Biochimica et Biophysica Acta 915 (1987) 357–370
Elsevier

BBA 32955

Primary and secondary structure of antifreeze peptides from arctic and antarctic zoarcid fishes

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(Received 26 May 1987)

Key words: Antifreeze peptide; Peptide structure; Amino acid sequence; Ice crystal formation; Cold water fish; (Zoarcid fish)

Antifreeze peptides were isolated from *Rhigophila dearborni*, an antarctic eel pout, and *Lycodes polaris*, an arctic eel pout (both from the family Zoarcidae). The primary structures of two peptides, one from each species, were determined using a combination of Edman degradation and mass spectrometric techniques. The two sequences show a high degree of homology with nearly 80% of the residues being identical. These peptides are also homologous to antifreeze peptides from a third eel pout which inhabits the north Atlantic Ocean. The CD spectra of all of these peptides are also very similar. Unlike the antifreeze peptides of cottid fishes, the structures of antifreeze peptides from zoarcid fishes appear to be highly conserved, despite the large geographic distances which separate the different species. The zoarcid peptides also appear to have structures very different from other fish antifreezes.

Introduction

Macromolecular antifreezes protect polar fishes from subfreezing temperatures by inhibiting ice growth in their body fluids. The mechanism by which these antifreezes function is not completely understood, but it is not merely a depression of the freezing point by colligative effects. Adsorption of antifreeze to the surface of the ice crystal has been proposed to result in the noncolligative lowering of the freezing point of water [1]. Evidence of adsorption of antifreezes to ice has been presented [1,2]. These studies show that several antifreezes, in contrast to other solutes, are not concentrated into the liquid phase as ice crystals grow in the solution. Antifreezes have also been shown to alter the crystal-growth habit of ice. Ice grown at high antifreeze concentrations (10 mg/ml) characteristically grows as long thin needles or spicules [1,3]. At low antifreeze concentrations (10^{-8} M), ice crystals develop crystal faces never observed when grown in solutions of other solutes [4]. These studies strongly suggest that these antifreezes act at the ice/water interface.

The structural components of the antifreezes which allow them to adsorb to the ice surface have not been unequivocally identified. The first antifreezes whose primary and secondary structures

Abbreviations: DABITC, 4-*N*,*N*-dimethylaminoazobenzene-4′-isothiocyanate; DABTH, 4-*N*,*N*-dimethylaminoazobenzene-4′-thiohydantoin; FAB, fast atom bombardment; PTH, phenylthiohydantoin; Peptides LP and RD, antifreeze peptides from *L. polaris* and *R. dearborni*, respectively.

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were studied were the glycopeptide antifreezes from antarctic fishes and the peptide antifreeze of the winter flounder [5–8]. These antifreezes are composed largely of alanine. Their primary structures, although different, result in a periodic placement of polar groups in both types of antifreeze. Chemical modifications of the polar groups were shown to eliminate antifreeze activity [9,10]. Models which incorporate the secondary structures of these antifreezes suggest that the spacings of the polar groups within the molecules are favorable for hydrogen bonding to the ice lattice [6,11]. The suggestion was made that all antifreezes might exhibit compositions similar to either the glycopeptides of antarctic fish or the winter flounder peptides [11]. Glycopeptide antifreezes which were nearly identical to those from antarctic fishes were identified in several arctic fishes [12–15]. Peptide antifreezes which showed many similarities to the winter flounder peptide were identified in the alaskan plaice and in two sculpins [11,16,17]. More recently, however, peptide antifreezes which differ significantly from the flounder peptide have been isolated from the sea raven and the ocean pout [18,19]. The structures of these peptides do not appear to fit the proposed model relating peptide structure and adsorption to ice. It is now becoming clear that a number of peptides with diverse structures are used by different fishes to accomplish the same function. With hopes of more clearly understanding the structure–function relationship in peptide antifreezes, we determined the primary structures of peptide antifreezes from one antarctic and one arctic ocean pout.

Materials and Methods

Peptide purification

Specimens of *Rhigophila dearborni* were caught in traps at depths of 400–500 m in McMurdo Sound, Antarctica (77°54′S, 166°40′E) during the austral summer (October–December) in water of −1.9°C. Blood was collected from the caudal vein with syringe and needle. After clotting at 4°C and centrifugation, the serum was pooled and dialyzed in Spectrapore 3 against 0.1 M Tris-HCl (pH 9.2) for 48 h. The dialyzed serum was applied to a DEAE ion-exchange column equilibrated with the same buffer. Protein was eluted from the column with a linear NaCl gradient (0–0.1 M in 0.1 M Tris-HCl (pH 9.2)) and was detected by absorbance at 230 and 280 nm. Each of the protein fractions eluted from the column was dialyzed, lyophilized, and assayed for the presence of antifreeze by determining the difference between the freezing and melting points of their aqueous solutions at a concentration of 10 mg/ml [3,20]. The largest antifreeze containing peak was further purified by HPLC on an Altex Ultrasphere ODS reverse-phase column (5μ, 4.6 × 250 mm). A linear acetonitrile gradient in triethylamine phosphate (pH 2.2) (0.1 M phosphoric acid adjusted to pH 2.2 with triethylamine) was used to elute the peptides which were detected by absorbance at 230 nm.

Lycodes polaris were caught between May 6 and May 20 in the Bering Sea off the coast of Alaska (57°33′9″N, 172°03′1″W) by bottom trawling at depths down to 140 m. The water temperature was −1°C. Blood samples were taken as described above. The serum was dialyzed in Spectrapore 3 against 2.5 mM Tris-HCl (pH 9.2) for 43 h and chromatographed on a DEAE ion-exchange column equilibrated with the same buffer. Protein was eluted with a NaCl gradient (0–0.1 M in 0.2 M Tris-HCl (pH 9.2)). Antifreeze activity was found in a shoulder which was poorly resovled from a large peak which contained most of the serum proteins. The fractions containing the antifreeze were reequilibrated with 2.5 mM Tris-HCl (pH 9.2) and chromatographed on a second DEAE column. Protein was eluted with a NaCl gradient (0–0.04 M in 2.5 mM Tris-HCl (pH 9.2)). Fractions were processed and assayed for antifreeze as described for the *Rhigophila* peptide.

