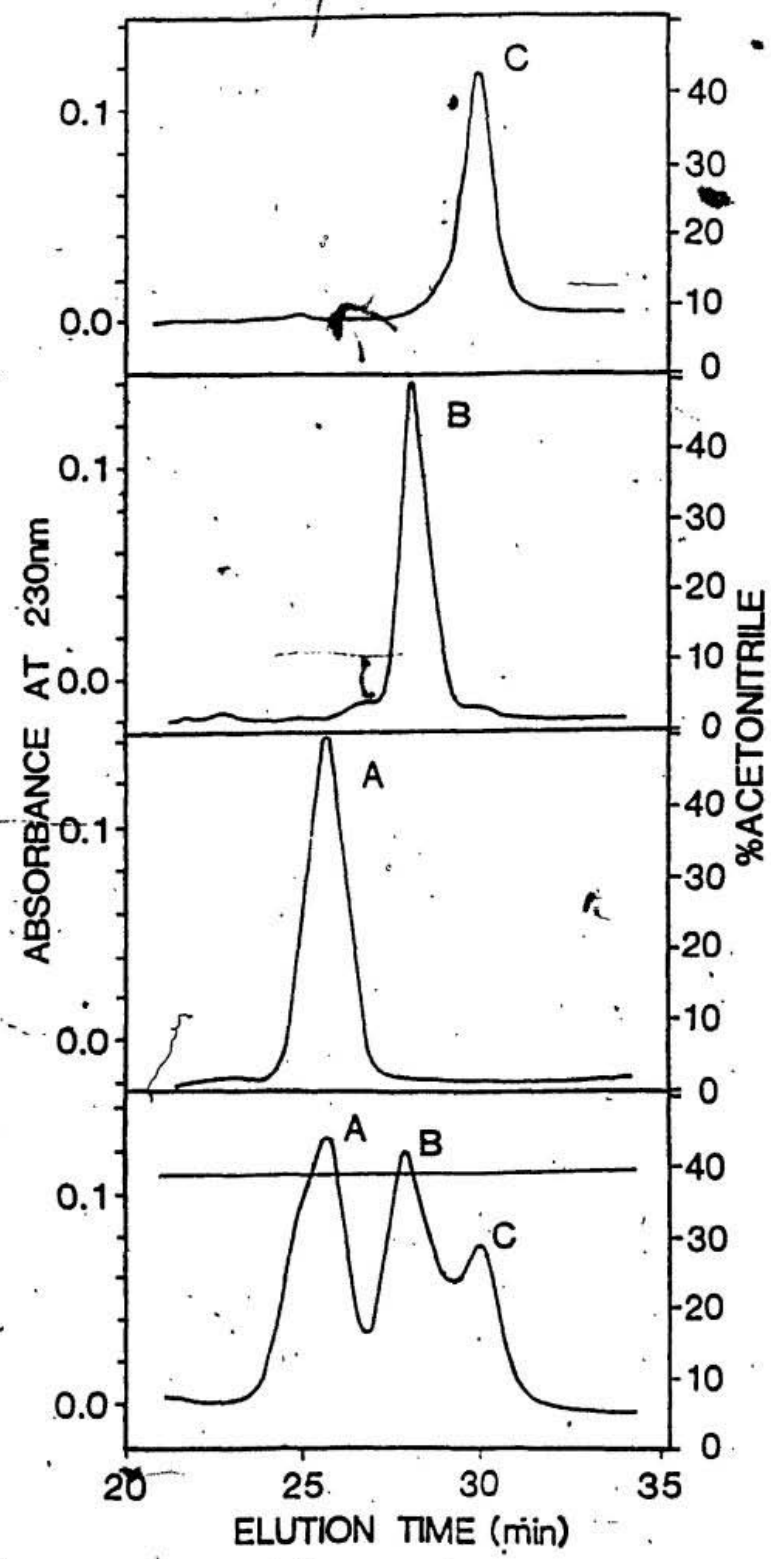
From the studies in chapter 1, it is apparent that there are at least three distinct components in SP-1, namely SP-1-A, SP-1-B and SP-1-C (see Figure 4), with similar amino acid compositions.

Further studies were carried out to characterize these three components, because their similar amino acid compositions would provide the basis for the comparative analyses of their sequences. A partial amino acid sequence of SP-1-A was established up to 41 residues. The structural homologies among SP-1-A, SP-1-B and SP-1-C were compared by tryptic and chymotryptic peptide mapping as well as their amino acid compositions.

2.1. MATERIALS AND METHODS

The starting materials were prepared by Sephadex G-75 gel-filtration column, QAE-Sephadex (A25) and SP-Sephadex (C-50) ion-exchange columns as well as reverse phase HPLC as described in the previous chapter. SP-1-A, SP-1-B and SP-1-C were collected from SP-1 and rechromatographed on HPLC with a less steeper gradient. As can be seen from Figure 12, they appear to be distinct and homogeneous.

Figure 12. Chromatography of SP-1 on reverse phase HPLC in 0.05 % TFA. Peaks were eluted with an acetonitrile gradient as indicated and designated, in order of elution times, as SP-1-A, SP-1-B and SP-1-C, respectively. The column was μBondapak™ C18 (7.8 mm I.D. x 30 cm) (Waters). The flow rate was 1 ml/min.


C
B
A
A
B
C
0.1
0.0
40
30
20
10
0
ABSORBANCE AT 230nm
%ACETONITRILE
20
25
30
35
ELUTION TIME (min)

2.1.1. ISOLATION OF CHYMOTRYPTIC PEPTIDES OF SP-1-A FOR EDMAN DEGRADATION STUDIES

1 mg of SP-1-A in 1 ml 0.2 M NH_4CO_3 was digested with 50 μg α-chymotrypsin at 37°C for 2 h (46). Then it was loaded on a C18 reverse phase HPLC column and the major peaks were collected.

Aliquots of the major peaks were hydrolyzed with 6 M HCl as described before (see 1.1.6.) and the rest of them were subjected to automatic Edman degradation on a Beckman 890C protein sequenator using the 0.1 M Quadrol program in the presence of polybrene (Pierce Chemical). After conversion in the presence of 25 % trifluoroacetic acid for 30 min at 70 °C, the phenylthiohydantoin derivatives of the amino acids were analyzed by reverse phase HPLC using a Beckman Standard PTH Identification kit.

2.1.2. PEPTIDE MAPPING AND AMINO ACID ANALYSES OF SP-1-A, SP-1-B AND SP-1-C BY TRYPSIN DIGESTION

Trypsin (2.5 μg) and TPCK (0.25 μg) were added to ocean pout AFP samples (SP-1-A, SP-1-B and SP-1-C, 500 μg each) which were dissolved individually in 500 μl of 0.2 M NH_4HCO_3, giving the ratio of the substrate:trypsin:TPCK 200:1:0.1. The samples were digested at 37°C for 2 h, lyophilized, and applied directly on a C18 reverse phase HPLC column (47, 48). Major components were collected and amino acid analyses were performed.

2.1.3. PEPTIDE MAPPING OF SP-1-A, SP-1-B AND SP-1-C BY CHYMOTRYPSIN DIGESTION

7.5 μg of α-chymotrypsin, 0.75 μg of TLCK and the sample of ocean pout AFP (SP-1-A, SP-1-B and SP-1-C, 1.5 mg each) were dissolved in 1.5 ml of 0.2 M NH_4HCO_3. After digestion at 37°C for 2 h, the samples were lyophilized and analyzed on a C18 reverse phase HPLC column.

2.1.4. THERMOLYSIN DIGESTION ON SP-1-A

Thermolysin (1.5 μg) was added to 300 μg of SP-1-A which was in 100 μl of 0.2 M N-ethylmorpholine (pH 8.6) containing 0.002 M $CaCl_2$. After digestion at 37°C for 3 h, the sample was lyophilized and applied to a C18 reverse phase HPLC column (49). The major components were collected from the HPLC column, and hydrolyzed with 6 M HCl for amino acid analyses.

2.1.5. PURITY OF REAGENTS

Trypsin, α-chymotrypsin, thermolysin (Type X), TPCK and TLCK were all purchased from Sigma Chemical Company (St. Louis, MO. USA). The other chemicals were of reagent grade.

2.2. RESULTS

2.2.1. SEQUENCES OF CHYMOTRYPTIC PEPTIDES FROM SP-1-A

Figure 13 gives the HPLC profile of the chymotryptic peptides of SP-1-A. The amino acid analyses of the major peaks are shown in Table 3. The data from the protein sequenator are as follows:

Figure 13. Chromatography of the chymotryptic peptides of SP-1-A on reverse phase HPLC in 0.05 % TFA. The sample was dissolved in 5 % formic acid and then injected into HPLC column. Peaks were eluted with an acetonitrile gradient as indicated and numbered 1 to 14 in order of elution time. The column was μBondapak^{TM} C18 (7.8 mm I.D. x 30 cm) (Waters). The flow rate was 1 ml/min.

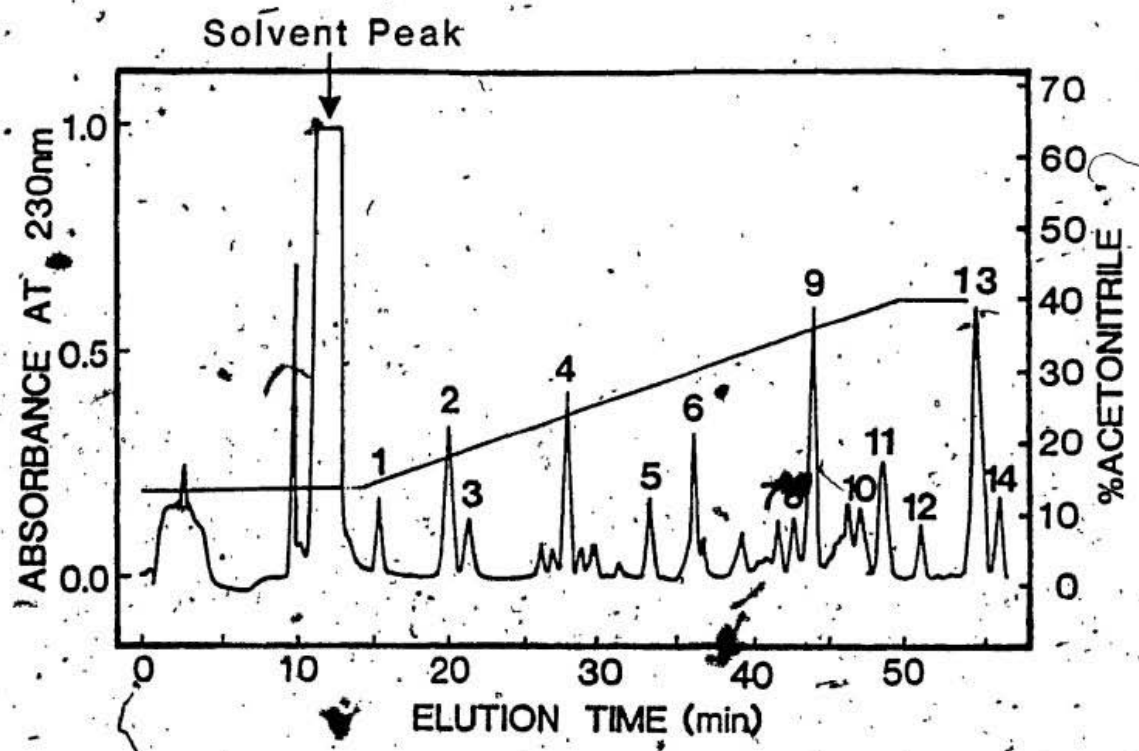

Solvent Peak
ABSORBANCE AT 230nm
1.0
0.5
0.0
70
60
50
40
30
20
10
0
%ACETONITRILE
1
2
3
4
5
6
7
8
9
10
11
12
13
14
0
10
20
30
40
50
ELUTION TIME (min)

Table 3. Amino Acid Analyses of the Chymotryptic Peptides of SP-1-A (yield in nmol)

	C3	C4	C5	C6	C9	C13
Asp	11.91(1)*	0.46(0)	1.05(0)	6.34(1)	1.22(1)	27.51(1)
Thr	10.40(1)	3.80(1)	0.00(0)	5.36(1)	2.33(2)	28.38(1)
Ser	0.00(0)	0.86(0)	5.27(1)	4.28(1)	0.00(0)	0.00(0)
Glu	22.78(2)	0.51(0)	24.12(3)	19.76(4)	1.13(1)	30.95(1)
Pro	12.60(1)	0.00(0)	1.11(0)	5.26(1)	1.20(1)	61.27(2)
Gly	9.39(1)	0.61(0)	10.72(1)	9.30(2)	0.72(1)	52.65(2)
Ala	11.85(1)	0.68(0)	10.54(1)	9.47(2)	0.30(0)	0.00(0)
Val	21.36(2)	4.96(1)	8.47(1)	12.84(3)	1.68(1)	25.98(1)
Met	0.00(0)	1.80(1)	4.19(1)	4.50(1)	0.00(0)	0.00(0)
Ile	0.00(0)	0.00(0)	9.07(1)	3.64(1)	1.40(1)	56.19(2)
Leu	0.00(0)	0.00(0)	0.00(0)	0.00(0)	0.00(0)	0.00(0)
Tyr	0.00(0)	2.97(1)	0.00(0)	0.00(0)	1.40(1)	0.00(0)
Phe	0.00(0)	0.00(0)	0.00(0)	0.00(0)	0.00(0)	25.00(1)
Lys	12.27(1)	3.76(1)	9.93(1)	9.20(2)	1.16(1)	24.45(1)
Total	(10)	(5)	(10)	(19)	(10)	(12)
nmol/res.#	11.00	3.50	7.50	4.50	1.20	27.51

* The figures in ( ) indicate the calculated numbers of residues
# This is the assumed average value of nmol corresponding to one residue in the calculations.

