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Structures of antifreeze peptides from the antarctic eel pout, *Austrolycicthys brachycephalus*

Chi-Hing C. Cheng and Arthur L. DeVries

Department of Physiology and Biophysics, 524 Burrill Hall – 407 S. Goodwin, University of Illinois, Urbana, IL (U.S.A.)

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The antarctic eel pout, *Austrolycicthys brachycephalus*, synthesizes two predominant antifreeze peptides (AFPs) which, based on purification yields, make up about 94 and 6%, respectively, of the antifreezes in its serum. The amino acid sequences of these two AFPs, AB1 and AB2, were determined using automated sequencing, and compositional analyses of peptide fragments from enzymatic digests, and verified by molecular masses obtained with Fast Atom Bombardment Mass spetrometry. Substantial homologies in amino acid sequence exist between the AFPs of *Austrolycicthys* and those of other Southern and Northern eel pouts. 72% of the residues of AB1, and 84% of AB2, are identical to those of an AFP from another antarctic eel pout, *Rhigophila dearborni*. Between AB1 and AB2, 83% of the residues are identical. Secondary structure data based on circular dichroism studies indicated AB1 to be a random chain, but a sharp thermal transition of CD spectra around 30 °C suggested the presence of definite secondary or tertiary structure.

Introduction

Many polar and cold-water fishes avoid freezing by synthesizing macromolecular antifreezes. These molecules are either glycopeptides or peptides [1]. The antarctic nototheniid fishes and several Northern cods produce antifreeze glycopeptides (AFGPs) of similar or nearly identical structure. They are polymers of various lengths of the glycotripeptide, alanyl-alanyl-threonine with the disaccharide, galactose-*N*-acetylgalactosamine linked to threonine [1,2]. Winter flounder and Alaskan plaice (flat fishes), short-horn sculpin and sea raven (cottids), and eel pouts and ocean pouts (zoarcids), produce antifreeze peptides (AFPs) which are highly varied in structure but similar in size. The AFPs of winter flounder, Alaskan plaice and sculpin are primarily helical peptides of repeating stretches of non-polar alanines separated by short segments of polar residues [1,3–6]. AFPs of sea raven contain high percentages of cystines, and possess β-structure [7,8]. The AFPs from the zoarcids, which include *Austrolycicthys brachycephalus*, comprise a third class of AFPs that differs from all known antifreezes in that they appear to have no defined structures [9,10].

Despite the diversity in primary and secondary structure, all antifreezes perform a common fucntion; at a concentration of 2% (w/v), they lower the freezing point of water by about 1.5 °C in the presence of a seed ice crystal, but do not affect the melting point [1]. In addition, single ice crystals grown in the presence of different antifreeze solutions showed similar crystal habit changes, i.e., complete inhibition of growth on the prism planes, and limited growth on the basal plane [11]. This evidence suggests a common mechanism of action, and that perhaps some common feature or structural component exists in the different antifreezes that allows them to function similarly. Adsorption of antifreeze molecules to ice surface resulting in inhibition of further ice growth has been proposed to explain the non-colligative lowering of the freezing point of water [12,13]. Evidence for adsorption has been reported [12,14,15], but precisely how adsorption is achieved, and the antifreeze structure responsible for it, remain to be elucidated. The sequences of the AFPs from the Atlantic ocean pout, *Macrozoarces americanus*, and two polar eel pouts, *Lycodes polaris* (Arctic) and *Rhigophila dearborni* (Antarctic), have been obtained and were found to be similar. But unlike any other fish antifreezes, these AFPs have no repeating sequences in the primary structure and the secondary structures ap-

Abbreviations: TEAP, triethylamine phosphate; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

The sequence in data this paper have been submitted to the EMBL/Genbank Data Libraries under accession number A30095.

Correspondence: A.L. DeVries, 524 Burrill Hall - 407 S. Goodwin, University of Illinois, Urbana, IL 61801 U.S.A.

pear to be random chains [9,10]. With the hope of further understanding the structure–function relationships of zoarcid antifreeze peptides, we purified and determined the sequences of two AFPs from the Antarctic eel pout, *A. brachycephalus* in this study.

Materials and Methods

Specimens of *A. brachycephalus* were caught in baited traps at depths of 720–740 meters in McMurdo Sound, Antarctica during the austral summer. Blood samples were collected from the caudal vein or artery with 30 gauge needle and 1 ml syringe, pooled, clotted at 4°C and centrifuged. Serum was removed and stored at −70°C until further purification.

Peptide purification

10 ml of pooled serum was loaded onto a Sephadex G-75 (Pharmacia) column (2.7 cm (i.d.) × 150 cm) equilibrated with 50 mM NH_4HCO_3. Protein was eluted from the column with the same buffer at a flow rate of about 0.5 ml/min, and the absorbances of 9 ml fractions at 230 and 280 nm were read. The fractions containing each absorbance peak were pooled and lyophilized to remove the volatile solvent. The dried material was then assayed for antifreeze activity at concentrations of 20 mg/ml according to the melting point-freezing point determination method of DeVries [16]. About 0.1 mg of the material from the antifreeze-containing peak was further analysed by HPLC with an analytical Ultrasphere (Altex) C_{18} reverse-phase column (4.6 × 250 mm) and a linear gradient of 0–90% acetonitrile in triethylamine phosphate (TEAP), pH 2.2 (0.1 M phosphoric acid titrated to pH 2.2 with triethylamine), over 60 min. Protein elution was monitored by absorbance at 220 nm, and was found to take place between 30–60% acetonitrile. Larger quantities of antifreeze material, about 6 mg per run, were then fractionated and purified with an Altex preparative C_{18} reverse-phase column (10 × 250 mm) and a 60 min linear gradient of 30–60% acetonitrile/0.1 M TEAP for better resolution. The two major fractions, AB1 and AB2, were dialysed separately in Spectrapor 3 (Spectrum Medical Industries) against distilled water. The dialysis tubing pore size (molecular weight mass cutoff, 3000 Da) was chosen based on an approximate molecular weight obtained with SDS gel electrophoresis for the G-75 purified antifreeze material. The dialysed fractions were lyophilized; about 2.6 mg of AB1 and 0.17 mg of AB2 were recovered for each 6 mg of antifreeze material applied to the C_{18} preparative HPLC column. The antifreeze activity of Ab1 and AB2 from HPLC purification was verified again using the method of DeVries [16].

