UNIVERSITI PUTRA MALAYSIA

THERMAL ADAPTATION AND CATALYTIC PROMISCUITY OF RECONSTRUCTED ANCIENT LIPASE FROM FAMILY I.3 BACTERIAL LIPOLYTIC ENZYMES

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By

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December 2020

Chair : Mohd Shukuri Mohamad Ali, PhD
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Enzymes are widely used in the chemical and biotechnological industries and this includes hydrolase. Hydrolases are a class of enzymes that demonstrate broad substrate specificity. One of the most valuable classes of hydrolases in biotechnological applications are lipolytic enzymes, which comprise lipases and esterases. The 3-dimensional structure of lipases and esterases displays the characteristic *α*/*β*-hydrolase fold, a general structural feature shared between all lipolytic enzymes. Family I.3 lipase is a member of the large group of Gram-negative bacterial true lipases. This lipase family is distinguished from other lipase families by the amino acid sequence and secretion mechanism. Little is known about the evolutionary process driving these differences. This study attempts to understand how the diverse temperature stabilities of bacterial lipases from family I.3 evolved. Trends in thermostability are complex. This work briefly addresses the answer to this problem by reconstructing a protein which is an ancestor to family I.3 lipases. To achieve that, eighty-three protein sequences sharing a minimum 30% sequence identity with Antarctic *Pseudomonas* sp. AMS8 lipase were used to infer phylogenetic tree. Using ancestral sequence reconstruction (ASR) technique, the last universal common ancestor (LUCA) sequence of family I.3 was reconstructed. LUCA structure was modelled using structure modelling software and undergo molecular dynamics simulation. Next, gene encoding LUCA was synthesized, cloned and expressed in *E. coli* system. Lastly, LUCA was refolded, purified and characterized. Molecular dynamics simulation indicates LUCA is stable at 70 ℃ for 75 ns simulation period. LUCA was expressed as inclusion bodies. Insoluble form of LUCA was refolded using urea dilution method. The refolded LUCA was purified to a purification fold of 8.0 and a recovery of 51.4%. The molecular weight was approximately ~70 kDa including polyhistidine tag. Interestingly, the purified LUCA exhibited an optimum temperature and pH at 70 ℃ and 10 respectively. Various mono and divalent metal ions increased or retained the activity of LUCA while only Ni^{2+} decreased

the activity. LUCA exhibited the highest activity towards C_{16} substrate followed by C_{10} substrate. Steady state kinetic study however showed a higher preference for C_{10} substrate over C_{16}. In addition, LUCA also demonstrated tolerance towards various organic solvents in 25% v/v concentration. Circular dichroism (CD) spectroscopy estimated the melting temperature of LUCA at 72 ℃. Catalytic promiscuity characterization of LUCA shows that LUCA is catalytically promiscuous. The finding from this study could support the understanding of environmental condition and wide reaction range of enzymes during ancient time. In summary, reconstructed ancestral enzymes have improved physicochemical properties that make them suitable for industrial applications and ASR technique can be employed as a general technique for enzyme engineering.

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PENYESUAIAN TERMA DAN PEMANGKINAN RAMBANG LIPASE PURBA YANG DIUBAHSUAI DARI ENZIM-ENZIM LIPOLITIK BAKTERIA KELUARGA I.3

Oleh

MOHAMAD FARIHAN AFNAN BIN MOHD ROZI

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Enzim digunakan secara meluas dalam industri kimia dan bioteknologi dan ini melibatkan hidrolase. Hidrolase adalah kelas enzim yang mempamerkan pengkhususan substrat yang luas. Salah satu kelas hidrolases yang paling berharga dalam aplikasi bioteknologi adalah enzim lipolitik, yang terdiri daripada lipase dan esterase. Struktur 3-dimensi lipase dan esterase memaparkan ciri-ciri lipatan *α*/*β*-hidrolase, iaitu ciri-ciri umum struktur enzim lipolitik. Keluarga I.3 lipase adalah ahli kumpulan besar lipase sebenar dari bakteria Gram-negatif. Keluarga lipase ini dibezakan daripada keluarga lain oleh urutan asid amino dan mekanisma rembesan. Tidak banyak yang diketahui tentang proses evolusi yang menyebabkan perbezaan ini. Kajian ini berusaha untuk memahami bagaimana kepelbagaian kestabilan suhu pelbagai lipase keluarga I.3 daripada bakteria telah berevolusi. Aliran kestabilan terma adalah kompleks. Kerja ini secara ringkasnya membicarakan jawapan kepada masalah ini dengan mengubah-suai satu protein yang merupakan moyang kepada lipase-lipase dari keluarga I.3. Bagi mencapai objektif ini, sebanyak lapan puluh tiga urutan protein yang berkongsi sekurang-kurangnya 30% identiti urutan dengan *Pseudomonas* sp. AMS8 lipase dari Antartika digunakan untuk menyimpulkan pokok filogenetik. Menggunakan teknik penyusunan semula keturunan leluhur (ASR), urutan leluhur umum universal (LUCA) keluarga I.3 telah diubahsuai. Struktur LUCA dimodel menggunakan perisian model struktur dan digunakan dalam simulasi dinamik molekul. Seterusnya, gen yang mengekod LUCA disintesis, diklon dan diungkapkan dalam sistem *E. coli*. Akhir sekali, LUCA dilipat semula, ditulenkan dan dicirikan. Simulasi dinamik molekul menunjukkan LUCA adalah stabil pada 70 ℃ untuk selama 75 ns tempoh simulasi. LUCA diungkapkan dalam bentuk jasad pencantuman. LUCA yang tidak larut dilarutkan menggunakan kaedah pencairan urea. Setelah ditulenkan LUCA yang dilipat semula menghasilkan lipatan penulenan 8.0 dan pemulihan 51.4%. Berat molekul adalah lebih kurang ~70 kDa termasuk tag polyhistidine. Menariknya, LUCA yang ditulenkan mempamerkan suhu optimum dan pH pada 70 ℃ dan 10 masing-masing. Pelbagai ion logam mono dan divalen

