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DESIGN, SYNTHESIS AND ACTIVITY STUDIES OF ANTIFREEZE PEPTIDE DERIVED FROM TYPE I SHORTHORN SCULPIN

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By

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DESIGN, SYNTHESIS AND ACTIVITY STUDIES OF ANTIFREEZE PEPTIDE DERIVED FROM TYPE I SHORTHORN SCULPIN

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October 2015

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Freezing water is rapidly fatal to most organisms as it deprives biological reactions of the aqueous medium they require due to the formation of ice crystals. However, a number of structurally different classes of antifreeze proteins (AFPs) allow cold-adapted organisms to survive in a sub-zero environment by inhibiting the growth of the ice crystals; phenomenons known as Thermal Hysteresis (TH) and Ice Recrystallization Inhibition (IRI). In this work, we study on the antifreeze activity of short protein fragments (peptides) instead of the whole protein and analyse their functional properties. These peptides are characterized by several fragments isolated from different regions of type I shorthorn sculpin, *Myoxocephalus scorpius* (SS-3) AFP; namely SC-C, SC-N, SC-M and two modified peptides (SC-MM and SC-NM). The peptides were predicted by PEP-FOLD to be an amphipathic Ŭ-helix with one face entirely composed of Alanine and other hydrophobic residues, assumed to be responsible for antifreeze activity. The peptides with more than 90% purity were synthesized using solid phase peptide synthesis with immobilized Fmoc-amino acids on rink amide resins as a starting point. Their antifreeze activity and interactions with water and ice crystals are analysed using osmometry techniques. It is shown that only peptide SC-C has no IRI activity, while peptide SC-N, SC-M and both modified peptides (SC-MM and SC-NM) have an activity of about 25 ï 45% of the native protein activity. These four peptides, which exhibit hexagonal shapes of ice crystal morphology in aqueous solution, indicate the presence of moderate antifreeze activity. The relationship between peptide activity and structure is explored by CD and FTIR spectroscopy. All designed peptides show moderate helical content (24-37%) concordant with FTIR results due to the presence of amide I at 1650 ï 1658 cm^{-1} and amide II at 1545 cm^{-1}, which are referred to be the characteristic of Ŭ- helices. Peptides SC-MM, SC-M and SC-NM demonstrate the highest activity that correlates with helicity content and hydrophobicity distribution in their structure. Furthermore, these peptides have been proved to be non-cytotoxic on 3T3 cell line with IC_{50} value >100 ɛg/mL and have a positive impact on ice crystal growth in ice cream mixes. Hence, this approach can create very promising possibilities for developing commercial potential peptide with antifreeze activity at a low cost.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah sarjana sains

REKA BENTUK, SINTESIS DAN AKTIVITI PEPTIDA ANTIBEKU DARI SHORTHORN SCULPIN JENIS I

Oleh

AZREN AIDA BINTI ASMAWI

Oktober 2015

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Pembekuan air akan mempercepatkan kematian kepada kebanyakan organisma kerana ia merubah tindak balas biologi di dalam medium akueus yang mereka perlukan kesan daripada pembentukan kristal ais. Walau bagaimanapun, beberapa kelas protein antibeku (AFPs) yang berbeza dari segi struktur membenarkan organisma adaptasi-sejuk untuk terus hidup dalam persekitaran sub-sifar dengan menghalang pertumbuhan kristal ais, fenomenon yang dikenali sebagai histerisis haba (TH) dan perencatan penghabluran semula ais (IRI). Dalam kajian ini, kami mengkaji aktiviti antibeku bagi segmen protein pendek (peptida) berbanding keseluruhan protein dan menganalisis fungsi peptide-peptida tersebut. Peptida ini dicirikan kepada beberapa jenis segmen pada kawasan yang berlainan daripada protein antibeku jenis I shorthorn sculpin, *Myoxocephalus scorpius* (SS-3) iaitu SC-C, SC-N, SC-M dan dua peptida yang telah diubahsuai (SC-MM dan SC-NM). Peptida-peptida ini telah diramalkan oleh PEP-FOLD membentuk Ŭ-heliks ampifatikdengan satu muka sepenuhnya terdiri daripada Alanina dan rantaian hydrophobic yang lain, dianggap bertanggungjawab untuk aktiviti antibeku. Peptida dengan ketulelenan lebih daripada 90% telah disintesis dengan menggunakan sintesis peptida fasa pepejal dan FMOC asid amino pada resin amida digunakan sebagai titik permulaan. Aktiviti antibeku dan interaksi antara air dan ais kristal telah dianalisis dengan menggunakan teknik osmometri. Kajian menunjukkan bahawa hanya peptida SC-C tidak mempunyai aktiviti perencatan penghabluran semula ais, manakala peptida SC-N, SC-M dan kedua-dua peptida yang telah diubahsuai (SC-MM dan SC-NM) mempunyai aktiviti kira-kira 25 - 45% daripada aktiviti protein yang asal. Keempat-empat peptida ini mempamerkan kristal ais berbentuk heksagon dalam larutan akueus dan ini menunjukkan kehadiran aktiviti antibeku tahap sederhana. Hubungan antara aktiviti peptida dan strukturnya telah dikaji dengan menggunakan CD dan FTIR spektroskopi. Semua peptida yang direka menunjukan kandungan heliks yang sederhana (24 - 37%) selari dengan keputusan FTIR disebabkan oleh kehadiran amida I pada 1650-1658 cm^{-1} dan amida II pada 1545 cm^{-1} yang menunjukan ciri Ŭ- heliks. Peptida SC-MM, SC-M dan SC-NM menunjukan aktiviti tertinggi dan mempunyai hubung kait dengan kadar kandungan Ŭ-heliks dan pembahagian hidrofobisiti

