Solution Structures, Dynamics, and Ice Growth Inhibitory Activity of Peptide Fragments Derived from an Antarctic Yeast Protein

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Abstract

Exotic functions of antifreeze proteins (AFP) and antifreeze glycopeptides (AFGP) have recently been attracted with much interest to develop them as commercial products. AFPs and AFGPs inhibit ice crystal growth by lowering the water freezing point without changing the water melting point. Our group isolated the Antarctic yeast Glaciozyma antarctica that expresses antifreeze protein to assist it in its survival mechanism at sub-zero temperatures. The protein is unique and novel, indicated by its low sequence homology compared to those of other AFPs. We explore the structure-function relationship of G. antarctica AFP using various approaches ranging from protein structure prediction, peptide design and antifreeze activity assays, nuclear magnetic resonance (NMR) studies and molecular dynamics simulation. The predicted secondary structure of G. antarctica AFP shows several α-helices, assumed to be responsible for its antifreeze activity. We designed several peptide fragments derived from the amino acid sequences of α-helical regions of the parent AFP and they also showed substantial antifreeze activities, below that of the original AFP. The relationship between peptide structure and activity was explored by NMR spectroscopy and molecular dynamics simulation. NMR results show that the antifreeze activity of the peptides correlates with their helicity and geometrical straightforwardness. Furthermore, molecular dynamics simulation also suggests that the activity of the designed peptides can be explained in terms of the structural rigidity/flexibility, i.e., the most active peptide demonstrates higher structural stability, lower flexibility than that of the other peptides with lower activities, and of lower rigidity. This report represents the first detailed report of downsizing a yeast AFP into its peptide fragments with measurable antifreeze activities.

Introduction

Sub-zero temperatures are fatal in most organisms by kinetically slowing down vital biochemical reactions, denaturing biomolecules, or rupturing cell membranes. In agreement with Darwin’s theory of natural selection, Antarctic and Arctic organisms, including plants, animals, fungi and bacteria, have developed a unique adaptive mechanism of survival by producing antifreeze proteins (AFPs) and antifreeze glycopeptides (AFGPs) [1]. Studies over several decades have revealed that AFPs and AFGPs act as biological inhibitors of ice crystal formation by depressing the water freezing point in a non-colligative manner [2,3], a process known as thermal hysteresis (TH) [4].

The first AFP was discovered in the blood of Antarctic fish over 40 years ago [5,6]. Over the past half century, more AFPs have been isolated from different organisms and are now classified into four major types: (1) type I AFPs are described as having Ala-rich protein sequences with amphipathic α-helical structures and varying sizes between 3.3 kDa and 4.5 kDa [7–10]; (2) type II AFPs are larger, globular folded proteins with multi-Cys residues bridged by disulphide bonds [11–13]; (3) type III AFPs are described as globular proteins with molecular weights of approximately 6 kDa [14–17]; and (4) type IV AFPs are α-helical in structure with multi-Glu (E) or Gln (Q) residues in their sequences [18]. In addition, type V AFPs have also been reported from insects and are known as hyperactive proteins [19].

Because of their unique function, AFPs have been proposed to be developed as for commercial products by several reports. For example, some of the current prospects regarding the use of AFPs...
include extending the expiry date of commercial food products such as frozen meat and yogurt [20], serving as a chemical adjuvant in cryosurgery [21], or supporting the preservation of tissues in transplant [22]. In addition, AFP also has promising utility in genetic engineering where it can be used to increase the cold tolerance of plants and fishes to allow their harvest in colder climatic conditions [23].

Kun and Mastai [24] hypothesized that smaller antifreeze molecules can act as useful molecular tools for zooming in on the significant portion of antifreeze proteins that contribute to their functionality. Garner and Harding [25] showed that the design of small peptides containing not less than 25 amino acid residues with antifreeze activity is possible. Interestingly, in type I AFPs, it is the α-helical structures of the protein that are responsible for the inhibition of ice crystal growth upon binding to the hydrophobic face of helices with water crystal [26]. Interestingly, not only the α-helical extent of a peptide should be judged but also the composition of the antifreeze peptide should be considered. For instance, though LL37 [27,28] completely α-helical in nature but it does not have any antifreeze activity. It has also been recently proven that reducing the helical content of AFP by shortening the peptide length results a reduction of the TH value [29]. These facts led us to focus on creating peptide segments with measurable antifreeze activity derived from native AFPs. Attributed to their simpler structure, peptides offer another advantage over large proteins because, in some cases, large protein-based antifreeze molecules do enhance and sustain cold tolerance for a long period of time, whereas in other cases, they do not [30], perhaps due to the complexity of the large protein affecting its stability. Therefore, it can be assumed that peptides may have an advantage over large proteins in terms of their application in areas of medicine, agriculture, and other commercial industries where ice crystal growth is a damaging factor [31].

An Antarctic yeast, Glaciozyma antarctica (previously known as Leucosporidium antarcticum) [32], that expresses an 18 kDa AFP with very low sequence identity to other AFPs (UniProtKB accession code D0EKL2). The sequence dissimilarity of this protein with other AFPs ignited our interest to unlock its structural and functional features, which is the main objective of this paper. The predicted secondary structure of the protein suggests that its α-helical structure is adopted by several small sequences of amino acids. Several peptide fragments were designed based on those sequential strings native to AFP that show α-helical secondary structure. The antifreeze activity of each peptide segment was evaluated by means of thermal hysteresis (TH) and ice recrystallization inhibition (IRI) assays. The peptides show a wide range of measurable antifreeze activities; thus, it has become necessary to correlate the peptide structures with their activities. The ensemble of solution phase structures of the individual antifreeze peptides was determined using NMR spectroscopy at an atomic resolution. The structural straightforwardness and helicity were observed to be the primary factors governing the antifreeze activity of a peptide. The extent of structural helicity in a peptide is proportional to its antifreeze activity.

Results

Design of antifreeze peptides

The yeast Glaciozyma antarctica is believed to survive in sub-zero temperature by employing antifreeze protein. This yeast has eight different genes that express various types of AFPs (unpublished data). At the moment, only one AFP gene has been completely characterized (UniProtKB accession code D0EKL2). The predicted secondary structure of G. antarctica AFP consisting of four α-helices and three β-strands (Figure 1A). The α-helical region of non-glycosylated native AFP has been suggested to be responsible for the inhibition of ice crystal growth by binding the hydrophobic face of the helices to the water crystal [33]. Therefore, the central hypothesis for this study is that the antifreeze activity of G. antarctica AFP relies on the α-helical segments of the protein. Small peptides are known to be able to be used as a molecular tool to mimic the biological activity of parent AFPs. As to test our hypothesis, peptide 1 with 25 amino acids in the sequence was designed based on helix-1 of the α-helical regions of G. antarctica AFP (Figure 1B). Similarly, peptides 2, 3 and 4 were also designed mimicking the other three α-helices in the protein (Figure 1B). Peptide 2, mimicking helix-2 in the protein, is composed of mainly hydrophobic residues and has been found to be insoluble in aqueous medium.

