UNIVERSITI PUTRA MALAYSIA

STRUCTURE AND FUNCTION OF NOVEL ANTIFREEZE PEPTIDES DERIVED FROM GLACIOZYMA ANTARCTICA ANTIFREEZE PROTEIN-1

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By

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STRUCTURE AND FUNCTION OF NOVEL ANTIFREEZE PEPTIDES DERIVED FROM *GLACIOZYMA ANTARCTICA* ANTIFREEZE PROTEIN-1

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July 2013

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Organisms living in cold environment produce antifreeze proteins (AFPs) which exhibit special functions as a result of cold adaptation. AFP is currently being identified in many organisms such as bacteria, plants, fish, and fungi that are exposed to freezing stress. This study aimed to create novel antifreeze peptides based on the three-dimensional structure of *Glaciozyma antarctica* antifreeze protein-1 (AFP-1). Computational prediction on the structure of AFP-1 suggests that the helical segments of this protein are responsible for antifreeze activity. Six peptides derived from the sequence of *G. antarctica* have been synthesized. The peptides show measurable antifreeze activity as quantitatively measured by thermal hysteresis (TH) assay and qualitatively by ice recrystallization inhibition (IRI) assay. Structure determination of antifreeze peptides was carried out based on spectroscopic data

obtained by using one dimensional and two dimensional ^{1}H-NMR (800 MHz) in elucidating the structures of obtained peptides. All antifreeze peptides showed increase of thermal hysteresis value which is relative to the increase of antifreeze peptides concentration until the saturation point of solution. Peptide 1m recored the highest antifreeze activity with TH value 0.097 ± 0.004 ^{o}C, almost similar to the parent protein AFP-1 (0.1^{o}C in concentration 0.1mM). Analysis of relationship between the peptide NMR structure and its activity showed that the peptides form alpha helical structure and the extent of peptide helicity greatly influences the activity of antifreeze peptides derived from *G. antarctica* AFP-1 segments.

Keywords: AFP, antifreeze peptides, freezing tolerance, ice morphology, ice crystal, recrystallization inhibition, thermal hysteresis, alpha helical structure, NMR

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

STRUKTUR DAN FUNGSI PEPTIDA ANTIBEKU BAHARU BERASASKAN PROTEIN ANTIBEKU-1 *GLACIOZYMA ANTARCTICA*

Oleh

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Organisma yang berhabitat dalam persekitaran iklim sejuk menghasilkan protein antibeku (AFP) yang berfungsi memberikan adaptasi organisma itu kepada persekitaran sejuk. Kini, AFP sudah dapat dikenal pasti pada pelbagai organisma iaitu bakteria, tumbuhan, ikan, dan kulat yang terdedah pada persekitaran beku lampau. Kajian ini bertujuan mencipta peptide baharu berasaskan struktur tiga dimensi protein antibeku-1 *Glaciozyma antarctica* (AFP-1). Struktur yang diperoleh melalui kaedah ramalan komputer mencadangkan bahawa segmen helikal pada AFP-1 berperanan untuk menghasilkan aktiviti antibeku. Enam peptida daripada jujukan AFP *G. antarctica* digunakan dalam kajian ini. Peptida yang dikehendaki menunjukkan aktiviti antibeku berdasarkan data kuantitatif histeresis suhu (TH) dan data kualitatif perencatan pembentukan kristal ais (IRI). Penentuan struktur peptida anti-beku diperoleh dengan menggunakan ^{1}H-NMR (800 MHz) satu dimensi dan dua dimensi. Semua peptida anti-beku menunjukkan peningkatan aktiviti histeresis suhu selaras dengan peningkatan konsentrasi larutan peptida anti-beku sehingga ke titik

tepu. Peptida 1m merekodkan aktiviti anti-beku paling tinggi antara semua sampel dengan nilai histeresis suhu 0.097 ± 0.004 ^{o}C yang mana hampir sama dengan protein AFP-1 (0.1 ^{o}C dalam kepekatan larutan 0.1 mM). Analisis perkaitan antara struktur NMR peptida yang dikehendaki dengan aktiviti anti-bekunya menunjukkan bahawa peptida antibeku memerlukan struktur alfa-helikal dan takat helikal tersebut amat mempengaruhi aktiviti peptida antibeku yang dicipta berasaskan daripada struktur *G. antarctica* AFP-1.