Polyacrylamide gel electrophoresis

SDS-gel electrophoresis was performed according to the method of Laemmli [21]. Gradient gels from 10 to 20% acrylamide were run at 25 mA constant current. Estimates of molecular weight of the pure peptide antifreezes were made from a standard curve of relative mobilities of Bio-Rad low-molecular-weight standards.

Amino-acid analysis

The antifreeze peptides were hydrolyzed in 6 M HCl in vacuo according to the method of Moore

and Stein [22]. Norleucine was added to each sample as an internal standard. After hydrolysis the samples were dried in vacuo over NaOH and analyzed on a single-column analyzer with ninhydrin detection, according to Gürtler [23].

Proteinase digestions and fragment isolation

Trypsin, *Staphylococcus aureus* (V8) proteinase, chymotrypsin and carboxypeptidase B digestions were done in 50 mM ammonium bicarbonate (pH 8–8.5) at 37 °C. Tryptic and carboxypeptidase B digestions were done with an enzyme/substrate ratio of 1 : 50 (w/w) for 4 h and 1 h, respectively. V8 proteinase digestions were done at an enzyme/substrate ratio of 1 : 30 (w/w) for 24 h, and chymotryptic digestions were done at an enzyme/substrate ratio of 1 : 100 (w/w) for 30 min. Carboxypeptidase A digestions were done in *N*-ethylmorpholine acetate buffer (pH 8.5) at an enzyme/substrate ratio of 1 : 100 (w/w) for 30 min at 25 °C after the method of Ambler [24]. This sample was acidified with 1 M acetic acid, dried in vacuo, and run on a single-column amino-acid analyzer. Cyanogen bromide digestions were done with a 30-fold excess of the reagent in 70% formic acid for 4 h at 50 °C.

Tryptic and V8 proteinase digest fragments were separated on either an Ultrasphere ODS (Altex) or a μBondapak (Waters Associates) reverse-phase HPLC column. The solvent system used on the Ultrasphere column was 0.2 M triethylamine phosphate (pH 2.2) with a linear acetonitrile gradient from 0–60% at 1% per min. Detection of the peptides was by absorbance at 230 nm. The acetonitrile was evaporated from the eluted peptides under a stream of nitrogen. The samples were applied to a C_{18} Sep-Pak cartridge (Waters Associates) and washed with triethylamine acetate (0.1 M acetic acid titrated to pH 4.5 with triethylamine) to remove the phosphoric acid. The peptides were then eluted with acetonitrile and dried under vacuum. The solvent system used on the μBondapak column was 5% acetic acid with *n*-propanol as the organic modifier. A concave gradient (number seven on a Waters Model 660 gradient programmer) was used going from 0–60% *n*-propanol. 1 ml fractions were collected. Aliquots of each fraction were spotted on Whatman 3MM paper and stained with cadmium/ninhydrin for detection of eluted peptides.

Cyanogen bromide fragments were isolated on the Ultrasphere ODS column with a linear gradient of acetonitrile (0–80%) in 0.1% trifluoroacetic acid.

Chymotryptic fragments were separated by thin-layer chromatography on Eastman cellulose layers in 2-butanol/3% NH_4OH, 3 : 1 (v/v). The edges of the plate were cut off and sprayed with fluorescamine in acetone to locate the peptides. The stained edges were realigned to the plate and each peptide was recovered by scraping the cellulose off the support and eluting the peptide into 200 μl of 0.1 M NH_4OH.

Peptide sequencing

Manual sequencing of the digest fragments was done using the 4-*N*,*N*-dimethylaminoazobenzene-4′-isothiocyanate (DABITC) method of Chang et al. [25] as modified by Allen [26]. The thiohydantoin derivatives (DABTH) were qualitatively identified on 2.5 × 2.5 cm polyamide thin layers. Leucine and isoleucine assignments were tentatively made by running the DABTH derivatives on 10-cm-polyamide layers in solvent 1 (H_2O/acetic acid, 2 : 1 (v/v)). The R_f values of these derivatives are slightly different [27]. The amino terminal sequences of the two native peptides were also determined by the dansyl-Edman method [28].

Fast-atom-bombardment (FAB) mapping was done according to the method of Morris et al. [29,30]. Sequence analysis by FAB mass spectrometry was done using 1 : 1 $^1H/^2H$ acetyl derivatives of the peptides as described by Morris et al. [30].

The DABTH derivatives of leucine and isoleucine are not well resolved on the polyamide layers, and accurate assignment of these two amino acids by this method is difficult. Mass spectrometric techniques also fail to distinguish these amino acids. The positions of these residues were therefore established by automated sequence analysis on a Applied Biosystems model 470A sequenator at the University of Illinois Biotechnology Center. The PTH (phenylthiohydantoin) derivatives were quantitatively identified by HPLC on a Applied Biosystems C_{18} reverse-phase column. Though the sequenator could potentially yield most of the

antifreeze sequence, identification beyond about 50 residues can be difficult for several reasons. Incomplete reactions in each cycle of Edman degradation leads to carry-over of residues from one cycle to the next. Since the yield of released amino acids decreases with each cycle as the carry-over is increasing, determining the signal from the noise becomes a problem. The yields of several amino acids, such as threonine and serine, are intrinsically low in Edman degradation, making their identification difficult. The carry-over problem also makes it difficult to ascertain when the C-terminus has been reached. For these reasons sequencing and overlapping of proteinase fragments remains an essential process.

Circular dichroism

CD spectra of peptide LP were obtained on a Jasco J-40A spectropolarimeter. The sample cell had a pathlength of 0.1 mm and was water-jacketed for temperature control. Spectra were taken at 10 C° intervals from 0 to 70° C. The peptide was dissolved in water at a concentration of 1.9 mg/ml. The molar ellipticity $[\theta]$ was calculated using the expression $[\theta] = \theta° m/10\ \lambda C$, where $\theta°$ is ellipticity in degrees, m is the mean residue weight, λ is the pathlength in cm, and C is the sample concentration in g/cm^3. The mean residue weight calculated from the sequence is 105.