Peptides 1m and 4m were designed based on the sequence of peptides 1 and 4 by replacing Leu19 with Glu for peptide 1m and Gln19 with Lys for peptide 4m (Figure 1B). These sequence modifications were performed to assist α-helical structure formation by adding salt bridges in the peptide sequences, which possibly occur at positions i, i+4 or i, i+7 between acidic residues (Asp or Glu) and basic residues (Arg, Lys or His) [25,34]. Due to these modifications, the modified peptides are expected to form more stable α-helical structure and subsequently have higher antifreeze activity. In addition, the choice of Glu to replace Leu19 in peptide 1m was made with careful consideration as the carbon chain length of the Glu side chain is similar to that of Leu (Figure 1B). The same consideration was applied for the replacement of Gln19 with Lys in peptide 4m (Figure 1B).

Evaluations of antifreeze activity

Throughout this work, peptides were solubilized in an unbuffered solution of pH 5.0. The presence of salts in the buffered solution may affect the accuracy of the antifreeze activity assay because saline condition is known to reduce the freezing point [23]. The assay protocol applied in this study is a simple re-crystallization method that enables us to observe ice crystal shape and morphology [35–37]. Furthermore, this re-crystallization method also allows us to calculate the TH value of an antifreeze peptide in a solution.

The ice recrystallization inhibition (IRI) assay for the peptide in low and high peptide concentrations (1 mM and 10 mM) was observed by measuring the growth of ice crystals after 3 h of incubation at −6°C (Figure 2 and Figure S1) [38]. Recombinant AFP without the signal peptide region from G. antarctica expressed in E. coli was used as a positive control. A scrambled peptide and a helical peptide, LL37 were used as negative controls. Our results show that native AFP and all five peptides (peptides 1, 1m, 3, 4, and 4m) did not completely arrest the ice crystal growth but demonstrated slow to moderate growth prior to a well-defined freezing point (Figure 2). These observations indicate that the four peptides (peptides 1, 1m, 4, and 4m) at both low and high concentration can inhibit ice growth better than peptide 3, which had the lowest IRI activity as marked by the presence of larger ice crystals between the non-modified and modified peptides (Figure 2). The order of antifreeze effectiveness on the ice crystal growth was found to be peptide 1m > peptide 1 > peptide 4m > peptide 4 > peptide 3 amongst all the designed peptides. Both negative controls did not arrest the ice crystal growth (Figure 2).

In addition to lowering the freezing point of water, antifreeze peptides also cause changes in the ice crystal morphology to a
hexagonal or similar shape and can completely arrest ice crystal growth at sufficiently high concentrations [39]. The interaction of peptides with the surface of ice crystals and the resulting change in crystal morphology can be studied using simple crystallization experiments. Antifreeze molecules are known to change ice crystal morphology due to their binding to the ice crystal surface. Figure 3A represents the light microscopy image of the positive control (recombinant G. antarctica AFP) showing a star-shaped ice crystal at the freezing point. In the presence of peptide 1m and peptide 4m, a hexagonal shape was formed due to the thermal hysteresis gap, which can be better observed by shrinking without growing into the melting point and the non-equilibrium freezing point of the solution (Figure 3B and 3C). On contrary, unbuffered solution at pH 5.0 without peptide showed zero hysteresis activity with non-restriction of ice crystal growth at 0°C (Figure 3D). It is interesting to observe that both negative controls, scrambled peptide (Figure 1B) and α-helical peptide, LL37 (Figure 1B) do not show any crystal morphology (Figure 3E and 3F). The purpose of using a scrambled peptide as negative control is to prove our hypothesis that the activity of our peptides is sequence-specific; a peptide with random sequence should not have antifreeze activity, which has been successfully proven in our study. Another negative control is LL37 peptide, an antimicrobial peptide that has been shown to adopt a-helical structure [27,28]. This peptide has been taken as the negative control (recombinant G. antarctica AFP). The amino acid mutation in the designed peptide is shown in red.

In the TH assay, two basic variables are monitored, i.e., peptide concentration and ice crystal growth rate [38]. We tested the antifreeze activity of five peptides at different concentrations, i.e., 1, 2, 4, 6, 8, and 10 mM. The development of single ice crystals was observed in terms of their growth rate at 1°C/minute for slow changes in the ice crystal shape and for the calculation of the TH value. Our results indicate that the antifreeze activity increases as the peptide concentration goes from low to high (Figure 3G). All peptides show measurable hysteresis activity, which is a non-equilibrium phenomenon, by depressing the solution freezing point. Peptide 1m at 10 mM concentration shows the highest activity with a TH value of ca. 0.11°C. At this concentration, the activity of peptide 1m is higher than that of its non-modified form (peptide 1), which indicates that the replacement of Leu19 by Glu as Glu19 can stabilize the helicity of peptide 1m due to salt bridge or hydrogen bond formation between Arg15 and Glu19. Glu19 can also form a salt bridge or hydrogen bond with Arg23. In a similar fashion, peptide 4m shows higher antifreeze activity than its non-modified form (peptide 4) at 10 mM concentration, which resulted due to the replacement of Glu19 by Lys as Lys19, which was able to stabilize the α-helix by forming a hydrogen bond/salt bridge between Lys19 and Asp23. Peptide 3 shows lower activity at various concentrations compared to peptides 1m and 4m (Figure 3G). If we consider the sequence of a peptide contributing significantly to its antifreeze activity, then peptide 3 is understood to mainly consist of hydrophobic residues. No inter residual hydrogen bond/salt bridge is expected to form that can stabilize its structure or help it to interact with the ice crystal surface. This reasoning explains why this peptide shows the least affinity to ice crystals and hence was found to have the least antifreeze activity.

Figure 3G is a salient picture (TH vs. peptide concentration) for the assessment of antifreeze activity of the series of peptides in this study. In the TH analysis, the peptide antifreeze activities were as follows: peptide 1m > peptide 4m > peptide 3. Peptide 1m is the most amphipathic in nature followed by peptide 4m, and finally, peptide 3 is mainly hydrophobic in nature. It is interesting to see that the antifreeze activity of a peptide directly connects to the 50% hydrophobicity-50% hydrophilicity nature of a peptide. The amphipathic nature essentially controls its affinity to ice crystals.
The activity of peptides in this study, however, is lower than that of its parent protein. Peptide 1, which shows some potential that the activity of derived peptides can be improved to match or even better than its parent protein by applying the correct strategy in peptide structural modification.