dalam struktur mereka. Selain itu, peptida ini telah dibuktikan tidak toksik dengan nilai IC_{50} > 100 ε g/mL dan mampu merencat pertumbuhan kristal ais dalam campuran ais krim. Oleh itu, pendekatan ini berpotensi dalam penghasilan peptida yang mempunyai aktiviti antibeku untuk dikomersialkan pada kos yang rendah.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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This is to confirm that:

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LIST OF ABBREVIATIONS

AFGPs	Antifreeze glycoproteins
AFP	Antifreeze protein
Boc	Tertiary-butyloxycarbonyl
DCM	Dichloromethane
DIEA	*N*,*N*-Diisopropylethylamine
DMF	*N*,*N*-Dimethylformamide
DTT	1,4-Dithiothreitol
DVB	Divinylbenzene
Fmoc	9-fluorenylmethyloxycarbonyl
GS-5	Grubby Sculpin antifreeze protein isoform 5
GS-8	Grubby Sculpin antifreeze protein isoform 8
HCTU	2-(6-Chloro-1H-benotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate
HMM	Hidden Markov model
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	1-hydroxy-1H-benzotriazole
IRI	Ice recrystallization inhibition
ISPs	Ice structuring proteins
Kcal	Kilocalorie
kDa	kiloDalton
M	Molar
MTT	Microculture tetrazolium
NMP	1-methyl-2-pyrrolidinone
PDB	Protein data bank
Psi	Per square inch
PVDF	Polyvinylidine Fluoride
RPMI	Roswell Park Memorial Institute medium
SA	Structural alphabet
SPPS	Solid phase peptide synthesis
SS-3	Shorthorn Sculpin antifreeze protein isoform 3
tBu	Tertiary-butyl
TH	Thermal hysteresis
THP	Thermal hysteresis protein
TIS	Triisopropylsilane

CHAPTER 1

INTRODUCTION

Water freezing is rapidly fatal to most organisms as it divests both biological reactions of the aqueous medium they require, causes denaturation of biomolecules and damages cell membrane due to the formation of ice crystals. However, organisms found in both polar and subpolar seawaters where the □W□H□P□S□H□U□D□W□X□U□H□V□□□D□U□H□□□F□R□Q□V□L□V□W□H□Q□W□O□ point, able to survive due to a unique adaptation. Over thirthy years ago, antifreeze protein (AFP) was discovered in *Antarctic notothenioids* by biologist Eastment and DeVries (1986), first established the essential role of such proteins to the survival of marine teleosts in icy seawater. This discovery initiated a field of challenging and exciting research which examines the proteins and their mechanisms by which they prevent or reduce damage to organisms that live in sub-zero temperatures.

About two decades ago, it was assumed that AFPs were synthesized in the liver, distributed into the blood pathways and secreted at appropriate levels into extracellular fluids (Deng *et al.*, 1997; Graham *et al.*, 2013). Initially, the effects of AFPs were thought to reduce the extracellular fluids temperature and prevent fish from being affected by icy conditions. However, scientists were led to re-evaluate this theory, when two antifreeze gene families were identified in the winter flounder. The first supplies the blood with antifreeze properties and is expressed in the liver, and the second protects cells and tissues that are directly contact with ice, which is mainly expressed by the gills and skin epithelia (Low *et al.*, 1998). Research has shown that apart from providing a defence against the effect of freezing to the whole organism and its external epithelia, mammalian cell membranes can also be protected from damage from cold by AFPs. Their role is thought to assist in physiological adjustment to lower temperatures (Fletcher *et al.*, 1999).

There are five classes of AFPs found in fish, based on their sequence similarities. Types I□IV have diverse structures (Baardsnes *et al.*, 1999; Harding *et al.*, 1999; Lillford & Holt, 1994; Wathen & Jia, 2005) and Type V has been identified as glycosylated protein (AFGP) (Bouvet & Ben, 2003; Lillford & Holt, 1994). Antifreeze proteins have subsequently been identified in other life forms that are exposed to sub-zero conditions in their natural habitat including bacteria, fungi, plants, and insects (Lorv *et al.*, 2014; Middleton *et al.*, 2012; Mok *et al.*, 2010). There has been more research carried out on Type I, but studies of the more complex Types II and III have also been undertaken. Currently, far less research has been carried out on AFGPs in cold water fish compared to AFPs, primarily due to their structural complexity and the complication of sourcing samples on which detailed studies can be conducted.