Amino acid composition

The antifreeze peptides and peptide fragments were hydrolysed in 1 ml of 6 M HCl under nitrogen at 110°C for 20 h. The hydrolysate was dried in a rotor evaporator, redissolved in sodium citrate buffer and analysed on a single-column analyser with ninhydrin detection [17].

Proteinase digestion and fragment isolation

Trypsin (Sigma) digestion using a 1:50 enzyme/substrate ratio was done in 50 mM NH_4HCO_3 (pH 8.1) at 37°C for 4 h. *Staphylococcus aureus* (V8) proteinase (Pierce Chemcial) digestion was done in 50 mM CH_3COONH_4 (pH 4.0), 1:35 enzyme/substrate ratio, at 37°C for 48 h [18]. After digestion, the digest fragments were separated by HPLC with an Altex Ultrasphere C_{18} reverse-phase column (4.6 × 250 mm) and a linear gradient of 0–100% acetonitrile with 0.1% trifluoroacetic acid over 60 min. The individual peaks were lyophilized to remove the volatile solvents, and then analysed for amino acid composition, and for molecular mass using Fast Atom Bombardment Mass Spectrometry (FAB/MS).

Molecular mass determination

Accurate molecular mass of the purified peptide and peptide fragments were determined by FAB/MS on a ZAB-HF spectrometer (VG Analytical, U.K.) with a xenon ionization source at the Mass Spectrometry Laboratory at the University of Illinois School of Chemical Sciences. Native peptides were dissolved in 0.1% TFA (about 30 μg in 1 μl per run), and applied onto thioglycerol matrix. Peptide fragments were dissolved in 0.1% TFA (about 10 μg in 1 μl per run), and applied onto a dithiothreitol/dithioerythritol matrix.

Peptide sequencing

About 3 nmol of AB1, and 1 nmol of AB2 were used for automated, gas-phase, N-terminal sequencing on an Applied Biosystems 470A sequenator at the University of Illinois Biotechnology Center. The PTH (pehnylthiohydantoin) derivatives of each cycle were separated by HPLC with a C_{18} reverse phase column, and identified by computerized comparisons of the peak elution times and areas of eluents with those of the preceding degradation cycle, and the elution profile of PTH-aa standards.

Circular dichroism

CD spectra were obtained on a Cary 60 spectropolarimeter that has been upgraded to a computer-aided AVIV Model 60DS unit. The sample cell had a path length of 0.1 mm, and was jacketed for temperature control. The peptide was dissolved in 250 mM NaCl at a concentration of 1.9 mg/ml. Ellipticity in degrees, $[\theta°]$, was recorded at 20 nm intervals, and three scans per sample temperature were averaged by computer. The molar ellipticity, $[\theta]$, was calculated using the equa-

tion: $[\theta] = \theta° m/10\lambda C$ in deg · cm^2 · dmol^{-1}, where m is the mean residue mass determined from the peptide sequence, λ is the path length in cm, and C is the sample concentration in mg/ml.

Results

Purifications

Fig. 1 shows the protein elution profile of *A. brachycephalus* serum on G-75. Only peak III was found to contain antifreeze activity, with a melting point-freezing point difference of 1.24°C at 20 mg/ml in 0.1% TFA (0.1% TFA facilitated dissolving the peptides, and it makes no contribution to the mp–fp difference). Reverse-phase HPLC further resolved peak III protein into two components, AB1 and AB2, as shown in Fig. 2. Both components were found to contain antifreeze activity; the mp–fp difference is 1.27°C for AB1 and 1.17°C for AB2 at 20 mg/ml. Based on peak areas, AB1 and AB2 constituted 88 and 12%, respectively, of antifreeze present in the serum. Based on yield, AB1 constituted 94%, and AB2, 6%. The HPLC elution profile also showed several other minute peaks which could represent variants of AB1 and AB2. But their peak areas were so small that AB1 and AB2 could be considered as the only major antifreeze peptides.

Molecular mass determination

SDS-polyacrylamide gel electrophoresis of antifreeze material from G-75 fraction III showed a single band of about 7000 Da. Mass determination with FAB/MS of AB1 and AB2 showed single molecular ions at mass number 6846 and 7002, respectively (Fig. 3). The peptide molecule often becomes protonated during FAB ionization, especially if it is dissolved in an acidic matrix. Therefore, the molecular mass of AB1 is 6845, and AB2 7001, 1 mass unit less than those of their respective molecular ions. The small difference in the molecular masses could not be resolved on SDS-PAGE, which accounts for the single band. Since AB1 is the predominant antifreeze peptide, it was subjected to detailed sequence analysis; the minor component AB2 was analysed for amino acid composition, and sequence by automated sequencing only.

