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Research Article

Isolation of an antifreeze peptide from the Antarctic sponge *Homaxinella balfourensis*

S. P. Wilkins [a], A. J. Blum [a], D. E. Burkepile [a], T. J. Rutland [b], A. Wierzbicki [b], M. Kelly [c] and M. T. Hamann [a,*]

[a] Department of Pharmacognosy NCNPR, School of Pharmacy, 400 C Faser Hall, University of Mississippi, Mississippi 38677 (USA), Fax 662 915 5730, e-mail: mthamann@olemiss.edu
[b] Department of Chemistry, University of South Alabama, Mobile, Alabama 36688 (USA)
[c] National Institute of Water and Atmospheric Research (NIWA) Let., Private Bag 109-695, Newmarket, Auckland (New Zealand)

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Abstract. Polar plants and animals survive in subzero waters (–2 °C) and many of these marine organisms produce antifreeze proteins (AFPs) to better adapt themselves to these conditions. AFPs prevent the growth of ice crystals which disrupt cellular membranes and destroy cells by inhibiting crystallization of water within the organism. The hydrophilic extract of an Antarctic sponge *Homaxinella balfourensis* exhibited a non-colligative freezing point depression effect on the crystal morphology of water. The extract was purified by repeated reverse phase high-pressure liquid chromatography, then assayed and shown to contain several AFPs. The major peptide was isolated, analyzed using matrix-assisted laser desorption ionization mass spectrometry and the partial structure of the peptide identified through amino acid sequencing. AFPs have potential applications in agriculture, medicine and the food industry.

Key words. Antifreeze protein; non-colligative freezing point depression; HPLC; matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI/TOF-MS).

Antifreeze proteins (AFPs) were first discovered in Arctic fish in which proteins were identified which lowered the freezing temperature of blood without increasing the osmotic pressure due to high salt concentrations which could be lethal to the fish [1]. The most common classes of AFP consist of antifreeze glycoproteins (AFGPs) and four subclasses (I–IV) of AFP [2]. AFGPs and class I AFPs both contain approximately 67% alanine with threonine providing the second most abundant amino acid in AFGPs. Threonine may be linked to a disaccharide moiety which is arranged in repeating tripeptide units (Ala-Ala-Thr-disaccharide)$_n$, a motif found in fish [3]. Examples of antifreeze proteins from plants have shown extreme heat stability [4], while those from insects have shown the greatest potency [5].

AFPs function non-colligatively by lowering the freezing point while leaving the melting temperature unchanged. There is no difference between the freezing-point and the melting point in colligative freezing point depressants. The level of non-colligative activity, as measured by the freezing point depression, can be significant and this activity can be as high as 500 times that of colligative activity on a molal basis [6]. In all classes of AFPs, the solution concentration of the protein in excess of the saturation level will not increase the level of activity significantly.

An AFGP has evolved from a trypsinogen gene in an Antarctic notothenioid. The small sequence divergence (4–7%) between notothenioid AFGP and trypsinogen

* Corresponding author.

genes indicates that the transformation of the proteinase gene into the novel ice-binding protein gene occurred quite recently, about 5–14 million years ago [7]. This is highly consistent with the estimated time for the freezing of the Antarctic Ocean, which is believed to have occurred between 10–14 million years ago. The conversion of the notothenioid trypsinogen gene to an AFGP gene is the first clear example of an old gene producing a new gene which encodes a protein with a totally different biological function [7].

There is also evidence of convergent evolution for genes encoding almost identical AFGPs in two unrelated fish with different ancestral genes [8]. As mentioned, the AFGP gene in Antarctic notothenioid fish evolved from a gene that encodes trypsinogen, a digestive enzyme. A similar AFGP was isolated from an Arctic cod although it shares no sequence identity with the trypsinogen gene. The Antarctic notothenioid and the Arctic cod belong to two different orders of fish that diverged some 40 million years ago, before the polar oceans froze [8].

AFPs have a wide range of potential applications. In agriculture, they are used to genetically engineer plants for resistance to frost damage. Cryomedicine could potentially use AFPs to decrease damage to organs frozen before transplant surgery [9]. There are also potential applications of AFPs in the frozen-food industry where AFPs could be used to decrease cellular damage. AFPs could also be used to decrease the recrystallization of water in stored frozen foods such as ice cream by inhibiting massive ice crystal growth that degrades the smooth texture of these products when they are warmed and recooled [10].