C3 : Gln-Val-Asn-Thr-Pro-Val-Ala-Lys-Gly-Gln

C6 : Ala-Glu-Met-Ser-Gln-Ile-Val-Gly-Lys-Gln-Val-Asn-Thr-Pro-Val-Ala

C5 : Ala-Glu-Met-Ser-Gln-Ile-Val-Gly-Lys

C4 : Met-Val-Lys-Thr-Tyr

C9 : Thr-Ile-Met-Pro-Asn-Met-Val-Lys-Thr

C13 : Glu-Gly-Lys-Thr

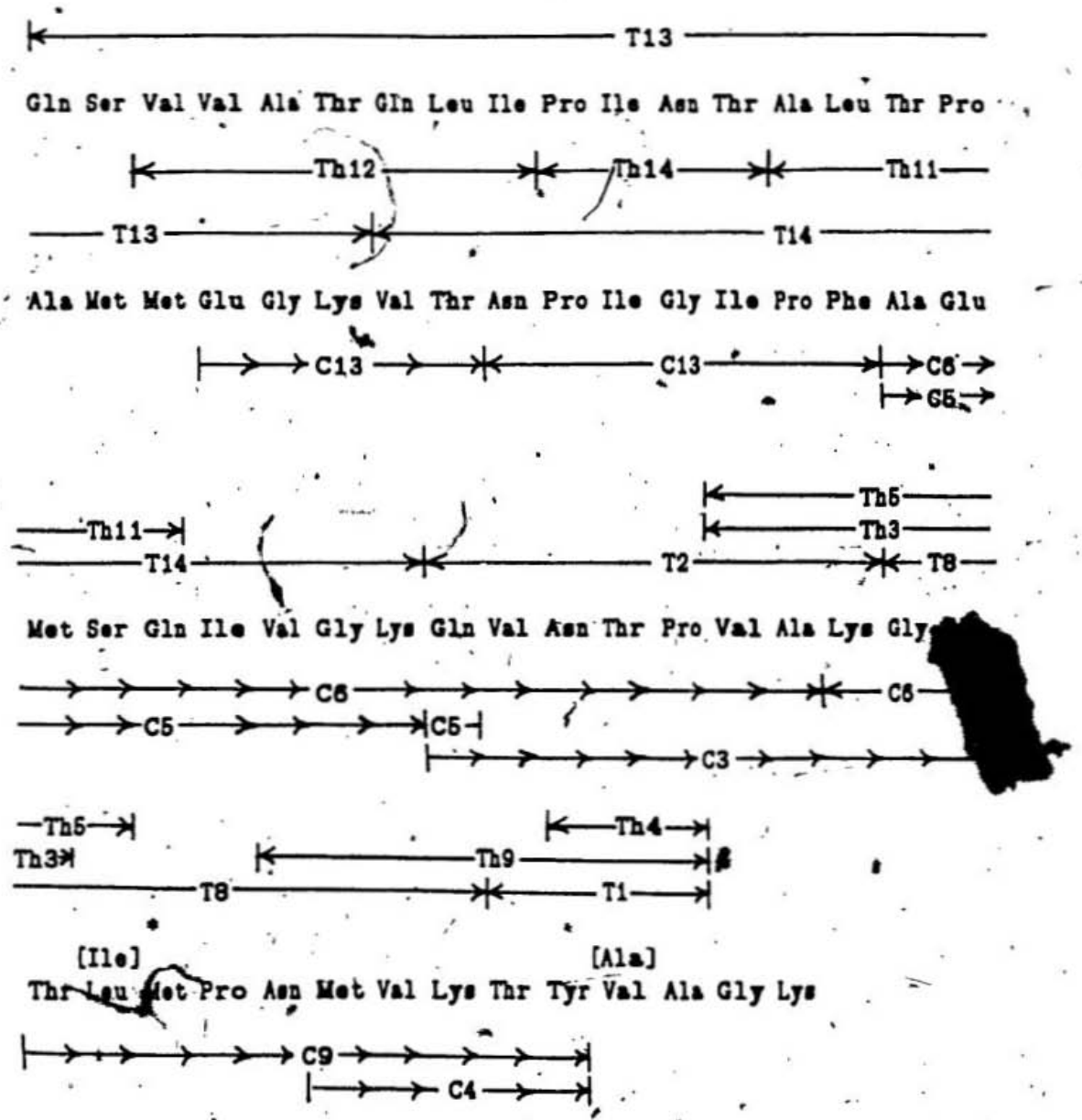
These results were provided by Mr. S. B. Joshi who operated the protein sequenator.

It is suggested that C5 is the N-terminal part of C6, C3 is the C-terminal part of C6 and C4 is the C-terminal part of C9.

The reason for choosing the above peptides for sequence analyses is that the amino acid analyses of only these were clear-cut, and their yields were sufficient for sequence analyses.

The cDNA coding for one of the ocean pout AFP precursors (clone No.77) has been sequenced, as will be discussed in more detail in the next chapter. Comparing the partial amino acid sequences from SP-1-A with the amino acid sequence obtained from the nucleotide sequence of the cDNA (Table 4), it seems that C13, C6 and C9 cover 41 amino acids among the overall 65 amino acids. The only difference between them is the replacement of an Ile and an Ala in SP-1-A to a Leu and a Val in the cDNA sequence at positions 53 and 62 respectively.

Table 4. Comparison of the Amino Acid Sequence of Clone No 77 and the Partial Sequences of SP-1-A



* The amino acids in [ ] are the ones in SP-1-A which are different from the corresponding ones in the cDNA.

2.2.2. RESULTS OF THE THERMOLYSIN DIGESTION ON SP-1-A

Figure 14 shows the HPLC profile of the thermolytic peptides of SP-1-A. There were 15 major peaks in the sample (Figure 14(b)). After comparing with the enzyme control (Figure 14(a)), the peak No.10 was identified as the enzyme peak. From the amino acid analyses of the remaining 14 components, some of them, namely Th3, Th4, Th5, Th9, Th11, Th12 and Th14 (Table 5), matched the SP-1-A sequence. These peptides are included in Table 4.

It should be mentioned that because of the possible cross-contaminations, the data in Table 5 for a given fragment may show "impurities" which were neglected in arriving at the numbers of residues. Also the value of the average nanomol/residue was dictated by the partial amino acid sequence data on SP-1-A and the nucleotide sequence of clone No.77.

2.2.3. TRYPTIC PEPTIDE MAPPING AND AMINO ACID ANALYSES OF SP-1-A, SP-1-B AND SP-1-C

As it can be seen in Figure 15(a), the tryptic peptides of SP-1-A were resolved by reverse phase HPLC into 14 major peaks. The amino acid compositions of six of these are shown in Table 6. The data were analyzed in the same manner as described for the thermolytic peptides (see 2.2.2.). These data were compared with the amino acid sequence of the peptide that is coded by the cDNA (clone No.77) and the 41-residue partial sequence of SP-1-A. T13, T14, T2, T8 and T1 in SP-1-A covered the overall sequence of the cDNA except 2 residues, Gly and Lys, at the C-terminal (Table 4). The results also show the differences between the amino acid sequences of SP-1-A and the peptide coded by

Figure 14. Chromatography of the thermolytic peptides of SP-1-A (Figure (b)) on reverse phase HPLC in 0.05 % TFA. The peaks were numbered 1 to 15 in order of their elution times. Figure (a) was the enzyme control. The column was μBondapak™ C18 (7.8 mm I.D. x 30 cm) (Waters). The other conditions were the same as in Figure 13.

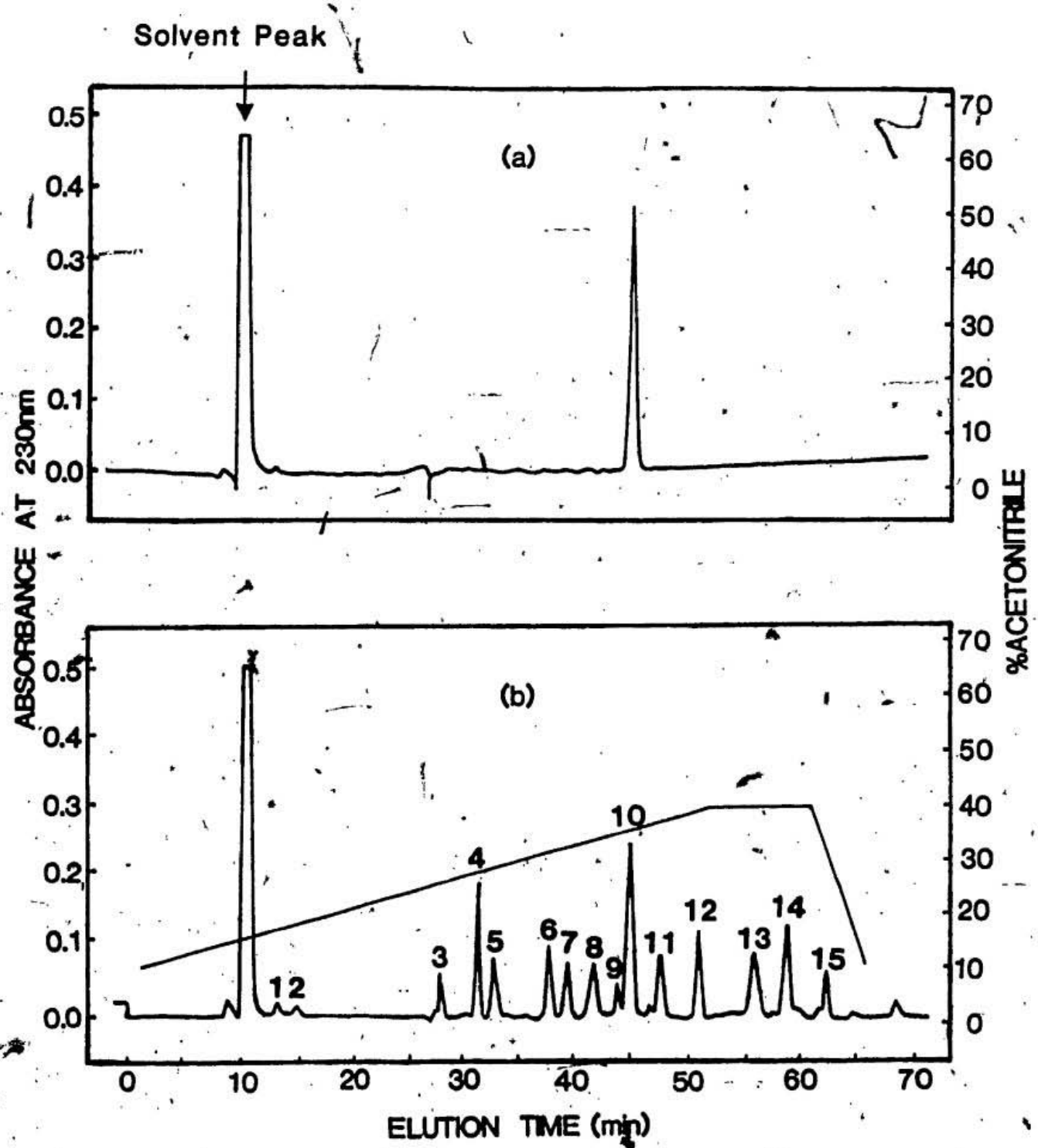

Solvent Peak
(a)
(b)
ABSORBANCE AT 230nm
%ACETONITRILE
ELUTION TIME (min)
0.5
0.4
0.3
0.2
0.1
0.0
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