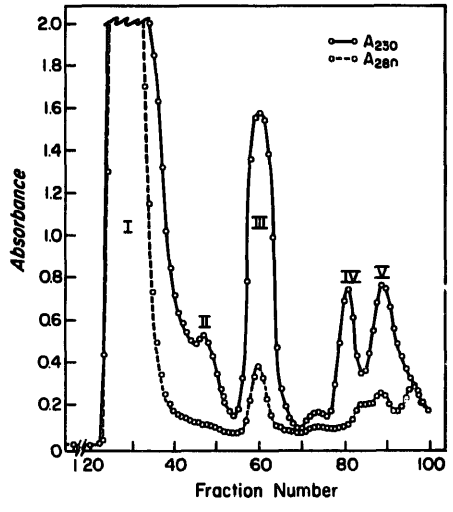

Fig. 1. Elution profile of *A. brachycephalus* antifreeze peptide on Sephadex G-75. 10 ml of serum was applied. ———, A_{230}; - - - - - -, A_{280}. The fractions within each absorbance peak were pooled and lyophilized. Freezing and melting points of the material from each peak were determined. Peak III was found to contain antifreeze protein, with a melting point-freezing point difference of 1.24°C.

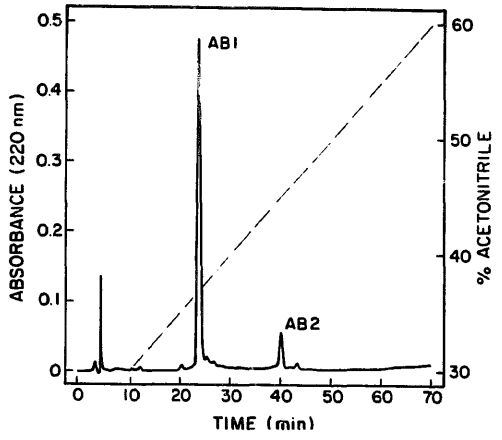

Fig. 2. HPLC purification of peak III antifreeze protein from Sephadex G-75 column. A linear gradient of 30–60% acetonitrile in 0.1 M triethylamine phosphate (pH 2.2) at a flow rate of 1 ml/min was used to elute the peptides after an initial column equilibration of 10 min at 30% acetonitrile. ———, A_{220}; - - - - - -, % acetonitrile.

Automated peptide sequencing

Sequences of AB1 and AB2 determined by automated peptide sequencing were shown in Table I. Residue assignment was possible until cycle 64 for both peptides. Three succeeding degradation cycles for each peptide showed no peak area increase for any particular amino acid residue, indicating residue 63 is likely the C-terminus of the peptides. The calculated molecular masses of AB1 and AB2 based on the sequences of

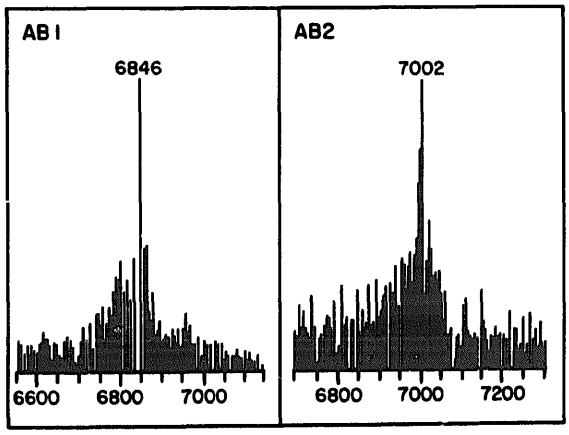

Fig. 3. FAB/MS spectra showing the protonated molecular ions ($M.H^+$) of *A. brachycephalus* antifreeze peptides AB1 and AB2. The molecular masses for AB1 and AB2 are 6845 and 7001, respectively.

TABLE I

Sequence of AB1 and AB2 from automated sequencing

Cycle	AB1		AB2	
	residue	yield (pmol)	residue	yield (pmol)
1	Thr	1685	Thr	366
2	Lys	4046	Lys	946
3	Ser	847	Ser	212
4	Val	2789	Val	501
5	Val	3248	Val	483
6	Ala	2550	Ala	647
7	Ser	523	Asn	555
8	Gln	2250	Gln	561
9	Leu	1689	Leu	377
10	Ile	1974	Ile	319
11	Pro	1529	Pro	200
12	Ile	1591	Ile	237
13	Asn	1255	Asn	384
14	Thr	440	Thr	131
15	Ala	1190	Ala	308
16	Leu	1109	Leu	178
17	Thr	523	Thr	80
18	Pro	1132	Leu	188
19	Ala	1054	Val	164
20	Met	1398	Met	220
21	Met	1735	Met	311
22	Lys	1349	Lys	162
23	Ala	1038	Ala	91
24	Lys	1361	Glu	91
25	Glu	493	Glu	144
26	Val	876	Val	82
27	Ser	178	Ser	25
28	Pro	611	Pro	54
29	Lys	791	Lys	69
30	Gly	535	Gly	66
31	Ile	518	Ile	49
32	Pro	546	Pro	54
33	Ala	501	Ala	4
34	Glu	293	Glu	54
35	Glu	319	Glu	77
36	Met	532	Ile	41
37	Ser	81	Pro	44
38	Lys	397	Arg	24
39	Ile	263	Leu	40
40	Val	313	Val	35
41	Gly	240	Gly	32
42	Met	343	Met	36
43	Gln	231	Gln	37
44	Val	278	Val	41
45	Asn	210	Asn	41
46	Arg	179	Arg	14
47	Ala	211	Ala	29
48	Val	244	Val	32
49	Asn	188	Tyr	17
50	Leu	174	Leu	31
51	Asp	114	Asp	23
52	Glu	85	Glu	16
53	Thr	70	Thr	6
54	Leu	142	Leu	18
55	Met	174	Met	22
56	Pro	126	Pro	18
57	Asp	74	Asp	14
58	Met	158	Met	21
59	Val	130	Val	16
60	Lys	109	Lys	9
61	Thr	31	Asn	14
62	Tyr	53	Tyr	5
63	Gln	60	Glu	9
64	?		?	