Materials and methods

General experimental conditions

Solvents for extraction and purification were certified grade from Fisher Scientific Co. or distilled from glass. High-performance liquid chromatography (HPLC) was performed using a Waters 510 system with a Waters 486 UV detector and gradient programmer.

Collection and extraction

Approximately 450 g of the sponge *Homaxinella balfourensis* was collected, lyophilized and frozen from McMurdo Sound, Antarctica in December 1993. The sponge was extracted three times with EtOAc/IPA 1:1 (lipophilic extract) followed by three times EtOH/H_2O 1:1 (hydrophilic extract).

Purification

Approximately 1 g of hydrophilic extract was subjected to reverse-phase HPLC using a Phenomonex, 10 × 250 mm, Jupiter C18 column containing 5 µm packing material with a pore size of 300 Å. An HPLC gradient with an H_2O/CH_3CN mobile phase was used to achieve elution and resolution of the water-soluble peptides. Typical elution times ranged from 30 to 90 min with a flow rate of 5 ml/min.

Freezing-point depressant assay

The freezing-point depressant activity was measured using a Clifton Nanoliter Osmometer with a Sony Video Printer [11]. Samples were prepared in a 0.1 M solution of ammonium bicarbonate buffer and were loaded into

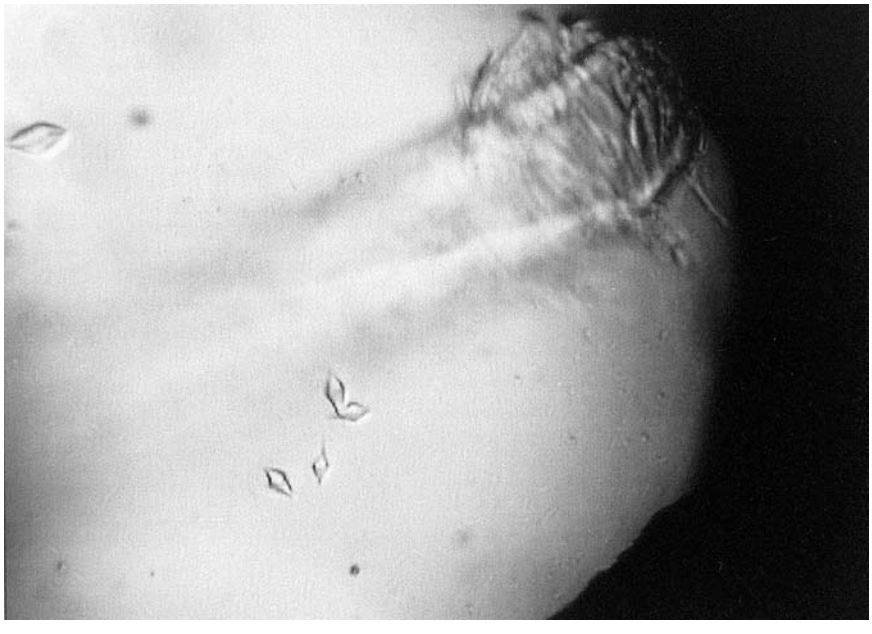

Figure 1. Cryomicroscopic image (magnification × 400) of ice grown in the presence of crude extract from the sponge *Homaxinella balfourensis* (120 mg/ml solution) above the freezing point. The characteristic bipyramidal morphology of ice crystals can be observed.

the sample holder of the nanoliter osmometer. The sample holder of the nanometer osmometer was a silver plate $6 \times 6 \times 1$ mm^3 that was bored with eight holes (diameter approximately 0.4 mm) that served as sample wells. The wells were filled with Cargille's type B immersion oil using a very fine capillary tube produced by drawing out a 10-µl micropipet over a Bunsen burner. Next, using the micropipet, a single droplet of antifreeze solution (less than 10 nl) was suspended in the center of the oil-filled well and covered with Cargille's type A immersion oil to prevent evaporation. The sample holder was then placed on the thermoelectrically controlled microscope stage (operating on the Peltier principle). The sample was then rapidly frozen to –40 °C. After freezing the entire solution, the temperature was slowly raised and the sample was thawed until only a single spherical-shaped ice seed crystal remained in the solution. From this point, the temperature was slowly decreased and the growth morphology of the single ice crystal was followed. Initially the ice crystal grew spherically, but it was rapidly transformed into a hexagonal bipyramid (fig. 1) with the presence of the non-equilibrium antifreezes. It remained unchanged until the freezing-point depression temperature was reached. The samples were determined to have antifreeze activity if crystal growth was entirely arrested for at least 1 h at a steady temperature just above freezing point. For comparison, figure 2 shows the spherical ice crystal morphology of the control grown in deionized water. Below the freezing point, crystal growth continued indiscriminately along the c-axis of the crystal (fig. 3), producing needle-like protrusions from the tips of the bipyramids. Eventually, the entire solution was frozen when the needle-like crystal reached the edges of the wells (fig. 4).