NMR studies of antifreeze peptides

Peptide 1 and peptide 4 are not considered in this study due to their less potentiality in antifreeze activity compared to that of their mutated analogues, 1m and 4m. One-dimensional 1H NMR spectra of the three peptides (peptides 1m, 3, and 4m) show large dispersions of the amide protons (7.7–9.0 ppm), demonstrating that the peptide adopts a folded conformation at low temperatures (Figure S2). These results motivated us to determine the three-dimensional structure of the peptides using NMR spectroscopy. The complete sequence-specific proton resonance assignments of all three peptides were achieved by the analysis of two-dimensional 1H-1H NOESY and TOCSY spectra [40]. To avoid the severe signal overlap of the NOE cross peaks observed using the Bruker Avance III 500 MHz NMR instrument, the experiment was further carried out using the Bruker DRX 800 MHz NMR magnet. The large numbers of NOE cross-peaks were observed to correlate with the backbone/backbone and backbone/side chain resonances of the three peptides (Figure 4 and S3). For the sequential analysis, the NOESY data unambiguously revealed sequential (CαH to NH i to i+1), medium range (CαH to NH i to i+2, i+3 and i+4) and sequential NH/NH NOEs, a pattern which was ultimately used as a basis for the development of distance restraints to calculate the NMR-derived ensemble of three-dimensional structures (Figure 4 and Figure S3). The data set describing the medium range NOEs completely outlines that all three peptides in the solution phase primarily reside in 3helical conformations (Figures 4 and 5). For peptide 1m, it is interesting to see that, from Ser3 to Arg23, almost all of the residues are capable of showing medium range (CαH to NH i to i+2, i+3 and i+4) NOEs, indicating higher order 3-helical stability of the middle portion of the peptide (Figure 5). In a similar fashion, the residues Lys2 to Ser30 of peptide 4m show medium range (CαH to NH i to i+2, i+3 and i+4) NOEs, defining a well-conserved structure of the central part of the peptide (Figure 5). In addition, due to presence of three Pro residues, Pro8, Pro11 and Pro14, the peptide bond between the residues X-P of peptide 4m showed cis/trans isomerization resulting two different sets of resonances (Figure 4).

Interestingly, only five residues, namely Ser7, Gly9, Leu14, Phe17 and Val20 in peptide 3 were able to produce j to i+3 NOEs in the aggregation and the aggregated protein product gets precipitated. That is why the measurement of antifreeze activity becomes impossible for that protein at higher concentration. The presence of multiple helical regions in the protein structure may explain the higher efficacy of parent protein compared to its derived peptides. Other than helical structures, 3-sheet region of the native G. antarctica AFP may also contribute to its antifreeze activity. We did not test the latter hypothesis as we only focused on the role of 3-helical structure on the activity of G. antarctica AFP. Admittedly, the activity of peptides in this study is less superior to that of its parent protein; however, there are several advantages of using peptides compared to their protein parents for future commercial applications. The modular nature of peptides allows for fine-tuning of activity and specificity by replacing single amino acid residue. Since peptides consist of amino acids, they retain the advantages of protein activity and selectivity, but in the same time peptides can be produced in industrial scale with larger quantity than proteins whose production is often marred by sensitivity of biomaterials used during production. We have shown that the activity of peptide 1 can be improved by fine-tuning its structure into peptide 1m, which shows some potential that the activity of derived peptides can be improved to match or even better than its parent protein by applying the correct strategy in peptide structural modification.
two-dimensional $^1$H-$^1$H NOESY spectrum (Figures 4 and 5). The numbers of contacts are highest in the case of peptide 1m and lowest in the case of peptide 3, which definitely signifies the higher order stability of peptide 1m compared to that of peptide 3. In peptide 1m, from Leu8 to His20, the NOEs range from 12 to 30, this establishes the high order of the structural stability (Figure S4). In peptide 4m, the number of NOEs varies from 5 to 16 in a consistent way (Figure S4). However, residues ranging from Gly9 to Thr15 and Phe17 to Val of peptide 3 show a large number of NOE contacts, ranging from 7 to 18 (Figure S4). Most of the residues in peptide 3 and 4m failed to produce large number of NOE contacts, indicating higher dynamics of these peptide in the solution phase (Figure S4).

The chemical shift deviations of the CαH resonances from their random coil conformation dictate the extent of secondary conformation of a peptide or protein [31]. The upfield shift of the CαH proton confirms the α-helical conformation when a stretch of at least four contiguous residues or a stretch of three adjacent residues showed the same kind of uniform deviation. It is also worthwhile to mention that all three different peptides (peptides 1m, 3, and 4m) have α-helical conformation as, for each of them, the $\Delta$Hα values of most of the residues were found to be negative (Figure 5).

Three-dimensional structures of antifreeze peptides

The three-dimensional structures of all three antifreeze peptides were determined based on the distance constraint derived from NOE-based intensities of intra-residual contacts. Among the three, peptide 1m is found to be the most stable and have the most uniform α-helical structure. From the $^1$H-$^1$H NOESY, the total number of NOEs is found to be 179 (Table 1). Of these, 80 NOEs are found to be sequential and 63 fall into medium range distances. Similarly, for peptide 3, the value of sequential and medium range NOEs is found to be 69 and 35, respectively. For peptide 4m, 24 medium range NOEs and 78 sequential NOEs are used for the structure calculation. The dihedral angles, both $\phi$ and $\psi$, are estimated using the program TALOS.

Figure 6 displays the superposition of all backbone atoms (N, Cα and C') of the 20 lowest energy structures of peptide 1m (Figure 6A), peptide 3 (Figure 6B), and peptide 4m (Figure 6C). The three-dimensional solution structures of all three peptides (1m, 3, and 4m) are well defined with average backbone RMSD values of 0.10±0.06 Å, 0.48±0.05 Å, and 0.33±0.10 Å, respectively (Table 1). The RMSD values for the heavy atoms are found to be 0.93±0.15 Å, 0.93±0.18 Å, and 0.90±0.13 Å for peptide 1m, peptide 3, and peptide 4m, respectively (Table 1).