Table 5. Amino Acid Analyses of the Thermolytic Peptides of SP-1-A
(yield in nmol)

	Th3	Th4	Th5	Th9
Asp	0.00(0)	0.00(0)	0.00(0)	1.08(1)
Thr	7.51(1)	0.00(0)	5.95(1)	1.53(1)
Ser	0.00(0)	0.00(0)	0.00(0)	0.60(0)
Glu	5.48(1)	0.00(0)	3.88(1)	0.37(0)
Pro	0.00(0)	0.00(0)	0.00(0)	0.00(0)
Gly	5.49(1)	0.00(0)	5.95(1)	0.64(0)
Ala	4.74(1)	11.22(2)	6.54(1)	2.30(2)
Val	5.16(1)	0.00(0)	6.11(1)	0.91(1)
Met	0.00(0)	0.00(0)	0.00(0)	0.85(1)
Ile	0.00(0)	0.00(0)	3.58(1)	0.00(0)
Leu	0.00(0)	0.00(0)	0.00(0)	0.00(0)
Tyr	0.00(0)	5.14(1)	0.00(0)	1.06(1)
Phe	0.00(0)	0.00(0)	0.00(0)	0.00(0)
Lys	5.94(1)	0.00(0)	5.83(1)	1.13(1)
Total	(6)	(3)	(7)	(8)
nmol/res.#	5.94	5.14	5.95	1.13

* The figures in ( ) indicate the calculated numbers of residues.
\# This is the asssumed average value of nmol corresponding to one residue in the calculations.

Table 5. Amino Acid Analyses of the Thermolytic Peptides of SP-1-A
(yield in nmol) (continued)

	Th11	Th12	Th14	---
Asp	0.96(0)*	3.05(1)	2.21(0)	---
Thr	0.00(0)	4.31(1)	2.20(0)	---
Ser	2.84(1)	0.15(0)	0.36(0)	---
Glu	8.87(2)	3.22(1)	0.29(0)	---
Pro	0.00(0)	2.41(1)	5.33(1)	---
Gly	0.80(0)	3.16(1)	4.77(1)	---
Ala	4.53(1)	0.89(0)	2.63(0)	---
Val	0.00(0)	4.71(1)	0.70(0)	---
Met	3.77(1)	3.89(1)	0.00(0)	---
Ile	0.32(0)	0.00(0)	9.86(2)	---
Leu	0.26(0)	0.67(0)	0.96(0)	---
Tyr	0.00(0)	0.00(0)	0.79(0)	---
Phe	3.47(1)	0.00(0)	0.00(0)	---
Lys	0.00(0)	2.62(1)	0.55(0)	---
Total	(6)	(8)	(4)	---
nmol/res.#	3.77	3.89	5.33	---

* The figures in ( ) indicate the calculated numbers of residues.
# This is the assumed average value of nmol corresponding to one residue in the calculations.

Figure 15. Chromatography of the tryptic peptides of SP-1-A (Figure (a)), SP-1-B (Figure (b)) and SP-1-C (Figure (c)) on reverse phase HPLC in 0.02 M TEAP, pH 3. Peaks were eluted with an acetonitrile gradient as indicated. The peaks were designated in all three cases according to their order of elution times respectively. The column was Ultrasphere ODS (4.6 mm I.D. x 25 cm) (Beckman). The flow rate was 0.5 ml/min.

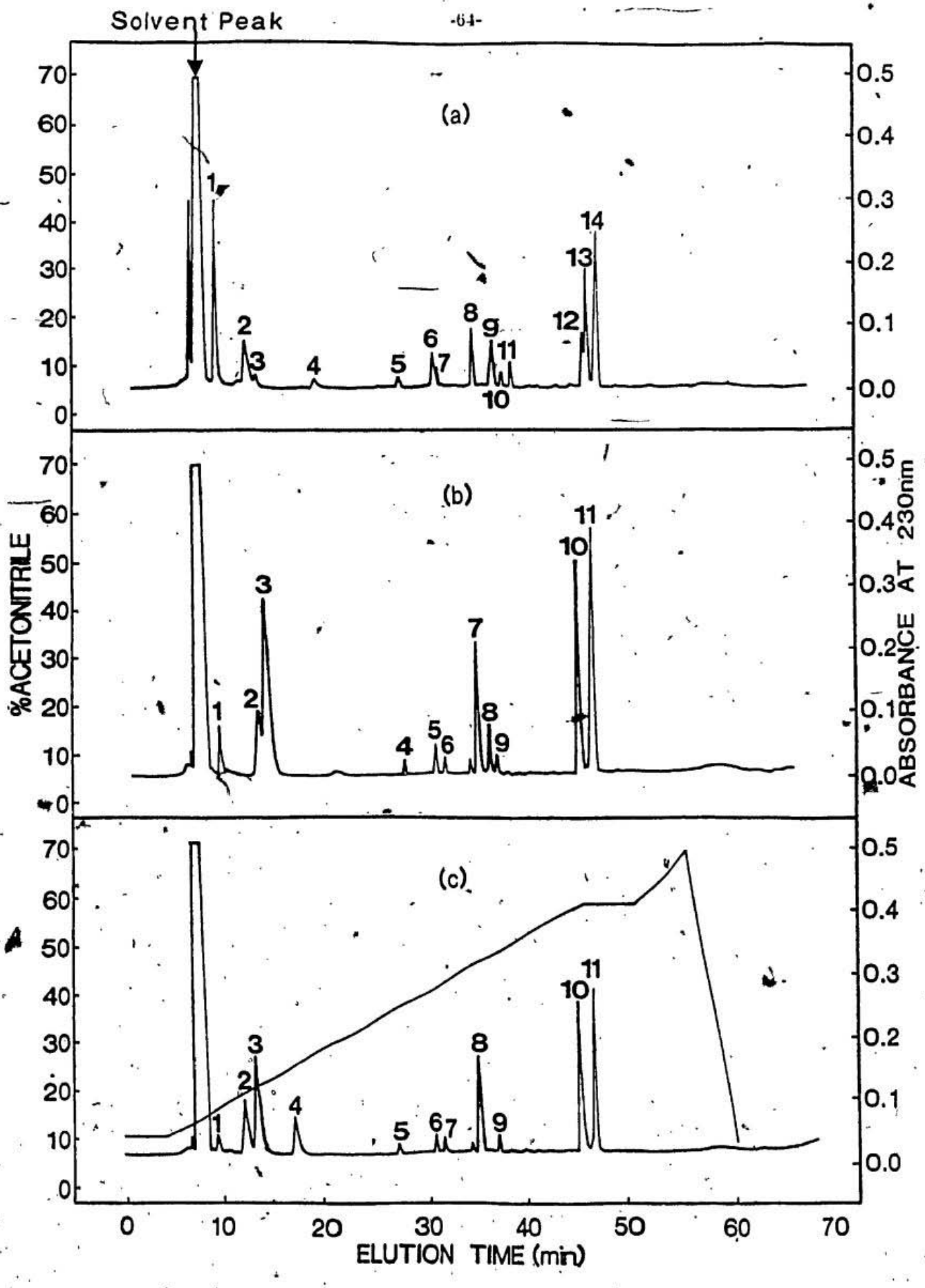

Solvent Peak
(a)
(b)
(c)
%ACETONITRILE
ABSORBANCE AT 230nm
ELUTION TIME (min)
0
10
20
30
40
50
60
70
0.0
0.1
0.2
0.3
0.4
0.5

Table 6. Amino Acid Analyses of the Tryptic Peptides
(yield in nmol)

	SP-1-A				
	T1	T2	T8	T13	T14
Asp	0.00(0)	4.63(1)	1.83(1)	2.13(1)	2.44(1)
Thr	4.94(1)	5.01(1)	1.95(1)	5.26(3)	2.60(1)
Ser	0.17(0)	0.23(0)	0.00(0)	1.97(1)	2.05(1)
Glu	0.00(0)	4.47(1)	1.70(1)	4.63(3)	4.30(2)
Pro	0.00(0)	4.51(1)	2.39(1)	4.73(3)	3.48(2)
Gly	0.00(0)	0.71(0)	1.73(1)	2.37(1)	3.97(2)
Ala	7.76(2)	4.42(1)	0.00(0)	2.97(2)	1.91(1)
Val	0.00(0)	7.51(2)	1.80(1)	3.82(2)	3.40(2)
Met	0.00(0)	0.00(0)	3.70(2)	3.05(2)	1.80(1)
Ile	0.00(0)	0.00(0)	1.35(1)	3.27(2)	5.15(3)
Leu	0.00(0)	0.00(0)	0.00(0)	2.91(2)	0.00(0)
Tyr	3.49(1)	0.00(0)	0.00(0)	0.00(0)	0.00(0)
Phe	0.00(0)	0.00(0)	0.00(0)	0.00(0)	1.66(1)
Lys	0.00(0)	4.06(1)	1.61(1)	1.50(1)	2.18(1)
Total	(4)	(8)	(10)	(23)	(18)
nmol/res.#	3.49	4.06	1.83	1.70	1.90

* The figures in ( ) indicate the calculated numbers of residues.
#This is the assumed average value of nmol corresponding to one residue in the calculations.

Table 6. Amino Acid Analyses of the Tryptic Peptides (yield in nmol) (continued)

	SP-1-B				
	T2	T3	T7	T10	T11
Asp	1.68(1)	1.00(0)	2.91(1)	2.48(1)	2.60(1)
Thr	2.12(1)	4.07(1)	2.91(1)	5.22(3)	3.01(1)
Ser	0.00(0)	0.00(0)	0.00(0)	1.57(1)	2.01(1)
Glu	1.72(1)	0.82(0)	2.48(1)	4.55(3)	4.50(2)
Pro	2.62(1)	0.51(0)	2.29(1)	4.64(3)	2.73(1-2)
Gly	0.24(0)	0.16(0)	3.08(1)	2.41(1)	4.45(2)
Ala	1.31(1)	3.42(1)	0.00(0)	3.86(2)	2.25(1)
Val	3.44(2)	4.32(1)	2.00(1)	3.62(2)	4.50(2)
Met	0.00(0)	0.00(0)	4.20(2)	3.00(2)	2.00(1)
Ile	0.00(0)	0.00(0)	0.00(0)	3.57(2)	5.85(3)
Leu	0.00(0)	0.00(0)	2.51(0)	2.91(2)	0.23(0)
Tyr	0.00(0)	2.21(1)	0.00(0)	0.00(0)	0.00(0)
Phe	0.00(0)	0.00(0)	0.00(0)	0.00(0)	1.67(1)
Lys	2.01(1)	0.00(0)	1.96(1)	1.63(1)	2.21(1)
Total	(8)	(4)	(10)	(23)	(17-18)
nmol/res.#	2.01	3.42	2.48	1.70	2.21

* The figures in ( ) indicate the calculated numbers of residues.
# This is the assumed average value of nmol corresponding to one residue in the calculations.