Mass spectrometry

A Perceptive Biosystems Voyager matrix-assisted laser desorption time-of-flight (MALDI/TOF) instrument was used to identify the mass of the peptide in an alpha cyano-4-hydroxycinammic acid matrix. The peptide was N-terminally sequenced on an Applied Biosystems 473A Protein Sequencer using standard Edman chemistry.

Results

Exhibition of non-colligative crystal morphology

Hydrophilic extracts of most of the common Antarctic invertebrates were analyzed for antifreeze activity. Solutions of each sample were made at an initial concentration of 30 mg/ml with 0.1 M ammonium bicarbonate buffer serving as the solvent. To determine the possible presence of antifreeze in these samples, crystal morphology tests were performed using the Clifton Nanoliter Osmometer (see Materials and methods). Thirty-eight species of Antarctic invertebrates and algae were assayed, and 14 species of sponge exhibited some level of non-colligative antifreeze activity. However, only one sample, from the sponge, *H. balfourensis,* showed strong non-equilibrium antifreeze activity, manifested as the formation of a characteristic bipyramidal ice morphology (fig. 1). Figure 1 shows the crystal morphology of an active antifreeze hydrophilic extract from *H. balfourensis* (120 mg/ml solution) above the non-equilibrium freezing point. Without the presence of antifreeze molecules, the negative control (0.1 M ammonium bicarbonate buffer) grows a spherical crystal versus the hexagonal bipyramid formed in the presence of non-colligative AFPs (fig. 2). Just below the non-equilibrium freezing point of the so-

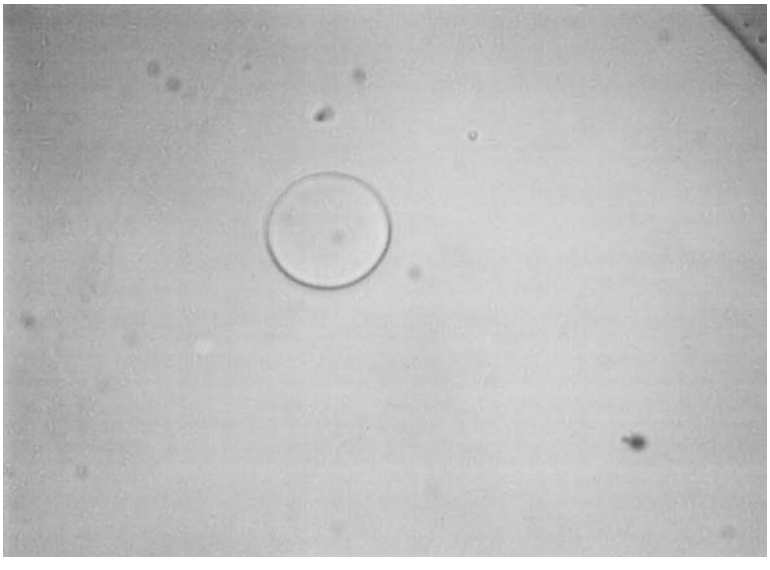

Figure 2. Cryomicroscopic image (magnification × 400) of ice grown without an antifreeze substance (negative control).

