UNIVERSITI PUTRA MALAYSIA

DIRECTED EVOLUTION OF RECOMBINANT C-TERMINAL TRUNCATED LIPASE FROM Staphylococcus epidermidis AT2 FOR ENHANCED THERMAL STABILITY

JIIVITTHA VENO

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DIRECTED EVOLUTION OF RECOMBINANT C-TERMINAL TRUNCATED LIPASE FROM *Staphylococcus epidermidis* AT2 FOR ENHANCED THERMAL STABILITY

By

JIIVITTHA VENO

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DIRECTED EVOLUTION OF RECOMBINANT C-TERMINAL TRUNCATED LIPASE FROM *Staphylococcus epidermidis* AT2 FOR ENHANCED THERMAL STABILITY

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Chairman: Professor Raja Noor Zaliha Raja Abd Rahman, PhD
Faculty: Biotechnology and Biomolecular Sciences

Lipases specifically thermostable lipases which are origin from microbial are desirable commercially as they are resilient and robust. In the industrial processes, lipases are expected to operate at temperatures above 40°C and could retain activity in organic solvents. However, screening of organic tolerant lipases with increased thermal property from organisms is time consuming for high yield. Therefore, the objective of this study is to evolve rT-M386 lipase using error-prone PCR to achieve thermostability and to study the effect of mutation on biochemical properties and structural conformations of the lipase. In the first screening, approximately 1500 positive colonies were obtained. From these thousands of colonies, 900 colonies were analyzed quantitatively and resulted in seven mutant clones which exhibited higher activity when compared to rT-M386. Out of seven mutants, G210C lipase demonstrated a remarkable improvement of the activity by 5-fold when compared with rT-M386 at 50°C. rT-M386 and G210C were purified concurrently using GST-affinity chromatography with the yield of 92.25% and 92.17%, respectively. A distinct single band with a molecular weight of 69 kDa was observed on SDS-PAGE. The purified G210C lipase showed a 20°C increased of the optimum temperature. Apparently, rT-M386 could not maintain its stability for 30 min at temperatures above 20°C as its decreasing drastically. Meanwhile, G210C presented an exceptional thermal stability profile as it can sustain its stability at 50°C with 100% of relative activity. In addition, G210C lipase displayed more prolonged half-life in the range of 40-60°C as compared to rT-M386. Both lipases exhibited optimal activity and stability at pH 8. G210C exhibited the highest activity in the presence of diethyl ether, isopropanol, DMSO and methanol at 50ºC compared to rT-M386. Structural analysis of rT-M386 and G210C lipases has demonstrated variations in structural conformation. CD spectral analysis revealed that G210C attained more structural stability compared to the

rT-M386 lipase and this supported the experimental findings. The structure was found more stable and compact as revealed by molecular dynamic simulation, suggesting its tolerance at elevated temperature. In conclusion, the results indicated that single residue substitution of G210C lipase contributes to the enhancement of thermal stability without adversely impacting the catalytic performance. rT-M386 lipase was successfully mutated via directed evolution strategy and the findings will be useful insight on the understanding of the structural-functional adaptation of thermostable lipases.

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

MUTASI SECARA RAWAK ATAS "RECOMBINANT C-TERMINAL TRUNCATED" LIPASE DARIPADA *Staphylococcus epidermidis* AT2 BAGI MENINGKATKAN KADAR STABILITI PADA SUHU TINGGI

Oleh

JIIVITTHA VENO

Jun 2017

Pengerusi: Professor Raja Noor Zaliha Raja Abd Rahman, PhD
Fakulti: Bioteknologi dan Sains Biomolekul

Lipase khususya termostabil lipase yang berasal dari mikrob adalah diminati secara komersial kerana mereka adalah berdaya tahan dan teguh. Dalam proses perindustrian, lipase dijangka beroperasi pada suhu melebihi 40°C dan dapat mengekalkan aktiviti dalam pelarut organik. Walau bagaimanapun, penyaringan lipase yang stabil terhadap pelarut organik dengan peningkatan profil termal daripada organisma memerlukan lebih masa untuk hasil lipase yang tinggi. Maka, objektif kajian ini adalah untuk memutasikan rT-M386 lipase dengan menggunakan teknik Reaksi Berantai Polimer beralat untuk mencapai termostabiliti dan untuk mengkaji kesan mutasi dari segi sifat biokimia dan bentuk struktur lipase. Dalam pemeriksaan pertama, kira-kira 1000-1500 koloni positif telah diperolehi. Daripada ribuan koloni, 900 koloni telah dianalisis secara kuantitatif dan menghasilkan hanya 7 klon mutan yang menunjukkan aktiviti yang lebih tinggi jika dibandingkan dengan rT-M386. Mutan G210C menunjukkan peningkatan aktiviti sebanyak 5 kali ganda jika dibandingkan dengan rT-M386 pada 50°C. rT-M386 dan G210C telah ditulenkan menggunakan GST-afiniti kromatografi dengan hasil 92.25% dan 92.17% masing-masing. Satu jalur yang jelas dengan jisim molekul 69 kDa diperhatikan pada SDS-PAGE. G210C lipase yang ditulenkan menunjukkan peningkatan suhu optima sebanyak 20°C. rT-M386 tidak dapat mengekalkan kestabilan selama 30 minit pada suhu yang lebih tinggi daripada 20°C. Sementara itu, G210C menunjukkan profil kestabilan terma yang luar biasa kerana ia boleh mengekalkan kestabilan konformasi pada 50°C dengan 100% daripada aktiviti relatif. Di samping itu, G210C lipase menunjukkan separuh hayat yang lebih panjang dalam lingkungan 40-60°C berbanding dengan rT-M386. Kedua-dua lipase menunjukkan aktiviti optimum dan kestabilan pada pH 8. G210C menunjukkan aktiviti tertinggi dalam diethyl ether, isopropanol, DMSO dan metanol pada 50ºC berbanding rT-M386. Analisis struktur

