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Properties of engineered antifreeze peptides

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Eight antifreeze-like peptides were produced by cleavage from engineered chimeric proteins. One was homologous to an antifreeze peptide of the winter flounder; the others differed in length and/or sequence. The homologous peptide and all those of equal or greater length were able to inhibit recrystallization. The longer peptides were so hydrophobic that their identification required modification of the usual protocols for high pressure liquid chromatography. Their elution positions were correlated to their hydrophobicities and their lengths. Additional naturally occurring antifreezes may be identifiable with this knowledge.

Antifreeze peptide; *Pseudopleuronectes americanus*; HPLC, reverse phase; Protein engineering; Ice, recrystallization

1. INTRODUCTION

Certain fish [1–3] and terrestrial arthropods [4] contain 'antifreeze' proteins that cause non-colligative depression of their body fluids' freezing point. The antifreeze proteins are generally induced by low temperatures and they constitute a significant adaptation to cold environments. The winter flounder [4] and Alaskan plaice [1] have antifreeze peptides in which an 11-amino acid unit ($TA??A_6?$) is repeated in tandem. Multiple species of such peptides are present. In winter flounder, the predominant species contain three $TA??A_6?$ repeats [5,6]. Less abundant peptide species may contain four [7,8] and five [9] repeats. Purification of the less abundant peptides has been problematic; their sequences are inferred from cDNA clones.

What function might be served by the multiplicity of antifreeze peptides? To address this, we have attempted to differentiate between the properties of antifreeze peptides of varying length and composition. A series of antifreeze analogue (Afa) sequences was designed and generated using synthetic DNA. Chimeric proteins, consisting of Afa peptides joined to staphylococcal protein A (Spa), were first produced in and purified from *E. coli* [10]. This paper describes the separation of the peptides from their Spa fusion partners, and reports the activities of the various peptides in inhibiting the recrystallization of ice [11–13]. We also correlate their chromatographic properties with properties predictable from their sequences.

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The peptides Afa2, Afa3, Afa4, Afa5 and Afa6 form a homologous series containing two, three, four, five and six $TA??A_6?$ repeats, respectively. Peptides $Afa3_{SB}$ and $Afa3_R$ differ from Afa3 by the presence of a salt bridge, or by the presence of a carboxy-terminal arginine, respectively; $Afa3_{SB/R}$ combines both types of difference. The properties of the various peptides are summarized in Table I. Relative to antifreeze species that occur naturally in winter flounder, $Afa3_{SB/R}$ is identical in sequence to one of the major species, and Afa4 and Afa5 are similar to probable minor species. However, the carboxy-terminal arginine residues of the analogues are unmodified, whereas the carboxy-termini of antifreeze peptides are amidated in the fish.

2. MATERIALS AND METHODS

2.1. *Treatment of chimeric proteins with cyanogen bromide*

The method derives from that of Gross [14]. Each reaction contained cyanogen bromide at 7 mg/ml, protein at 9.5 mg/ml, and 0.1 M HCl. After 16 h incubation at 24°C, reaction mixtures were evaporated to dryness under vacuum. The products were redissolved in 50 mM Tris-HCl (pH 7.5) to a final protein concentration of 47.5 mg/ml. Examination of the reaction products by SDS-PAGE showed that between 50 and 75% of the chimeric protein was cleaved.

2.2. *Filtration of peptides*

Solutions were passed through Centricon 10 filters (Amicon-W.R. Grace, Danvers, MA) to remove proteins larger than approximately 10 kDa. Peptides present in the filtrates were subsequently concentrated by retention on Centricon 3 filters.

2.3. *Calculation of hydropathy*

We calculated the mean hydropathic index of all residues in a peptide, using standard values [15]. However, in peptides where the carboxy-terminal residue was non-polar (in all such cases, alanine), we adjusted the value of that residue's hydropathy to −3.5 (the same as that for aspartic and glutamic acid residues) to reflect its content of a hydrophilic carboxyl group. We were aware that this adjustment would improve the correlation between hydropathic index and elution

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position. No adjustments were made for amino-terminal residues because aspartic acid, the amino-terminal residue of every peptide, is already assigned a hydropathy reflective of hydrophilic residues.