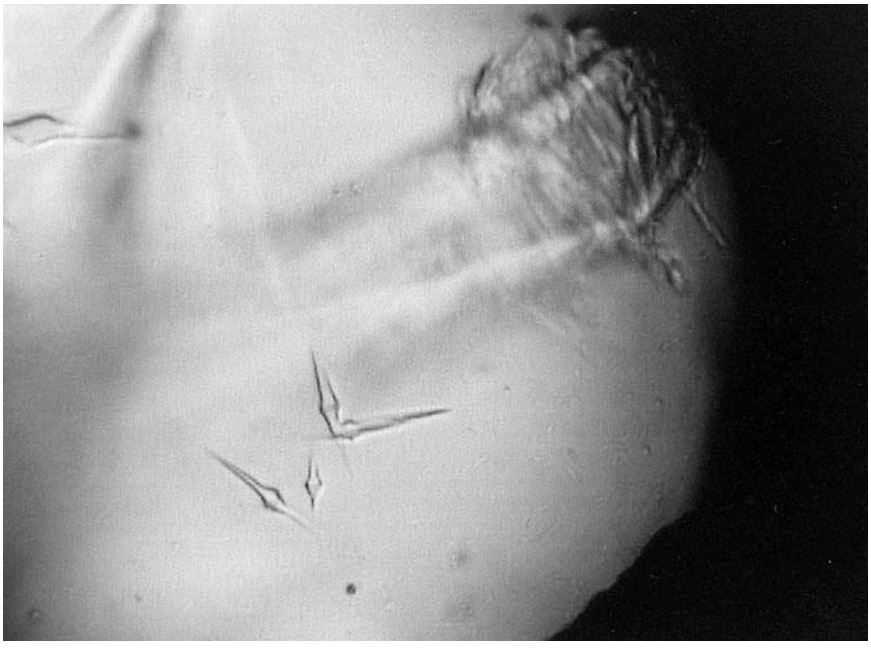

Figure 3. Cryomicroscopic image (magnification × 400) of ice grown in the presence of crude extract from the sponge *H. balfourensis* (120 mg/ml solution) just below the freezing point. Characteristic growth along the c-axis of ice crystals can be seen.

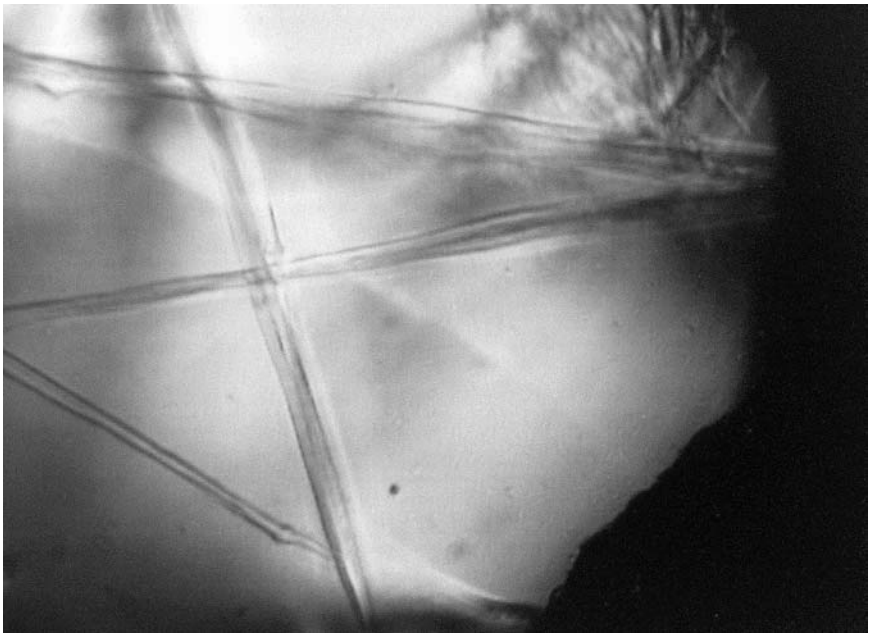

Figure 4. Cryomicroscopic image (magnification × 400) of ice grown in the presence of crude extract from the sponge *H. balfourensis* (120 mg/ml solution) below the freezing point. Further unrestricted growth along the c-axis of ice crystals leads to the total freezing of the solution.

