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ANALYSIS OF FISH ANTIFREEZE POLYPEPTIDES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

We have isolated antifreeze polypeptides (AFPs) from four species of fish, the winter flounder, shorthorn sculpin, sea raven and ocean pout. Except for apparent similarities in their activity, comparison of structural information so far has led to the classification of these AFPs into three distinct groups. Each AFP occurs as a family of polypeptides of similar molecular weights and charges, and we have encountered some difficulties in resolving them by conventional fractionation techniques. However, with the application of C_{18} and C_3 reversed-phase high-performance liquid chromatography (HPLC) in triethylamine phosphate buffer, pH 3.0 or 0.05% trifluoroacetic acid and acetonitrile gradient, we were able to fractionate AFPs into at least seven components for winter flounder, one major and several minor components for shorthorn sculpin, two for sea raven, and 8–11 for ocean pout. The recovery after reversed-phase HPLC of these peptides was estimated as 82–85%. In the case of winter flounder, the two major components differ only by an aspartic acid ↔ glutamic acid replacement. We have now used HPLC successfully to monitor AFP heterogeneity, induction of these AFPs by hormones, and their analysis due to seasonal, population and geographical variations.

INTRODUCTION

The blood plasma of a variety of teleost fishes from polar and subpolar oceans contain antifreeze glycoproteins (AFGPs) or antifreeze polypeptides (AFPs). These macromolecules, which lower the freezing temperatures of body fluids below that of the surrounding waters, are essential for the survival of these species inhabiting the ice-laden environment[1,2,3].

Because of the cold Labrador current, many species of fish inhabiting the coastal waters of Newfoundland and Labrador produce antifreeze proteins and peptides in the winter. These include winter flounder[4,5], shorthorn sculpin[6], sea raven[7], and ocean pout[8]. AFPs from these species occur as multiple components of similar size and charge. Early attempts to fractionate these AFPs by conventional protein fractionation procedures had not been completely satisfactory. Discrepancies in the

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number and size of these peptides have been reported by different laboratories[3,9].

Reversed-phase HPLC, because of its superior resolving power, has enabled us to fractionate these peptides satisfactorily. Besides their isolation and characterization, HPLC provides us with an invaluable and indispensable tool for the analysis of these AFPs due to seasonal and geographical variation, preferential induction by hormones, and their heterogeneity.

The present communication summarizes our experiences with the use of reversed-phase HPLC for these materials.

MATERIALS AND METHODS

Sample preparation

The isolation of partially purified AFP from winter flounder[5], shorthorn sculpin[6], sea raven[7], and ocean pout[8] was described in our early publications. Briefly, the fish blood was clotted in the presence of phenylmethanesulfonyl fluoride and centrifuged. The serum was chromatographed on a Sephadex G-75 column (86 × 1.5 cm I.D.) in 0.2 *M* NH_4HCO_3. Active fractions (monitored with a freezing point osmometer, Advanced Instruments, Needham Heights, MA, U.S.A.) were rechromatographed on the same column and lyophilized. The lyophilized powders were the starting materials for HPLC.

Liquid chromatography system

HPLC systems from two commercial suppliers were used. The Beckman Model 334 HPLC system included a Hitachi variable-wavelength detector, a C-RIA data recorder and 2 Beckman Model 110-A pumps. Water HPLC System included 2 Model 6000 A pumps, Model 481 absorbance detector, Model 730 data module, and Model 680 automatic controller.

Chromatography and columns

Both the aqueous and organic solvents were filtered separately through Millipore filters (GS 0.22-μm for aqueous, and HF 0.45-μm for organic solvents). Solvents were saturated with nitrogen and stored in amber bottles prior to use. The columns used were Altex Ultrasphere ODS (5-μm, 25 cm × 4.6 mm I.D.), Altex Ultrapore RPSC (7.5 cm × 4.6 mm I.D.), Waters μBondapak C_{18} (30 cm × 7.8 mm I.D.), and a C_2 column (25 cm × 4.6 mm I.D.). The last column was packed in our laboratory using C_2 (ethyl) silica obtained from Sigma (pore size 60 Å, particle size 5 μm average).