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I certify that an Examination Committee has met on.....to conduct the final examination of Syed Hussinien Hielmie Shah bin Said Amin Shah on his thesis entitled "**Structural and Functional Studies of Novel Antifreeze Peptides Derived From *Glaciozyma Antarctica* Antifreeze Protein-1**" in accordance with the Universities and University College 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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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

/

SYED HUSSINIEN HIELMIE SHAH BIN SAID AMIN SHAH

Date: 4 July 2013

TABLE OF CONTENTS

		Page
ABSTRACT		ii
ABSTRAK		iv
ACKNOWLEDGEMENTS		vi
APPROVAL		vii
DECLARATION		ix
LIST OF TABLES		xii
LIST OF FIGURES		xiii
LIST OF APPENDICES		xiv
LIST OF ABBREVIATIONS		xv
CHAPTER		
1.	**INTRODUCTION**	1
	1.1. Research Objectives	3
2.	**LITERATURE REVIEW**	4
	2.1 Antifreeze Proteins (AFPs)	4
	2.2 AFPs Mechanism of Action	6
	2.3. Type I AFP	9
	2.4 AFPs from Fungi	9
	2.5 Potential Application of AFPs	11
	2.6 Antifreeze Peptides	12
3.	**MATERIALS AND METHODS**	15
	3.1 Materials	15
	3.2 Methods	17
	3.2.1 Peptide Synthesis and Analysis	17
	3.2.2 Ice-recrystallization Inhibition (IRI) Assay	17
	3.2.3 Thermal Hysteresis (TH) Assay	18
	3.2.4 Ice Morphology Study	19
	3.2.5 Antifreeze Peptide Structure Determination	20
4.	**RESULTS AND DISCUSSION**	22
	4.1 Peptide Structure Predictions and Modifications	22
	4.2 Antifreeze Activity	26
	4.3 NMR Interpretation of Antifreeze Peptides Structure from *G. antarctica* AFP Segments	37
	4.4 Three Dimensional Structures of Antifreeze Peptides	43
5.	**CONCLUSION**	49

BIBLIOGRAPHY 51
APPENDICES 56
BIODATA OF STUDENT 62
LIST OF PUBLICATIONS 63

LIST OF TABLES

Table		Page
4.1	Sequences of designed peptides used in this study	27
4.2	Characteristics of Gly, Ala and Cys in comparison to Pro.	47

LIST OF FIGURES

Figure		Page
2.1	Ice crystal stasis in the presence of winter flounder AFP	4
2.2	Proposed mechanisms of growth inhibition based on Kelvin effect	7
2.3	Interaction of AFP with ice molecules and water molecules in AFP type III.	8
4.1	(A) The model of AFP-1 from *G. antarctica* (B) Four (4) alpha-helical structures in the sequences which involve in AFP-water interactions.	24
4.2	IRI results of negative and positive controls	30
4.3	Ice recrystallization inhibition (IRI) assay results	30
4.4	Salient picture (TH vs peptide concentration) for the assessment of antifreeze activity of the series of peptides concentrations	33
4.5	Change of ice crystal morphology of positive control solution	33
4.6	Ice crystal stasis in the presence of antifreeze peptides from peptide 1m and 4m	34
4.7	Ice morphology of negative control and peptide 3	35
4.8	One-dimensional NMR spectrum of three different peptides at 10 ^{o}C.	38
4.9	Superimposed TOCSY and NOESY peaks of the peptides and their assignments	40
4.10	NOE cross-peaks for peptides 1m (A); peptide 3 (B); peptide 4m (C)	41
4.11	NOE contacts (number of NOEs vs residue)	42
4.12	Model structures of antifreeze peptides: (A) Peptide 1m; (B) Peptide 3; (C) Peptide 4m.	45

LIST OF APPENDICES

Appendix		Page
1	The instruments used for TH assays are set of Microscope Olympus BX51, with temperature controller	56
2	Analytical HPLC chromatogram of peptide 1	57
3	Analytical HPLC and MS profile of peptide 1m	57
4	MS profile of peptide 2	58
5	Analytical HPLC chromatogram of peptide 3	58
6	MS profile of peptide 3	59
7	Analytical HPLC chromatogram of peptide 4	59
8	MS profile of peptide 4	60
9	Analytical HPLC chromatogram of peptide 4m	60
10	MS profile of peptide 4m	61

LIST OF ABBREVIATIONS

AFP	Antifreeze protein
G.antarctica	*Glaciozyma antarctica*
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhause Effect Spectroscopy
TOCSY	Total Correlation Spectroscopy
^{1}H-NMR	Proton nuclear magnetic resonance
Glu	Glutamic acid
Gln	Glutamine
FDA	U.S Food and Drug Administration
GRAS	Generally recognized as safe
PG	Propylene Glycol
IgE	Immuglibilin E
TH	Thermal hysteresis
Thr	Thrionine
Asp	Aspartic acid
Leu	Leucine
Ser	Serine
Val	Valine
Ala	Alanine
E. coli	*Escherichia coli*
G. antarctica	*Glaciozyma antarctica*
IRI	Ice recrystallization inhibition

