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TAILORING PEPTIDOMIMETICS ANTIFREEZE PROTEIN FROM EXOTIC ANTARCTIC MARINE

(Pengubahsuaian Protein Antibeku Peptidomimetik daripada Hidupan Eksotik Marin Antartika)

Mohd Basyaruddin Abdul Rahman[1, 2]*, Azren AidaAsmawi[1,2], Emilia Abdulmalek[1,2], Abu Bakar Salleh[1], Bimo Ario Tejo[3]

[1]*Enzyme and Microbial Technology Research Centre (EMTech)*
[2]*Department of Chemistry, Faculty of Science*
Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
[3]*Center for Infectious Diseases Research,*
Surya University, Jalan Boulevard Gading Serpong Blok O/1,
Scientia Business Park, Tangerang 15810, Indonesia

**Corresponding author: basya@upm.edu.my*

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Abstract

Antifreeze proteins (AFPs) are synthesized by various cold-adapted organisms to enable them to survive in subzero environment. The unique role of AFPs recently attracted enormous interest to develop them as commercial products. In this work, we have studied the antifreeze activity of short helical protein fragments (peptides) instead of the entire antifreeze protein of Antarctic yeast *Glaciozyma antarctica*. Several short peptide segments were designed according to amino acid sequence of helical region of AFP-1 *G.antarctica*, which are assumed to be involved in its antifreeze activity. We have demonstrated that short peptide segments derived from yeast AFP possess antifreeze activity and result in modification of the ice crystals growth rates and habits. This strategy has enabled the preparation of short AFP with high antifreeze activity in large amount of quantities at a low cost further opens the chance of developing the commercial potentials of AFPs.

Keywords: Antarctic yeast, antifreeze protein (AFP), *Glaciozyma antarctica*, ice recrystallization inhibition (IRI)

Abstrak

Protein antibeku (AFPs) disintesis oleh pelbagai adaptasi-sejuk organisma untuk membolehkan ia bertahan di persekitaran suhu bawah takat beku. Peranan unik AFPs telah menarik minat yang besar bagi tujuan produk komersil. Di dalam kajian ini, kami telah mengkaji aktiviti protein antibeku berlingkar yang pendek (peptida) daripada protein antibeku Antartika yis *Glaciozyma antarctica*. Beberapa segmen peptida pendek direka mengikut jujukan asid amino berlingkar AFP-1 *G.antarctica*, yang dianggap terlibat dalam aktiviti antibeku. Kami telah menunjukkan bahawa segmen – segmen peptida pendek yang diperolehi daripada yis AFP mempunyai aktiviti antibeku dan menyebabkan pengubahsuaian kadar pertumbuhan dan tabiat hablur ais. Strategi ini telah membolehkan penyediaan AFP pendek dengan aktiviti antibeku yang tinggi dalam jumlah kuantiti besar pada kos yang rendah seterusnya membuka peluang untuk membangunkan potensi komersial AFPs.

Kata kunci: yis Antartika, protein antibeku (AFP), *Glaciozyma antarctica*, perencatan penghabluran ais (IRI)

Introduction

The effect of sub-zero temperatures on organisms can cause extensive damage due to the presence of ice crystals. If freezing persists, intracellular water passes into extracellular spaces and leads to cell dehydration. Despite this,

various species such as plants, fish, insect, bacteria and fungi are able to survive in the coldest and most southerly continent of the world, Antarctica, where it was once thought conditions would render it uninhabitable. DeVries [1] and his colleagues [2] were the first to reveal the mechanisms through which species of a thriving marine inhabiting the polar oceans at freezing temperatures are able to survive. Although these species have high levels of salt ions as compared to temperate species, the presence of these ions accounted for only about half of the observed water freezing point depression in their body fluid. From the analyses, it is clear that the primary defense in these organisms is attributed to a class of highly specialized biomolecules known as antifreeze proteins or AFPs [3].

Antifreeze activity has been speculated to be a result of ice recrystallization inhibition process. AFPs inhibit the growth of ice crystals by binding to ice surfaces and disrupt the normal propagation of the ice crystal by restricting the growth in the areas where AFPs cover the ice surface [4]. This leads to a curvature of the surface of the ice crystal, increasing the ratio of the surface area to volume, and resulting in a ratio which is no longer thermodynamically supportive of spontaneous ice growth. The potential for an ice crystal to grow is therefore arrested by this mechanism [5]. Most exciting is that AFPs are now leading to promising commercial applications such as improving the texture of ice-cream [6], extending the half-life of frozen dough [7] and as far as cryosurgery [8]. Another interesting development in the application of AFPs is their potential for the cryopreservation of cells, tissues and membranes from damage at low, but not freezing temperatures [9].

