UNIVERSITI PUTRA MALAYSIA

DIRECTED EVOLUTION OF AMS8 LIPASE TOWARDS ENHANCED ACTIVITY AND STABILITY AT LOW TEMPERATURE

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DIRECTED EVOLUTION OF AMS8 LIPASE TOWARDS ENHANCED ACTIVITY AND STABILITY AT LOW TEMPERATURE

By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

DIRECTED EVOLUTION OF AMS8 LIPASE TOWARDS ENHANCED ACTIVITY AND STABILITY AT LOW TEMPERATURE

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February 2017

Chairman : Mohd Shukuri Mohamad Ali, PhD
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Cold active lipases have huge biotechnological prospects due to their high catalytic activity at low temperature. Generally, cold active lipases demonstrates high specific activity at low temperature and rapidly denatured in moderate range of temperature due to their thermosensitive nature. However, the factor that contributes to this cold adaptation properties are still vague. AMS8 lipase is a Family 1.3 lipase produced by Antarctic *Pseudomonas* sp exhibits minimum activity at low temperature. The aim of this study is to evolve AMS8 lipase with enhanced activity and stability at low temperature and to study the effect of the amino acid substitution on the biochemical features of this lipase. The mutant library of AMS8 lipase was generated by error-prone PCR. Mutant M15 lipase was selected as it has the highest lipolytic activity at 20°C. M15 lipase was sequenced and two mutation points were identified which are R259C and V342E. *In silico* studies of this mutant has predicted that M15 lipase has increased structural flexibility compared to the native enzyme. Mutant M15 lipase was purified using gel filtration chromatography method. Biochemical characterization has revealed that M15 lipase has an optimum temperature at 20°C and highly stable at 10°C. M15 lipase was optimally active at pH 6 and stable within a small range of pH, 6-10. The catalytic activity of the mutant was boosted in the presence of Ca^{2+} and Na^{+}. Moreover, M15 lipase was found to be tolerant towards hydrophobic organic solvents and demonstrated great specificity towards long-chain pNP esters and optimum activity was observed in pNP-laurate. Secondary structure analysis of M15 lipase revealed that the enzyme has attained more structural flexibility compared to the wild type. In conclusion, AMS8 lipase was successfully mutated via directed evolution strategy and the findings will be useful insight on the understanding of the cold active lipases properties.

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

EVOLUSI TERARAH ATAS AMS8 LIPASE BAGI MENINGKATKAN KADAR AKTIVITI DAN STABILITI PADA SUHU RENDAH

Oleh

PIREYA THARASENI ARULU

Februari 2017

Pengerusi : Mohd Shukuri Mohamad Ali, PhD
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Lipase tahan sejuk merupakan enzim yang mempunyai kadar aktiviti pemangkin yang sangat tinggi pada suhu rendah dan oleh sebab itu, lipase tahan sejuk amat berpotensi dalam bidang industri bioteknologi. Enzim ini mempunyai kadar aktiviti degradasi yang tinggi pada suhu rendah tetapi dinyahaktifkan walaupun pada suhu yang sederhana. Bagaimanapun, faktor-faktor yang menyumbang kepada ciri-ciri penyesuaian dengan suhu rendah ini masih tidak jelas. AMS8 lipase yang dikategorikan dalam Family I.3 adalah disintesis oleh *Pseudomonas* Antartika, mempunyai kadar aktiviti pemangkin yang rendah pada suhu rendah. Eksperimen ini bertujuan untuk memutasikan AMS8 lipase supaya stabil dan bertindak pada kadar yang tinggi pada suhu rendah. dan untuk menganalisa kesan mutasi terhadap ciri-ciri biokimia enzim tersebut. Mutan-mutan AMS8 lipase dihasikan dengan menggunakan teknik Reaksi Berantai Polimer berralat. Mutan M15 lipase telah dipilih kerana ia mempunyai kadar aktiviti yang tinggi pada suhu 20°C. Urutan amino asid mutan M15 lipase telah dikenalkan pasti dan didapati dua residue telah bermutasi iaitu R259C dan V342E. Analisa struktur mutan ini melalui perisian komputer telah menunjukkan bahawa struktur mutan mempunyai fleksibiliti yang lebih tinggi daripada struktur asal AMS8 lipase. Seterusnya, mutan M15 lipase telah ditulenkan dan ciri-ciri biokimia enzim ini telah dikaji. Enzim ini berfungsi pada kadar optima pada suhu 20°C dan menunjukkan kestabilan yang sangat baik pada 10°C. M15 lipase juga aktif pada pH 6 dan stabil pada pH 6-8. Kadar aktiviti M15 lipase telah meningkat dalam kehadiran Ca^{2+} dan Na^{+}. Tambahan pula, M15 lipase menunjukkan kestabilan yang tinggi dalan pelarut organik hidrofobik dan juga menunjukkan kadar hidrolisis yang tinggi bagi substrat berantai panjang dan aktivitivi maksima telah dilihat pada pNP-laurate. Struktur sekunder M15 lipase telah dianalisa dan didapati bahawa struktur M15 lipase adalah lebih fleksibel berbanding struktur AMS8 lipase. Kesimpulannya, AMS8 lipase telah berjaya dimutasi melalui teknik evolusi terarah. Penemuan kajian ini telah membolehkan pemahaman yang mendalam mengenai ciri-ciri lipase tahan sejuk.