these 63 residues are 6845 and 7001, respectively, which are in complete agreement with those determined with FAB/MS, indicating that the sequences are accurate. However, there are amino acids with identical molecular masses such as Lys and Gln (146 Da) and Ile and Leu (131 Da), which if wrongly assigned in the sequence will still lead to same molecular masses for the peptide. Amino acid composition analyses of the native peptide and of peptide fragments from limited enzymatic digests were carried out to check the accuracy of the sequence obained from automated sequencing.

Proteinase digestion and peptide fragment analyses of AB1

Fig. 4A and B showed the HPLC purification of the trypsin and V8 proteinase digest fragments of AB1, respectively. Six tryptic and four V8 fragments were obtained. The four V8 fragments, V1 to V4, together

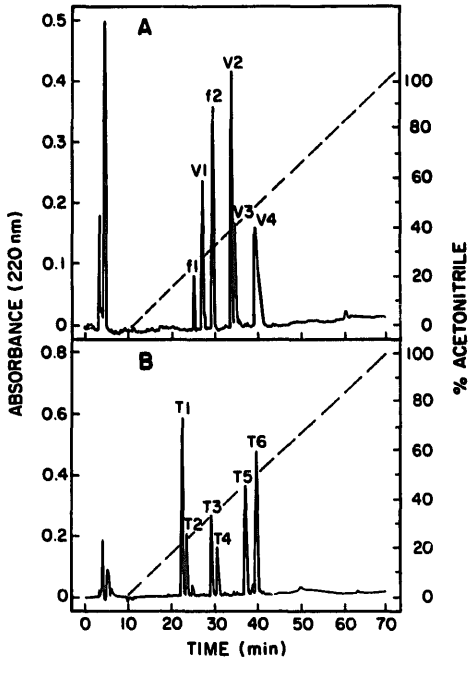

Fig. 4. HPLC purification of peptide fragments of peptide AB1 after limited enzymatic digest, using a linear gradient of 0–100% acetonitrile with 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. (A) Elution profile of V8 proteinase fragments, V1–V4. Peaks f1 and f2 were partially digested peptides. (B) Elution profile of tryptic fragment, T1–T6. ———, A_{220}; - - - - - -, % acetonitrile.

TABLE II

Amino acid composition analysis of tryptic fragments of AB1

Amino acid	T1			T2			T3			T4			T5			T6		
	mol%	No.[a]	No.[b]	mol%	No.[a]	No.[b]	mol%	No.[a]	No.[b]	mol%	No.[a]	No.[b]	Mol%	No.[a]	No.[b]	mol%	No.[a]	No.[b]
Asx										13.06	1	1	23.73	3	3	5.29	1	1
Thr	30.36	1	1										7.91	1	1	10.52	2	2
Ser				14.80	1	1	10.71	1	1							9.29	2	2
Glx	38.84	1	1	23.46	1	1	25.45	2	2	14.61	1	1	8.31	1	1	5.35	1	1
Pro				21.57	1	1	11.83	1	1				7.77	1	1	9.36	2	2
Gly							13.13	1	1	15.73	1	1						
Ala							7.48	1	1				8.85	1	1	17.39	3	3
Val				20.10	1	1				23.01	2	2	15.82	2	2	7.40	2	2
Met							5.61	1	1	10.85	1	1	4.42	1	1	7.91	2	2
Ile							12.75	1	1	9.41	1	1				10.95	2	2
Leu													15.55	2	2	10.94	2	2
Tyr	30.80	1	1															
Lys				20.15	1	1	13.04	1	1				7.64	1	1	5.56	1	1
Arg										13.32	1	1						

[a] Number of residues estimated from the amino acid composition.

[b] Number of residues based on sequence of AB1.

TABLE III

Amino acid composition analysis of V8 proteinase fragments of AB1

Amino acid	V1			V2			V3			V4		
	mol%	No.[a]	No.[b]	mol%	No.[a]	No.[b]	mol%	No.[a]	No.[b]	mol%	No.[a]	No.[b]
Asx				10.39	1	1	18.67	3	3	4.91	1	1
Thr				16.45	2	2				11.82	3	3
Ser	8.20	1	1				4.56	1	1	7.11	2	2
Glx	20.98	2	2	10.82	1	1	12.93	2	2	8.99	2	2
Pro	18.89	2	2	8.87	1	1				8.37	2	2
Gly	1.16	1	1				5.60	1	1			
Ala	10.53	1	1				6.27	1	1	16.14	4	4
Val	9.92	1	1	9.31	1	1	20.27	3	3	7.49	2	2
Met				15.15	2	2	8.80	2	2	7.55	2	2
Ile	9.92	1	1				4.30	1	1	8.21	2	2
Leu				10.17	1	1	6.90	1	1	8.90	2	2
Tyr				9.10	1	1						
Lys	10.40	1	1	9.74	1	1	6.00	1	1	10.46	2	3
Arg							6.27	1	1			