2.4. *High pressure liquid chromatography (HPLC)*

Chromatography was performed on reverse-phase C_{18} and C_4 columns, using continuous buffer gradients. The eluent was monitored by simultaneously recording absorbance at 216 nm and 280 nm. Initially, acetonitrile was utilized as the organic eluent (the buffer system varying from 84.9% water, 15% acetonitrile, 0.1% trifluoroacetic acid at loading to 10% water, 89.9% acetonitrile, 0.1% trifluoroacetic acid at the end of elution). After difficulties described in section 3, we then utilized only C_4 columns and a 2:1 mixture of propan-2-ol and acetonitrile ('2I:A') as the organic eluent (the buffer system varying from 74.9% water, 25% 2I:A and 0.1% trifluoroacetic acid at loading to 99.9% 2I:A and 0.1% trifluoroacetic acid at elution). All results presented were obtained with the latter system. We express elution characteristics in terms of the proportion of organic solvents present at the time of elution (they therefore fall between 25% and 99.9%). Solvents were HPLC grade, obtained from J.T. Baker Chemical Co., Philipsburg, NJ. Trifluoroacetic acid (Sequanal Grade) was obtained from Pierce Chemical Co., Rockford, IL.

2.5. *Estimation of protein and peptide concentrations*

Concentrations were estimated by measuring the areas below A_{216} peaks observed on the chart recorder and comparing these to the peak areas produced by eluting known quantities of pure proteins under identical conditions. A_{280} readings were not utilized because the antifreeze peptides lack aromatic residues and absorb poorly at 280 nm.

2.6. *Amino acid analysis and protein sequencing*

Amino acid analysis of peptide Afa4 was carried out by the UCD Protein Structure Laboratory (Davis, CA). After hydrolysis of the peptide in 6 M hydrochloric acid, amino acids were separated by ion-exchange chromatography and quantitated by a ninhydrin detection system.

Sequencing of the amino-terminus of peptide Afa4 was performed by the Biomolecular Resource Center at UCSF (San Francisco, CA). Samples were subjected to Edman degradation using an Applied Biosystems (Foster City, CA) 470A gas-phase sequencer. The PTH derivatives were identified and quantitated by reverse-phase HPLC using an on-line Applied Biosystems 120A PTH analyzer.

2.7. *Assays of recrystallization-inhibiting activity*

Activity levels were estimated by observing the recrystallization of 'splat cooled' samples [16] from a 2-fold dilution series of each preparation, as described [10].

3. RESULTS

3.1. *Identification of antifreeze peptides by HPLC*

To liberate antifreeze (Afa) peptides from chimeric Spa–Afa proteins [10], we treated the latter with cyanogen bromide (CNBr). The liberated Afa peptides should range in size and sequence: their characteristics are compared in Table I. CNBr treatment cleaves preferentially at peptide bonds carboxy-terminal to Met residues [14], and each chimeric protein contained a single internal Met residue immediately amino-terminal to its antifreeze moiety. Therefore, CNBr cleavage was expected to liberate intact Spa and Afa domains. We attempted to identify the free Spa and Afa domains by performing HPLC on the CNBr-treated samples.

When utilizing acetonitrile as the organic eluent, we could only find the free peptides $Afa3_{SB}$ and $Afa3_{SB/R}$ as novel peaks. Most of the other peptides would be relatively more hydrophobic than $Afa3_{SB}$ and $Afa3_{SB/R}$, so we reasoned that the buffer system might have failed to elute them. Therefore we changed the organic eluent to a 2:1 mixture of isopropanol and acetonitrile ('2I:A'). Spa–Afa chimeric proteins were subjected to HPLC with the 2I:A buffer system to determine their elution positions, which are reported by stippled bars in Fig. 1. The Spa–X protein, which has a sequence similar to that expected of a free Spa domain [10], was also analyzed. This enabled us to understand, in subsequent work, which peaks were due to uncleaved chimeric proteins and free Spa domains.