There are five types of AFPs which have already been identified: AFP type I, II, III and IV. Type I AFPs are known as amphipathic α-helical structure with alanine-rich sequence sized between 3.3 kDa to 9 kDa -R, type II AFPs with molecular mass ranging from 11 kDa to 24 kDa exists as large globular proteins comprising multi-cysteine residue in their structure [10-12], type III AFPs are recognized as β-sandwich globular proteins with molecular weight around 6 kDa [13-16], type IV AFPs are rich with glutamate (E) or glutamine (Q) residues in sequence form a bundle of with α-helical [17], while type V AFPs were recently reported and known as hyperactive proteins isolated from insects [18].

Instead of using an antifreeze protein, this study will focus on application of the peptide segments of AFP. Because of the simpler structure of antifreeze peptides over antifreeze proteins, it can be used as "molecular tools" to investigate each sequence in peptide structure which performs a function in the antifreeze protein [19]. The α-helical structures are believed to play a major role in inhibiting of ice crystal in Type-I AFPs, and this require the binding of hydrophobic face helices with water crystals [20]. These facts led us to design and study antifreeze activity of short peptides isolated from Antarctic yeast, *Glaciozyma Antarctica* that produced an 18 kDA of AFP I. The predicted three-dimensional structure of AFP I display several α-helical conformations in its structure expected to have biological activity as their native protein.

Materials and Methods

Peptides

Peptide 1, peptide 1m, peptide 2, peptide 3, peptide 4 and peptide 4m, were bought from GL Biochem, Shanghai, China. Each peptide was supplied HPLC purified >90% purity and was used without further purification. The molecular weight of each peptide was analyzed using ESI LC-MS.

Peptide design

Four peptides namely peptide 1, 2, 3 and 4 were derived from α-helical regions in the predicted structure of *G. antarctica* AFP-1. Further modifications were made in peptides 1 and 4 by introducing salt bridges in their structure to improve their α-helical conformation. A scrambled peptide was designed based on the systematic scrambling of peptide 1m and was used as a negative control. Their sequences are shown in Table 1. Structure of the design peptides were then predicted using online server; PEP-FOLD [21]. The basic principle behind PEP-FOLD is that the three dimensional model is built based on the concept of hidden Markov model (HMM) derived structural alphabet (SA) of 27 four amino acids letters. The SA letter profiles of each peptide sequences were first predicted and subsequently assembled with the fragments according to the sOPEP coarse-grained force field.

Table 1. List of peptide used in this work

Peptide	Sequence
1	MRSNFHPLAASFIVRCAFLHSRRFT
1m	QRSNFHPLAASFIVRCAFEHSRRFT
2	RRFTDSLFQLLSSLISLTSAATAID
3	TGNVGLSPGLSTALTGFTLVPVEDH
4	VKGRIDAPDFPSSPAILGQAATDVVAAWKS
4m	VKGRIDAPDFPSSPAILGKAATDVVAAWKS
Scrambled	QSSFHNTRFAEHFARALSPRIVCFR

Antifreeze activity determination

Ice crystals growth in 30 % sucrose solution was studied using a temperature-controlled freezing stage (Model THM 600 Linkham Scientific Instrument, UK) connected with a temperature controller unit (Model TMS 94, Linkham Scientific Instrument, UK). The freezing stage was incorporated on top of the conventional microscope stage (Olympus BX51, USA). In ice recrystallization experiment, 1.5 µL mixture of 1 mM peptide solution and 30 % sucrose (1:1 ratio) was sandwiched between two microscope glass coverslip. The sample was added inside the freezing stage and the temperature was adjusted from 20°C to – 40°C at the rate of 100°C/min. The temperature of the sample was then slowly increased from – 40°C to – 6°C at the rate 10°C/min and was maintained for 3 hours. The irruptive of ice recrystallization phase was occurred, generating a very fine of ice nuclei with different size in different sample. A reduce number and growth of ice crystals size in peptide solution after 3 hours incubation period would be indicative of the peptide efficiency in inhibiting ice crystal growth [23]. The sample microscopic images were captured at the end of incubation period at 100x magnification. The mean size of the ice crystals formed was calculated using the Cell^F software.