[a] Number of residues estimated from amino acid composition.
[b] Number of residues based on sequence of AB1.

account for the entire sequence of the native peptide, as will be shown below. Peaks f1 and f2, although they showed high absorbance on HPLC, gave very low amino acid yields when analysed for composition, and therefore are not included in the peptide fragment analyses. Based on the sequence of AB1 from automated sequencing, there are seven possible trypsin cleavable sites (Lys at residues number 2, 22, 24, 29, 38 and 60, and Arg at 46) to give eight tryptic fragments, and four possible V8 proteinase cleavable sites (Glu at residues number 25, 34, 35 and 52) to give four fragments and 1 free Glu. The results of the amino acid analysis of the tryptic and V8 fragments are shown in Tables II and III, and those of the molecular mass determination by FAB/MS in Figs. 5 and 6, respectively. By comparing the mass from FAB/MS and the amino acid composition of the digest fragments with the calculated mass and the amino acid composition of the possible fragments based on the AB1 sequence, the positions of the digest fragments in the native peptide were identified. The alignment of the digest fragments was constructed as shown in Table IV. The amino acid compositions and molecular masses of the digest fragments, and those of the corresponding possible fragments based on AB1 sequence are in com-

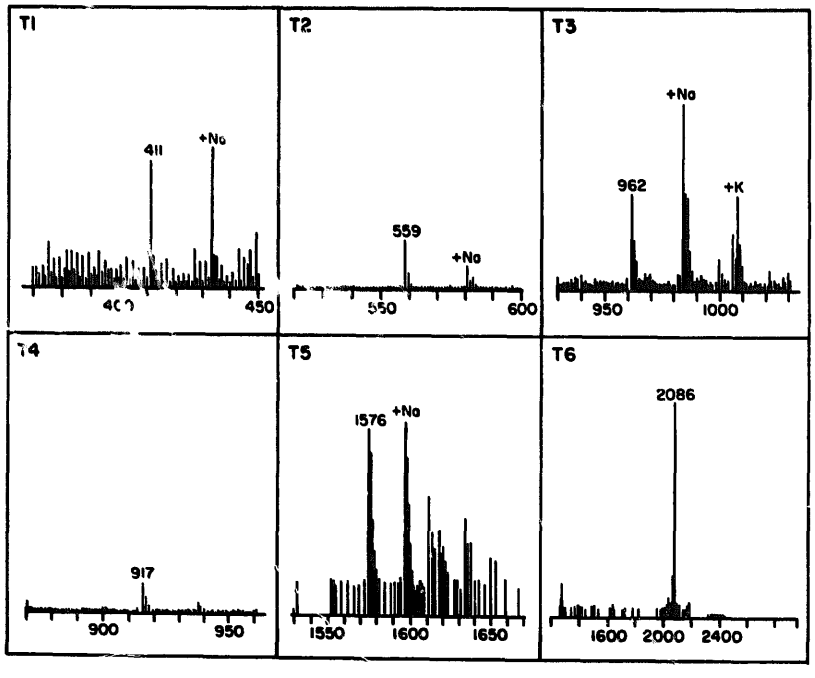

Fig. 5. FAB/MS spectra showing the molecular ions ($M.H^+$) of the tryptic fragments, T1–T6, of peptide AB1.

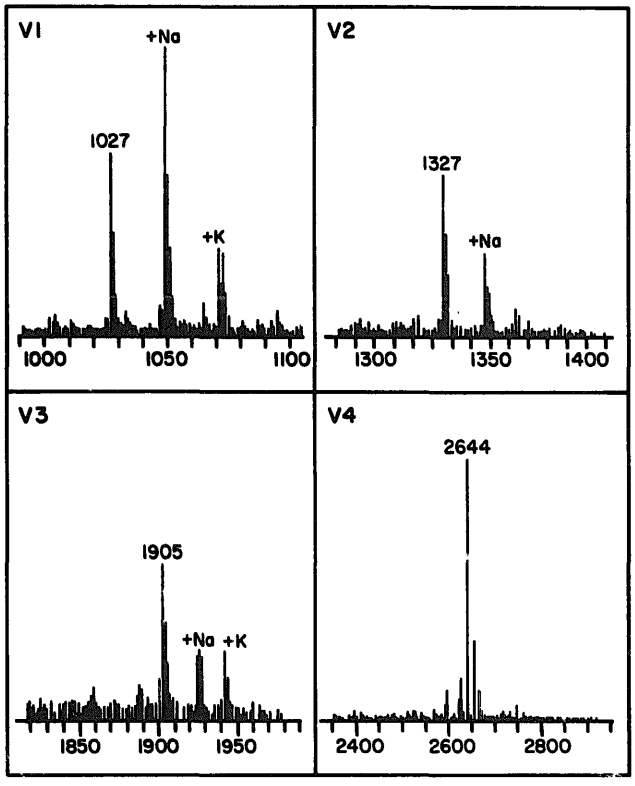

Fig. 6. FAB/MS spectra showing the molecular ions ($M.H^+$) of the V8 proteinase fragments, V1–V4, of peptide AB1.

