UNIVERSITI PUTRA MALAYSIA

SYNTHESIS AND ACTIVITY EVALUATION OF SHORT ANTIFREEZE PEPTIDES OF TYPE I SHORTHORN SCULPIN ANTIFREEZE PROTEIN

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By

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Organisms living in cold environments are adapted to tolerate the freezing cold conditions and survive at subzero temperature by evolving antifreeze proteins (AFPs). AFPs bind to ice crystals and inhibit ice crystals growth and change their morphology. AFPs have function to depress the freezing point in non-colligative manner, resulting in a difference between the freezing point and the melting point of these organisms' aqueous fluids, a property known as thermal hysteresis (TH). They are also capable of inhibiting ice recrystallization in the frozen state. This phenomenon called ice recrystallization inhibition (IRI). The main aim of this study is to synthesize short antifreeze peptides from shorthorn sculpin antifreeze protein and analyze the functional properties of these peptides. The analysis of the antifreeze activity of the short peptides was studied in order to investigate the role of each segment in the overall antifreeze activity of shorthorn sculpin AFP.

The peptides were designed based on shortening the parent peptide into three fragments which represent different regions of shorthorn sculpin AFP. The synthesis of these peptides was performed following solid phase peptide synthesis method. The antifreeze activity of each peptide was determined experimentally by performing TH and IRI activity assay. The secondary structure of the short antifreeze peptides was determined by circular dichroism spectroscopy. Using solid phase peptide synthesis method yielded peptides with at least 68% purity. Antifreeze activity assay revealed that the three peptide fragments were devoid of TH activity. Peptides SC1 and SC2 demonstrated moderate IRI and ice structuring morphology activities, while SC3 showed no activity. Secondary structure analysis indicated that the helical content was reduced with increasing temperature. The results indicated that there is no correlation between the helical content and antifreeze activity of the short peptides. As a conclusion, synthesizing short peptide fragments based on shortening shorthorn sculpin antifreeze protein (SS3) would results in short peptide segments that behave as ice modifiers rather than efficient ice inhibitors or true antifreeze peptides.

Abstrak tesis yang dikemukakan kepada Senat of Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

SINTESIS DAN PENILAIAN AKTIVITI PEPTIDA PENDEK PROTEIN ANTIBEKU JENIS 1 DARIPADA SHORTHORN SCULPIN

Oleh

AHMAD OMAR ABDELAZIM SULIMAN WARRAD

Disember 2013

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Organisma jenis hidup dalam persekitaran sejuk dapat menyesuaikan diri dalam keadaan sejukbeku dan berupaya hidup pada suhu bawah sifar dengan merembeskan jenis protein antibeku (AFPs). AFPs mengikat pada kristal ais dan merencat pertumbuhan serta mengubah morfologinya. AFPs berfungsi dengan menurunkan takat beku ais secara tak koligatif, dan menyebabkan perbezaan antara takat beku dan takat lebur akues dalam organisma tersebut. Ciri-ciri ini dikenali sabagai histerisis terma (TH). AFPs juga berupaya untuk merencat penghabluran semula ais dalam keadaan beku. Fenomena ini dikenali sebagai perencatan penghabluran semula ais (IRI). Tujuan utama penyelidikan ini adalah untuk sintesis peptide pendek daripada protein antibeku shorthorn sculpin dan mengkaji fungsi peptide tersebut. Penilaian aktiviti antibeku peptida pendek dikaji untuk menentukan peranan setiap segmen dalam keseluruhan aktiviti antibeku shorthorn sculpin AFP.