RESULTS AND DISCUSSION

Representative HPLC profiles of different AFP on a Waters μBondapak C_{18} column in 0.05% trifluoroacetic acid–acetonitrile gradient is shown in Fig. 1. Flounder AFP was resolved mainly into three components, sea raven into two, shorthorn sculpin into one major plus several minor peaks and ocean pout into at least eleven components. Except for the minor peaks in the shorthorn sculpin, all the other peaks in these profiles showed antifreeze activities, as measured by a nanoliter osmometer (Clifton Technical Physics, Hartford, N.Y., U.S.A.). The amino acid composition of

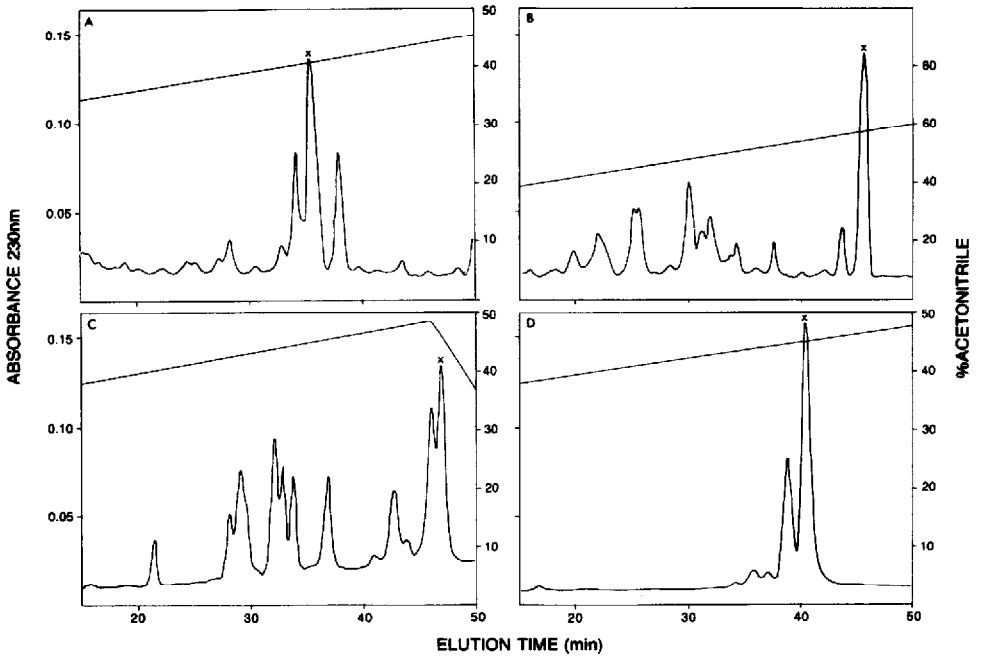

Fig. 1. Analysis of fish antifreezes by reversed-phase HPLC. Columns used: Waters μBondapak C_{18} (30 cm × 7.1 mm I.D.); flow-rate: 1 ml/min; with 0.05% trifluoroacetic acid–acetonitrile. Approximately 20–50 μg of samples were applied. (A) Winter flounder, (B) shorthorn sculpin, (C) ocean pout, and (D) sea raven.

the major peak (marked as × in Fig. 1) in each of the four species of fish is shown in Table I. None of the AFP contains carbohydrates. Both flounder and sculpin AFP contain an abundance of alanine (approximately 60% of total amino acids). On the other hand, the sea raven AFP is rich in half-cysteine while the ocean pout AFP is neither rich in alanine nor in half-cystine. It appears that there are at least three distinct classes of polypeptide antifreezes, each having distinct structural features[4–9].