Table 6. Amino Acid Analyses of the Tryptic Peptides (yield in nmol) (continued)

	SP-1-C					
	T2	T3	T4	T8	T10	T11
Asp	1.08(1)	0.21(0)	0.00(0)	1.93(1)	1.92(1)	1.40(1)
Thr	1.30(1)	2.37(1)	2.35(1)	1.69(1)	3.71(3)	1.24(1)
Ser	0.00(0)	0.00(0)	1.20(0)	0.00(0)	1.30(1)	1.47(1)
Glu	1.00(1)	0.00(0)	0.99(0)	1.53(1)	3.57(3)	2.90(2)
Pro	1.77(1)	0.00(0)	0.00(0)	0.90(1)	3.34(3)	2.04(2)
Gly	0.00(0)	0.16(0)	3.91(1)	2.07(1)	1.80(1)	2.47(2)
Ala	0.82(1)	1.85(1)	3.46(1)	0.00(0)	2.90(2)	1.49(1)
Val	2.54(2)	1.65(1)	2.56(1)	8.94(1)	2.60(2)	2.70(2)
Met	0.00(0)	0.00(0)	0.00(0)	2.92(2)	?(2)	0.72(1)
Ile	0.00(0)	0.00(0)	0.00(0)	0.00(0)	2.99(2)	3.85(3)
Leu	0.00(0)	0.00(0)	0.00(0)	1.26(1)	2.76(2)	0.00(0)
Tyr	0.00(0)	0.94(1)	2.73(1)	0.00(0)	0.00(0)	0.00(0)
Phe	0.00(0)	0.00(0)	0.00(0)	0.00(0)	0.00(0)	0.87(1)
Lys	1.27(1)	0.00(0)	0.00(0)	0.90(1)	0.90(1)	1.10(1)
Total	(8)	(4)	(5)	(10)	(23)	(18)
nmol/res.#	1.27	1.65	3.20	1.53	1.30	1.24

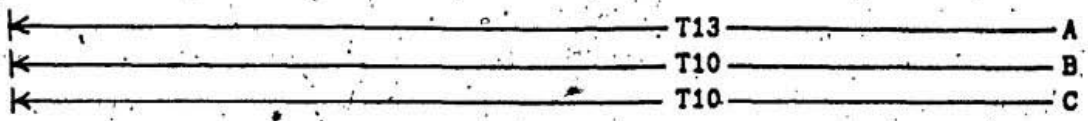
* The figures in ( ) indicate the calculated numbers of residues.
# This is the assumed average value of nmol corresponding to one residue in the calculations.

clone No.77. The former contains Ile and Ala at positions 53 and 62 respectively while the latter contains Leu and Val respectively at these same positions. These differences between the two sequences were identical to those obtained by the study of the chymotryptic digestion.

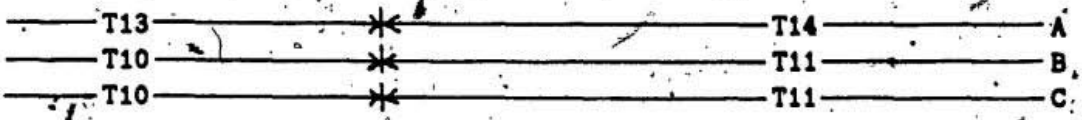
Comparing the tryptic peptide maps of SP-1-A, SP-1-B and SP-1-C obtained by reverse phase HPLC (see Figure 15) showed that these peptides had a lot of similarities. For instance, based on their elution times on HPLC, T2, T8, T13 and T14 in SP-1-A corresponded to T2, T7, T10 and T11 in SP-1-B and T2, T8, T10 and T11 in SP-1-C.

Upon comparing the amino acid analyses of the tryptic peptides of SP-1-A, SP-1-B and SP-1-C (Table 6), it was seen that an Ala and an Ile in SP-1-A (T1 and T8) replaced a Val and a Leu in SP-1-B (T3 and T7) and SP-1-C (T3 and T8). SP-1-C T4 has been found to be unique. Neither SP-1-A nor SP-1-B show any peaks at the elution time equal to that of SP-1-C T4 on the HPLC. The amino acid analysis of SP-1-C T4 showed that it contained one residue of each of Thr, Gly, Ala, Val and Tyr. The amino acid compositions of the remaining peaks were very similar. The tryptic fragments of SP-1-B and SP-1-C were matched with the peptide coded by clone No.77, thus showing that all three peptides possessed close similarities. They seem to differ only in that SP-1-C had one more Gly extended at its C-terminal (Table 7). Tentatively, it can be concluded that SP-1-A, SP-1-B and SP-1-C have very similar compositions and sequences with minor variations as discussed above.

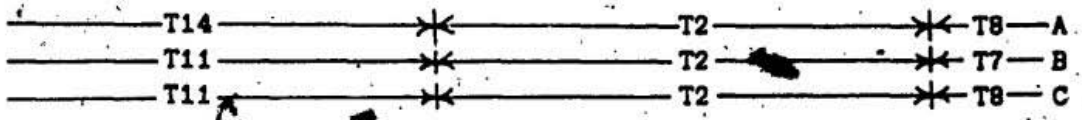
Table 7. Alignment of the Tryptic Peptides of SP-1-A (A), SP-1-B (B) and SP-1-C (C) with the Amino Acid Sequence of Clone No.77



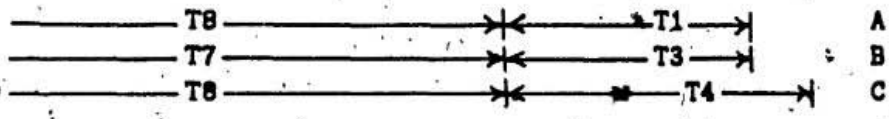
Gln Ser Val Val Ala Thr Gln Leu Ile Pro Ile Asn Thr Ala Leu Thr Pro



Ala Met Met Glu Gly Lys Val Thr Asn Pro Ile Gly Ile Pro Phe Ala Glu



Met Ser Gln Ile Val Gly Lys Gln Val Asn Thr Pro Val Ala Lys Gly Gln



[Ile] [Ala]
Thr Leu Met Pro Asn Met Val Lys Thr Tyr Val Ala Gly Lys

* The amino acids in [ ] are the ones in SP-1-A which are different from the corresponding ones in the cDNA, SP-1-B and SP-1-C.

2.2.4. CHYMOTRYPTIC PEPTIDE MAPPING OF SP-1-A, SP-1-B AND SP-1-C

The α-chymotryptic peptides of SP-1-A, SP-1-B and SP-1-C were analyzed on reverse phase HPLC in parallel. As shown in Figure 16, there were a lot of similarities among them.

2.3. SUMMARY

1. The primary structures of SP-1-A, SP-1-B and SP-1-C are very similar. This was shown by peptide mapping and amino acid analyses of both tryptic and chymotryptic digests.

2. The amino acid sequences of SP-1-A, SP-1-B and SP-1-C differ in three positions based on the data from both protein chemistry and molecular biology:

   replacement of an Ile in SP-1-A by a Leu in SP-1-B and SP-1-C;

   replacement of an Ala in SP-1-A by a Val in SP-1-B and SP-1-C;

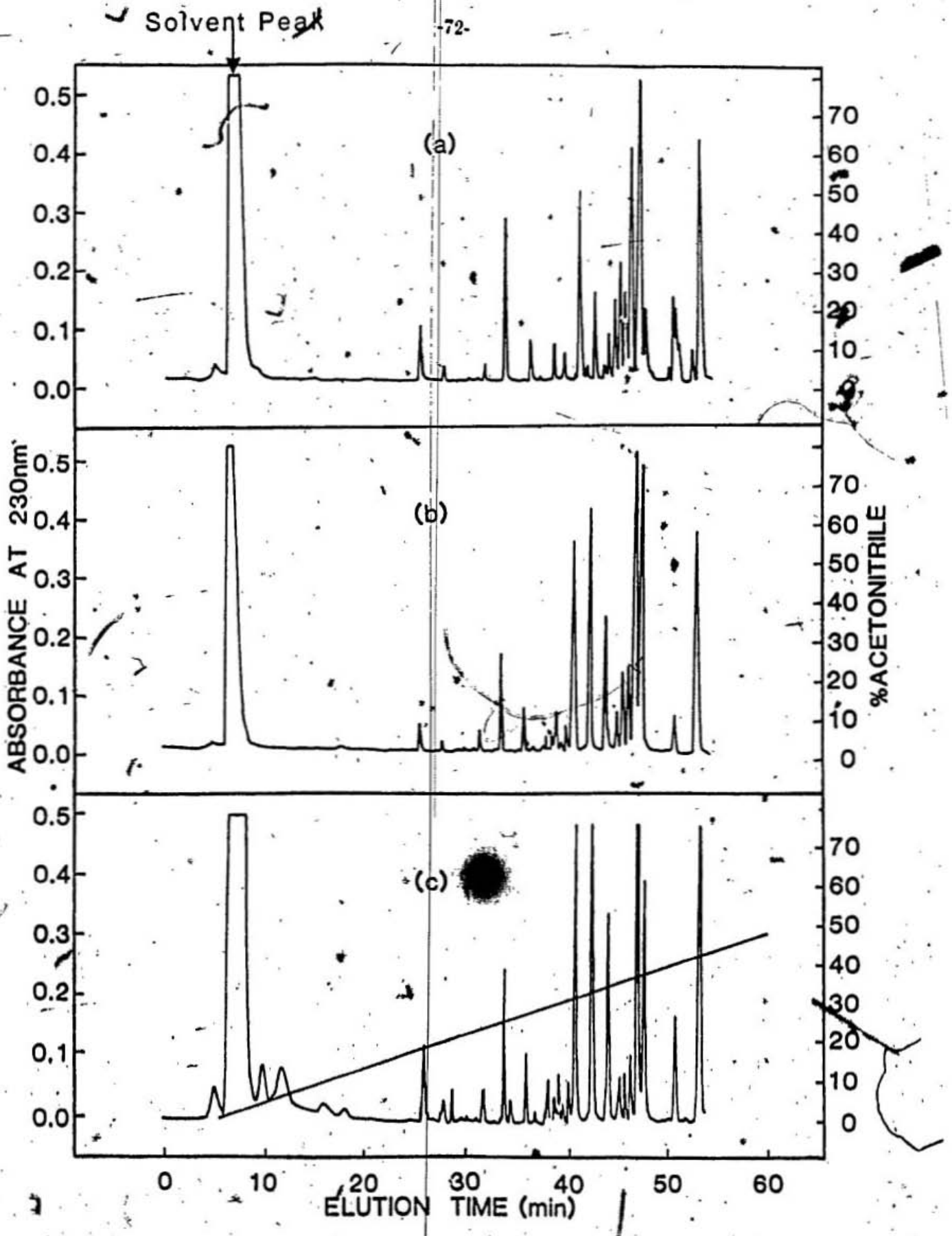
   possession of an extra Gly residue by SP-1-C at its C-terminal

   as compared to SP-1-A and SP-1-B.

   Thus, it may be concluded that SP-1-A and SP-1-B are each 63 amino acid residues long and SP-1-C is 64 residues long.

3. Unlike the AFP from flounder and sculpin which contain repeated sequences, no repeated structure can be recognized in the amino acid sequences of SP-1-A, SP-1-B and SP-1-C, suggesting that ocean pout AFP represent a new different type of fish antifreeze polypeptides.

Figure 16. Chromatography of the α-chymotryptic peptides of SP-1-A (Figure (a)), SP-1-B (Figure (b)) and SP-1-C (Figure (c)) on reverse phase HPLC in 0.02 M TEAP, pH 3. Peaks were eluted with an acetonitrile gradient as indicated. The column was Ultrasphere ODS (4.6 mm I.D. x 25 cm) (Beckman), and the flow rate was 0.5 ml/min.


Solvent Peak
(a)
(b)
(c)
ABSORBANCE AT 230nm
%ACETONITRILE
ELUTION TIME (min)
0.0
0.1
0.2
0.3
0.4
0.5
0
10
20
30
40
50
60
70

Chapter 3

Sequencing Of The cDNA Coding For One Of The Ocean Pout AFP Precursors

Protein sequence determination can only provide the primary structure of the mature protein. In order to determine the structure of one of the ocean pout AFP biosynthetic precursors, techniques of molecular biology were employed. A cDNA coding for an ocean pout AFP precursor was isolated and purified from a hybrid recombinant plasmid. This was then cloned into phage M13 mp8. The sequence of the cDNA was determined by the dideoxy chain termintion method.

3.1. MATERIALS AND METHODS

A cDNA coding for an ocean pout AFP precursor was prepared previously in our laboratory using the same methodology as for winter flounder (24). The cDNA was inserted into the PstI site of plasmid pBR 322 by poly G-C linking. This hybrid recombinant plasmid was used to transform *E. coli* HB101.