Potential application of AFPs in medicine and industry has stimulated many interests, due to its unique properties that would be useful where low temperature storage is required and ice crystallization needs to be avoided. Protecting blood platelets and human organs are typical biomedical applications (Nishijima *et al.*, 2014), and in the food industry applications include improving the texture of frozen foods (Wathen & Jia, 2005; Zhang *et al.*, 2007). Associated with genetic engineering, more complex applications of AFPs include adapting plant species so they can better withstand cold (Duman & Wisniewski, 2014). Nevertheless, the exact mechanisms by which AFPs offer protection at low temperature are still not fully understood. One of the main features is the relative flatness of the ice-binding face and more hydrophobic than on other protein surfaces (Garner & Harding, 2007; Sönnichsen *et al.*, 1996). Research has identified that in some cases it is due to the presence of regular array of threonine residues (Lin *et al.*, 2011). Up till now, researchers continue to try and solve the enigma by attempting to model the effects, investigate new AFP structures, and identify indicators that will lead to an understanding of the exact mechanisms behind the effect.

To date, the most widely applied antifreeze agent in frozen food production and other consumer goods is known as propylene glycol. It is designated as □µ□J□H□Q□H□U□D□O□O□\□□ □U□H□F□R□J□Q□L□V□H□G□□ □D□V□□ □V□D□I□ Administration, which classifies it as an additive for use in food. Although no extensive tests on humans have been carried out, based on its extensive use in the food industry, propylene glycol has received its GRAS status. However, □(□Q□Y□L□U□R□Q□P□H□Q□W□□□&□D□Q□D□G□D□□□K□□be toxic for□O□D□V□V□L□ □K□D□U□P□I□X□O□¶□□□□ □3□H□U□V□R□Q□D□O□□ □F□D□U□H□□ □S□U□R□G□X been associated with incidences of skin allergy (Holyoak *et al.*, 2011). In addition, allergic symptoms in children such as asthma, rhinitis and eczema, have been reported as potential effects of propylene glycol by Choi *et al.* (2010), and glycol ethers have been implicated in Immunoglobulin E (IgE) sensitisation. These findings have spurred scientists into finding replacement of edible antifreeze agents that are derived from natural sources. Replacing the chemical compounds by naturally-occurring antifreeze proteins from nature is currently not economically viable or sustainable due to the complexity of the large protein. Therefore, the area of interest in this study will focus on the application of the peptide segments of AFPs instead of the whole protein. A major benefit of using antifreeze peptides rather than proteins is the smaller □D□Q□W□L□I□U□H□H□]□H□□□P□R□O□H□F□X□O□H□V□□□F□D□Q□□□D□F□W□□ approach brings new potential to understanding the important sequences in antifreeze protein, as low-cost peptide preparation can take place in large quantities.

The design of small, structured peptides that have an antifreeze effect is reported to be possible by Garner and Harding (2007). Inhibiting the growth of ice crystals in solution is attributed to the helical structures of AFPs. The □P□H□F□K□D□Q□L□V□P□□□L□V□□□E□\□□□W□K□H□□□E□L□Q□G□L□Q□J□□□R□I (Garner & Harding, 2007; Harding *et al.*, 1999; Sönnichsen *et al.*, 1996). Introducing a lactam bridge (*i*, *i*+4), achieves stability in the helical structure of the peptides (Garner & Harding, 2007). The effect on thermotropic properties of

a model membrane by short segments of Type I AFP are reported by Kun and his colleague (2008). In their study, the AFP segments are able to stabilize the liquid crystal (LC) phase compared to the gel phase, where the hydrophobic interaction in the model membrane core is important for the stabilization. Ice crystals can be sustained at the similar size for hours or days by an antifreeze peptide solution, which has a similar hysteresis phenomenon, in a non-colligative manner (Kristiansen & Zachariassen, 2005).

The focus of this study is to identify the activity of short segments of Type I AFP, which is isolated from the shorthorn sculpin, SS-3. The hypothesis is that the short segments of AFP protein may preserve antifreeze activity and have a substantial effect on ice crystallization and solution-freezing points. The study targets the synthesis of short sequences (<18 amino acids) and explore their antifreeze activity by structure–function correlation, based on the known antifreeze activity of the SS-3 protein. It is anticipated that the outcome of this study will provide additional knowledge on the native protein antifreeze mechanism. The objectives of this study are:

1. To design and synthesize antifreeze peptides derived from different segments of Type-I shorthorn sculpin AFP, SS-3.
2. To elucidate the secondary structure of peptides using infrared spectroscopy (FTIR) and circular dichroism (CD).
3. To determine the antifreeze activity of designed peptides by thermal hysteresis (TH) and ice recrystallization inhibition (IRI) assays.
4. To evaluate the cytotoxicity potential and antifreeze ability of peptides on ice cream.

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□=□K□D□Q□J□□□□ □:□□□□□□ □□□□ □/□D□X□U□V□H□Q□□□□ □5□-□□ □$□□□□ □□ helical peptides with KAAK motifs have antifreeze and ice crystal morphology modifying properties. *FEBS Letters*, 455, 372□376