Table I

Predicted properties of antifreeze peptides

Peptide	Repeat units*	Salt bridge[a]	Arg[37] residue[b]	Length[c]	M_r[d]	Adjusted hydropathy[e]
Afa2	2			26	2,145	0.673
Afa3	3			37	3,042	0.851
$Afa3_R$	3		+	37	3,127	0.824
$Afa3_{SB}$	3	+		37	3,143	0.554
$Afa3_{SB/R}$	3	+	+	37	3,228	0.527
Afa4	4			48	3,939	0.948
Afa5	5			59	4,836	1.008
Afa6	6			70	5,733	1.050

*Number of 11-residue consensus repeats in peptide.
[a] The presence of a salt bridge is denoted by a plus (+) sign.
[b] The presence of a C-terminal arginine residue is denoted by a plus (+) sign.
[c] Length in aminoacyl residues.
[d] Molecular mass.
[e] Adjusted hydropathic index for entire peptide molecule.

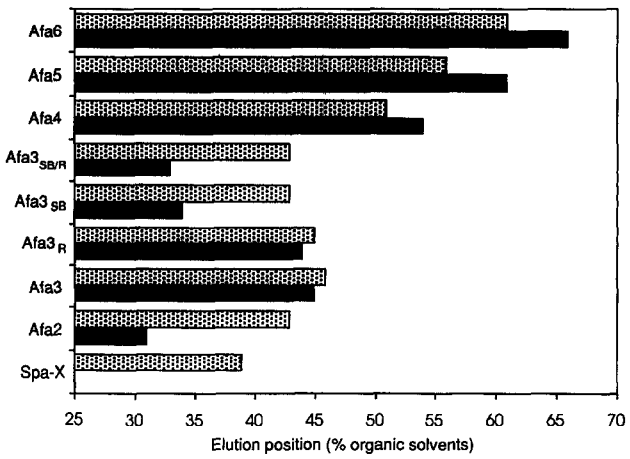

Fig. 1. The HPLC elution positions of peptides, denoted by the right end of each filled bar. The cognate chimeric protein's elution position is denoted by the right end of each adjacent stippled bar. Spa–X is similar to the Spa portion of the chimeric proteins; its elution position is also shown.

CNBr-treated preparations were now subjected to HPLC utilizing the 2I:A buffer system. Novel peaks were observed in the A_{216} spectra of samples corresponding to Afa2, $Afa3_{SB}$, $Afa3_{SB/R}$, Afa4, Afa5 and Afa6. The new peaks (unlike those due to free Spa and the Spa–Afa proteins) had no counterparts in the A_{280} spectra. This was to be expected for Afa peptides, since they contain no aromatic residues.

The above analysis still did not identify novel peaks attributable to Afa3 and $Afa3_R$. We suspected that Afa3 and $Afa3_R$ might co-elute with other proteins. Therefore, the CNBr-treated samples were filtered to remove proteins larger than 10 kDa before HPLC. Filtration of the $Afa3_{SB/R}$ sample, as a positive control, demonstrated that the filtrate was depleted for other proteins but still contained free $Afa3_{SB/R}$. The filtered preparations of Afa3 and $Afa3_R$ were now observed to give A_{216} peaks without A_{280} counterparts, at elution positions previously obscured by their corresponding chimeric proteins: we inferred that these peaks represented the free peptides. The elution positions of all the putative free Afa peptides are reported as solid bars in Fig. 1.

3.2. *Purification of antifreeze peptides*

Peptides Afa4, Afa5, and Afa6 were purified simply by collecting the appropriate fractions from HPLC elutions. However, the CNBr reaction mixtures containing Afa3, $Afa3_R$, $Afa3_{SB}$, $Afa3_{SB/R}$, and Afa2, were first passed through low molecular weight cut-off filters. Part of each filtrate was analyzed by SDS-PAGE to confirm the absence of the larger proteins, and the remainder was subjected to HPLC with collection of the peak containing the peptide.