The solution structure of peptide 1m is characterized by an α-helical conformation spanning from Arg2 to Arg22 (Figure 6A, middle panel). However, the long α-helical structure of peptide 1m bends at the N-terminal region of the helix, around Phe5-His6-Pro7 (Figure 6A, middle panel). This bent conformation could be due to the combined structural effect induced by the presence of Ser3 and Asn4 consecutively at the N-terminus of the peptide. It is to be noted that these residues exhibit a lower propensity for α-helical structures [42]. Close inspection of the three-dimensional structure of peptide 1m clearly indicates that both termini of the peptide are rich in polar residues, whereas the central region is enriched with primarily hydrophobic and aromatic residues. The architecture of peptide 1m is tuned in such a way that the terminal ends can be the hands involved in solvating the molecule, whereas the intrinsic straightforward structural pattern of the central part is well maintained by the hydrophobic residual interactions. The additional electrostatic interactions reinforce a strong base in the structural stability of the peptide. In the triad unit Arg15-Glu19-Arg23, i to i+4 side chain/side chain electrostatic interactions govern the structural stability to a huge extent (Figure 6A, middle panel). Interestingly, Phe12-Arg15 and Phe18-Arg22 interact (π-cation type interaction) at the

![Figure 3. Ice crystal morphology and Thermal hysteresis.](image-url)
central part of the peptide, maintaining the three-dimensional framework of the peptide (Figure 6A, middle panel). The interesting composition of peptide 1m attempts to keep it in a positively charged micro-environment, which keenly helps to solubilize it in the solvent core (Figure 6A, bottom panel). This feature is manifested from the residual electrostatic potential of the peptide (Figure 6A, bottom panel). The adaptive Poisson-Boltzmann surface of charge distribution showed the peptide to be mostly positively charged all across its framework.

In comparison with the straightforward structural pattern of peptide 1m, peptide 3 is structurally less straightforward. Although the structure is α-helical, there are two twists in the long helical construction, Pro8 and Thr18, providing an “S” type shape of the peptide (Figure 6B, middle panel). The difference in the structure actually arises due to the sitting of amino acids in the wrong sequence context. The Pro8 governs the first twist assisted by the combined structural neighboring effect of Gly5, Leu6, and Ser7 (Figure 6B, middle panel). In a similar fashion, the deformity at the tail of the C-terminus is brought in by the presence of Thr18. The effect of Thr18 in the structural deformity is imposed by its neighboring residues, such as Thr15, Gly16 and Phe17 which are able to form a β-branched structure (Figure 6B, middle panel) [42]. One very interesting feature in the sequence of peptide...
Table 1. Summary of structural statistics for the 20 final structures of three antifreeze peptides.

| Distance restraints                      | Peptide 1m | Peptide 3 | Peptide 4m |
|------------------------------------------|------------|-----------|------------|
| Intra-residue (|i–j| = 0)         | 35         | 29        | 42         |
| Sequential (|i–j| = 1)         | 80         | 69        | 78         |
| Medium-range (2| |j| | =4)         | 63         | 35        | 24         |
| Long-range (|i–j| =5)         | 01         | 00        | 00         |
| Total                                     | 179        | 133       | 144        |

Angular restraints

| Deviation from mean structure (Å)         | Peptide 1m | Peptide 3 | Peptide 4m |
|------------------------------------------|------------|-----------|------------|
| ϕ                                        | 23         | 22        | 26         |
| ψ                                        | 23         | 22        | 26         |

Distance restraints from violations (≥ 0.3 Å)

| % Residues in the most favorable and additionally allowed regions | Peptide 1m | Peptide 3 | Peptide 4m |
|----------------------------------------------------------------|------------|-----------|------------|
| % Residues in the generously allowed Region                   | 100        | 100       | 100        |
| % Residues in the disallowed region                           | 0          | 0         | 0          |

Ramachandran plot for mean structure

| % Residues in the disallowed region | Peptide 1m | Peptide 3 | Peptide 4m |
|------------------------------------|------------|-----------|------------|
| % Residues in the generously allowed Region | 0          | 0         | 0          |

Table 1. Summary of structural statistics for the 20 final structures of three antifreeze peptides.

3 is the absence of cationic amino acid residues in the central part of the structure. The structure is basically stabilized by the residual hydrophobic interactions (Figure 6B, middle panel). The electrostatic potential surface demonstrates that this peptide is mainly neutral at its central part, whereas the C- and N-termini are enriched with negative and positive charges, respectively (Figure 6B, bottom panel).

The “L” shaped α-helical structure of peptide 4m is due to the presence of three Pro residues, Pro8, Pro11, and Pro14 (Figure 6C, middle panel). The positioning of three consecutive Pro residues is gapped with two amino acids, providing a unique structural element within the long helical framework. This structure creates a kink at the positions of the three Pro residues and thus makes it “L” shaped. Peptide 4m is enriched with four positively charged residues, Lys2, Arg4, Lys19, and Lys29, and three negatively charged residues, Asp6, Asp9, and Asp23. The structure is neutral at its central part, whereas the C- and N-termini are exposed to the solvent.

Infrared spectroscopy

The α-helical nature of all the three peptides comes visible clearly from the FT-IR spectrum where peptide 1m, 3, and 4m gives characteristic amide I and amide II vibrational bands. The backbone conformation as related to amide I peak is found to be coming at 1658 cm⁻¹, 1649 cm⁻¹ and 1662 cm⁻¹ for peptide 1m, 3, and 4m, respectively (Figure 7A-C) [43]. Such C = O stretching vibration peaks generally corresponds to α-helix secondary structure. In contrast, amide II band is conformationally more sensitive. Unlike peptide 1m and 4m the absence of a distinct up-rise in the amide I peak of peptide 3 might be associated to the two helical twists in the helical construction as because of Pro8 and Thr18. A similar kind of loop is appearing in the amide II region at around 1541 cm⁻¹ peak. The broadening of the peak for all the three peptides in amide II region is attributed to the extended conformations at termini mainly for peptide 1m and 4m. Such region of amide II peaks are shown in highlighted symbols. There is a prominent hump near 1548 cm⁻¹ in all the three peptides in the amide II region (1510–1580 cm⁻¹). Such infrared bands results from the N-H bending vibration (40–60%) and from C-N (18–40%) and C–C stretching vibration (10%). Comprehensively the IR spectrum is in good agreement with the NMR derived secondary structure of peptides.

CD spectroscopy

Figure 7D–F shows far UV CD spectra of peptide 1m, 3, and 4m, respectively. The far UV CD spectra of peptide 1m and 4m is characterized by two negative CD bands, one at ~206 nm and another at ~222 nm, which clearly demonstrates that both the peptides exist in alpha helical conformation. However, there is a decrease in the ellipticity values of the diagnostic CD bands of peptide 4m in comparison to peptide 1m. This observation depicts a partial loss in the helical structure of peptide 4m. In contrast, the same CD spectrum of peptide 3 consists of two negative minima, at ~209 nm and ~215 nm. The minima at
209 nm is broadened which signifies the large extent of dynamics in the alpha helical content. These fluctuations in the CD band clearly indicate a conformational switch from helical to random coil structure in a dynamic state.