We have compared and analysed the elution profiles of these AFPs on different reversed-phase columns using two different aqueous buffers. In the case of flounder AFP, the best resolution was achieved with an Altex Ultrasphere ODS column in a triethylamine phosphate buffer, pH 3.0, and acetonitrile (Fig. 2). The peptides can be resolved into seven active components[10]. The two major components, No. 6 and No. 8, differ in their amino acid composition[9] by only one amino acid (glutamic acid *vs.* aspartic acid). Amino acid sequence determination has shown that instead of a simple replacement, it actually involves the alternation of three separate positions where $Lys_{18} \rightarrow Ala$, $Glu_{22} \rightarrow Lys$, $Ala_{26} \rightarrow Asp$ from component 6 to 8 (Fig. 3). The evidence of these substitutions will be reported in a separate publication[11]. It is significant that HPLC can resolve these two components containing 37 amino acids and differing by only one methylene group ($-CH_2-$). On our previous fractionation procedures, where QAE-Sephadex was used, these two components were eluted as a single peak[9]. The above methodology has enabled us to clarify the discrepancies

TABLE I

AMINO ACID COMPOSITION OF FISH ANTIFREEZE POLYPEPTIDES

The amino acids are expressed in mol-%; ND means not determined.

Amino acid	*Winter flounder (mol%)*	*Shorthorn sculpin (mol%)*	*Sea raven (mol%)*	*Ocean pout (mol%)*
Asx	11.5	3.2	10.7	8.6
Thr	10.7	4.8	7.9	7.8
Ser	3.0	2.8	8.2	4.6
Pro	–	2.5	6.7	11.0
Glx	3.0	5.4	9.1	8.4
Gly	0.3	2.8	8.1	5.4
Ala	60.6	56.7	14.4	9.6
half Cys	–	–	7.6	–
Val	–	–	1.2	15.5
Met	–	2.5	5.4	5.9
Ile	–	2.1	1.7	5.8
Leu	5.6	4.9	6.2	9.9
Tyr	–	–	1.2	1.9
Phe	–	–	2.0	0.7
Lys	2.7	8.1	2.1	2.4
His	–	–	2.5	–
Arg	2.7	2.3	2.3	4.5
Trp	–	–	2.8	N.D.
Mol.wt.	3300	6000	12,000	6000

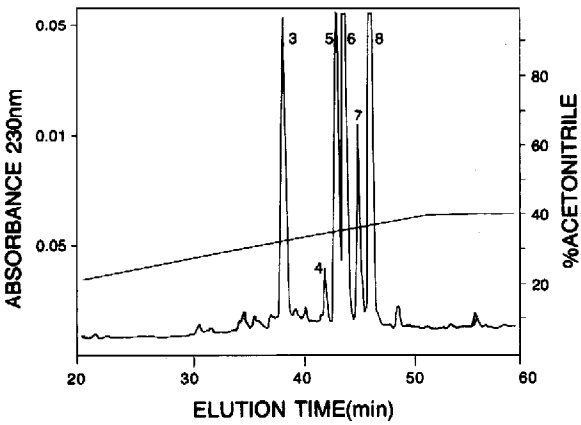

Fig. 2. Analysis of winter flounder AFP by reversed-phase HPLC. Column used: Altex Ultrasphere ODS column (25 cm × 4.6 mm I.D.); flow-rate 1 ml/min with triethylamine phosphate buffer, 0.01 *M* (pH 3.0) and acetonitrile. The numbering of polypeptides is based on ref. 10. Component 9, which is eluted much later, is not shown in this figure.

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                    1               5                   10

Component 6 ... NH2 - Asp-Thr-Ala Ser Asp Ala Ala Ala Ala Ala Ala Leu
Component 8 ... NH2 - Asp-Thr-Ala Ser Asp Ala Ala Ala Ala Ala Ala Leu

                                  15                  20
                          Thr Ala Ala Asn Ala Lys Ala Ala Ala Glu Leu
                          Thr Ala Ala Asn Ala Ala Ala Ala Ala Lys Leu
                               25                  30
                          Thr Ala Ala Asn Ala Ala Ala Ala Ala Ala Ala
                          Thr Ala Asp Asn Ala Ala Ala Ala Ala Ala Ala
                           35
                          Thr Ala Arg - COOH
                          Thr Ala Arg - COOH
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Fig. 3. Amino acid sequences of the two major flounder AFP (No. 6 and No. 8). The C-terminal glycine is absent in the mature peptide; its presence has been derived from the nucleotide sequence of mRNA[9].

in the number and size of active AFP between De Vries[3,4] and our laboratories[5,10] and to eliminate geographical and population variations as a source of these differences[12].