Samples of the purified peptides were subjected to HPLC again to check purity and obtain a quantitative estimation of their concentrations. The set of A_{216} elution spectra is shown in Fig. 2. In addition, we subjected Afa4 to two further analyses to test its identity: analysis of amino acid content, and amino-terminal sequencing. Afa4 was selected as the best single representative because its size and its elution position fall in the middle of the range of the eight antifreeze peptides. The measured and predicted amino acid contents of Afa4 are given in Table II. The correspondence between predicted and measured values was good, and the unusual composition of Afa4 (particularly its high content of Ala) makes such a correspondence very unlikely to occur by chance. Five rounds of amino-terminal sequencing of the Afa4 preparation inferred the sequence DTASD, in perfect correspondence with prediction. These analyses strongly suggest that Afa4 is the major component in the Afa4 preparation, and thus lend credence to the assignment of the other HPLC peaks to free antifreeze peptides.

The activities of the different antifreeze peptides were compared. Because of the limited quantities available, we tested for the inhibition of ice recrystallization, an

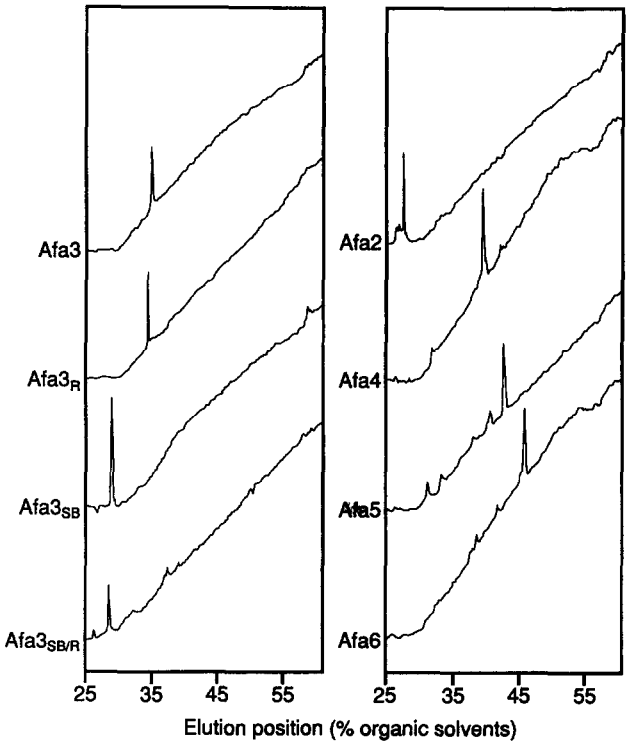

Fig. 2. The A_{216} elution spectra of the purified Afa peptides. Baselines increase linearly because of absorbance by acetonitrile at 216 nm. Approximately 6 μg of peptide was loaded for each chromatography run.

Table II

Amino acid analysis of presumptive Afa4 peptide

Amino acid	Mole %	
	Measured	Predicted
Ala	67.1	70.8
Arg	0.3	0.0
Asx	10.8	10.4
Cys	0.0	0.0
Glx	0.9	0.0
Gly	0.4	0.0
His	0.1	0.0
Ile	0.3	0.0
Leu	6.7	6.2
Lys	0.5	0.0
Met	0.1	0.0
Phe	0.0	0.0
Pro	0.2	0.0
Ser	2.2	2.1
Thr	9.5	10.4
Tyr	0.0	0.0
Val	1.0	0.0

Trp was not measured; Asp and Asn were measured together and are reported as Asx; likewise, Glu and Gln were measured together and are reported as Glx. The predicted sequence of Afa4 is DTASDAAAAAALTAANAAAAAALTAANAAAAAALTAANAAAAAAATAA.