Molecular dynamics simulation

The NMR-derived solution structures of the antifreeze peptides, peptides 1m, 3, and 4m, were uniformly treated in the molecular dynamics simulation at constant temperature and volume to understand their internal dynamics in the water medium. The dynamics simulation is actually a tool to unravel the atomic integrity in a molecular framework. Peptide 1m was found to be mainly α-helical and very well packed. The backbone dynamics were found to be very stable in the solvated system, and RMSD values for the backbone for peptide 1m were found to be within 1.0 Å from 500 ps to 1200 ps (Figure 8A). The side chain dynamics for peptide 1m were also found to be very much dynamic in nature, varying the RMSD values from 4 to 6 Å (Figure 8A). Pro8 and Thr18 are the key amino acid residues that affect the α-helical structure of the peptide and create two kinks in the peptide structure. These two residues majorly reinforce the bending of the helicity and generate an “S” shaped structure. The radius of gyration in RMSD for peptide 3 fluctuates widely, meaning that the molecule is very dynamic in nature and from time to time, its helicity is changed (Figure 8C). The backbone dynamics for peptide 4m were found to be moderately flexible with RMSD values ranging from 2 to 3 Å (Figure 8A). The side chain dynamics for peptide 4m were also found to be moderately dynamic in nature with RMSD values from 2.9 to 4.2 Å compared to the initial NMR-derived structure (Figure 8B). The presence of three repeats of Pro residues (Pro1, Pro11, Pro14) with an interval of two amino acids in a sequence (ProxxProxxPro) in a i to i+3 manner generates a unique structural element in peptide 4m’s structure that alters the long α-helical structure to an “L” shaped one. The radius of gyration in RMSD for peptide 4m...
fluctuates moderately with an RMSD of 13 to 14 Å (Figure 8C). From the dynamics data, it is well understood that peptide 1m is very stable in water and forms a straightforward long α-helical structure, whereas peptides 4m and 3 are not stable in the water core. The relative water interactions of all three peptides are as follows: peptide 1m: water... peptide 4m: water... peptide 3: water.

The comparisons were found to be well matched with their experimental antifreeze activity assays. The differences in electrostatic iso-surfaces as calculated by APBS are shown diagrammatically in the supplementary material (Figure S6). Figure 8D, 8E, and 8F represent the ensembles of five high resolution structures at the 0.25, 0.5, 0.75, 1.0, and 1.2 ns time scales for peptide 1m, peptide 3, and peptide 4m, respectively. The ensemble structures demonstrate that the side chains of peptide 1m are well converged, whereas those of peptides 3 and 4m are found to be more dynamic (Figure 8D–F).

Comparative study of the antifreeze activity of peptides from high resolution NMR

To determine whether there is any change in the structure of three consecutive antifreeze peptides present in normal vs super cooled water, we examined the one-dimensional 1H NMR spectra of these peptides. Thus, a series of one-dimensional 1H NMR spectra were recorded at various temperatures ranging from 25°C to −2°C (Figure 9) to understand the change of helicity with respect to temperature. The interesting feature in decreasing the temperature is that all of the resonances (aromatic, Hα, other protons and methyl protons) spanned in the 1H NMR spectra are broadened in three consecutive peptides, peptides 1m, 3, and 4m (Figure 9). This finding indicates that the three peptides become more α-helical in nature at low temperatures. For clarity, only the Hα proton region (3.6 to 4.8 ppm) in the spectrum is selected to monitor the change of helicity of the given peptides. It is worthwhile to mention that the Hα resonances are particularly sensitive indicators of protein secondary structure [44]. Broadening of the line width in this region suffices to prove qualitatively that the peptide is interacting with the ice-water surface.

The Hα region of peptide 1m becomes broader and, at the same time, shifts toward a higher field as the temperature is kept low. This finding signifies that the peptide becomes more α-helical in nature with a lowering the temperature; hence, it is understood that the peptide has more side chain interactions in water near the freezing point. However, interestingly, the broadening of the Hα peaks dictates the higher viscosity of the water molecules as the temperature drops. This selectively portrayed more interactions between the peptide and the semi-frozen water. Similarly, the Hα resonances of peptide 3 showed a broadening of the peaks as the temperature diminishes. Additionally, the Hα resonances shifted toward the higher field with a decrease in the temperature. Broadening of Hα resonances depicts a clear-cut picture of increasing interactions of the peptide with semi frozen water. Peptide 4m also demonstrated its antifreeze activity by the signature of its proton NMR footprint. Both broadening and changes of the chemical shift of Hα resonances are found; hence, it is noted that the structure of peptide 4m becomes more α-helical in nature as the temperature decreases. Peptide 4m also becomes more prone to interacting with semi frozen water molecules at

Figure 7. Secondary structure determination using fourier transform IR and CD spectroscopy. α-helical structure of peptide (A) 1m, (B) 3 and (C) 4m using fourier transform IR spectroscopy. The far UV CD spectra of peptide (D) 1m, (E) 3 and (F) 4m in water (pH 5.0) with a peptide concentration of ca. 25 μM. doi:10.1371/journal.pone.0049788.g007
lower temperatures. A comparative extent of the broadening helped us to categorize the three antifreeze peptides by the efficiency of their antifreeze activities. Broadening is more profound in peptide 1m, moderate in peptide 4m, and least for peptide 3. This conclusion from the NMR data in conjunction with other low resolution spectroscopic data matches quite well with the experimental data from the TH assay.

Discussion

To our knowledge, this work is the first report on the rational design of peptides having measurable antifreeze activity based on the structure of a yeast AFP. There are very few records characterizing the antifreeze activity of bacteria and fungi species. Previously, the psychrophilic fungi Coprinus psychromobius and Typhula ishikariensis have been reported by Hoshino et al. [45], as these species are able to produce unique AFP extra-cellularly. In another work, Gilbert et al. [46] reported the isolation of 866 bacterial isolates from an Antarctic lake, 187 of which showed antifreeze activities. A very recent study by Lee et al. [47] demonstrated that the planar β-sheet structure can be involved in the AFP-ice crystal interaction to suppress the freezing point of ice.

Garner and Harding [23] have conveyed the prospective in a report that the design of small structured peptides with antifreeze activity is possible. The helical structures of ACFs have been suggested as being responsible for the inhibition of ice crystal growth by the binding of the hydrophobic face of the helices to the ice crystal [26]. The length of the peptide also plays an important role, with at least 25 residues being required for antifreeze activity [25–29], even though Kun and Mastai [24] reported that shorter peptides (11–13 amino acids) show about 60% of the measurable antifreeze activity of their 37-residue-long parent peptides. Nevertheless, most studies on antifreeze peptides are based on at least 25-residue-long peptides [26,48–50]. All peptides used in this study are 25-residues long except peptides 4 and 4m with 30 amino acids each, with molecular sizes ranging from 2.7 to 3.1 kDa. The peptides used in this study are almost similar in size with other natural antifreeze peptides, such as winter flounder type 1 AFP with a molecular size between 3.3 and 4.5 kDa [7,14].