Results

Purifications

Fig. 1 illustrates the ion-exchange purification of *R. dearborni* serum. Each of the first five peaks was found to contain antifreeze activity (melting point minus freezing point ranged from 0.5 to 0.8 C° at 10 mg/ml in H_2O). Fraction 2, the largest antifreeze-containing peak, was further purified by reverse-phase HPLC (Fig. 2). Several components were resolved. The major peak is the antifreeze peptide designated RD and is the subject of this sequence analysis. The lesser components were not analyzed further.

The first ion-exchange purification of peptides from *L. polaris* gave poor resolution of antifreeze peptides. The reason for the poor resolution is uncertain, but is probably a result of the high initial ionic strength (0.2 M Tris) and the steepness of the NaCl gradient. The second purification, done in 2.5 mM Tris with a much shallower gradient, resolved three major peaks and is shown in Fig. 3.

Analysis of fraction 1 on the reverse-phase column did not indicate the presence of other peptides in this fraction (Fig. 4), suggesting that this fraction was homogeneous. Subsequent tests for homogeneity supported this conclusion. This peptide will be referred to as LP. Fractions 2 and

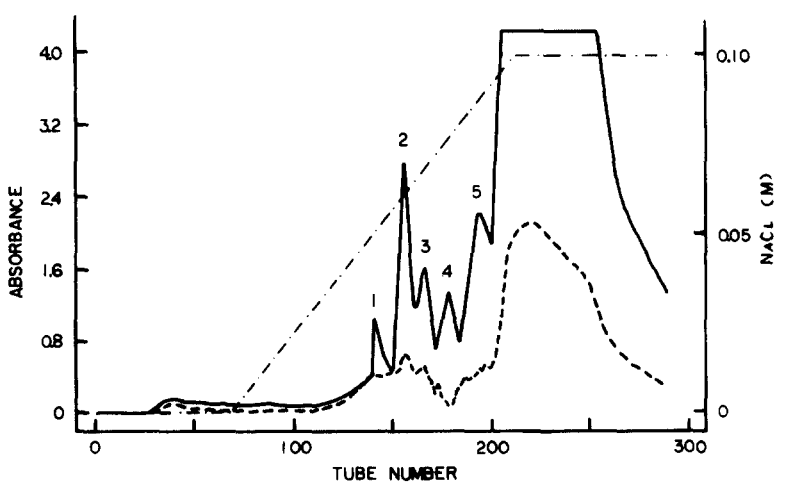

Fig. 1. Ion-exchange purification of *R. dearborni* antifreeze peptide. 100 ml dialyzed serum chromatographed on a DEAE-cellulose column (4.4×100 cm) at a flow rate of 1.7 ml/min. — — —, A_{280}; ———, A_{230}; -·-·- NaCl concentration.

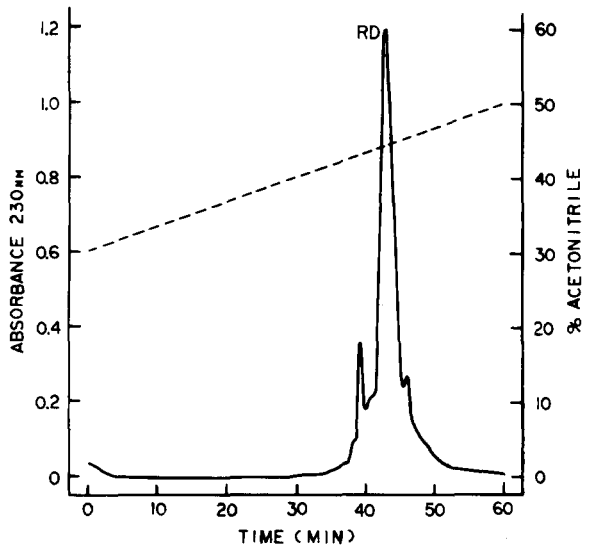

Fig. 2. HPLC purification of fraction 2 from *Rhigophila* ion-exchange column. Peptides eluted in linear gradient (30–50%) of acetonitrile in 0.1 M triethylamine phosphate (pH 2.2) at 0.33%/min at a flow rate of 1 ml/min. ———, A_{230}; — — —, percent acetonitrile.

3 also contained antifreeze activity but were not further analyzed.

The antifreeze activities of peptides LP and RD were measured by the method described previously. At concentrations of 10 mg/ml in water,

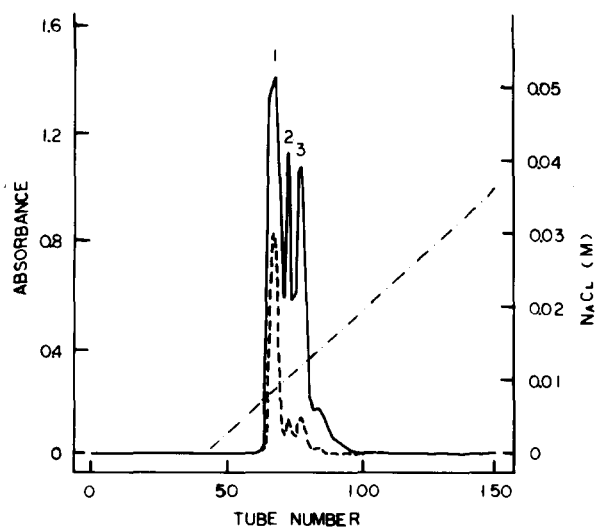

Fig. 3. Ion-exchange purification of *L. polaris* antifreeze peptide. 100 ml dialyzed serum chromatographed on a DEAE-cellulose column (4.4×100 cm) at a flow rate of 1.7 ml/min. — — —, A_{280}; ———, A_{230}; -·-·-, NaCl concentration.

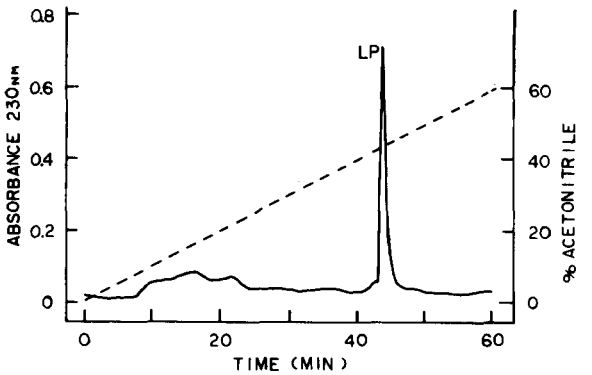

Fig. 4. HPLC analysis of fraction 1 from *Lycodes* ion exchange purification. Peptides eluted in linear gradient (0–60%) of acetonitrile in 0.1 M triethylamine phosphate (pH 2.2) at a flow rate of 1 ml/min. ———, A_{230}; — — —, percent acetonitrile.

peptides LP and RD prevented ice growth down to temperatures of −0.78 and −0.76 °C, respectively.