activity displayed by antifreeze proteins at lower concentrations than those required for measurement of freezing point depression [16]. A titration scheme was employed [10]: a 2-fold dilution series of each peptide was assayed for recrystallization inhibition, in order to define the minimum inhibitory concentration (mic) of each. The highest concentrations tested were 96 μg/ml. Table III reports the range of mic values obtained for each peptide over three separate experiments. The variability precludes precise quantitative comparisons. However, it is apparent that Afa2 lacks activity and that all the other peptides are active. Further, it appears that the longer peptides are more active than Afa3 and its variants, reminiscent of a similar trend among the corresponding chimeric proteins. Contrary to expectation, the data did not provide evidence that free peptides possess significantly higher activities than their parent molecules. We suspect that much of the variability in measured activities could have been due to varying efficiencies of redissolving the peptides after frozen storage.

We attempted to correlate the HPLC elution positions of the free peptides with two predictable properties: hydropathic index (mean hydropathy per residue) and length. Although the correlation with hydropathic index (Fig. 3a) appeared good, it was imperfect because Afa2 elutes before $Afa3_{SB}$ and $Afa3_{SB/R}$. This anomaly cannot be ascribed to our arbitrary choice of the hydrophobicity scale of Kyte and Doolittle [15]: the hydrophobicity scales of Argos et al., Chothia, Eisenberg, Janin, von Heijne and Blomberg, and Wolfenden (reviewed by Eisenberg [17]) all estimate Afa2 as more hydrophobic than $Afa3_{SB}$ and $Afa3_{SB/R}$. The anomalous elution position of Afa2 probably indicates that peptide length is a factor in elution behavior. Elution position appeared to correlate negatively with the reciprocal of peptide length (Fig. 3b) but this correlation of course could not predict the different elution positions of Afa3, $Afa3_R$, $Afa3_{SB}$ and $Afa3_{SB/R}$. By trial and error, we found a formula combining the variables of hydropathy and length into a single variable which correlated well with the elution positions of all peptides (Fig. 3c):

$$\text{(Hydropathic index)} - \frac{16}{\text{(Peptide length in residues)}}$$

An alternative formula produces a combined variable which also has a good correlation to elution position (data not shown):

$$\text{(Hydropathic index)} \times \frac{\text{(Peptide length in residues)}}{\text{(Peptide length in residues)} + 40}$$