The protein isolated from G. antarctica is predicted to have a globular shape. The X-ray crystallographic studies failed to elucidate the architecture of the molecule, whereas NMR did not provide any clues or the sub structural insights. The main reason for not elucidating the structure using NMR and X-ray is probably due to the protein's structural elements. It has enormous susceptibility to be aggregated in low concentration in the aqueous medium.

The modifications of peptides 1 and 4 into peptides 1m and 4m by replacing Leu19 with Glu (peptide 1m) and Glu19 with Lys (peptide 4m), respectively, were performed with careful considerations of several factors that might improve the activity of an antifreeze peptide. First, it has been shown that helical structure is important for the activity of antifreeze peptides [33], and the stability of the helical structure of the peptides can be enhanced by introducing an i to i+4 lactam bridge plus the N- and C-capping residues [37] or a salt bridge placed on the hydrophilic face of the peptide [34]. Second, the presence of Glu and Leu in peptides 1m and 4m, respectively, may increase the hydrophilicity of the peptide, which could influence the antifreeze-ice interaction. Several studies have reported that the interaction between antifreeze peptides and water molecules occurs by hydrophilic interaction [25,26,31,36,39,51]. Molecular dynamics simulation applied in another study showed that the binding of the hydrophilic surface of the peptide to water molecules provides a layer of unstructured water molecules that stops ice crystals from growing further [52]. Another advantage of having more acidic or basic amino acids in the peptide sequence is to minimize the challenges during the synthesis process and also to overcome the solubility problem of the peptide in water.

To determine the structure-activity correlation for the designed antifreeze peptides, we determined the three-dimensional structure of peptides 1m, 3, and 4m in solution using high resolution NMR spectroscopy (Figure 6). It is noteworthy to mention that the curved structures of these antifreeze peptides are very common in nature, in particular antimicrobial peptides and peptides in the membrane exhibit this type of structure [53–55]. The importance of helix straightforwardness was very well demonstrated earlier, particularly in AFP type 1 [56,57]. The geometrical straightforwardness (Figure 10) helps in stabilizing the peptide on ice crystal plane. The straightforwardness of the peptide 1m derived from the high resolution NMR is quite similar to that of the X-ray crystal structures of winter flounder AFP (1WFB.pdb) (Figure 10A) and its mutated analogue (1JSB.pdb) (Figure 10B). It has been suggested that AFP type 1 undergoes an equilibrium between

Figure 8. Molecular Dynamics simulation data. (A) A plot of RMSD for backbone vs. time (1.2 ns) for peptide 1m (black), peptide 3 (red) and peptide 4m (blue). (B) A plot of RMSD for side chain vs. time (1.2 ns) for peptide 1m (black), peptide 3 (red) and peptide 4m (blue). (C) A plot of RMSD for radius of gyration vs. time (1.2 ns) for peptide 1m (black), peptide 3 (red) and peptide 4m (blue). Overlay of the snapshots of (D) peptide 1m, (E) peptide 3 and (F) peptide 4m at 250, 500, 750, 1000, and 1250 ps. These images were produced using the program Chimera. doi:10.1371/journal.pone.0049788.g008
The mystery of the mechanism of the antifreeze activity of this type of peptide is under investigation. To unravel the underlying mechanism of antifreeze activity, a significant adsorption inhibition mechanism was proposed. Antifreeze proteins are cited as binding irreversibly to ice rather than migrating with the ice-water interface. Further crystal growth is restricted to the free, unblocked surface between the adsorbed, surface-bound 'impurities' and leads to an increase in the curvature of the ice-water interface in these regions [17]. Knight et al. showed that AFP can bind with the oxygen atom of a primary plane of ice by Thr and Asp side chains, donating hydrogen bonds [58]. Wen and Laursen investigated hydrogen bonding between AFP and the ice surface and found that Thr and Asp side chains bound to the oxygen atoms of ice water on the \( \{20 - 21\} \) plane of ice in the \( <01 - 12> \) direction [59]. In contrast, the electric dipole of a polypeptide was proposed to play a substantial role in determining its antifreeze activity [60]. When an AFP is aligned on the ice surface, it attempts to make an electrical macro-dipole where the N terminal is positively charged and the C terminal is negatively charge. The potential difference due to the existence of the charge reinforces the proper alignment of the peptide on the ice surface. The assembly of AFP on the ice surface is actually dictated by the interactions of the macro-dipole of the AFP and the dipoles of the water molecules in the surface. Taken together, five main factors govern the antifreeze activity of AFPs. They play very crucial roles in inhibiting the ice crystal growth in water, as follows: (i) Ala-rich AFPs induce highly helical conformations such that they can sit very well on the ice crystal surface, (ii) macro-dipoles induced by the AFPs inhibit crystal growth, (iii) amphiphilicity of the helix, (iv) the presence of charged polar residues, and (v) torsional freedom of the side chains in the AFP facilitate hydrogen bonding to the ice surface.

Figure 11 describes the efficacy of peptides 1m, 3, and 4m attaching to the ice crystal surface. It has been already shown from the IRI assay (Figure 2) that peptide 1m inhibits ice crystal formation more efficiently compared to peptides 3 and 4m. From the NMR-derived structure and MD simulation and also CD spectroscopy, it has been clear that peptide 1m retains a straightforward, long \( \alpha \)-helical structure. Peptide 1m is found to be the least dynamic among the three peptides. It is observed that the hydrophobic residues, particularly Thr, Leu, Ser, and Ile are directly involved in the interaction with the ice crystal surface. The structure of peptide 3 is very dynamic in nature and adopts an “S” shape in solution. Whereas peptide 4m retains an \( \alpha \)-helical conformation, its dynamics fall between those of peptides 1m and 3. IRI, as well as the MD simulation of NMR-derived structures of the peptides, confirm as peptide 1m is the least dynamic and straightforward \( \alpha \)-helical in nature, it can sit on the ice surface very efficiently (Figure 11). In contrast, peptide 3 with “S” shape and varied dynamics cannot be accommodated on the ice surface for long time. The structure and dynamics of peptide 4m are found to be in the middle of those of peptides 1m and 3. The “L” shaped structure of peptide 4m does not allow it to interact with the ice surface in a significant manner, thus reducing its antifreeze activity.

Here, we used the knowledge of sequence dependence on the structure of the peptides. We observed that Pro and Gly residues remarkably change the \( \alpha \)-helical structure, reinforcing the antifreeze activity. Discarding Pro and Gly residues from the peptide sequence can probably deliver well-conserved \( \alpha \)-helical structures of the peptides, which can have significant antifreeze activity. Our hypothesis is that the antifreeze activities of a small peptide situated in different environments, alone or placed within...
a protein, is preserved. The methodology is unique. We are in the process of gaining knowledge of structure-sequence context relationships that may lead us to discover novel antifreeze peptides in near future for industrial and medical use.