Polyacrylamide gel electrophoresis

The homogeneity of peptides RD and LP was determined by SDS-polyacrylamide gel electrophoresis. A single band corresponding to an apparent molecular weight of about 6700 was observed in both fractions (Fig. 5).

Amino-terminal analysis

Amino-terminal analysis of the two peptides was done by both the dansyl-Edman and the DABITC methods as a second test of homogeneity. Two-dimensional thin-layer chromatography qualitatively identified a single amino terminus for each peptide, suggesting that both peptides were homogeneous. Contaminants with the same amino terminus would not be detected by a single cycle of Edman degradation. Eleven subsequent cycles of Edman degradation gave no indication of heterogeneity in either peptide. The DABITC method identified both amino termini as asparagine. The dansyl-Edman method requires an acid hydrolysis, and asparagine is converted to aspartic acid. The amino terminus of both peptides was identified as aspartic acid by this method as expected.

Sequence analysis was continued from the amino terminus by both manual sequencing techniques. The sequences obtained are shown in Table I. Only twelve residues were clearly identified

TABLE I

AMINO-ACID SEQUENCES OF DIGEST FRAGMENTS

Digest fragment	Sequence	Mass	Observed FAB molecular ions
Peptide LP			
N-terminal			
dansyl	BKASVVABZLIPI–	–	–
DABTH	NKASVVANQLLPINTAITLVMMRAEV–	–	–
PTH [a]	NKASVVANQLIPINTALTLVMMRAEV–	–	–
Chymotrypsin			
CT1 DABTH	QVNRAV–	–	–
PTH	–QVNRAV–		
Trypsin			
T1 DABTH	GYAPQ	534	–
PTH	–GY–		
T2 DABTH	LVGLQVNR	897	898
PTH	–LVGLQVNR–		
T3 DABTH	AEVVTPAGLPAEDIPR	1633 (1134) [b]	1634 (1135) [b]
PTH	–AEVVTPAGIPAEDIPR–		
T4 DABTH	AVLIGTTLMPDMVK	1487	1488
PTH	–AVLIGTTLMPDMVK–		
V8 proteinase			
V1 DABTH	–MVKGYAPQ–	892	–
PTH	–MVKGY–		
V2 DABTH	VVTPAGLPAGEDIPRLV–	–	–
PTH	–VVTPAGIPAGEDIPRLV–		
Peptide RD			
N-terminal			
dansyl	BKASVVABZLIPI–	–	–
DABTH	NKASVVANQLLPINTAITLIMM?AEV–	–	–
PTH	NKASVVANQLIPINTALTLIMMKAEV–	–	–
Trypsin			
T1 DABTH	NY?	?	–
PTH	NYE		
T2 DABTH	LIGMQVNG	929	930
PTH	IIGMQVNG–		
t3 DABTH	AEVVTPMGLPAEDIPR	1693 (966) [b]	1694 (967) [b]
PTH	–AEVVTPMGIPAEDIPR–		
T4 DABTH	AVPIGTTLMPDMVK	1471	1472
PTH	–AVPLGTTLMPDMVK–		
V8 proteinase			
V1 DABTH	MVKNY?	?	–
PTH	–MVKNYE		
V2 DABTH	VVTPMGLPAEDIPRLI–	–	–
PTH	–VVTPMGIPAEDIPRII–		

[a] PTH sequences are from Table II. The sequences corresponding to the fragment sequences are shown for comparison only.

[b] The masses in parentheses correspond to mass-spectrometric fragment ions observed in both the spectra of purified peptides and in the digest FAB map.

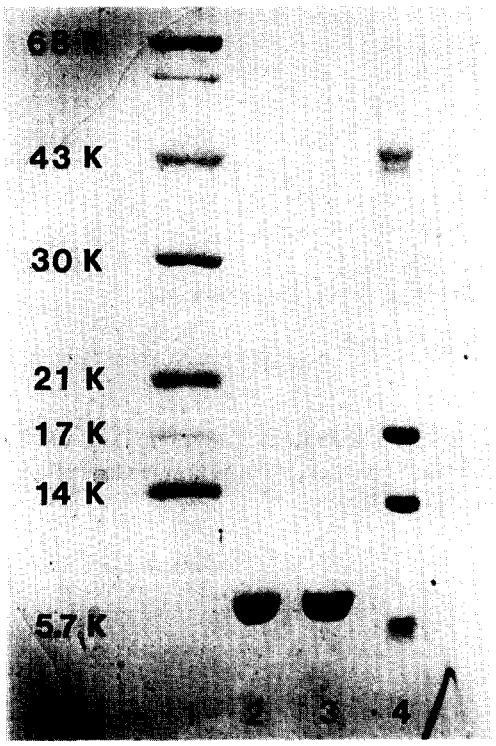

Fig. 5. SDS-polyacrylamide gel electrophoresis of peptides RD and LP. Gel is 10–20% gradient gel. Lanes: (1) Bio-Rad low-molecular-weight standards; (2) LP, 20 μg; (3) RD, 20 μg; (4) standards of ovalbumin, myoglobin, lysozyme and insulin.

by the dansyl-Edman method, while the DABITC method provided information as far as residue 26 in both peptides. As noted earlier, the dansyl-Edman technique cannot differentiate aspartic and glutamic acids from the amidated forms asparagine and glutamine (residues 1, 8 and 9) and accurate assignment of leucine and isoleucine is difficult by the DABITC method (e.g., residues 11 and 17). Identification of PTH derivatives in the automated sequenator allows correct assignments of these residues to be made.