TABLE V

Comparison of amino acid compositions of AB1 and AB2 determined by amino acid analysis and sequence data

Amino acid	AB1			AB2		
	mol%	No. of residues estimated from analysis	No. of residues from sequence	mol%	No. of residues estimated from amino acid analysis	No. of residues from sequence
Asx	8.76	6	5	10.54	7	6
Thr	7.75	5	5	6.35	4	4
Ser	6.99	5	4	4.28	3	2
Glx	11.75	8	7	11.00	8	8
Pro	7.62	5	5	10.18	7	5
Gly	3.46	2	2	4.28	3	2
Ala	9.92	6	6	7.75	5	5
Cys	–	–	–	–	–	–
Val	10.02	7	7	11.01	8	8
Met	8.78	6	6	6.45	5	5
Ile	5.80	4	4	6.11	4	4
Leu	6.47	4	4	9.68	6	6
Tyr	1.44	1	1	2.87	2	2
Phe	–	–	–	–	–	–
Lys	9.44	6	6	6.58	5	4
His	–	–	–	–	–	–
Arg	1.60	1	1	2.93	2	2

plete agreement. Two small possible tryptic fragments of two residues each, i.e., Thr-Lys- at positions 1 and 2, and -Ala-Lys- at positions 23 and 24, were lost in the HPLC purification of the trypsin digest of AB1, probably during the isocratic equilibration before the gradient started. V8 proteinase did not appear to cleave at the carboxyl side of Glu at position 34, such that fragment V1 has two consecutive Glu residues at its C-terminus. The amino acid composition of fragment V1 showed two Glx residues; the exact molecular mass of the V1 fragment and the unambiguous assignment of PTH-Glu by automated sequencing assured that residue 34 is Glu and not Gln. T1 and V2 are the respective C-terminus fragmnet of AB1 from the tryptic and V8 digestion. Their amino acid compositions and masses agreed with those of the corresponding possible fragments based on AB1 sequence, thus confirming that residue number 63 was indeed the C-terminus residue of the native peptide. The data from peptide fragment analyses therefore verified the accuracy of the peptide sequence obtained by automated sequencing.

TABLE IV

Amino acid sequences and alignment of digest fragments of AB1

Digest fragment	Residue No.	Sequence [a]	Calculated mass	M.H.$^+$ [b] (FAB/MS)
V4	1–25	TKSVVASQLIPINTALTPAMMKAKE-	2643	2644
T6	3–22	-SVVASQLIPINTALTPAMMK-	2085	2086
T2	25–29	-EVSPK-	558	559
V1	26–35	-VSPKGIPAEE-	1026	1027
T3	30–38	-GIPAEEMSK-	961	962
V3	36–52	-MSKIVGMQVNRAVNLDE-	1904	1905
T4	39–46	-IVGMQVNR-	916	917
T5	47–60	-AVNLDETLMPDMVK-	1575	1576
V2	53–63	-TLMPDMVKTYQ	1326	1327
T1	61–63	-TYQ	410	411

[a] Sequences based automated sequencing.

[b] Mass of protonated molecular ion.

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                1                                   10                                  20
AB1       THR-LYS-SER-VAL-VAL-ALA-SER-GLN-LEU-ILE-PRO-ILE-ASN-THR-ALA-LEU-THR-PRO-ALA-MET-
AB2       THR-LYS-SER-VAL-VAL-ALA-ASN-GLN-LEU-ILE-PRO-ILE-ASN-THR-ALA-LEU-THR-LEU-VAL-MET-
      1                                    10                                  20
RD    ASN-LYS-ALA-SER-VAL-VAL-ALA-ASN-GLN-LEU-ILE-PRO-ILE-ASN-THR-ALA-LEU-THR-LEU-ILE-MET-

               21                                  30                                  40
AB1       MET-LYS-ALA-LYS-GLU-VAL-SER-PRO-LYS-GLY-ILE-PRO-ALA-GLU-GLU-MET-SER-LYS-ILE-VAL-
AB2       MET-LYS-ALA-GLU-GLU-VAL-SER-PRO-LYS-GLY-ILE-PRO-ALA-GLU-GLU-ILE-PRO-ARG-LEU-VAL-
                                          30                                  40
RD        MET-LYS-ALA-GLU-VAL-VAL-THR-PRO-MET-GLY-ILE-PRO-ALA-GLU-ASP-ILE-PRO-ARG-ILE-ILE-

               41                                  50                                  60
AB1       GLY-MET-GLN-VAL-ASN-ARG-ALA-VAL-ASN-LEU-ASP-GLU-THR-LEU-MET-PRO-ASP-MET-VAL-LYS-
AB2       GLY-MET-GLN-VAL-ASN-ARG-ALA-VAL-TYR-LEU-ASP-GLU-THR-LEU-MET-PRO-ASP-MET-VAL-LYS-
                                          50                                  60
RD        GLY-MET-GLN-VAL-ASN-ARG-ALA-VAL-PRO-LEU-GLY-THR-THR-LEU-MET-PRO-ASP-MET-VAL-LYS-