Materials and Methods

All the peptides (1, 1m, 2, 3, 4, 4m, scrambled and LL37) studied in this project were purchased from GL Biochem, Shanghai, China, with 98% purity. The molecular weights of these peptides were confirmed by ESI mass spectrometry.

Secondary structure of G. antarctica antifreeze protein

The sequence of G. antarctica AFP consisting of 177 amino acids was taken from UniProtKB (accession code D0EKL2). PSI-BLAST [61,62] and CLUSTALW [63] analyses showed low percentages of sequence identity (less than 30%) between G.
*antarctica* AFP and other antifreeze proteins that rendered homology modeling based protein structure prediction impossible. The secondary structure of *G. antarctica* AFP was predicted using a mixture of hyper threading and *ab initio* computational methods [64,65]. The uniqueness of this protein was confirmed by the fold recognition/threading method based on a similarity search in the secondary structure of the query protein and known proteins in the Protein Data Bank. Three methods were used for the similarity search: mGenThreader [66], 3DPSSM [67], and FUGUE [68], and none of these three methods showed any significant fold similarity between *G. antarctica* AFP and any protein in the Protein Data Bank.

**Peptide design**

The peptides were designed based on the amino acid sequence of the helical regions in the predicted structure of *G. antarctica* AFP. Four peptides were derived from the sequence of *G. antarctica* AFP without modification (peptide 1–4), whereas two peptides (peptide 1m and 4m) were peptides 1 and 4, respectively, with residual modifications expected to form additional salt bridges and result in an enhancement of the propensity of stable helix formation. The sequence of peptide 1m was derived from peptide 1 with Leu19Glu replacement to allow the formation of i, i+4 or i, i-4 helix-stabilizing salt bridges with neighboring residues Arg15 or Arg23. Another modification was Met1 to Gln to avoid the possibility of Met oxidation, thus potentially increasing peptide stability. Peptide 4m was a modified form of peptide 4 with a Gln19Lys replacement to allow the formation of an i, i+4 salt bridge with residue Asp23. Peptide 2 was insoluble in water due to its hydrophobicity. Therefore, this peptide was dropped from all experiments. The sequences of all peptides used in this study are listed in Figure 1.

**Ice recrystallization inhibition (IRI) assay**

All peptide samples were prepared in unbuffered solution (pH 5.0) and were then diluted with 30% sucrose in a 1:1 ratio. A volume of 1.5 μL of diluted sample was sandwiched between two 15 mm diameter circular glass cover slips. The sandwiched sample was cooled to −70°C using the programming unit and then maintained at −6°C for 3 h [38]. After the incubation period, ice crystals were observed under a light microscope attached to a temperature control machine. Finally, a comparison of the recrystallization of ice was made by comparing to control samples (30% sucrose without AFP). The same protocol was applied to the positive control, which was native *G. antarctica* AFP expressed in *E. coli* system. Two peptides were used as the negative controls: (i) a scrambled peptide derived from peptide 1m with systematic scrambling to nullify the presence of any α-helix-strengthening salt bridges, and (ii) an antimicrobial peptide (LL37) that has been shown to adopt α-helical structure [27].

**Thermal hysteresis (TH) assay**

The synthesized peptides, were dissolved in an aqueous solution of pH 5.0 at different concentrations, i.e., 1, 2, 4, 6, 8 and 10 mM. The positive control was native *G. antarctica* AFP (0.1 mM). The TH assay was carried out by dropping 1 μL of peptide solution onto a glass slide and placing it at the center of the temperature controller system (THM 600S, Linkam Scientific Instruments, Surrey, UK). The peptide-containing sample was heated to 20°C and then chilled to −40°C at a rate of 100°C/min. The sample was heated again to −5°C at 100°C/min, and then the heating rate was decreased to 1°C/min until one single ice crystal was formed with a diameter of approximately 10 μm, which was appropriate to observe the modification of ice crystal shapes. The temperature was then decreased slowly (at 1°C/min) to observe the ice crystal growth. The TH value was calculated by subtracting the temperature at which a single ice crystal formed from the temperature at which the ice crystal stopped growing. Ice crystal morphology changes were observed and recorded using computer software (cellD, Olympus, Hamburg, Germany) connected to the microscope. The same protocol was applied to the positive control.

**Crystallography study**

The effects of antifreeze peptides on the crystal morphology and crystallization kinetics were determined by cryomicroscopy based on a previous method [24]. A drop (1 μL) of the peptide solutions or positive control solution was frozen on a microscope slide with an average cooling rate of 1°C/min, from room temperature (20°C/min) to −5°C/min, before the temperature was adjusted until a single ice crystal obtained at 1°C on a cooling stage (THM 600S, Linkam Scientific Instruments, Surrey, UK) under a microscope (Olympus BX51, Olympus, Hamburg, Germany).

**NMR experiments**

Two-dimensional NMR experiments were carried out on both Bruker Avance DRX spectrometer operating at 800 MHz (Bio-molecular NMR Laboratory, University of Kansas, USA) and 500 MHz (Bose Institute, Kolkata, India). The peptides were dissolved in 600 μL of 90% H2O: 10% D2O, and the pH was adjusted to 5.0. Data collection was accomplished using the time proportional phase incrementation (TPPI) method at 15°C. Two-dimensional TOCSY (mixing time, 80 ms) and Nuclear Overhauser Effect Spectroscopy (NOESY) experiments were carried out for the sequential assignment and structure calculation, respectively. Three different mixing times, 150, 200, and 250 ms, were used for the NOESY experiments. The NOESY experiment was performed with 456 increments in t1 and 2K data points in t2 using excitation sculpting for residual water suppression [69]. The spectral width was normally 12 ppm in both dimensions. After 16 dummy scans, 80 scans were recorded per t1 increment. After zero-filling in t1, 4K (Δ2) × 1K (Δt) data matrices were obtained. All 1H chemical shifts were referenced using DSS (2,2-dimethyl-2-silapentane-5-sulfonate sodium salt) as an internal standard (0 ppm). The two-dimensional NMR data were processed by TopSpin software suite (Bruker, Switzerland) and analyzed using the program SPARKY [70]. The interaction of the peptide with the solvent water was examined by recording a series of one-dimensional proton NMR spectra using Bruker Avance III 500 MHz spectrometer with decreasing the temperature from 25°C to −2°C.