Tryptic fragment sequencing

FAB mapping of tryptic digests of peptide LP indicated molecular ions at *m/z* 898, 1135, 1488 and 1634 (Fig. 6A). After carboxypeptidase B digestion, the molecular ions were shifted to *m/z* 742, 979, 1360 and 1478 (Fig. 6B). These mass differences indicate C-terminal arginine residues for the tryptic fragments at *m/z* 898, 1135 and 1634, and C-terminal lysine for the fragment at *m/z* 1488. Tryptic fragments from RD were observed at *m/z* 930, 967, 1472 and 1694 (Fig. 7). These ions were shifted to *m/z* 774, 811, 1344 and 1538 by carboxypeptidase B digestion, indicating C-terminal arginine for the fragments at *m/z* 930, 967 and 1694 and C-terminal lysine for the fragment at *m/z* 1472.

Four tryptic fragments were isolated from each antifreeze peptide by HPLC (Fig. 8). The sequences of these fragments are shown in Table I. Some of the leucine and isoleucine assignments from DABTH derivatives again were shown to be inaccurate by the identification of PTH derivatives. Identical sequences for peptides T2, T3 and T4 were obtained independently by interpretation of the mass spectrometric fragment ions of the individual peptides [29]. Three of the signals in Fig. 6A were interpreted as molecular ions in the

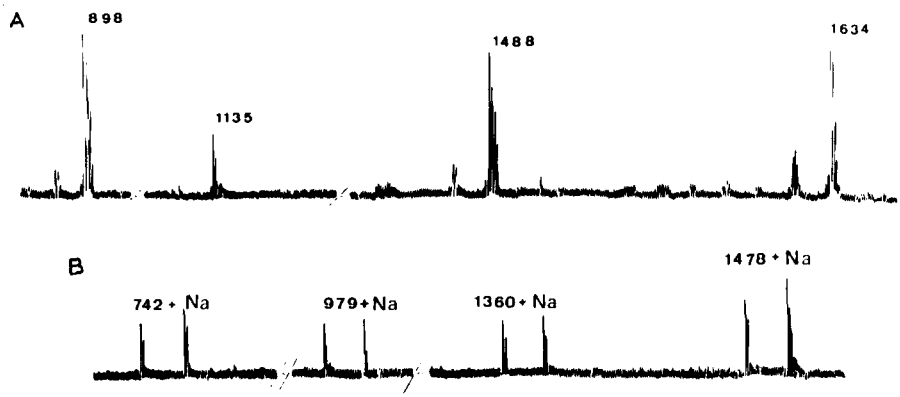

Fig. 6. FAB map of tryptic digestion of peptide LP. (A) Before digestion with carboxypeptidase B. (B) After digestion with carboxypeptidase B.

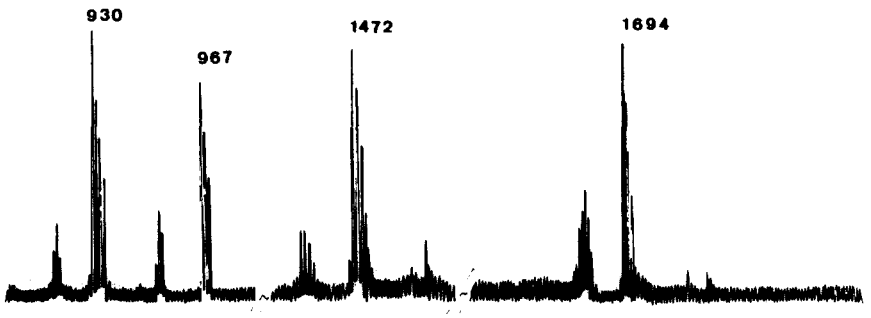

Fig. 7. FAB map of tryptic digestion of peptide RD.

observed FAB maps. The ion at m/z 1135 in Fig. 6A corresponds to a C-terminal fragment ion of peptide T3 via cleavage at the Thr–Pro bond. Similarly, a C-terminal fragment ion of T3 in peptide RD gives rise to the ion observed at m/z 967 in the FAB map.

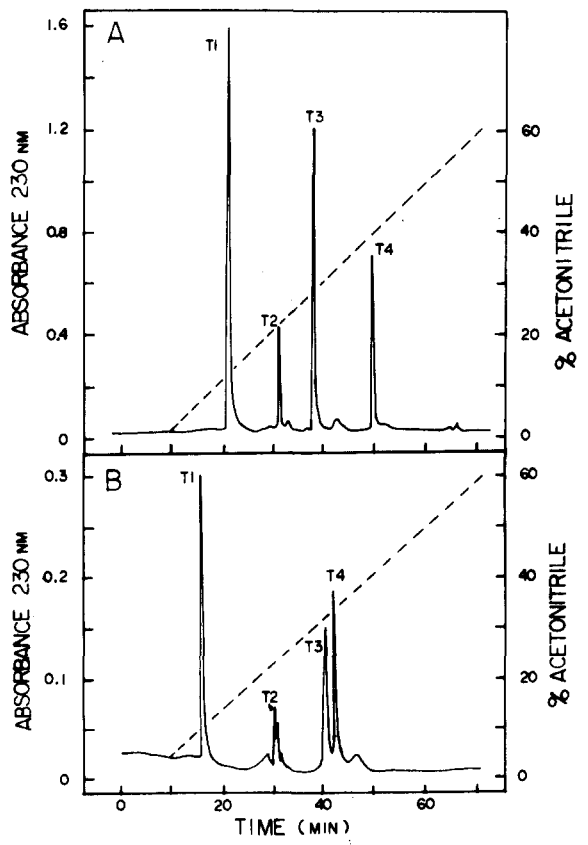

Fig. 8. HPLC purification of tryptic fragments from peptides LP and RD. (A) Elution profile obtained from a digestion of 2.6 mg of peptide LP. (B) Elution profile obtained from a digestion of 0.5 mg of peptide RD. ———, A_{230}; — — —, percent acetonitrile.