HOHAHA	Homonuclear Hartmann-Hahn
NOESY	Nuclear Overhauser enhancement and exchange spectroscopy
TPPI	Time-proportional phase incrementation
Arg	Arginine
Lys	Lysine
His	Histidine
Met	Methionine
Pro	Proline

CHAPTER 1

INTRODUCTION

Freezing at sub-zero temperature always causes damage to cellular organisms. The freezing phenomenon occurred by inhibiting biological and chemical reaction in their natural medium. The freezing also affect normal concentration of elements in plasma, denatures organisms' biomolecules and ruptures cell membranes (Harding, 1999). However, several species of fish, plants, arthropods, fungi, and bacteria in Antarctic and Artic poles are able to survive in temperatures below freezing point (Duman and Olsen, 1993). These organisms produce antifreeze proteins which play the role of inhibitor toward the ice crystal formation by depressing the freezing point.

The first antifreeze protein (AFP) was discovered in the blood of Antarctic fish over 40 years ago (Scholander *et al.*, 1957; DeVries, 1971). To date, there are five types of AFPs classified based on their metal dependencies, molecular sizes, secondary and tertiary structures, and ice-binding plane (Davies and Sykes, 1997). Type I AFPs are described as alanine-rich protein sequence with α-helical structure and sized between 3.3 kD and 4.5 kD (Duman and Devries, 1974; Duman and deVries, 1976; Hew *et al.*, 1985). Type II AFPs are described as globular proteins containing multi-cysteine residue with five disulfide bonds (Ng *et al.*, 1986; Slaughter *et al.*, 1981; Ng and Hew, 1992). Meanwhile, type III antifreeze proteins are described as globular proteins with molecular weight around 6 kD (Jia *et al.*, 1995; DeLuca *et al.*, 1996; Sonnichsen *et al.*, 1996). Type IV AFPs have α-helical proteins structure with multi glutamate (Glu) or glutamine (Gln) residues in sequence (Deng *et al.*, 1997). The last

one is type V AFPs, which were discovered from insects and known as hyperactive proteins from its source (Liou *et al.*, 2000).

Because of their unique function, AFPs have been proposed to be developed as for commercial products. For example, some of the current prospects regarding the use of AFPs include (Griffith and Ewart, 1995), artificial rain and surgical preservation (Arav *et al.*, 1994; Payne and Wilson, 1994; Chao, *et al.*, 1996; Kun and Mastai, 2007). In more advance application, AFP could be applied to increase cold tolerance of plant through genetic engineering (Fan *et al*., 2002). In food industry, propylene glycol (PG) is the most common antifreeze agent used in food and other consumer products nowadays. PG is classified as "*generally recognized as safe*" (GRAS) by FDA. Propylene glycol (PG) was never really tested directly in human, but GRAS status was given based on common use of PG in foods. There are increasing concerns about propylene glycol toxicity since it has been used as common household chemicals. Choi *et al.* (2010) suggested that propylene glycol and glycol ethers (PGEs) may induce allergic symptoms, asthma, rhinitis and eczema in children, as well as IgE sensitization respectively. Therefore, a new edible antifreeze agent derived from natural sources is needed.

It has been reported that several types of antifreeze proteins exist in *Glaciozyma antarctica* (Hashim *et al*., 2013), This study has been focused on the synthesis of the peptide segments derived from the sequence of *G. antarctica* AFP with measureable antifreeze activity. From practical aspects point of view, peptide has been known as a better alternative due to less adverse reaction in body than proteins. Other obvious advantage of using antifreeze peptides over the use of antifreeze protein is that the smaller antifreeze molecules can act as "molecular tools" to study

the most important sequences, which play role in the antifreeze proteins (Kun and Mastai, 2007).

Specifically, the central hypothesis for this study is that the antifreeze activity of *G. antarctica* AFP relies on the helical regions of the protein. In this study, several novel antifreeze peptides have been synthesized based on helical part of *G. antarctica*. This approach enables us to study the ice-binding mechanism of antifreeze proteins.

1.1 Research Objectives

The main objective of this work is to experimentally identify the structure and functions of novel antifreeze peptide derived from *G. antarctica* AFP. Therefore, this study embarks on the following objectives:

1. to design and synthesize novel antifreeze peptides derived from the helical regions of AFP that may have ice inhibiting activity;
2. to study antifreeze activity of *G. antarctica* AFP-derived peptides sequences and enhance the antifreeze activity by amino acid replacements; and
3. to determine the structure of the antifreeze peptides by nuclear magnetic resonance techniques.

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