The separation of sea raven AFP (mol. wt. 13,000) into two components differs from our earlier procedure, which involved ion-exchange chromatography and polyacrylamide gel electrophoresis[7]. Resolution on the C_3 column is comparable with that on the Waters μBondapak C_{18} column. However, we were unable to elute the protein from the Altex Ultrasphere ODS column, presumably because of much stronger binding to this packing material.

The ocean pout AFP gave the most complex picture. It showed at least eleven components in all columns analysed, except C_2. This observed heterogeneity is neither an artifact due to proteolytic degradation of the sample nor due to pooling of different serum samples. The serum contained the protease inhibitor phenylmethanesulfonyl fluoride during isolation. The HPLC profile of AFP from an individual animal was identical with the one from pooled samples[8]. Furthermore, tryptic peptide mapping of the three components with elution time 31.5, 32.2 and 33.2 min., (Fig. 1c) showed structural homology, but not complete identity[13]. However, one cannot rule out microheterogeneity due to *in vivo* post-translational modification or processing. Such a modification has been observed in flounder AFP, where component 5 (Fig. 2) is a cleavage product of component 6, in which its C-terminal arginine has been removed[10]. Besides the multiplicity of polypeptides, ocean pout is unique because it contains significant concentrations of AFP in the summer[14]. The role of AFP in these warmer temperatures, if any, is presently unknown. A comparison of the

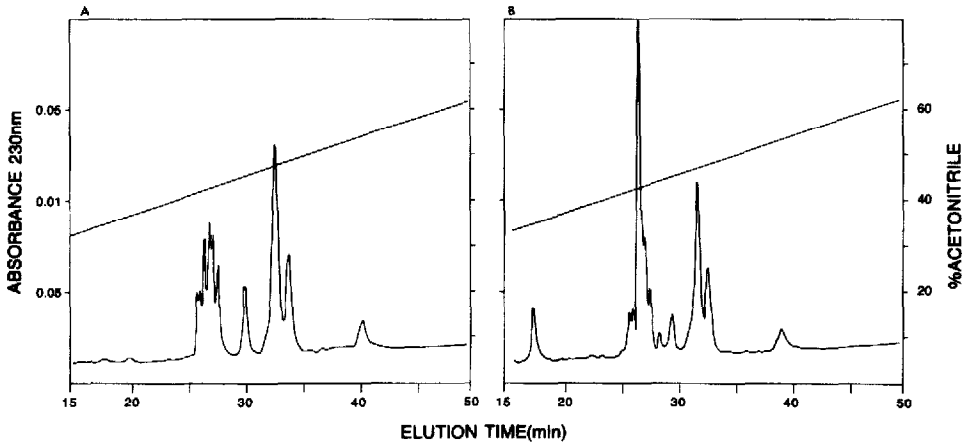

Fig. 4. HPLC comparison of ocean pout AFP isolated from (A) winter, (B) summer. The column used was Altex Ultrapore RPSC, flow-rate 0.5 ml/min with 0.05% trifluoroacetic acid–acetonitrile.

HPLC profiles between a summer *vs.* winter sample is shown in Fig. 4. There is a definite difference in these two profiles. However, the fact that most of the peaks are eluted in the same position would argue against the non-specific degradation of the summer sample.

In summary, the reversed-phase HPLC, has provided us with a powerful analytical tool for demonstrating the heterogeneity of various fish AFPs, their structural characterization as well as for solving some complex biological puzzles. We anticipate that the methodology will continue to be used successfully in our studies of these polypeptides.

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