3.1.1. PREPARATION OF THE PLASMID DNA WITH THE cDNA INSERT

After screening, clone No.77 has been identified as containing the cDNA coding for an ocean pout AFP precursor. It was cultured in 500 ml of LB medium (10 g Difco tryptone, 5 g Difco yeast extract, 5 g NaCl and 1 g glucose in a litre of water, pH 7-7.2) at 37°C, 220 RPM overnight. The cells were harvested by centrifugation at 6 K RPM at 4°C for 10 min, resuspended in 15 ml of 50 mM Tris-HCl (pH 8), 25 % sucrose and digested with 40 mg of lysozyme at 4°C for 10 min, followed by addition of 2.5 ml of 0.5 M EDTA (pH 8) and incubation at 4°C for 5 min. Then it was mixed slowly with 25 ml of 0.5 mM Tris-HCl (pH 8), 62.5 mM EDTA, 0.2 % Triton-X 100, incubated at 4°C for 10 min and recentrifuged at 30 K RPM at 4°C for 45 min with "brakes off". The supernatant had the plasmid DNA, while the pellet on the bottom contained the cell chromosomal DNA. To get rid of the proteins in the supernatant, phenol and chloroform extractions were carried out three times, with the volumes of phenol and chloroform equal to that of the sample each time. Then it was centrifuged at 5 K RPM for 10 min at room temperature, and the supernatant was precipitated overnight at -20°C with one tenth volume of 3 M NaOAC (pH 5) and 2.5 volumes of 95 % ethanol. The plasmid DNA was pelleted by spinning down at 5 K RPM for 30 min at 4°C, rinsed with 70 % ethanol twice, lyophilized briefly and resuspended in TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA). The sample was digested with DNAase-free RNAase at a concentration of 10 μg per ml at room temperature for 1 h. 1 ml of the RNAase-treated plasmid DNA was layered on the top of 4 ml of 1 M NaCl in TE buffer, and the 5 ml tubes were centrifuged

at 40 K RPM at 20°C for 6 h using a Beckman SW 50.1 rotor. The plasmid DNA sedimented to the bottom, while the oligo-ribonucleotides remained in the supernatant (50).

3.1.2. PURIFICATION OF THE PLASMID DNA

The plasmid DNA thus prepared was found to be contaminated by the cell chromosomal DNA, as shown by 0.8 % agarose gel electrophoresis. A 0.8 % low melting point agarose gel was used to purify the plasmid DNA. The sample was mixed with the 6 x sample buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol, 40 % sucrose in water) and loaded on a 0.8 % low melting point agarose gel in TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA). Both the gel and the buffer contained 0.5 μg per ml of ethidium bromide. After overnight electrophoresis at 35 V at 4°C, the plasmid DNA was well separated from the chromosomal DNA contaminants.

Plasmid DNA was electroeluted into a sheet of dialysis membrane (Cellulose Dialyzer Tubing, M.W. cut-off 12,000, Fisher Scientific) inside of a trough which was cut directly in front of the leading edge of the plasmid DNA band. The membrane trough was filled with TBE buffer and the electrophoresis was carried out at 100 V, until no more DNA came from the gel. The current was reversed for 2 min at 140 V, followed by sample collection from the membrane trough.

In order to remove the ethidium bromide from the DNA sample, it was extracted with an equal volume of 1-butanol three times, followed by phenol and chloroform extractions (three times each) and ether extraction. The DNA was

precipitated overnight at -20°C with one tenth volume of 3 M NaOAC (pH 5) and 2.5 volumes of 95 % ethanol (51).

The purified plasmid DNA was pure, as judged by electrophoresis on an agarose gel (0.8 %).

3.1.3. ISOLATION OF THE INSERTED cDNA FRAGMENT

The plasmid DNA was cut using the restriction enzyme PstI. 100 µl of the plasmid DNA were mixed with 20 µl of 10 x core buffer (500 mM Tris-HCl, pH 8, 100 mM $MgCl_2$, 500 mM NaCl), 30 µl of the enzyme (9 units per µl) and 50 µl of water and digested at 37°C for 2 h. An aliquot of 2 µl was analyzed on a 0.8 % agarose gel using uncut pBR 322 as a marker. The gel electrophoresis showed that the plasmid DNA was cut into two bands, indicating that the digestion was complete.

The digest was extracted with phenol and chloroform to remove the enzyme and then precipitated with ethanol at -20°C overnight.

A 5 % polyacrylamide slab gel was employed to isolate the insert. The gel electrophoresis was carried out at 150 V in TBE buffer at room temperature until the xylene cyanol in the sample buffer reached the bottom edge of the gel. The gel was stained with ethidium bromide (0.5 µg per ml). The cDNA band in the gel was cut out under UV light, transfered into a dialysis tube and electroeluted in 0.5 x TBE buffer for 2 h at 200 V. The current was reversed for 5 min. The sample was collected, filtered through glass-wool, extracted with butanol, phenol, chloroform and ether and precipitated with ethanol at -20°C overnight (52).

3.1.4. CLONING OF THE cDNA INSERT INTO M13 mp8

3.1.4.1. LIGATION OF THE cDNA AND M13 mp8

1. Restriction Cleavage of mp8 with PstI

Due to the presence of PstI sites on both ends of the cDNA, the vector mp8 was cut with PstI as well. 4 µl of mp8 (RF form, 100 ng per µl) were mixed with 2 µl of PstI (9.5 units per µl), 2 µl of 10 x core buffer and 12 µl of water and digested at 37°C for 3 h.

2. Ligation of the PstI cut mp8 and the cDNA

20 ng of the PstI cut mp8 were ligated with 52 ng of the cDNA in 1 x ligation buffer (70 mM Tris-HCl, pH 7.5, 7 mM $MgCl_2$, 0.07 mM ATP) with 0.9 unit of T4 ligase in the presence of 0.1 mM ATP at 16°C overnight. A religation of the cut or linearized mp8 by itself was included to check the purity of the mp8 and the T4 ligase.

3.1.4.2. TRANSFORMATION OF *E. Coli* JM101 BY LIGATED M13 mp8

1. Preparation of the competent Cells of JM101 by $CaCl_2$ Treatment

A freshly grown culture of 50 ml JM101 (absorbance (650 nm) about 0.4, in YT medium which had 8 g Difco tryptone, 5 g Difco yeast extract and 5 g NaCl in a litre of water) was harvested by centrifugation at 5 K RPM for 5 min. The cells were resuspended in 20 ml of 50 mM $CaCl_2$, kept at 4°C for 30 min, recentrifuged and resuspended in 5 ml of 50 mM $CaCl_2$.

2. Transformation through Heat Shock Procedure

T and 2 ng of the ligated mp8 were mixed with 0.2 ml of the competent cells, incubated at 4°C for 45 min and then at 42°C for 2 min (heat shock), and added 10 µl of 100 mM IPTG, 50 µl of 2 % X-gal, 200 µl of exponentially grown JM101 in YT medium and 2.5 ml of melted YT soft agar (6 g agar in 1 litre of YT medium). The mixtures were poured into YT agar plates (15 g agar in 1 litre of YT medium). After hardening, the plates were incubated at 37°C overnight. A set of controls, such as the original RF form mp8, linearized mp8 and religated mp8, were also included to transform JM101.

3.1.5. PURIFICATION OF SINGLE-STRANDED (ss) DNA FROM THE COLOURLESS TRANSFORMED PLAQUES

After overnight culture, colourless transformed plaques were picked up from the YT plates, transfered to 1 ml of low Tris buffer (0.02 M Tris-HCl pH 7.5, 0.02 M NaCl, 0.001 M EDTA) individually and a drop of chloroform was added. The samples were kept at room temperature for 1 h and stored at 4°C.

It was found necessary to replate the plaques to avoid any possible contaminants from blue plaques. 2 µl of the stock phage solution were mixed with 10 µl of 100 mM IPTG, 50 µl of 2 % X-gal, 200 µl of exponentially grown JM101 in YT medium and 2.5 ml of melted YT soft agar. The mixtures were poured into YT agar plates. After hardening, the plates were incubated at 37 °C overnight.

The purified colourless plaques were used for ss DNA preparation. The colourless plaque was picked up, transfered to an exponentially grown JM101 culture (1.5 ml, absorbance (660 nm) about 0.4, in 2 x YT) and incubated at 37°C for 5 h with vigorous shaking. Then it was centrifuged for 2 min to remove the cell pellet and the supernatant containing the secreted phages was allowed to precipitate with 300 µl of 20 % PEG (20 % PEG in 2.5 M NaCl) for 0.5 h at room temperature. After centrifugation at room temperature for 15 min, the supernatant was discarded and the PEG on the side wall of the tube was removed with a Q tip or a kimwipe. The pellet was resuspended in 200 µl of low Tris buffer, extracted with phenol and chloroform, and then precipitated with ethanol at -20°C overnight. After centrifugation, the pellet was lyophilized briefly and

resuspended in 50 μl of low Tris buffer. 5 μl from it was analyzed on a 0.8 % agarose gel in TBE buffer to check the yield and the purity of the ss DNA. Usually, ten individual plaques were picked up at one time.

3.1.6. ORIENTATIONS OF THE ss DNA BY FIGURE 8 FORMATION TEST

2 μl (out of 50 μl) of ss DNA from one clone were mixed with that of another clone in the presence of 4 μl of buffer I (100 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 50 mM NaCl) and 1 μl of buffer II (80 % glycerol, 1 % SDS, 0.2 % bromophenol blue) in a capillary pipet. It was boiled for 3 min, incubated at 65-67°C for 15 min, slowly equilibrated to room temperature in a 10 ml water bath in about 20 min and analyzed on a 0.8 % agarose gel in TBE buffer in parallel with ss DNA and RF mp8.

3.1.7. PRELIMINARY SCREENING OF THE ss DNA BY SINGLE LANE TRACKING

This was the way to check the identities of individual ss DNA, using essentially the same experimental procedures as those for DNA sequencing (which will be discussed in more detail in the next section) except that only a single chain terminator (ddG) was incorporated and that different samples were analyzed in parallel on a DNA sequencing gel. Clones with the same sequences would give the same G band patterns.

3.1.8. DNA SEQUENCING BY DIDEOXY CHAIN TERMINATION METHOD

3.1.8.1. ANNEALING REACTION OF THE PRIMER AND THE ss DNA TEMPLATE

The ss DNA prepared above was used as a template in the reaction. At first, a test was set to determine the optimal ratio of template to primer using varying concentrations of the primer from 0.5 μl to 3 μl, 5 μl of template, 1 μl of 10 x Hin buffer (66 mM Tris-HCl pH 7.4, 66 mM β-mercaptoethanol; 0.5 mM NaCl) and water to make up total volume of 10 μl. The primer used was 15 bases long: 5' CCCAGTCACGACGTT 3'.

The actual concentration of the template was not measured because of its small amount. The concentration of the primer (obtained from another laboratory) was unknown either. The important factor is their ratio which was optimized.

The samples were sealed in capillary pipets, boiled for 3 min, incubated at 65-67°C for 15 min and equilibrated to room temperature in a 10 ml water bath in about 20 min.

3.1.8.2. CHAIN EXTENSION REACTIONS

To an annealed mixture, 15-20 μCi of [α-^{32}P]dATP (3200 mCi per mmole in tricine) and 1 μl of 20 μM dATP were added. The solution was divided into 4 parts in Eppendorf (1.5 ml) microfuge tubes, each of which contained a mixture of dNTPs and one ddNTP. DNA polymerase (Klenow fragment) (1 μl, 0.2 unit) was added to the side wall of each tube, and the reaction was started by brief

centrifugation to mix the reagents. After standing at room temperature for 15 min, 1 µl of 0.5 mM dATP was added to each tube, and then after a further 15 min each reaction was stopped by the addition of 9 µl of stopping solution (0.3 % bromophenol blue, 0.3 % xylene cyanol, 10 mM EDTA, pH 8 in deionized formamide).

The reaction mixtures were identified as Amix, Tmix, Gmix and Cmix depending on whether they contained ddATP, ddTTP, ddGTP or ddCTP. The dNTP mixtures used in these reactions were different, and had the following compositon.

	Amix	Tmix	Gmix	Cmix
0.5 mM dTTP	20 µl	1 µl	15 µl	15 µl
0.5 mM dGTP	20 µl	15 µl	1 µl	15 µl
0.5 mM dCTP	20 µl	15 µl	15 µl	1 µl
10 x Hin buffer	20 µl	15 µl	15 µl	15 µl

The concentrations of the ddNTP solutions were: ddATP, 0.2 mM; ddTTP, 1mM; ddGTP, 0.4 mM; ddCTP, 0.4mM. Preliminary tests were made to determine the optimal volume ratio of each ddNTP to its appropriate dNTP mixture. ddNTP were used in volumes between 0.4 and 1 µl, and dNTP mixtures between 1 and 1.6 µl.