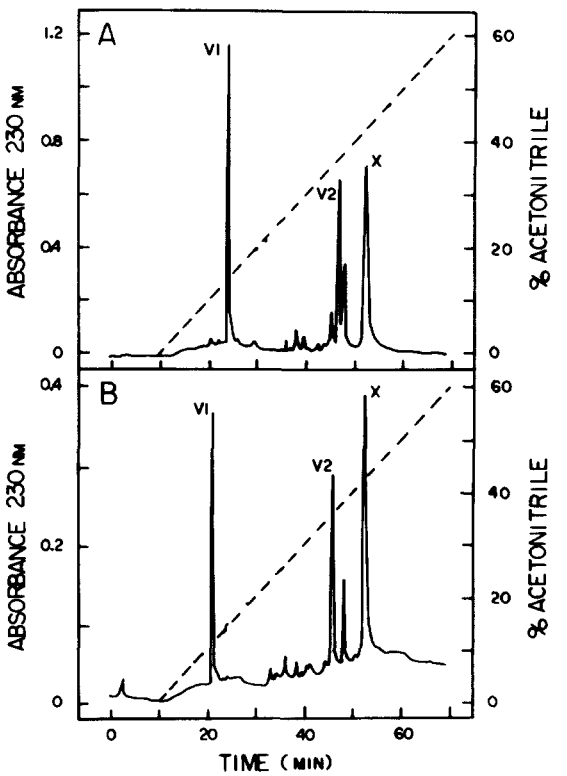

Fig. 9. HPLC purification of V8 proteinase fragments from peptides LP and RD. (A) Elution profile obtained from a digestion of 2.3 mg of peptide LP. Only fragments V1 and V2 were sequenced. Peak X is undigested LP. (B) Elution profile obtained from a digestion of 0.6 mg of peptide RD. Only fragments V1 and V2 were sequenced. Peak X is undigested RD. ———, A_{230}; — — —, percent acetonitrile.

V8 proteinase digestions

Proper alignment of the tryptic fragments requires peptides whose sequences overlap the ends of the tryptic fragments. V8 proteinase fragments were, therefore, isolated as shown in Fig. 9. The sequences of two of these fragments from both LP and RD are shown in Table I. No sequence data were obtained from the other V8 fragments.

Reconstruction of sequences

The amino-terminal sequences clearly overlap with fragment T3 in both peptides. This overlap allows proper placement of T3 and firmly establishes the amino terminal 39 residues in both LP and RD. Fragment V2 identifies T3 as being immediately amino terminal to T2. This overlap extends the known sequence to residue 47 for both peptides. Fragment V1 shows that T4 is amino terminal to T1. Fragment T1 in LP was determined to be the C-terminus of the peptide from several pieces of evidence. First, assuming that the obtained sequence was complete, it was the only tryptic fragment found lacking lysine or arginine as the C-terminus. Amino-acid analysis of this fragment after acid hydrolysis showed nearly equal amounts of of glycine, alanine, proline, tyrosine and glutamic acid, with no other amino acids (data not shown). This suggests that the sequence

TABLE II

AUTOMATED SEQUENCING OF LP AND RD

Cycle	LP		RD	
	residue	yield (pmol)	residue	yield (pmol)
1	Asn	1700	Asn	2400
2	Lys	300	Lys	2400
3	Ala	2000	Ala	2400
4	Ser	500	Ser	300
5	Val	1700	Val	2400
6	Val	1600	Val	2400
7	Ala	1600	Ala	2000
8	Asn	400	Asn	2400
9	Gln	40	Gln	2400
10	Leu	1200	Leu	2400
11	Ile	1200	Ile	2000
12	Pro	1000	Pro	1550
13	Ile	920	Ile	1500
14	Asn	850	Asn	1400
15	Thr	200	Thr	200
16	Ala	800	Ala	1400
17	Leu	740	Leu	1450
18	Thr	150	Thr	200
19	Leu	600	Leu	1200
20	Val	720	Ile	1260
21	Met	550	Met	1000
22	Met	680	Met	1300
23	Arg	620	Lys	700
24	Ala	466	Ala	1000
25	Glu	300	Glu	400
26	Val	?	Val	1000
27	Val	700	Val	1300
28	Thr	80	Thr	280
29	Pro	360	Pro	625
30	Ala	400	Met	420
31	Gly	250	Gly	550
32	Ile	270	Ile	600
33	Pro	320	Pro	600
34	Ala	320	Ala	500
35	Glu	230	Glu	300
36	Asp	200	Asp	350
37	Ile	200	Ile	650
38	Pro	160	Pro	480
39	Arg	234	Arg	260
40	Leu	130	Ile	400
41	Val	240	Ile	720
42	Gly	154	Gly	330
43	Leu	240	Met	480
44	Gln	160	Gln	250
45	Val	170	Val	450
46	Asn	100	Asn	246
47	Arg	160	Arg	160
48	Ala	170	Ala	260
49	Val	184	val	350
50	Leu	148	Pro	246
51	Ile	117	Leu	272
52	Gly	57	Gly	200
53	Thr	10	Thr	20
54	Thr	10	Thr	20
55	Leu	84	Leu	200
56	Met	67	Met	250
57	Pro	50	Pro	20
58	Asp	30	Asp	150
59	Met	63	Met	200
60	Val	52	Val	150
61	Lys	50	Lys	100
62	Gly	21	Asn	100
63	Tyr	10	Tyr	50
64	?	–	Glu?	–

TABLE III

COMPARISON OF AMINO-ACID COMPOSITIONS OF ANTIFREEZE PEPTIDES LP AND RD DETERMINED BY AMINO-ACID ANALYSIS AND SEQUENCE DATA