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I certify that a Thesis Examination Committee has met on 14 February 2017 to conduct the final examination of Pireya Tharaseni a/p Arulu on her thesis entitled "Directed Evolution of AMS8 Lipase Towards Enhanced Activity and Stability at Low Temperature" 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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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVATIONS

A_{600}/$O.D_{600}$	Absorbance/optical density at 600 nm
bp	Base pair
Da	Dalton
DMSO	Dimethyl sulfoxide
epPCR	Error-prone PCR
FFA	Free fatty acid
g	Relative centrifugal force
g	Gram
h	hour
IPTG	isopropyl β-D- Thiogalactopyranoside
kDa	kilo Dalton
L	Liter
M	Molar
mM	Milimolar
mg	Miligram
mg/mL	Miligram per mililiter
min	Minute
nm	Nanometer
PCR	Polymerase chain reaction
ps	Picosecond
rpm	Rotation per minute
RTX	Repeat in toxin
s	Second
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

U	Unit
U/mL	Unit per mililiter
µL	microlitre
µmol	micromole
w/v	Weight per volume
v/v	Volume per volume
V	Voltage

CHAPTER 1

INTRODUCTION

The emergence of protein science is strikingly influenced by the discovery of the recombinant DNA and genetic engineering. Being a crucial part of the biotechnological advances, protein science embraces large scope of studies including molecular biology, genetics, cell biology, evolution, structural elucidation and functional properties of enzymes. Enormous attention and curiosity drawn towards this field of study has lead to invention of various techniques to alter and modify protein sequence, which can be distinctly categorized as directed evolution and rational design (Arnold, 1998).

Directed evolution is an established technique of protein engineering that generates astonishing range of genetic library with novel properties. Accumulation of beneficial mutants diversity followed by meticulously designed screening and selection strategies is requisite in isolating a mutant with desired properties (Tracewell and Arnold, 2009). Several techniques of generating a promising molecular library are widely demonstrated such as DNA shuffling, chemical mutagenesis, error-prone PCR and mutator strain (Aguinaldo and Arnold, 2002; Nannemann et al., 2011). Over the years, abundant number of enzymes are being engineered via directed evolution with unique functional properties and specificities. Among them, lipases are largely been evolved to boost their thermostability, cold activity, regio-, stereo- and enantioselectively, substrate specificity, and solvent tolerance (Bassegoda et al., 2012; Goomber et al., 2016).

Lipases are ubiquitous enzyme that are widely acclaimed for their broad range of catalytic activity (Borrelli and Trono, 2015). Lipases are naturally synthesized by plants, animals and microorganisms, especially bacteria, yeasts and fungi (Treichel et al., 2010). Owing to their versatility and broad prospective in biotechnological industries, bacterial lipases are one of the largely studied group of lipases (Hasan et al., 2006). Each bacterial lipases are basically distinct in their adaptation features and functional properties. One of this kind is the cold active lipases. Cold active lipase are kinetically unique from other type of lipases as they exhibit excellent catalytic efficiency at low temperature. Their nature of being highly thermoliable is one of the key factors drawing this enzyme to be largely used in industrial application (Feller, 2013). However, factors that contribute to this unique properties of cold active lipases are still being experimented and varies in different type of lipases.

Therefore, AMS8 lipase, which was previously isolated from *Pseudomonas* sp bacteria was engineered via directed evolution to enhance the activity and stability of the enzyme at low temperature. The objectives are as follows:

a) To evolve AMS8 lipase by error-prone PCR
b) To identify the mutation points and purify the mutated AMS8 lipase
c) To analyze the mutant biochemically, biophysically and via *in silico* approaches

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