               61      63
AB1       THR-TYR-GLN
AB2       ASN-TYR-GLU
                  64
RD        ASN-TYR-GLU
```

Fig. 7. Comparison of amino acid sequence of peptides AB1, AB2 and peptide RD from a closely related fish *Rhigophila dearborni* determined by Schrag et al. [10]. Identical sequences between AB1 or AB2 and RD are underlined.

Amino acid analysis of AB2

Since the automated sequencing used in this study proved to be highly reliable for AB1, only amino acid composition was performed for the minor antifreeze peptide, AB2, for verification of its sequence determined by automated sequencing. The results of amino acid composition based on amino acid analysis and peptide sequence for both AB1 and AB2 were shown in Table V. There is good agreement between the compositions determined from the two methods. A comparison of the peptide sequences of AB1 and AB2, and of RD, an antifreeze peptide from the closely related fish *Rhigophila dearborni* determined previously [11], is shown in Fig. 7.

Circular dichroism

Fig. 8A shows the CD spectra for AB1 obtained at temperatures of 0 to 60 °C. The spectra were corrected for solvent absorption noise; 250 mM NaCl instead of water was used as the solvent to approximate the osmolarity the antifreeze peptide encounters naturally in the fish serum. The 0 °C spectrum most approximates that of the peptide in its native and natural state. It shows no α-helix or β-sheet characteristics; its specific shape, a slight positive band at about 220 nm, and a strong negative band at about 190 nm, is generally taken to represent that of a random chain. However, the CD spectra at elevated temperatures suggest the existence of definite secondary or tertiary structure. The spectra

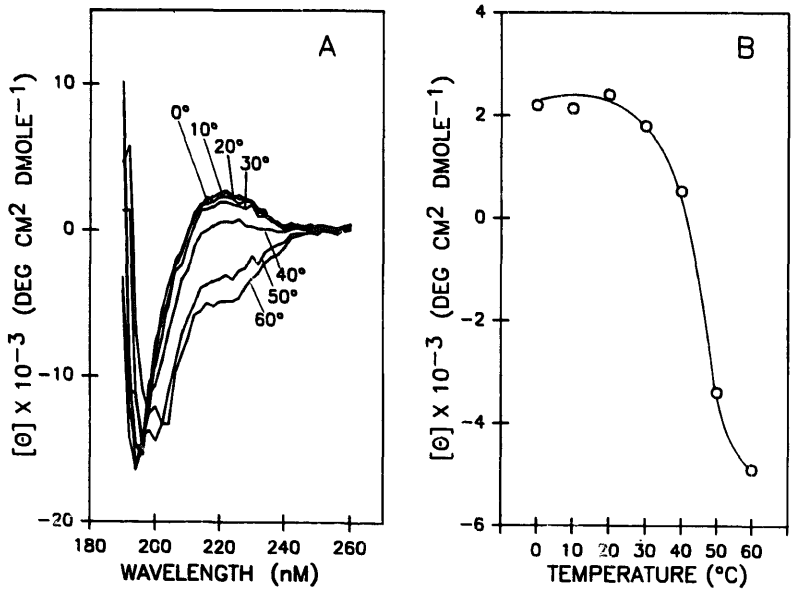

Fig. 8. (A) Circular dichroism spectra of peptide AB1 at 0 to 60 °C. The peptide was dissolved in 250 mM NaCl at 1.9 mg/ml. (B) Thermal denaturation of peptide AB1 as measured by changes in the magnitude of molar ellipticity [θ] at 224 nm.

changed little as the temperature was raised from 0 and 30 °C, but showed a sharp transition between 30 and 50 °C. Fig. 8B shows the molar ellipticity at 224 nm for the different temperatures. The sharp transition suggests a 'melting' of some structured portion of the native peptide.

Discussion

The antarctic zoarcid fish, *A. brachycephalus*, has primarily two antifreeze peptides (AFPs) in its blood, AB1 and AB2, the amino acid sequences of which were determined in this study. AB1 constitutes 94% of the AFP present based on yields from HPLC purification. *A. brachycephalus* therefore can be considered to synthesize predominantly one major AFP. According to peak areas in HPLC purification, AB2 constitutes 12% of the total AFPs, instead of 6% based on purification yield. The over-estimation is probably due to the presence of two residues of tyrosine in AB2 versus one residue in AB1, which augmented the absorbance of AB2 at 220 nm. The sequencing of AB1 and AB2 was greatly aided by the highly accurate method of molecular mass determination by Fast Atom Bombardment Mass Spectrometry, which has an error of ± 1 Da, particularly in deciding whether one has reached the C-terminus of the native peptide in automated sequencing. There is an unavoidable 1% carry-over per degradation cycle leading to noise peaks, in addition to decrease in the yield of PTH-derivative, such that unambiguous assignment of residues becomes difficult towards the C-terminus. The comparison between mass determined by FAB/MS and the calculated mass based on sequence provides an invaluable check on the accuracy of the sequence. FAB/MS is usually used for analyses of smaller molecular mass peptides or peptide fragments [19]; it has been demonstrated in this study that it can also be successfully used for determination of molecular masses of larger peptides.

A. bracycephalus is a member of the family Zoarcidae, which also includes *Rhigophila dearborni*, also from the antarctic, the arctic *Lycodes polaris*, and the North-Atlantic *Macrozoarces americanus*, all of which produce antifreeze proteins to avoid freezing. Each fish synthesizes more than one AFP with only minor variations in amino acid composition [10,20]. There are at least 12 AFP components in *M. americanus* [20], three major AFPs and at least four minor ones in *R. dearborni* [1], and at least 10 in *L. polaris* (Cheng and DeVries, unpbulished data). *A. brachycephalus* therefore differs from the other zoarcids in that it synthesizes predominantly one AFP, i.e., AB1.

The sequences for one of the AFPs of *R. dearborni* and *L. polaris* [10], and of *M. americanus* [9] have been reported. There is substantial sequence homology, in terms of number of identical residues, among all the zoarcid AFPs, including the *A. brachycephalus* AFPs reported here. Schrag et al. [10] showed that, interestingly, there was stronger sequence homology (80% identical residues) between the two eel pouts, *Rhigophila* and *Lycodes*, inhabiting opposite polar waters, than between the two Northern species, *Lycodes* and *Macrozoarces* (60% identical residues). *Austrolycicthys* and *Rhigophila* are closely related Antarctic species; therefore, it is not surprising that their AFP sequences are similar. 72% of the residues of AB1 and 84% of AB2 are identical to those of *Rhigophila* AFP. However, as shown in Fig. 7, this homology exists only if the *Austrolycicthys* AFP sequences are shifted one position C-terminus-ward with respect to the *Rhigophila* AFP sequence. It is likely that the genes encoding each of these two AFPs are similar, and that this one position shift in the peptide may represent some post-translational modification. Between AB1 and AB2, 83% of the residues are identical.