Peptida-peptida telah direka berdasarkan pemendekan peptida induk kepada tiga serpihan yang merangkumi kawasan shorthorn AFP sculpin yang berbeza. Sintesis peptida telah dilakukan berdasarkan kaedah sintesis fasa pepejal peptida. Penentuan aktiviti setiap peptide protein antibeku telah dilakukan berdasarkan analisis TH dan ujian aktiviti IRI. Struktur sekundar peptida antibeku pendek telah ditentukan oleh spektroskopi dikroisme bulat. Penggunaan kaedah sintesis fasa pepejal peptida menghasilkan peptida dengan sekurang-kurangnya 68% tulen. Ujian antibeku mendedahkan bahawa tiga serpihan peptida adalah tanpa aktiviti TH. Peptida SC1 dan SC2 menunjukkan IRI sederhana dan morfologi aktiviti penstrukturan ais, manakala SC3 menunjukkan tiada aktiviti. Analisis struktur sekunder menunjukkan bahawa kandungan heliks berkurangan dengan peningkatkan suhu. Keputusan juga menjelaskan bahawa tidak ada korelasi antara kandungan heliks dan aktiviti antibeku peptida pendek. Kesimpulannya, sintesis serpihan peptida pendek berdasarkan pemendekan protein antibeku shorthorn sculpin (SS3) akan menyebabkan segmen peptida pendek tersebut untuk bertindak sebagai pengubah ais tetapi bukannya sebagai perencat ais efisien ataupun antibeku peptida yang sebenar.

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I certify that a Thesis Examination Committee has met on 17 December 2013 to conduct the final examination of Ahmad (Omar Abdelazim) Suliman Warrad on his thesis entitled "Synthesis and Activity Evaluation of Short Antifreeze Peptides of Type I Shorthorn Sculpin Antifreeze Protein" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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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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DECLARATION

Declaration by graduate student

I hereby confirm that:

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LIST OF ABBREVIATIONS

3D	Three-dimensional
4Ac-rSS3	Acetylated recombinant version of shorthorn sculpin antifreeze protein isoform 3
A.A	Amino acid
AFGPs	Antifreeze glycoproteins
AFP	Antifreeze protein
AFP9	Antifreeze protein from winter flounder isoform 9
AFPs	Antifreeze proteins
AP	Alaskan plaice
Boc	Tertiary-butyloxycarbonyl
CE	Capillary electrophoresis
ddH_2O	Deionized distilled water
DIC	Diisopropylcarbodiimides
DIEA	*N,N*-Diisopropylethylamine
DMF	*N,N*-Dimethylformamide
DTT	1,4-Dithiothreitol
DVB	Divinylbenzen
EPL	Expressed protein ligation
ESI	Electrospray ionization
Fmoc	9-fluorenylmethyloxycarbonyl
GS5	Grubby sculpin antifreeze protein isoform 5
GS8	Grubby sculpin antifreeze protein isoform 8
HCTU	2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate
HF	Hydrogen fluoride
HMM	Hidden Markov model

HOAt	1-hydroxy-7-azabenzotriazole
HOBt	1-hydroxy-1H-benzotriazole
HPLC6	Antifreeze protein from winter flounder isoform 6
HPLC8	Antifreeze protein from winter flounder isoform 8
hyp-type I	Hyperactive type I antifreeze protein from winter flounder
IRI	Ice Recrystallization Inhibition
ISPs	Ice structuring proteins
Kcal	Kilocalorie
KCN	Potassium cyanide
kDa	kiloDalton
NCL	Native chemical ligation
NH4PA	Ammonium polyacrylate
NMP	1-methyl-2-pyrrolidinone
^{o}C	Degree Celsius
Pbf	Pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl
PDB	Protein Data Bank
Psi	Per square inch
PVDF	Polyvinylidine Fluoride
Rpm	Rotation per minute
rSS3	Recombinant version of shorthorn sculpin antifreeze protein isoform 3
SA	Structural alphabet
SPPS	Solid Phase Peptide Synthesis
SS3	Shorthorn sculpin antifreeze protein isoform 3
SS8	Shorthorn sculpin antifreeze protein isoform 8
sSS3	Synthetic version of shorthorn sculpin antifreeze protein isoform 3

tBu	Tertiary-butyl
TFA	Trifluoroacetic acid
TFE	Trifluoroethanol
TH	Thermal hysteresis
TIS	Triisopropylsilane
trityl	Triphenylmethyl
Trt	Trityl
YTAFP	Yellow tail flounder antifreeze protein
ρ	Density

CHAPTER 1

INTRODUCTION

Freezing is a fatal problem for organisms living in low-temperature climates as it divests biological reactions of the aqueous medium they need, due to the formation of ice crystals, which can enter their circulation and seeding further ice growth resulting in a significant damage to their cells and tissue (Gibson, 2010). Despite that, certain fish species were able to survive in polar and subpolar oceans even though the temperature drops down to -1.9^{o}C, which it is below the freezing point of their body fluids (-0.8^{o}C) (DeVries, 1983).