meningkatkan atau mengekalkan aktiviti LUCA sementara hanya Ni^{2+} menurunkan aktiviti. LUCA mempamerkan aktiviti tertinggi ke arah substrat C_{16} diikuti oleh substrat C_{10}. Kajian kinetik keadaan kekal bagaimanapun menunjukkan keutamaan yang lebih tinggi untuk substrat C_{10} berbanding C_{16}. Di samping itu, LUCA juga menunjukkan toleransi terhadap pelbagai pelarut organik dalam kepekatan 25% v/v. Spektroskopi "*circular dichroism*" (CD) menganggarkan takat lebur LUCA pada 72 ℃. Pencirian pemangkinan rambang LUCA menunjukkan bahawa LUCA mempunyai aktivititi pemangkinan rambang. Penemuan dari kajian ini dapat menyokong pemahaman tentang keadaan persekitaran dan pelbagai tindak balas rambang enzim pada zaman purba. Kesimpulannya, enzim-enzim purba mempunyai penambahbai-kan dari segi sifat fisiokimia yang membuatkan mereka sesuai untuk aplikasi-aplikasi industri dan ASR boleh digunakan sebagai teknik asas untuk kejuruteraan enzim.

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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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LIST OF ABBREVIATIONS

%	Percent
°C	Degree Celsius
α	Alpha
β	Beta
ε	Epsilon
µg	Microgram
µL	Microliter
µM	Micromolar
A	Absorbance
APS	Ammonium persulfate
ASR	Ancestral sequence reconstruction
bp	Base pair
cm	Centimeter
DNA	Deoxyribonucleic acid
et al	And friends
g	Gram
h	Hour
kb	Kilobase pair
kDa	Kilo Dalton
L	Liter
LUCA	Last universal common ancestor
m	Meter
MD	Molecular dynamics
mg	Miligram

min	Minute
mL	Mililiter
nm	Nanometer
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
R2	Correlation coefficient
Rgyration	Radius of gyration
RMSd	Root Mean Square deviation
RMSf	Root Mean Square fluctuation
s	Second
SASA	Solvent accessible surface area
TAE	Tris-acetate-EDTA
Taq	Thermus aquaticus
TEMED	Tetramethylethylenediamine
Tm	Melting temperature
×	Times
×g	Times gravity

CHAPTER 1

INTRODUCTION

Enzymes are widely used in the chemical and biotechnological industry, being essential biocatalysts in diverse areas such as pulp and paper, food and beverage, cosmetic, detergent, clothing, and pharmaceutical (Gurung et al., 2013). Recently, commercial enzymes had been implemented for biofuel production and natural gas conversion (Chapman et al., 2018). Currently, hydrolases are the most popular enzymes in biotechnology (Gurung et al., 2013). Lipases are long chain fatty acid ester hydrolases, while also being the most valuable hydrolase due to various biotechnological applications (Ramnath et al., 2017). Family I.3 lipase is a member of the large group of Gram-negative bacterial true lipases and distinguished from other families by the amino acid sequence and secretion mechanism (Angkawidjaja & Kanaya, 2006). Family I.3 lipases are composed of two domains, a domain with active site and another domain which possesses RTX-signature that binds Ca^{2+} (Amada et al., 2000; Welch, 2001). These features of lipase family are unique, making their ancestry origin interesting to be studied.

Developments in protein engineering over the past decades have enabled enzymes to be evolved *in vitro* for properties that favor the required process conditions, and to obtain enzyme variants with altered substrate specificity or enantioselectivity (Dalby, 2011; Hibbert et al., 2005; Osbon & Kumar, 2019). Despite the advances of protein engineering technology, the limitation of these engineered enzymes is still a serious barrier in the chemical industry. Nowadays no methodology seems to be able to enhance, for instance, the temperature and pH operability, the expression level or the specific activity of enzymes, all at once. In the past years, the ancestral sequence reconstruction technique (ASR) has been used in the study of genes, proteins and enzymes evolution. Surprisingly, reconstructed ancestral enzymes displayed better thermal stability, improved pH response, enhanced activity and promiscuous to chemicals (Alcalde, 2017; Barruetabeña et al., 2019; Devamani et al., 2016; Eick et al., 2012; Gaucher et al., 2008; Manteca et al., 2017; Perez-Jimenez et al., 2011; Plach et al., 2016; Risso et al., 2013; Zakas et al., 2017). This technique is based on the evolution theory, which states that groups of organisms change over time so that descendants differ structurally and functionally from their ancestor (Costa, 2009).

It is an interesting question whether the preferences for different amino acids at a given site in a specific protein (lipase) either changing during evolution or remain essentially constant. A trend temperature adaptation (hot to cold and vice versa) of specific proteins (lipase) is not yet established due to the limited information from the structural and genome aspect. In this study, it is hypothesized that ancient lipase is more promiscuous and thermostable compared to its modern descendants. Hence, this study aimed:

1. To reconstruct the ancestor of family I.3 bacterial lipase via ancestral sequence reconstruction.
2. To determine the 3-D structure of reconstructed lipase via computational approach.
3. To clone and express the reconstructed gene encoding the ancient lipase.
4. To purify and characterize the recombinant ancestral lipase.

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