3.1.8.3. GEL ELECTROPHORESIS

The DNA sequencing gel was 6 % polyacrylamide in TBE buffer containing 8 M urea (Bis : acrylamide = 20 : 1). A 5 % gel was also used.

In order to avoid the phenomenon of "smiling face" in the gel, a sheet of aluminium plate (3 mm thick) with size similar to that of the glass plates was attached to the outside glass plate.

2 μl of each extension reaction was loaded on the gel after it had been boiled for 3 min. Four extension reactions of each template were analyzed on the gel in parallel at the same time. The electrophoresis was carried out at a constant current of 25 mA which required a voltage of 1,200 to 1,400 and gave a gel surface temperature of around 60°C.

Three loadings were made which ran for 2 h, 4 h and 6 h respectively.

As soon as the electrophoresis was finished, the gel was transfered to a sheet of 3 MM Whatman paper, covered with Saran Wrap, dried on a gel dryer (Dual Temperature Slab Gel Dryer, Model SE 1125B from Bio-Rad Laboratories) under reduced pressure at 80°C for 45 min and autoradiographed at -20°C overnight using Kodak X-ray film (X-Omat RP film, XRP-1). The film was developed next morning and the sequence was read from it (53, 54, 55, 56, 57, 58, 59).

3.1.9. PURITY OF REAGENTS

All the glassware and solutions used were either autoclaved or filter-sterilized. Tryptone, yeast extract and agar were purchased from Difco Laboratories (Detroit, MI. USA). Lysozyme, IPTG and X-gal were from Sigma Chemical Company (St. Louis, MO, USA). Agarose (Ultra Pure DNA Grade), bromophenol blue, xylene cyanol FF and the chemicals for polyacrylamide gel electrophoresis, such as, acrylamide, Bis, TEMED, ammonium persulphate, were "Electrophoresis-Purity Reagents" and purchased from Bio-Rad Laboratories (Richmond, CA. USA). RNAase and T4 ligase were from Boehringer Mannheim GmbH (West Germany). Low melting point agarose (Electrophoresis Grade), urea (Ultra Pure Enzyme Grade), PstI, DNA polymerase (Klenow fragment), RF M13 mp8 and λDNA-Hind III markers were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, USA). The 15-base primer for DNA sequencing was from Pharmacia P-L Biochemicals, Inc. (Milwaukee, WI. USA) and the [α-^{32}P] dATP was from New England Nuclear (Boston, MA. USA). All the other chemicals were of reagent grade.

3.2. RESULTS

3.2.1. PURIFICATION OF THE PLASMID DNA

Figure 17 shows a comparison of the impure plasmid DNA and the purified plasmid DNA on a 0.8 % agarose gel in 1 x TBE buffer. The purification was successful.

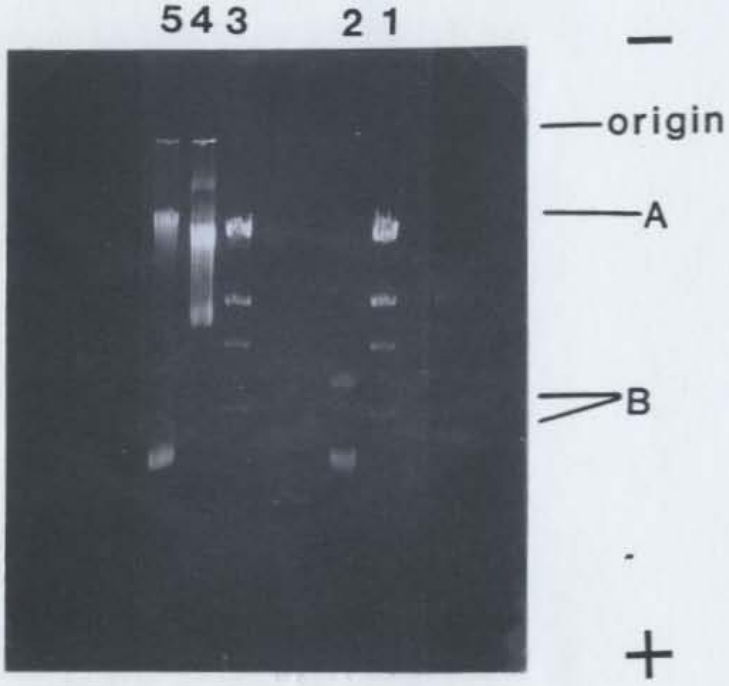


Figure 17. Analysis of the unpurified and purified plasmid DNA on a 0.8 % agarose gel in TBE buffer. The electrophoresis was carried out at 30 V overnight. Lanes No.1 and No.3 were λDNA-Hind III markers. Lanes No.2 and No.5 were the purified and unpurified plasmid DNA respectively. Lane No.4 was E3 plasmid DNA. Positions A and B correspond to the cell chromosomal DNA and the plasmid DNA respectively.

3.2.2. THE SIZE OF THE INSERTED cDNA

Figure 18 shows the plasmid DNA which had been digested with PstI at 37°C for 2 h in parallel with the λDNA-Hind III markers and the undigested plasmid DNA.

Figure 19 shows the pure cDNA fragment on a 0.8 %-agarose gel in TBE buffer in parallel with λDNA-Hind III markers.

From Figure 18 and Figure 19, it appears that the cDNA fragment was a bit smaller than the 560 base pair fragment in λDNA-Hind III markers, as indicated by the fact that it ran slightly faster than the 564 base pair fragment.

3.2.3. RESULTS OF cDNA SEQUENCING

Approximately twenty colourless transformed plaques were picked up. Figure 20 shows the purity of some of the single-stranded DNA on a 0.8 % agarose gel.

By "figure 8 formation" test (Figure 21), and G lane tracking, it was shown that clones No.2, No.11, No.13 and No.15 were the same with poly G tail, while clone No.6, No.12 and No.16 were shown to be the same with poly G tail and the first and the second groups were complementary to each other.

The results from the ratioing tests of the primers to the templates showed that 2 μl of primer and 5 μl of template were the optimal pair. Similarly, the tests checking the ratios of ddNTP to dNTP indicated that the following pairs were the optimal ones:

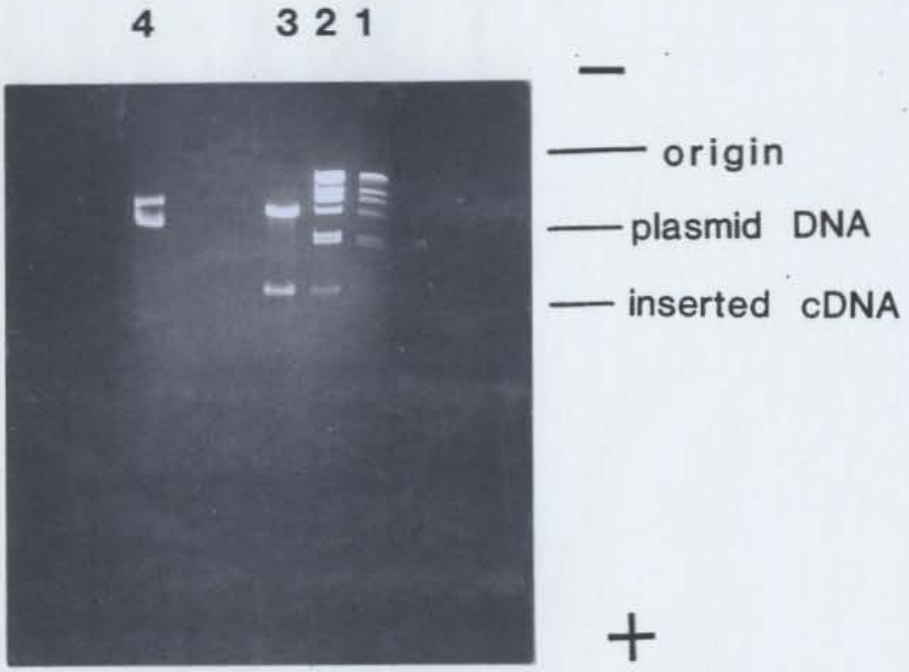


Figure 18. Analysis of the PstI digested plasmid DNA on a 0.8 % agarose gel in TBE buffer. Lanes No.1 to No.4 correspond to λDNA-Hind III markers, λDNA-Hind III markers, sample after digestion and sample without digestion respectively. The gel electrophoresis was carried out at 90 V for 1.5 h.

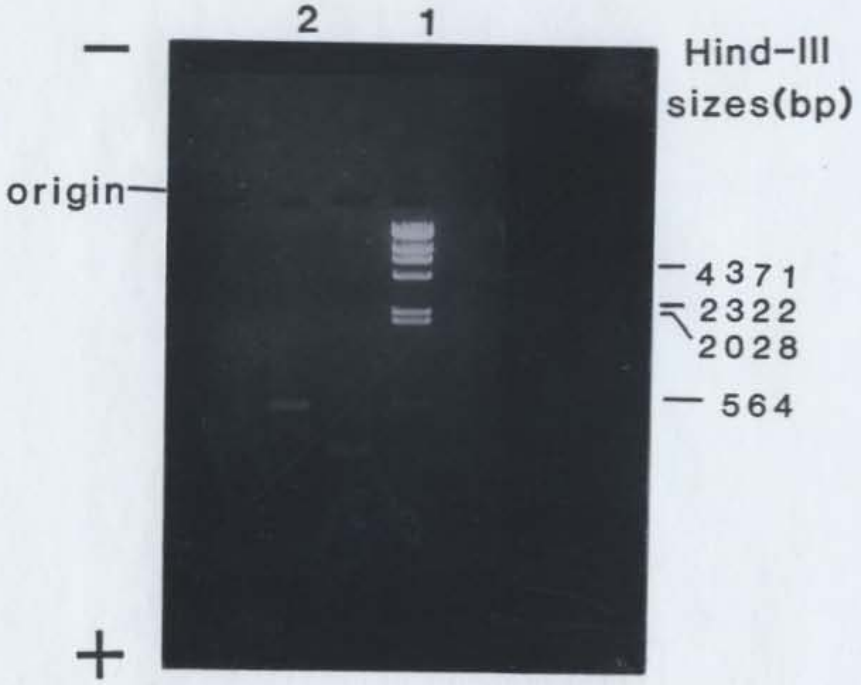


FIGURE 19. Analysis of the cDNA fragment in parallel with λDNA-Hind III markers on a 0.8 % agarose gel in TBE buffer. Lane No.1 was the λDNA-Hind III markers, No.2 was the cDNA fragment. The gel was run at 90 V for 2 h.

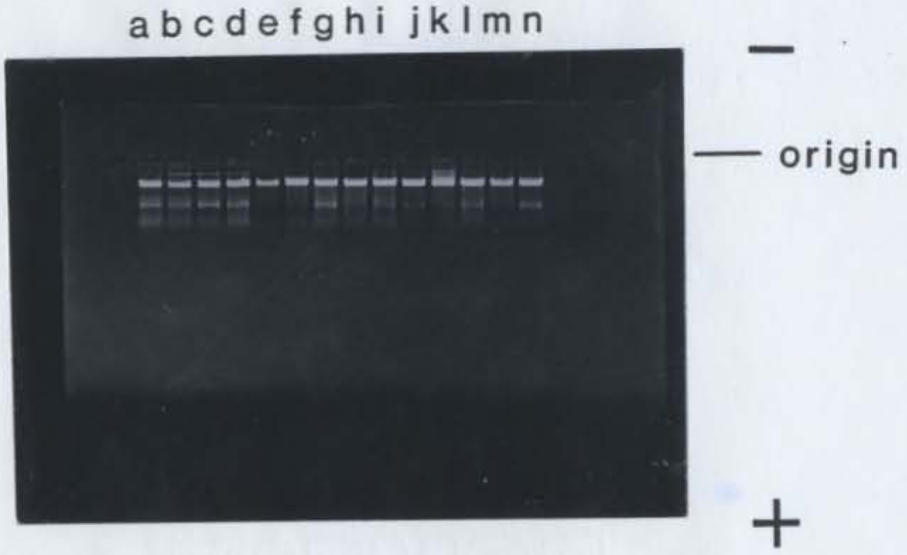


Figure 20. Analysis of ss DNA of the cDNA on a 0.8 % agarose gel in TBE buffer. Lanes "a to n" correspond to clone No.2, No.2, No.3, No.3, No.6, No.6, No.11, No.11, No.12, No.12, No.16, No.16, No.20 and No.20 respectively. The gel electrophoresis was carried out at 90 V for 1 h.