As an adaptation to this environmental stress, polar fish species have elevated levels of dissolved solutes such as sodium chloride in their blood plasma relative to temperate species, leading to a reduction in the freezing point. However, the analysis of the blood of Antarctic fish showed that colligative effect of dissolved salts and sugars accounted only for 40 – 50% of the freezing point depression of the blood (Duman and DeVries, 1975). The remaining 50 – 60% were found to be due to the presence of Antifreeze Proteins (AFPs), which have the ability to depress the freezing point of body fluids in a noncolligative manner, allowing them to survive in sea water at subzero temperature where they should freeze and die (Cheng, 1998). AFPs help freeze-tolerant organisms by unique antifreeze activities. Thermal hysteresis (TH) and ice recrystallization inhibition (IRI) are the mostly measured activities. Other activities are ice crystal's shape control and interaction with ice nucleators.

The initial AFP was isolated from the blood plasma of *Antarctic notothenioidae* (DeVries and Wohlschlag, 1969). After its first discovery in fish, AFPs has been reported in vertebrates, invertebrates, plants, bacteria, and fungi (Venketesh and Dayananda, 2008). AFPs from fish species, have been classified into five types based on its sequence and structural characteristics, AFP type I, II, III, IV and antifreeze glycoproteins (AFGPs). As a result of their unique properties, AFPs can be applied in different areas such as frozen-food technology to extend the expiry date of frozen products (Griffith and Ewart, 1995), enhancing the freeze-resistance in animals and plants to increase the yield of the products (Breton *et al.,* 2000), improving the cryopreservation of cells, tissues, and organs (Fletcher *et al.,* 2001) and in cryosurgery for cancer treatment (Koushafar *et al.,* 1997).

AFPs are obtainable in good quantities and purity from natural sources via recombination technique. However, the isolation and purification processes are costly and labor-intensive. As a promising alternative method, modern chemical protein synthesis technologies represent an effective alternative research tool. By chemical synthesis; various proteins have been synthesized in the same native functional form with high purity.

Recently, short antifreeze peptide segments have been introduced as a promising approach to study the activity of AFPs. Applying short segments of AFPs can be helpful in exploring the mechanism of action for the natural AFPs. They could be considered as "molecular tools" or "nano-indicators" which could reveal which part of the protein is most responsible for the activity and help in understanding the action

of different AFPs functions (Kun *et al.,* 2008). Small antifreeze peptide may play a significant role in increasing the commercial potential of AFPs in many applications by reducing the cost of synthesizing and purifying the entire native AFPs.

Most of the short peptide segments studies have been carried out on type I antifreeze protein from winter flounder because it is characterized by a high helical content, and have unique 11-residue repeat sequences (Kun and Mastai, 2007). However, there are no data available regarding the activity of short peptide segments of type I AFP from shorthorn sculpin.

1.1 Problem Statement

Unlike other type I AFPs, shorthorn sculpin AFP is less studied because of the low antifreeze activity, which could be due to the presence of a unique N-terminal region with unknown function to the mechanism of action and a hydrophobic C-terminal region with high content of Ala residues. In addition, there are no data available regarding the antifreeze activity of short peptide segments derived from shorthorn sculpin AFP. Therefore, shorthorn sculpin AFP was selected in this study to investigate the activity of short peptide segments and the role of each segment in the overall antifreeze activity of the native peptide.

1.2 Research Objectives

The main aim of this study is to synthesize short antifreeze peptides from shorthorn sculpin antifreeze protein and analyze the functional properties of these peptides. Therefore, the specific objectives of this study were:

1. To synthesize short antifreeze peptides derived from type I shorthorn sculpin antifreeze protein.
2. To study the antifreeze activity of the peptide segments by thermal hysteresis, ice recrystallisation inhibition and ice crystal morphology assays.
3. To investigate the role of each peptide segment on the overall antifreeze activity of the native peptide.
4. To predict the structure of the peptides using circular dichroism.

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