Amino acid	LP			RD		
	mol%	number of residues estimated from amino-acid analysis	number of residues from sequence data	mol%	number of residues estimated from amino-acid analysis	number of residues from sequence data
Asx	9.33	6	6	11.18	7	7
Thr	5.34	5	5	7.07	4	5
Ser	1.73	1	1	1.90	1	1
Glx	7.90	5	5	8.36	5	5
Pro	10.26	7	6	9.78	6	6
Gly	5.97	4	4	4.98	3	3
Ala	11.30	7	7	8.86	6	6
Cys	–	–	–	–	–	–
Val	12.74	9	9	9.54	6	7
Met	6.77	5	4	10.52	7	6
Ile	7.54	5	5	10.28	7	7
Leu	10.28	7	7	8.19	5	5
Tyr	1.15	1	1	1.33	1	1
Phe	–	–	–	–	–	–
Lys	2.92	2	2	5.06	3	3
His	–	–	–	–	–	–
Arg	4.49	3	3	2.96	2	2

```
                                        10                                  20
LP   ASN-LYS-ALA-SER-VAL-VAL-ALA-ASN-GLN-LEU-ILE-PRO-ILE-ASN-THR-ALA-LEU-THR-LEU-VAL-
RD   ASN-LYS-ALA-SER-VAL-VAL-ALA-ASN-GLN-LEU-ILE-PRO-ILE-ASN-THR-ALA-LEU-THR-LEU-ILE-

                                        30                                  40
LP   MET-MET-ARG-ALA-GLU-VAL-VAL-THR-PRO-ALA-GLY-ILE-PRO-ALA-GLU-ASP-ILE-PRO-ARG-LEU-
RD   MET-MET-LYS-ALA-GLU-VAL-VAL-THR-PRO-MET-GLY-ILE-PRO-ALA-GLU-ASP-ILE-PRO-ARG-ILE-

                                        50                                  60
LP   VAL-GLY-LEU-GLN-VAL-ASN-ARG-ALA-VAL-LEU-ILE-GLY-THR-THR-LEU-MET-PRO-ASP-MET-VAL-
RD   ILE-GLY-MET-GLN-VAL-ASN-ARG-ALA-VAL-PRO-LEU-GLY-THR-THR-LEU-MET-PRO-ASP-MET-VAL-

LP   LYS-GLY-TYR-ALA-PRO-GLN
RD   LYS-ASN-TYR-GLU
```

Fig. 10. Comparison of amino-acid sequences of peptides LP and RD.

determined for T1 was complete. Carboxypeptidase A digestion of the native antifreeze peptide released an amino acid which chromatographed on amino-acid analysis like serine. This is the expected elution position of glutamine [31]. Finally, if our assignment of T1 as the C-terminus is correct, cyanogen bromide digestion should produce a fragment with the sequence VKGYAPQ and a molecular weight of 761. As expected, a molecular ion at m/z 762 was observed in FAB maps of cyanogen bromide digests, and amino-acid analysis of one purified CNBr fragment showed equimolar amounts of valine, lysine, glycine, tyrosine, alanine, proline and glutamic acid (data not shown). This evidence clearly establishes fragment T1 in LP as the C-terminus of the native peptide and defines the sequence of the 19 C-terminal residues. T1 also appears to be the C-terminus of peptide RD. Manual sequencing of fragments T1 and V1 failed to identify any residues beyond the tyrosine residue, but the automated sequenator weakly indicated the presence of a glutamic acid residue. Amino-acid analysis of T1 showed only aspartic acid, tyrosine and glutamic acid in equimolar amounts. A CNBr fragment composed of equimolar amounts of valine, lysine, aspartic acid, tyrosine and glutamic acid was also isolated. These data clearly established the C-terminal sequence of RD as –VKNYE.

The amino terminal 47 residues and the C-terminal 19 residues of peptide LP account for nearly all of the mass of the peptide as estimated from polyacrylamide gels and amino-acid analysis. The most obvious way to complete the sequence is to place the 19 amino-acid C-terminal sequence immediately C-terminal to the 47th residue. One chymotryptic fragment, whose sequence is shown in Table I, was found, which proves that this is the correct alignment. This establishes the complete 66 residue sequence of LP. Although no chymotrypsin digestions were done on RD, the similarity between the two antifreeze peptides suggested the same alignment of N-terminal and C-terminal sequences.

Table II shows the results of the automated sequencing of peptides LP and RD. The yields of amino acids identified at each cycle are observed to decline until no clear assignment can be made. The valine assignment at cycle 26 in peptide LP was deduced from the unusually large yield of valine at cycle 27, which probably was the result of carry-over valine from cycle 26. With the exception of some previously mentioned leucine and isoleucine assignments and the failure of the sequenator to reach the C-terminus of peptide LP, the sequences obtained are identical to those deduced from manual sequencing and mass spec-

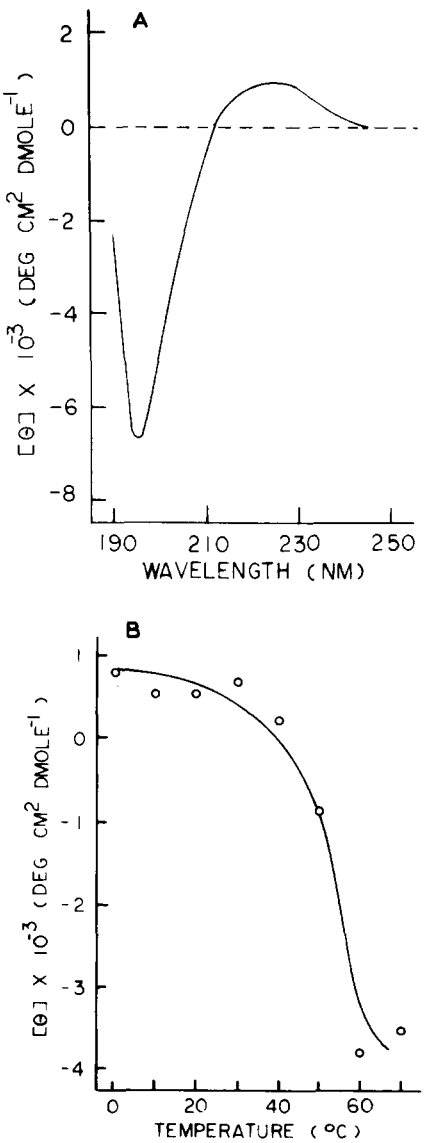

Fig. 11. (A) CD spectrum of peptide LP. The peptide was dissolved in water at a concentration of 1.9 mg/ml. Temperature was 0 °C. (B) Thermal denaturation of peptide as measured by CD. [θ] is the magnitude of the CD band at 225 nm.

trometry. The assignment of glutamic acid at cycle 64 in peptide RD has been confirmed by amino-acid analysis of digest fragments. The amino-acid compositions of LP and RD, as determined from the sequences and by amino-acid analysis, are shown in Table III. The compositions derived from the two methods are in excellent agreement. The two antifreeze sequences are compared in Fig. 10.

The CD spectrum obtained for peptide LP is shown in Fig. 11A. There is no indication of α-helix or β-sheet. The spectrum is very similar to those obtained from glycopeptide antifreezes [7,32]. These spectra have been interpreted as indicative of a random structure of a left-handed 3-fold helix. The spectrum of peptide LP, however, undergoes a rather sharp transition as the temperature is raised (Fig. 11B). This suggests strongly that peptide LP does adopt some folded tertiary structure. The glycopeptide antifreezes show no evidence of this type of conformational transition.