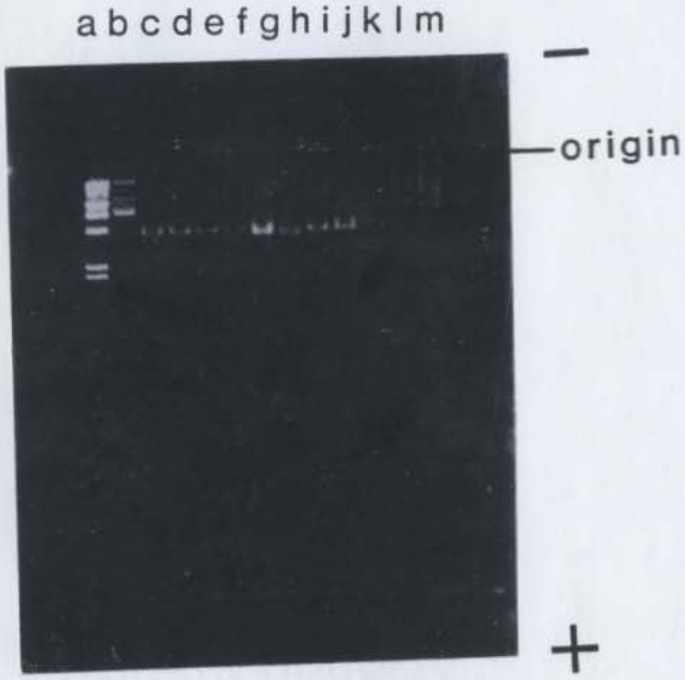


Figure 21. Result of the figure 8 formation test. Samples were annealed with clone No.2. The lanes "a to m" correspond respectively to λDNA-Hind III markers, mp8 (RF), clones No.2, No.3, No.11, No.13, No.15, No.1, No.5, No.19, No.6, No.12 and No.16. The gel electrophoresis was carried out at 90 V for 2 h.

	A mix	T mix	G mix	C mix
dd NTP	0.40 μl	1.00 μl	1.00 μl	0.66 μl
d NTP	1.60 μl	1.00 μl	1.00 μl	1.34 μl

They were chosen for annealing and extension reactions respectively in the sequencing experiments.

Clones No.2, No.11 and No.12 were sequenced. Figure 22 shows part of the X-ray film of a sequencing gel from clone No.11. From comparisons with the protein sequence data in chapter 2, it was seen that clones No.2 and No.11 started from the 5' end, while clone No.12 started from the 3' end. The direct reading from the sequencing gel of clone No.12 was converted to the complementary counterparts (A-T, G-C). There was an overlapping region of about 20 bases between clone No.2 (or 11) and clone No.12 (Table 8).

From Table 8 we can see that:

The overall sequence obtained in the present study is 539 bases with a Pst I site CTGCAG at both ends. Next to the Pst I site, there is a poly G region (hexadecamer) at 5' end and a poly C region (eicosamer) at 3' end. Therefore, the size of the cDNA estimated by 0.8 % agarose gel electrophoresis was quite close to its actual size.

The overall sequence includes a coding region of an 87 amino acid residue precursor for an ocean pout AFP, and two untranslated regions at 5' and 3' ends.

Figure 22. Autoradiograph of part of the sequencing gel from clone No.11. The four channels, A, T, G and C, correspond to the four individual extension reactions. * indicates the overlapping nucleotide sequence between the first and the second loadings.

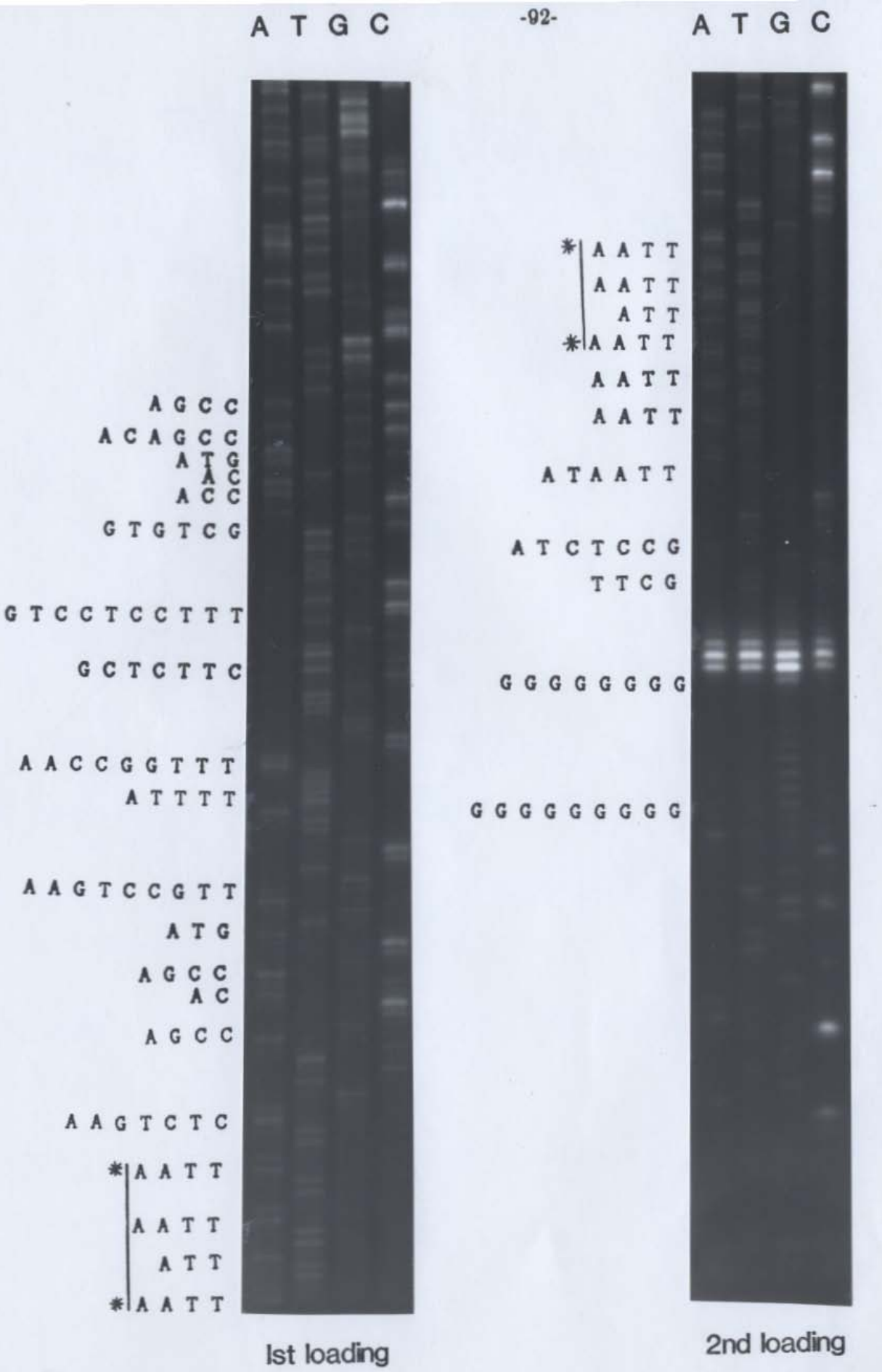

A T G C
A G C C
A C A G C C
A T G
A C
A C C
G T G T C G
G T C C T C C T T T
G C T C T T C
A A C C G G T T T
A T T T T
A A G T C C G T T
A T G
A G C C
A C
A G C C
A A G T C T C
* A A T T
A A T T
A T T
* A A T T
1st loading
A T G C
* A A T T
A A T T
A T T
* A A T T
A A T T
A A T T
A T A A T T
A T C T C C G
T T C G
G G G G G G G G
G G G G G G G G G
2nd loading

Table 8. The cDNA Sequence of Clone No.77 and Its Amino Acid Sequence

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. . . T T G G C T G C A G G G G G G G G G G G G G G G G T T C G A T
                P s t I
C T C C G A T A A T T A A T T A A T T A A T T A T T A A T T A A T T A A
          STOP                STOP                STOP
G T C T C A G C C A C A G C C A T G A A G T C C G T T A T T T T A A C C
                              M e t L y s S e r V a l I l e L e u T h r
G G T T T G C T C T T C G T C C T C C T T T G T G T C G A C C A C A T G
G l y L e u L e u P h e V a l L e u L e u C y s V a l A s p H i s M e t
A C A G C C A G C C A G T C C G T G G T G G C C A C C C A G C T G A T C
T h r A l a S e r G l n S e r V a l V a l A l a T h r G l n L e u I l e
C C C A T A A A T A C T G C C C T G A C T C C G G C G A T G A T G G A G
P r o I l e A s n T h r A l a L e u T h r P r o A l a M e t M e t G l u
G G G A A G G T G A C C A A C C C A A T A G G C A T C C C G T T C G C G
G l y L y s V a l T h r A s n P r o I l e G l y I l e P r o P h e A l a
G A G A T G T C C C A A A T A G T G G G G A A G C A A G T G A A C A C G
                              *                 &
G l u M e t S e r G l n I l e V a l G l y L y s G l n V a l A s n T h r
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CCAGTGGCTAAGGGCCAAACCCTCATGCCAAACATG

ProValAlaLysGlyGlnThrLeuMetProAsnMet
                    Ile (SP-1-A)

                                  G
GTGAAAACGTACGTCGCGGGAAAGTAGTTCTGAGGG

ValLysThrTyrValAlaGlyLysSTOP    STOP
          Ala (SP-1-A)

TGCCAAGGAGCTTCTTCCCAAAACCAAAAGAAGAAA

TGCCCCCCTCTCACAATTAACCTTGTTTTTGTCACAA

ACCCAAGTCTGTCCGGATGTTAACTGAACATGTCAA

AACCTGTGGAGACTGGTGAGATTTGATGGTCTGAAA

AGATAAAGCCTATAAATCCCCCCCCCCCCCCCCCCC

CGACGTCGGTTCGAA
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Pst I

* * indicates the overlapping region of clone No.2 and No.12 (for details see 3.2).

As mentioned in the previous chapter, the amino acid sequence of SP-1-A matches the cDNA coding sequence closely except for two replacements. In one, an Ile at position 53 in SP-1-A is replaced by a Leu in the cDNA, and in the other, an Ala at position 62 in SP-1-A is replaced by a Val in the cDNA. On the other hand, the cDNA sequence matches the amino acid compositions of SP-1-B and SP-1-C closely, as mentioned in chapter 2. The difference between SP-1-B and SP-1-C is due to the presence of one more Gly at the C-terminal end of SP-1-C.

3.3. SUMMARY

Several points can be summarized from Table 8 and the previous chapter:

1. Ocean pout AFP are synthesized via large precursors.

2. The cDNA coding for ocean pout AFP components SP-1-B and SP-1-C includes an 87 amino acid residue coding region and two untranslated regions at both 5' and 3' ends.

3. The 87 residue coding region consists of a 22 amino acid residue presequence and a 65 residue mature protein. The presequence which starts at Met is rich in hydrophobic amino acids, such as Leu and Ile. The mature protein region starts at Gln and ends at Lys. There is no prosequence in the biosynthetic precursor of ocean pout AFP (personal communication with Dr. Hew)

4. Post translational modifications may be involved in the synthesis of the AFP, as indicated by the fact that all of SP-1-A, SP-1-B and SP-1-C lack at their C-terminal ends Lys which is present in the cDNA sequence.

5. SP-1-B and SP-1-C differ from each other in that the latter has one more Gly at its C-terminal end, which may also be explained by a post translational modification.

6. SP-1-A differs from SP-1-B and SP-1-C in that one Ile and one Ala in SP-1-A at positions 53 and 62 (in the mature sequence) are replaced by Leu and Val in SP-1-B and SP-1-C.