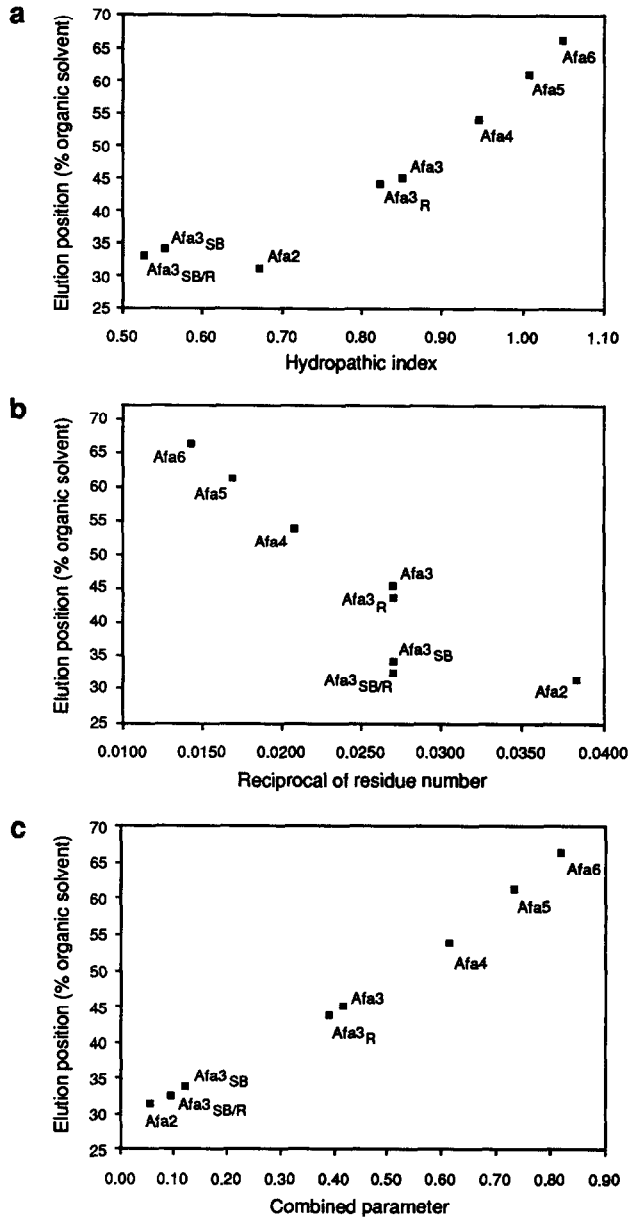

Fig. 3. Scatter-chart correlations of observed elution positions of Afa peptides (ordinate) with known parameters (abscissa). (a) Correlation with adjusted hydropathy. (b) Correlation with the reciprocal of peptide length (residues^{-1}). (c) Correlation with the combined parameter, (adjusted hydropathy) − (16/peptide length). (The factor of 16 in this function is empirically derived.)

Table III

Titration of recrystallization inhibition activities of free Afa peptides

Peptide	Range of mic* estimates (μg/ml)
Afa2	No inhibition[a]
Afa3	13–36
$Afa3_R$	12–39
$Afa3_{SB/R}$	6–28
$Afa3_{SB}$	8–40
Afa4	4–9
Afa5	5–47
Afa6	3–9

*mic, minimum inhibitory concentration for complete inhibition of recrystallization.

[a] The highest concentration tested was 96 μg/ml.

4. DISCUSSION

A series of peptides analogous to the antifreeze peptides of winter flounder was generated by site-specific cleavage of engineered proteins synthesized in *E. coli*. The peptides were identified by HPLC on the basis of two criteria. First, each gave a novel peak, the position of which varied with the peptide's composition, in the A_{216} elution spectrum. Second, each lacked absorbance at 280 nm, unlike the material giving rise to other peaks in the A_{216} elution spectrum, but as expected for a peptide lacking aromatic side chains. The identification was further supported by amino acid analysis and amino-terminal sequencing of one of the peptides.

All the antifreeze peptides, except that containing only two repeats of the 11-amino acid unit, showed activity as defined by the ability to inhibit the recrystallization of ice. This supports conclusions drawn previously concerning the minimum number of ice-interaction residues or minimum helix length required for antifreeze activity [18]. The peptides' activities were of the same order as those of their parent chimeric proteins [10] on a mass basis, and may be lower on a molar basis. This accords with the observation that the activity of an insect antifreeze was increased by the addition of an antibody directed against it [19]. It was suggested that increasing the mass of the ice-binding molecule may increase its effectiveness at inhibiting ice growth.

The HPLC protocol used to identify the peptides differs from that more commonly used with reverse-phase columns by employing a 2:1 mixture of isopropanol and acetonitrile as the organic eluent, rather than just acetonitrile. In addition, we utilized C_4 columns rather than the more hydrophobic C_{18} columns. We had previously been unable to identify the longer, more hydrophobic peptides when employing the standard protocol.

The HPLC elution positions of the peptides correlated with two of their predicted properties: mean hydropathy (per residue), and length (in residues). We obtained functions empirically, combining hydropathy and length, that appeared adequate for predicting HPLC elution positions. These may be of value in identifying longer peptides in the winter flounder and other fish producing similarly alanine-rich antifreeze peptides. Because our data were used to optimize the combined functions, they do not prove the validity of this treatment. However, the treatment is realistic: it is reasonable to expect molecular adsorption not only to depend on the mean hydrophobicity of a peptide but also to become stronger in longer molecules with more sites available for interaction with the substrate.

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