Discussion

The sequence homology of these two peptides is obvious. Only nine differences are found in the first 63 residues. Four of these differences involve the conservative interchange of the hydrophobic amino acids, leucine, isoleucine and valine, and a fifth conserves the positive charge at residues 23 where lysine in RD is substituted for the arginine found in LP. Hew et al. [19] have reported two families of antifreeze peptides from *Macrozoarces americanus*, designated 'QAE' and 'CM' families, distinguished by chromatographic and immunological properties. Li et al. [33] have reported the sequence of a CM-type peptide deduced from the cDNA sequence. Interestingly, the peptide from *Lycodes* (from the Bering Sea) shows much stronger homology to the peptide from the antarctic *Rhigophila* (80% of residues identical) than to that of *Macrozoarces*, another northern species (60% of residues identical). The amino-acid compositions of peptides RD and LP more closely resemble the 'QAE' family than the 'CM' family. Perhaps amino-acid sequences from 'QAE' peptides would more closely resemble peptides RD and LP.

The CD spectrum from peptide LP is very similar to those reported for peptides from *Macrozoarces* [34]. The thermal transition also appears similar. The similarities in the primary and secondary structures of these peptides suggests that the antifreeze structure within the family Zoarcidae, unlike that of the Cottidae, is highly conserved.

The zoarcid peptides show little similarity in structure to the other known fish antifreezes. Evidence suggests, however, that all of these antifreezes function by a common mechanism. All of the antifreezes are characterized by their ability to prevent the growth of ice crystals at temperatures below the equilibrium freezing point of the solution. An ice crystal formed in a solution of glycopeptide antifreezes (10 mg/ml in H_2O) does not grow until the temperature of the solution is lowered to approx. $-0.8\,^{\circ}C$. Although some variations in the effectiveness of the different peptide antifreezes is observed, all of them inhibit the growth over similar temperature ranges. At antifreeze concentrations of 10 mg/ml in water, reported temperatures of ice growth range from approx. $-0.5\,^{\circ}C$ for flounder peptides [10] to approx. $-0.78\,^{\circ}C$ for zoarcid peptides (this paper). When the temperature of the antifreeze solutions is lowered to a point where the ice crystals begin to grow, the growth occurs with a characteristic crystal habit. The ice crystals grow as long fine needles or spicules. This crystal habit has been observed in the presence of all known fish antifreezes. One study reports that low concentrations (10^{-8} M) of glycopeptide and winter flounder antifreezes cause the development of ice-crystal faces indexed as $10\bar{1}x$ [4]. Subsequent studies have shown that all known fish antifreezes cause this type of crystal growth (Knight, C.A., personal communication). In this regard, it appears that all antifreezes work by a similar mechanism.

Adsorption of the antifreezes to the ice surface has been proposed to inhibit the ice-crystal growth. Evidence of adsorption of antifreezes to ice has been presented [1], and models relating antifreeze structure and adsorption to ice have been proposed [6,11]. These models are based on matches in spacings of polar groups in the antifreezes and oxygen atoms in the ice lattice, which presumably allow hydrogen bonding of the antifreeze to the

crystal surface. The model proposed for the winter flounder peptide has been the most thoroughly developed. The primary structure of this peptide consists of a periodic arrangement of clusters of polar residues separated by long sequences of alanine. The α-helical secondary structure places all of the polar side-chains on one side of the helix, resulting in an amphiphilic structure with a hydrophilic and a hydrophobic face. Within each cluster of polar residues, potential hydrogen bonding side-chains are separated by 4.5 Å, a spacing which matches the spacing of oxygen atoms along the *a*-axis of the ice lattice. A recent study of the primary structure of an antifreeze peptide from the shorthorn sculpin suggests that a similar arrangement can be found in this peptide [35].

The primary structure of the glycopeptide antifreezes also shows a periodic arrangement of polar groups, because every third residue is a glycosidically linked threonine residue [5,36]. The uncertainty about the secondary structure has made determination of lattice matches difficult. CD spectra have been interpreted as indicative of random coil and left-handed three-fold helix [7,32]. A three-fold helix is an appealing possibility as it would place all of the carbohydrate moieties on one side of the helix, resulting in an amphiphilic molecule similar to the winter flounder peptide. Hydrogen bonding between the disaccharides and the ice lattice could then result in adsorption. A random conformation would most likely not result in an amphiphilic structure, but hydrogen bonding between the carbohydrate and the ice lattice could still occur. Chemical modification of the disaccharides is known to eliminate antifreeze activity. An alternative model of glycopeptide function involves a lattice match between alternate carbonyl groups in a completely extended peptide backbone and oxygen atoms along the *c*-axis of ice [11]. The function of the carbohydrate may be to maintain this extended conformation.

The models proposed above suggest that a periodic arrangement of hydrogen-bonding groups and/or a folding pattern which results in an amphiphilic molecule may be important in the function of antifreezes. Neither of these conditions are obvious in the zoarcid peptides. The primary structure shows no distinct periodic placement of charged or polar residues. The CD spectrum of peptide LP is similar to those interpreted as random coil or left-handed three-fold helix. The distinct transition observed in the spectrum would seem to preclude the random-coil interpretation. Threefold helices are typical of collagen and polyproline II. The two antifreezes show no sequence homology with collagen, making it difficult to imagine this type of secondary structure for the antifreezes. Secondary structure predictions by the methods of Chou and Fasman [37] and Garnier et al. [38] suggest that significant β-structure may be present, but this is not obvious in the observed CD spectrum. Even if one assumes that the predictions are correct, the predicted folding patterns do not produce any regular arrangement of hydrogen bonding groups. Adsorption of the antifreeze to ice may not require either a periodic placement of hydrogen-bonding groups or an amphiphilic structure. Several polar groups capable of hydrogen bonding to ice located randomly somewhere along the length of the chain may be all that is required. Chemical modification studies may identify residues essential for the function of the zoarcid peptides. Further work on the three-dimensional structure of these peptides would also be of great interest.

Acknowledgement

This work was supported in part by grant NSF DPP 85-15266 (to A.L.D.).

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