DISCUSSION

Ocean pout AFP can be isolated and separated successfully using Sephadex G-75 gel-filtration chromatography, QAE-Sephadex, SP-Sephadex ion-exchange chromatography and reverse phase HPLC. The main AFP components used in the present study, SP-1-A, SP-1-B and SP-1-C, were isolated with a high level of homogeneity by the above procedures.

Ocean pout AFP comprise a family of at least twelve active components of nearly identical size (about 6,000 daltons) and similar amino acid compositions which fall into two separate groups, named QAE and SP, based on their behaviour on ion-exchange chromatography and amino acid compositions.

Clone No.77 whose amino acid sequence matches the amino acid compositions of the tryptic peptides of both SP-1-B and SP-1-C is assumed here to code for the biosynthetic precursors of SP-1-B and SP-1-C. The sequence of clone No.77 has been compared with that of clone No.69 which was sequenced in Dr. P. L. Davies' laboratory in Queen's University. It is found that these two differ only at two positions (Table 9), C and T in clone No.77 substitute A and C in clone No.69, which leads to substitutions of a Leu and a Val in clone No.77 to an Ile and an Ala in clone No.69. Coincidently, the sequence of clone No.69 matches that of SP-1-A (Table 9), and it is assumed here that clone No.69 codes for the biosynthetic precursor of SP-1-A. The above suggests that in ocean pout, at least some of the antifreeze polypeptides are coded by different antifreeze genes. We can probably say safely that the multiple family of the ocean pout AFP is coded by a multiple family of AFP genes. From the evolution point of

Table 9. Sequence Comparison of Clone No.77 and No.89

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. . . . TTGGCTGCAGGGGGGGGGGGGGGGGTTCGAT
            Pst I

CTCCGATAATTAATTAATTAATTATTAATTAATTAA
      STOP          STOP          STOP

GTCTCAGCCACAGCCATGAAGTCCGTTATTTTAACC
                  MetLysSerValIleLeuThr

GGTTTGCTCTTCGTCCTCCTTTGTGTCGACCACATG
GlyLeuLeuPheValLeuLeuCysValAspHisMet

ACAGCCAGCCAGTCCGTGGTGGCCACCCAGCTGATC
ThrAlaSerGlnSerValValAlaThrGlnLeuIle

CCCATAAATACTGCCCTGACTCCGGCGATGATGGAG
ProIleAsnThrAlaLeuThrProAlaMetMetGlu

GGGAAGGTGACCAACCCAATAGGCATCCCGTTCGCG
GlyLysValThrAsnProIleGlyIleProPheAla

GAGATGTCCCAAATAGTGGGGAAGCAAGTGAACACG
GluMetSerGlnIleValGlyLysGlnValAsnThr
```

Ile (SP-1-A)
A (Clone No.69)
↑

CCAGTGGCTAAGGGCCAAACCCTCATGCCAAACATG

ProValAlaLysGlyGlnThrLeuMetProAsnMet

Ala (SP-1-A)
C (Clone No.69)
↑ G

GTGAAAACGTACGTCGCGGGAAAGTAGTTCTGAGGG

ValLysThrTyrValAlaGlyLysSTOP STOP

TGCCAAGGAGCTTCTTCCCAAAACCAAAAAGAAGAAA

TGCCCCCCTCTCACAATTAACCTTGTTTTTTGTCACAA

ACCCAAGTCTGTCCGGATGTTAACTGAACATGTCAA

AACCTGTGGAGACTGGTGAGATTTGATGGTCTGAAA

AGATAAAGCCTATAAATCCCCCCCCCCCCCCCCCCC

CGACGTCGGTTCGAA

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view, the conclusion that the multiple gene family encoding the ocean pout AFP components came from gene duplication events can be made. In addition, the ocean pout AFP genes corresponding to clone No.77 and No.69 must have come from recent duplication events.

From the amino acid analyses, QAEA, SP-1-A, SP-1-B and SP-1-C contain fourteen to fifteen amino acids, have a modest Ala content, and lack any half-cystine residues. Thus, this differs from flounder and sculpin AFP which have a high Ala content (about 60 mol %) (24, 25, 21, 27); and, also, sea raven AFP which have an extraordinarily high content of half-cystine (about 8 mol %) (20). The amino acid sequences of the AFP from flounder, sculpin and ocean pout (clone No.77) have been compared (Table 10). As mentioned in the introduction, the AFP from flounder and sculpin show remarkable similarities in terms of their primary and secondary structures, including the presence of the eleven amino acid residue repeats in both fish. Ocean pout AFP, on the other hand, fail to show any similarity to them. This confirms the early report that besides the AFGP, flounder AFP and sea raven AFP, the ocean pout AFP represent a fourth class of antifreeze, having its own distinct features (22).

Further structural studies carried out on SP-1-A, SP-1-B and SP-1-C have shown that their primary structures are very similar, as indicated by peptide mapping and amino acid analyses of both tryptic and chymotrytic digests. But differences do exist. The assumed amino acid sequences of SP-1-A, SP-1-B and SP-1-C which are based on results from both protein chemistry and molecular biology show that differences exist at at least three positions. C and T in clone

Table 10. Amino Acid Sequences of Some Antifreeze Polypeptides

Flounder AFP

a
#6
Asp Thr Ala Ser Asp Ala Ala Ala Ala Ala Ala Leu Thr Ala Ala Asn Ala Lys Ala Ala Ala Glu Leu Thr Ala Ala Asn Ala Ala Ala Ala Ala Ala Ala Thr Ala Arg

b
#6
Asp Thr Ala Ser Asp Ala Ala Ala Ala Ala Ala Leu Thr Ala Ala Asn Ala Ala Ala Ala Ala Lys Leu Thr Ala Asp Asn Ala Ala Ala Ala Ala Ala Ala Thr Ala Arg

c
AFP-3
Asp Thr Ala Ser Asp Ala Ala Ala Ala Ala Ala Ala Leu Thr Ala Ala Asx Ala Ala Ala Ala Ala Ala Lys Leu Thr Ala Asx Asx Ala Ala Ala Ala Ala Ala Ala Thr Ala Ala

Sculpin AFP

d
SS-8
Met Asn Gly Glu Thr Pro Ala Gln Lys Ala Ala Arg Leu Ala Ala Ala Ala Ala Leu Ala Ala Lys Thr Ala Ala Asp Ala Ala Ala Lys Ala Ala Ala Lys Ala Ala
Ala Ile Ala Ala Ala Ala Ala Ser Ala

d
SS-8
Met Asp - Ala Pro Ala Ala Arg Ala Ala Ala Lys Thr Ala Ala Asp Ala Leu Ala Ala Ala Lys Lys Thr Ala Ala Asp Ala
Ala Ala Ala Ala Ala Ala Ala

Ocean Pout AFP

Clone 77
Gln Ser Val Val Ala Thr Gln Leu Ile Pro Ile Asn Thr Ala Leu Thr Pro Ala Met Met Gln Gly Lys Val Thr Asn Pro Ile Gly Ile Pro Phe Ala Glu Met Ser
Gln Ile Val Gly Lys Gln Val Asn Thr Pro Val Ala Lys Gly Gln Thr Leu Met Pro Asn Met Val Lys Thr Tyr Val Ala Gly Lys

a) Davies *et. al* (24)

b) Pickett *et. al* (25)

c) DeVries *et. al* (60)

d) Hew *et. al* (28)

No.77 replace A and C in clone No.69, which causes the replacements of a Leu and a Val in SP-1-B and SP-1-C to an Ile and an Ala in SP-1-A. SP-1-C has one more Gly than SP-1-A and SP-1-B at its C-terminal. So, SP-1-A and SP-1-B are 63 amino acid residues long, while SP-1-C is 64 residues long.

AFP isolated from individual ocean pout caught in Newfoundland and New Brunswick during winter months were analyzed on reverse phase HPLC for comparison. Both HPLC profiles are similar, if not identical, indicating that there is no significant populational polymorphism. AFP from winter flounder inhabiting different waters have been studied (45); the results in that study were similar to those obtained from the ocean pout in the present investigation.

Ocean pout AFP from winter and summer months have been analyzed on reverse phase HPLC also. It appears that the AFP contents in both winter and summer are very similar, even though the relative amounts of one or two components are higher in summer than in winter. However, there is no significant seasonal polymorphism.

Clone No.77 which is assumed to code for SP-1-B and SP-1-C consists of two untranslated regions at both 3' and 5' ends and a precursor region coding for a 22 amino acid residue presequence and a 65 residue mature protein. The presequence, or "signal peptide" as it is normally called, is rich in Leu and Ile. Similarly in winter flounder, AFP are also synthesized via large precursors (24, 25). However, the biosynthetic precursor of ocean pout AFP, unlike that of flounder AFP, has no prosequence (personal communication with Dr. Hew).

Post translational modifications may be involved in the synthesis of the ocean pout AFP. This is shown by the fact that SP-1-A, SP-1-B and SP-1-C lack Lys at their C-terminal ends despite the fact that the cDNA (both clones No.77 and No.69) show Lys at their C-terminal ends. A similar situation has been reported in flounder AFP (24, 25) in which the Gly at C-terminal has been removed by a post translational modification.

The documentation of a new type of AFP from ocean pout has added additional complexity to the mechanism of action of the antifreezes. Apart from their ability to exhibit thermal hysteresis, there is no evidence to suggest that the AFGP and AFP operate via a common mechanism. The difference in their carbohydrate content, in their primary and secondary structures, as well as in the presence of disulphide bonds, have made it difficult to propose such a common mechanism. A well known example of different proteins performing similar biological functions via separate mechanisms is that of the proteases. It has been well established that serine proteases and subtilisin function via different mechanisms and have different genetic origins, that is, they evolved by convergent evolution, even though they all hydrolyze peptide bonds. Purely from the view of amino acid sequence homology or difference, and types of secondary structures, it seems that AFGP, flounder (or shorthorn sculpin) AFP, sea raven AFP, and ocean pout AFP may come by convergent evolution and function via separate mechanisms. However, the above speculation is tentative. The three dimensional structures of these antifreezes would be better indicators for their genetic origins and functional mechanisms, because proteins function via their conformations. It would be quite probable for these antifreezes to have different three dimensional

structures, because the types of secondary structures they possess have been shown to be quite different (see introduction for details).

Much information has been obtained on ocean pout AFP in the present investigation. However, some questions still remain to be answered.

The present and earlier investigations have shown that there are at least twelve active components in the ocean pout AFP which fall into two distinct groups, named QAE and SP. Only three components, SP-1-A, SP-1-B and SP-1-C, have been studied in some detail. The amino acid sequence of the N-terminal part of SP-1-A, the overall amino acid sequences of SP-1-B, SP-1-C and of the remaining components, especially those of the QAE group which is different from the SP group, are still to be confirmed or elucidated. Hopefully, the study of their primary structures will lead to some new thoughts on the mechanism of the antifreeze action.

A further step to understand the functional mechanism of the ocean pout AFP can be made by chemical modification studies. By chemically modifying some of the groups in the ocean pout AFP and looking at their effects on the antifreeze activity, it may be possible to locate the crucial amino acid residues for the AFP activity in the AFP molecules, and therefore, establish the functional mechanism. This may be a prospective project.

The elucidation of the three dimensional structures of the ocean pout AFP will be very helpful to our understanding of their functional mechanism(s). Also, it will provide evidence to support or dispute the suggestion that AFGP, flounder

AFP, sea raven AFP, and ocean pout AFP have different genetic origins and functional mechanisms.

Another interesting question is how the ocean pout AFP level is controlled and regulated. It is known that the ocean pout AFP level shows annual cycling, but this seasonal control of AFP biosynthesis is not tight. This is indicated by the fact that, in comparison to flounder, there is a considerably larger amount of AFP in ocean pout serum even in summer (22). It has been reported that in the case of the winter flounder, the pituitary gland, photoperiod and water temperature play major roles in the regulation of the AFP biosynthesis (31, 32, 33). Obviously, these three factors do not contribute as much in the control of the ocean pout AFP biosynthesis. In the long run, it will be worthwhile to find out the genomic organization and structure of the ocean pout AFP DNA; that is, the structure of exons and introns, the number of gene copies and the arrangement of the multiple AFP gene family, and identify the regulatory gene sequences and the molecular basis for the seasonal expression of the AFP genes.

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