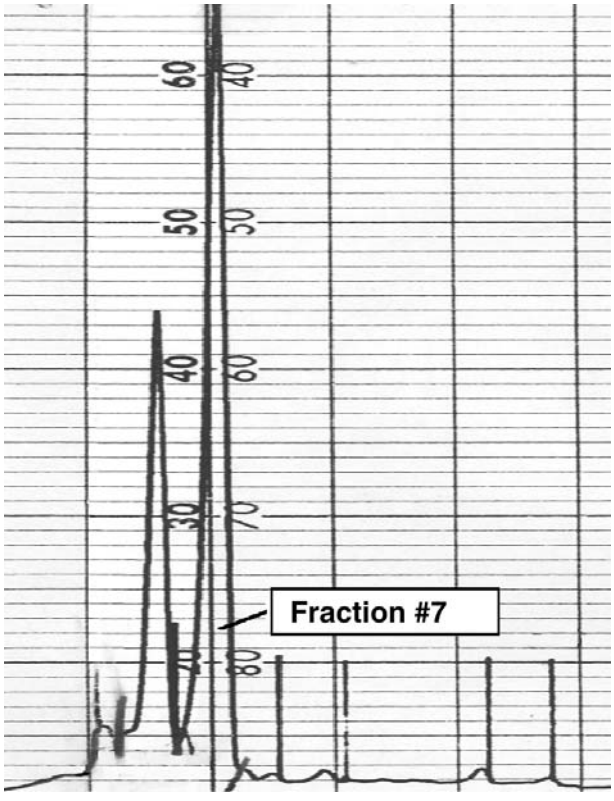

Figure 5. Reverse-phase HPLC chromatogram showing the AFP from *H. balfourensis*.

lution, ice crystal morphology shows limited growth along the c-axis (long axis of the bipyramid), as seen in figure 3. As the temperature is lowered even further, this growth continues indiscriminately (fig. 4), eventually leading to the total freezing of the solution. This type of ice crystal growth has only been observed for the true non-equilibrium AFPs [6]. To quantify the antifreeze activity; we attempted to measure the freezing-point depression of the hydrophilic extract from *H. balfourensis.* Due to a very limited amount of available material, we could not analyze the freezing-point depression dependence on the peptide concentration, which is customarily performed for this type of analysis [11]. Instead, submilligram amounts of the extract were dissolved in several microliters of a 0.1 M solution of ammonium bicarbonate and the freezing-point depression was determined following a well-established standard procedure, using the Nanoliter Osmometer [11]. The lowest freezing-point depression obtained using this method was 0.65 °C. This value is for material that is part of a crude extract or an incompletely purified mixture. The freezing-point depression values for highly purified AFPs isolated from polar invertebrates maybe considerably greater. The approximate concentration of the hydrophilic extract in this measurement was 500 mg/ml. However, the concentration of the antifreeze active peptide in this sample, according to our estimates, was probably much less that 10% per weight.

Peptide purification

The sponge *H. balfourensis* was collected from McMurdo Sound, Antarctica in December 1993. Approximately 450 g of the lyophilized sponge yielded 1 g of an aqueous extract. From the water-soluble extract, 95% of the inactive metabolites could removed by eluting isocratically under pure aqueous conditions using RP-C18 preparative HPLC. A gradient from 100% H_2O–100% CH_3CN was used to fractionate the remaining material on the column. These fractions were assayed for freezing-point depressant activity and the active fractions were found to elute with 90% H_2O/10% CH_3CN. This active peptide mixture (4 mg) was further purified with an HPLC gradient of 100% H_2O–90% H_2O/CH_3CN on an RP-C18 column. The fractions were assayed and the activity was concentrated in four fractions (100–500 µg each) which eluted between 93%–91% H_2O/CH_3CN. These fractions were assayed, and established that a single fraction was the most active and eluted with 91%–90% H_2O in CH_3CN. Figure. 4 exhibits the formation of bipyramidal crystals with a 10 µg/µl solution of the four most active AFP fractions. Final purification of the most active peptide was accomplished isocratically on RP-C18 with 95 : 5 H_2O/IPA (Fig. 5).

Mass spectrum and sequential structural data

This peptide was analyzed by MALDI/TOF mass spectrometry providing a molecular weight of 2457.32 which was in the same mass range as many previously described AFPs. A partial sequence was identified as R-Pro-His-Gln-Ser-R-Gly-Ala-Gln-Arg, with R-representing the unknown amino acid chains in the peptide. The identification was accomplished through Edman degradation amino acid sequencing at the Macromolecular Lab, Department of Biochemistry, Colorado State University.

Discussion

This is the first report of an AFP from a marine invertebrate and the first example of what may be the incorporation of atypical amino acid residues into an AFP. Marine invertebrate-derived peptides commonly include unusual amino acids and this, combined with a shortage of material, resulted in our failure to obtain a complete amino acid sequence. Efforts to isolate sufficient material to complete a full sequence and stereochemistry determination using a combination of two-dimensional nuclear magnetic resonance spectrometry and traditional se-

quencing techniques are currently underway in our laboratories. Should the detailed structure elucidation of this molecule reveal that it indeed incorporates atypical amino acids, it would represent a new class of AFP. Such class of AFP would clearly provide valuable insight into the structure-activity relationship for AFPs and would have particular significance in the cryopreservation of tissue due to the added resistance to metabolism.

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