Results and Discussion

The ability of Antarctica yeast *Glaciozyma antarctica* to survive in freezing temperature is believed by employing antifreeze proteins. Currently, only AFP-1 gene sequence has been successfully characterized and expressed from the yeast [22]. The large antifreeze protein (AFP) from this *antarctica* yeast shows very low homology as compared to other AFP genes [23]. Interestingly, the predicted three-dimensional structure of AFP-1 sequence shows the presence of several α-helical conformations in its structure. Garner and Harding [20] put forward the idea to design small structured peptides with antifreeze properties and suggested that the α-helical structures which contained both hydrophilic and hydrophobic region are important and may responsible for the antifreeze activity [24].

Peptides 1, 2, 3 and 4 were designed representing the helices presence in the AFP-1 while peptide 1m and 4m were modified version of peptide 1 and 4. The solubility of peptides 1, 1m, 3, 4 and 4m in an unbuffered solution at pH 5.0 was good. However, peptide 2 was dropped from this study due to poor insolubility in water. Modification were made in peptides 1 and 4 sequences and produced peptides 1m and 4m to improve helical structure formation by the use of lactam bridges, which might form at positions either *i, i*+4 or *i, i*+7 between basic and acidic residues. In addition, peptide solubility in water will increase in the presence of more basic and acidic amino acids in the peptide sequence. Besides, it has been recently reported that, reducing the helicity content of AFP results a reduction of the antifreeze activity [24]. Therefore, through the amino acids sequence alterations, the fixed α-helical structures of modified peptides are predicted to be more stable and consequently exhibit higher antifreeze activity.

The three dimensional structure of peptides are predicted to exists as single, amphipathic, α-helix by PEP-FOLD. The prediction can only provide an indication of how certain peptide conforms in an aqueous solution. According to the results, peptide 1m is structurally predicted being more straightforward helix, while both peptides 3 and 4m are structurally less straightforward since there are slightly helical twist can be seen in their structure. As shown in Figure 1, the cross-section view of 3D conformation clearly shows the amino acid distribution in the peptides structure. All peptides have hydrophobic face in their structure. A clear distinct region of hydrophilic and flat

hydrophobic region may be observed in peptide 1m (Figure 2). Although the exact mechanism which attributed to the action of antifreeze by these peptides is remain elusive, it is expected that it may follow a mechanism similar to that of antifreeze activity of type-1 AFPs as found in organisms that live in freezing temperatures [25]. It is well accepted that flat-faced proteins in hydrophobic region may contribute to the binding mechanism of these proteins to ice crystals [20].

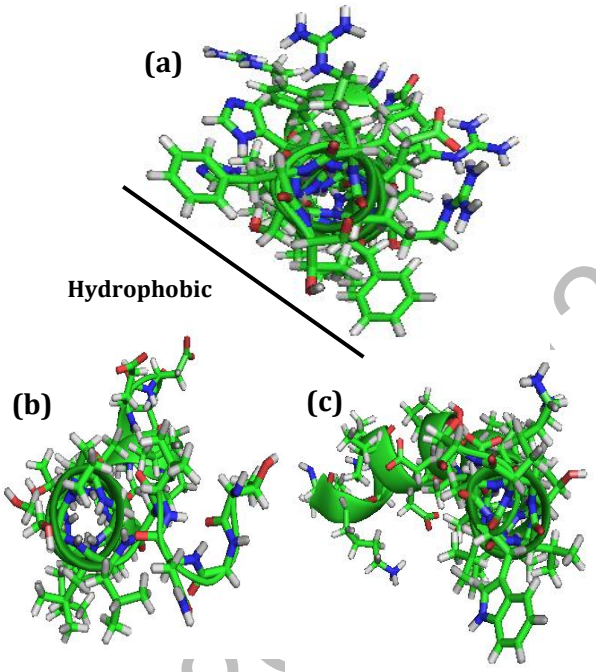

Figure 1. Cross-section view of 3D conformation of (a) peptide 1m, (b) peptide 3 and (c) peptide 4m, using PEP-FOLD and PyMOL showing hydrophobic region. Carbon: green, hydrogen: white, nitrogen: blue, oxygen: red, sulphur: yellow.