**Peptide structure determination**

The NOE cross-peak intensities from the two-dimensional NOESY spectra acquired with a mixing time of 150 ms were classified qualitatively as strong, medium, and weak, which was then translated to upper bound distance limits of 2.8, 3.5, and 5.0 Å, respectively. The lower bound distance was restricted to 2.0 Å to avoid van der Waals repulsion. In addition, backbone dihedral angle (φ and ψ) restraints were derived from TALOS [71] using Hα chemical shifts of the designed peptides [72]. These predicted dihedral angle constraints were used for structure calculation with a variation of ±20° from the average values. The DYANA program (version 1.5) [73] was used for structure calculation. However, to refine the structure, several rounds of structure calculation were carried out based on the NOE violations, and the distance constraints were adjusted accordingly. A total of 100 structures were calculated, and 20 conformers with the lowest energy values were selected to present the NMR ensemble. The
 stereo chemical quality of the structures was determined using the program PROCHECK-NMR [74].

**Infrared spectroscopy**

FT-IR spectra for all three peptides, Peptide 1m, 3 and 4m were recorded on a Fourier transform infrared spectrometer FTIR-8400S (Shimadzu FTIR Spectrometer) at room temperature. The samples are analyzed in the single beam optical system using Germanium-coated KBr plate as beam splitter equipped with temperature controlled high sensitivity detector (DLATIGS detector). The powder form of peptides were mixed with KBr-powder, placed in a sample cup and then processed for spectrum measurement (diffuse reflectance method) at a resolution of 4 cm\(^{-1}\). Data sampling in the instrument was done with He-Ne laser. The diffuse reflected spectra were converted into transmission spectra for comparison purpose using Kubelka-Munk conversion spectra in the IR software [75]. The concept of Fourier self deconvolution is based on the assumption, that a spectrum of single bands is broadened in the liquid or solid state. The deconvoluted spectrum was fitted with Gaussian band shapes by an iterative curve fitting procedure.

**Circular Dichroism**

Secondary structure of peptides, 1m, 3 and 4m were determined using far UV CD method. Peptides were dissolved in water (pH 4.5), CD data were collected using a Jasco-715 spectropolarimeter. The far UV CD spectra were obtained over a range of 200–260 nm using a quartz cell of 2.0 mm path length at 10°C. For each analysis, three scans were accumulated and averaged. All CD spectra were corrected by subtraction of baseline. The corrected CD data obtained in millidegree (\(\Delta\)) were converted to molar ellipticity in deg.cm\(^2\).dmol\(^{-1}\).

**Molecular dynamics simulation of NMR-derived structures of antifreeze peptides**

The molecular dynamics simulation for all antifreeze peptide structures was carried out using CHARMM version-34b1 [76]. The standard CHARMM protein and nucleic acid residue topology and parameter files were used [77]. The initial energy minimization for all peptides was accomplished using 500 steepest descent steps followed by Adopted Basis Newton-Raphson steps up to their convergence. Each system was solvated using a TIP3P [78] water model with a 12 Å distance between the box edge and the peptides, and the peptide-water system was neutralized using counter ions as required. Water molecules closer than 2.8 Å from any atom of solute or counter ions were deleted. The solvated system was then subjected to energy-minimization, keeping the solute fixed, for 1000 Adopted Basis Newton-Raphson steps, to allow the water molecules and counter ions to orient themselves around the solute. All three systems were processed for a cycle of heating (100 K to 300 K) – constant temperature (300 K) – cooling (300 K to 100 K) for 20 ps each phase. The cycle was repeated three times. The method was adapted with a vision to achieve favourable global minima for all three peptides. Before the production run for dynamics simulation, each system was again heated up to 300 K for 20 ps and then equilibrated at constant temperature and pressure for the next 40 ps so as to provide sufficient time to distribute the velocity equally among all atoms. Hence, in this way, the above process proceeded for a time scale of 240 ps. SHAKE [79] was applied with a tolerance of 1.0e-10 to constrain the bonds involving hydrogen atoms, enabling a 2 fs integration time step to be used. Molecular dynamics simulation at a constant temperature and volume was carried out with the help of Newton’s equation of motion using the Leap Verlet algorithm. Particle Mesh Ewald (PME) [80] was used to handle electrostatic interactions. A nonbonded cutoff of 16 Å was used, and the coordinates were saved after each 1 ps. The NMR-derived structures of each peptide underwent molecular dynamics simulation of a 1.2 ns time scale. The structures at the end of the simulation, i.e., the last 300 ps, were found to be convergent within 1 to 1.5 Å rmsd deviations. The electrostatic iso-surfaces of the final structures for each peptide after finishing the molecular dynamics simulation were compared with their NMR-derived initial structures. Adaptive Poisson Boltzmann Solver [81] was used to calculate the iso-surfaces with a range of -15 K/eT to +15 K/eT.

**Theoretical model for ice crystal-peptide interaction**

The structural models of molecular interactions between ice crystal and peptides 1m, 3, and 4m were built using rigid docking computational method implemented in HEX 6.3 program [82]. The energy minimization was performed to remove the steric constraints and the lowest energy conformations were selected.

**Supporting Information**

**Figure S1** Ice re-crystallization inhibition (IRI) assay results. The growth of ice crystal in the presence of 1 mM peptide was observed for 3 h at the temperature of -6°C. (A) Unbuffered water solution (pH 5.0) without peptide, (B) peptide 1, (C) peptide 1m, (D) peptide 3, (E) peptide 4, (F) peptide 4m. The segmented bar represents 100 μm.

**Figure S2** Low field region of the one-dimensional proton NMR spectra of peptide 1m (top), peptide 3 (middle) and peptide 4m (below).

**Figure S3** Selected region of two-dimensional 1H-1H NOESY spectra of peptide 1m (left panel) and peptide 3 (right panel).

**Figure S4** Bar diagram showing the NOE contacts for each residue of peptide 1m, peptide 3, and peptide 4m.

**Figure S5** Fluorescence quenching of peptide 4m by acrylamide in water, pH 5.0. The Stern-Volmer constant (Ksv) for the peptide is 36.55 M\(^{-1}\).

**Figure S6** APBS calculation per residues of peptide 1m (A) NMR structure and (B) after 1.2 ns MD; Peptide 3 (C) NMR structure and (D) after 1.2 ns MD; Peptide 4m (E) NMR structure and (F) after 1.2 ns MD. Blue color indicates the negatively charged amino acid residue whereas the positively charged residues are marked by blue color.

**Acknowledgments**

This work is dedicated to the late Omar Pozan (Universiti Putra Malaysia), who died during the mission in Antarctica.

**Author Contributions**

Conceived and designed the experiments: SHHS RKK AB SC BAT. Performed the experiments: SHHS RKK AAA MB RNZAR AB SC BAT. Analyzed the data: AB SC BAT SHHS RKK RNZAR. Contributed reagents/materials/analysis tools: MBAR ABS AMAM NMM AB SC BAT. Wrote the paper: AB SC BAT.
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