A common characteristic of the sequences of zoarcid AFPs and distinguishes them from other classes of antifreezes, is the even distribution of most amino acids. The amino acids are all aliphatic except for one or two aromatic ones. AB1 of *Austrolycicthys*, *Rhigophila*, and *Lycodes* AFPs contain a single aromatic amino acid, namely, tyrosine (Ref. 10 and this paper), AB2 contains two tyrosines, while *Macrozoarces* AFP has one tyrosine and one phenylalanine [9]. It is unclear what, if any, the significance of the single aromatic residue may be. It was suggested that asymmetric placement of Tyr and Phe in the *Macrozoarces* AFP is involved in some tertiary structure of the peptide [21].

Austrolycicthys AFPs, like the other zoarcid AFPs apparently lack clearly recognizable secondary structures based on circular dichroism studies. The CD spectrum of AB1, resembles that of *Lycodes* [10], as well as that of *Macrozoarces* [21]. The general shape of the spectrum is interpreted to be that of a random chain. Secondary structure predictions using the method of Chou and Fasman [22], however, indicated presence of some α-structure and significant β-structure, but these are not apparent in the CD spectrum. The sharp thermal transition in the CD spectra between 30 to 50 °C, however, argues for the presence of some higher order structure.

A mechanism of adsorption–inhibition has been proposed to explain the antifreeze action, in that adsorption of antifreeze to ice surface inhibits ice-crystal growth [12]. The structure of antifreeze is thought to be important in the adsorption–inhibition process. One mode of antifreeze adsorption to ice probably involves a lattice match between the polar groups in the antifreeze and the oxygen atoms in the ice crystals, allowing hydrogen bonding [13]. This model is best exemplified in the AFPs that have amphiphilic α-helical secondary structures. The most thoroughly studied helical AFP is

that of the winter flounder [3–5,23], which consists of periodic placement of the polar side-chains and non-polar ones on opposite sides of the α-helix. The polar side-chains are 4.5 Å apart, which matches the distance between oxygen atoms parallel to the *a*-axes in the ice lattice [13]. Dipole –dipole interaction between the helix and the ice lattice is thought to be important in the initial alignment of the peptide on the ice [24]. Other fishes that have AFPs with high helical content include the short horn sculpin [1,6] and Alaskan plaice (DeVries, unpublished data). A possible lattice match also exists in the antifreeze glycopeptide (AFGP) between alternate carbonyl groups of the peptide backbone, and the oxygen atoms parallel to the *c*-axis of the ice crystal [1,13].

However, this lattice match model does not seem to apply to the zoarcid AFPs which contain no repeating sequences or obvious secondary structure. Nevertheless, AFPs of *Austrolycicthys* and other zoarcids, like other known APFs and AFGPs, function in a similar way, i.e., they lower the freezing point of a solution, i.e., the temperature of ice growth in the presence of a seed crystal, without affecting the melting point. This suggests to us that all antifreezes function by a common mechanism. Results from our recent studies using single ice crystals grown in the presence of different AFPs and AFGPs provided supporting evidence. Irrespective of the antifreeze type (at concentrations of 10 mg/ml), at temperatures between the melting and freezing points (freezing point being the temperature at which spicular ice growth is observed), there was complete inhibition of growth on the prism faces, and limited growth on the basal plane resulting in the appearance of hexagonal pits as growth layers were laid down [11]. Growth on the basal plane stops when the basal plane becomes completely pitted, or when the crystal assumes the shape of a hexagonal bipyramid [11]. The inhibition of growth on the prism faces presumably is a result of antifreeze adsorption on those faces. Preliminary results from our experiments with large single-crystal ice hemispheres grown slowly from dilute solution (10^{-4}%, w/w) of various antifreezes showed that, the *Austrolycicthys* AFPs preferentially adsorbed on the primary prism plane $\{10\bar{1}0\}$ as do all the AFGPs from the Antarctic cod, *Dissostichus mawsoni,* the short-horn sculpin AFPs adsorbed on the secondary prism plane $\{11\bar{2}0\}$, and the winter flounder and sea raven on two different pyramidal planes, $\{20\bar{2}1\}$ and $\{11\bar{2}1\}$, respectively [25]. None of the antifreezes where found to adsorb on or near the basal plane {0001}, which accounts for the characteristic spicular ice or *c*-axis growth in the presence of antifreeze when the temperature is lowered below the freezing point. It is unclear at this point why different antifreezes have a different preferred plane of adsorption, but the common end results is that growth parallel to the *a*-axes is inhibited.

From this study and others [21], it is clear that circular dichroism cannot provide conclusive data on the secondary and teritary structures of the AFPs of *Austrolycicthys* and other zoarcids, which are important in the understanding of how antifreezes work. Other methods such as X-ray crystallogrphy and two-dimensional Nuclear Magnetic Resonance [26] might give more definitive answers. The relative high yield of AB1 based on the purification methods used in this study, about 25 mg per 10 ml serum, will make attempts at X-ray crystallography and two-dimensional NMR studies possible.

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