Throughout this work, to ensure on the accuracy of antifreeze activity determination, all peptide samples used were dissolved in unbuffered solution at pH 5.0, as salt presence in a solution would elevate the antifreeze activity due to its ability to reduce the freezing point [26]. In this work, to obtain a quantitative estimation of antifreeze activity, ice recrystallization inhibition (IRI) was investigated by observing the ice crystals size after 3 hours of incubation time at – 6°C at low peptides concentration. Figure 2 and Table 2 show IRI results for 1 mM of designed peptide. To investigate the ability of the peptides in ice growth inhibition, the results were compared with a negative control, namely scrambled peptide. The results obtained show that, all five designed peptides could not completely arrest the ice crystal growth. Nevertheless, by comparing IRI results of negative control, diminutive ice crystal size can be clearly seen. The designed peptides demonstrated moderate growth of ice crystals growth. The order of antifreeze activity of peptides at 1 mM concentration based on the size of ice crystal growth was found to be highest in modified peptide 1m and peptide 4m followed by peptide 1, peptide 4 and peptide 3. Interestingly, both modified peptide (peptides 1m and 4m) able to arrest ice growth better than their original peptide (peptides 1 and 4) due to the mutations of Leu19Glu and Gln19Lys in their sequence.

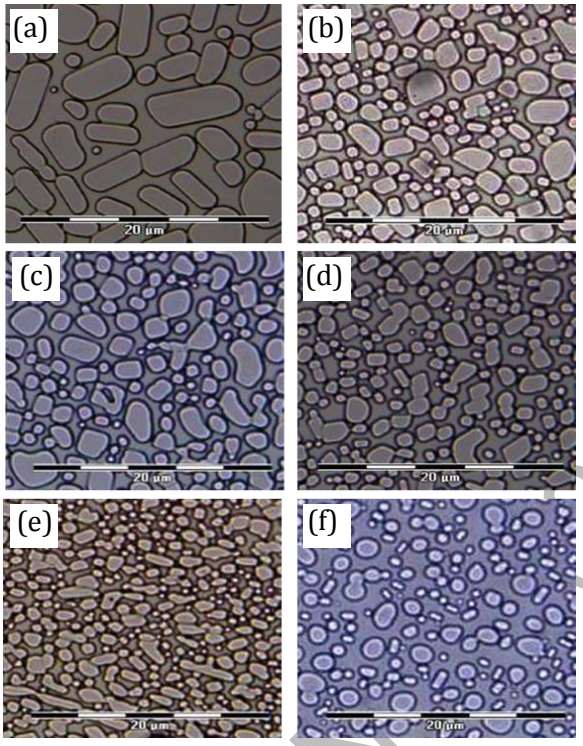

Figure 2. Ice recrystallization inhibition (IRI) of 1 mM peptide assays results. The growth of ice crystals were observed after 3 h incubation at the temperature of -6 °C. (a) scrambled peptide, (b) peptide 1, (c) peptide 3, (d) peptide 4, (e) peptide 4m, (f) peptide 1m.

Table 2. Mean of ice crystal size in 30% sucrose solution containing peptide

Sample	**Mean ice crystal diameter ± SD (µm)**
30% sucrose + 1 mM peptide 1	6.37 ± 2.61
30% sucrose + 1 mM peptide 3	9.27 ± 4.61
30% sucrose + 1 mM peptide 4	7.66 ± 2.94
30% sucrose + 1 mM peptide 1m	4.50 ± 2.42
30% sucrose + 1 mM peptide 4m	5.57 ± 2.21
30% sucrose + 1 mM scrambled peptide	17.22 ± 3.42

The results reported here appear to support the hypothesis that short helical fragments of antifreeze protein should be able to mimic the biological activity of its parent protein by modifying the ice crystals growth and habit. The short designed peptides appear to have similar adaptability which required for antifreeze activity. It was also evident that the hydrophobicity and straightforwardness of helical peptide were crucial for their ice crystal modification capabilities [3]. In this respect, modified peptides (peptide 1m and 4m) possessing a high degree of helicity due to the formation of salt bridges between acidic residues and basic residues exhibited the greatest inhibitory activity than less helical peptide.

Conclusion

As a conclusion, our results proved that a well-conserved α-helical with distinctive hydrophobic and hydrophilic region in the peptide structure, reinforcing the antifreeze activity. The antifreeze activity was found to be peptide 1m > peptide 4m > peptide 1 > peptide 4 > peptide 4. The success of our strategy of using short segments of yeast AFP may enhance potential to develop applications using the short antifreeze peptide properties via routine design and synthesis in cost-effective commercial production.

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