lipase rT-M386 dan G210C telah memaparkan variasi dalam konformasi struktur. Analisis CD spektrum mendedahkan bahawa G210C mencapai struktur yang lebih stabil berbanding dengan rT-M386 dan ini menyokong dapatan eksperimen. Struktur ini didapati lebih stabil dan padat seperti yang didedahkan oleh simulasi dinamik molekul, menunjukkan toleransi pada suhu tinggi. Kesimpulannya, keputusan menunjukkan bahawa satu penggantian residu G210C lipase menyumbang kepada peningkatan kestabilan haba tanpa memberi kesan negatif ke atas tahap pemangkinan. rT-M386 lipase telah berjaya bermutasi melalui strategi mutase secara rawak dan penemuan ini telah membolehkan pemahaman yang mendalam mengenai adaptasi struktur-fungsi lipase termostabil.

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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:

Raja Noor Zaliha Raja Abd. Rahman, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Mohd Shukuri Mohamad Ali, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Nor Hafizah Ahmad Kamarudin, PhD
Senior lecturer
Centre of Foundation Studies for Agricultural Science
Universiti Putra Malaysia
(member)

_____
ROBIAH BINTI YUNUS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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Signature: _____ Date: _____

Name and Matric No.: Jiivittha Veno, GS37107

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LIST OF ABBREVIATIONS

A280	Absorbance at 280 nm
A595	Absorbance at 595 nm
A600/ OD600	Absorbance/ optical density at 600 nm
A410	Absorbance at 410 nm
bp	base pair
x*g*	relative centrifugal force
g	gram
g/L	gram /Litre
h	hour
IPTG	isopropyl β-D- Thiogalactopyranoside
kDa	kilo Dalton
K	Kelvin
kB	kilo base pair
L	Liter
M	Molar
mA	mili ampere
mAU	mili absorbance unit
mM	milimolar
mdeg	mili degree
min	minute
ml	milliliter
mg/L	milligram/Liter
nm	nanometer
ns	nanosecond
OD	optical density
ps	picoseconds
rpm	rotation per minute
s	second
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
µmole	micromole
µL	microlitre
U/ mL	Unit per milliliter
U/ mg	Unit per milligram
v/ v	Volume per volume
w/ v	Weight per volume
V	Voltage

CHAPTER 1

INTRODUCTION

Lipases are versatile due to its capability to react in both aqueous and non-aqueous environments and as biocatalyst there are considerably economical compared to conventional chemical reactions. Thermostability of an enzyme is closely associated to its expression, folding, activities and functions, thereby thermal stability is fundamental property of an enzyme (Jemli, Ayadi-Zouari, Hlima, & Bejar, 2016). Thermostable lipases can be exploited in food processing, biopolymer; organic synthesis, biodiesel production, oleochemical and pulp industries (Andualema & Gessesse, 2012; Chakravorty, Parameswaran, Dubey, & Patra, 2011; Haki & Rakshit, 2003).

Organic tolerant lipases with increased thermal property are needed for industrial lipase-catalyzed reactions as they can increase the conversion rate of lipid substrates especially with the high melting point, resist the chemical modifications by elevated temperature and prevent contamination by microorganism (Bornscheuer & Pohl, 2001; S. Sharma & Kanwar, 2014). Lipases from *Candida antarctica and Burkholderia cepacia* are most well known in detergent and organic synthesis industrial applicants (Gunasekaran & Das, 2005). Although, many researches have characterized thermostable microbial lipases but there are only few lipases were reported to possess both thermostable and organic solvent-tolerant properties.

Up to now there are no studies described on thermostable lipases from *Staphylococcus* sp. and they are rarely exploited in the industrial processes because of their relatively lower catalytic activities and stabilities under conditions that required for industrial applications such as in high temperatures, non-aqueous solvents or extreme pH values (Cherif, Mnif, Hadrich, Abdelkafi, & Sayadi, n.d.). The highly produced staphylococcal lipases can be engineered to alter its properties for better performance in the practical applications in the aspect of quality and quantity (Horchani et al., 2012).

To expand the functionality of the lipase as well to explore the structure-function relationship, attempts had been made to evolve lipase that could overcome its limiting factors for industrial preference. Currently, recombinant DNA technology and protein engineering techniques have empowered lipases with wide enzymatic properties to compete with other well-established chemical technologies. Chemical compounds are environmentally hazardous unlike the harmless use of enzymes in the industry.

Over the years, researchers are intended to study the intrinsic stability of thermophilic proteins that render them to thermo-stabilization. The most commonly predicted factors are associated with their primary structure, hydrophobic interactions among the hydrophobic residues, formation of salt bridges due to the charged residues, hydrogen bonds between surrounding water molecules and the surface exposed hydrophilic

residues, packing efficiency, loop stabilization and defense against covalent destruction (Siddiqui, 2015; Trejo, 2013; Villeneuve, Muderhwa, Graille, & Haas, 2000).

Even though many thermostable lipases have been extensively studied on several aspects for years and commercialized, there are still numerous unresolved clear factors which influence the thermal stabilization of lipases correlating the role of sequence or amino acid compositions and structural conformations. Therefore, directed evolution which is leading to *in silico* comparison studies between thermostable and non-thermostable lipases could assist in the discovery of these factors. Eventually, this can bring out the effortless ways in protein engineering to produce lipases with remarkable characteristics based on industrial preferences. Recently, many enzymes were being engineered via directed evolution to own unique specificities and functionalities such thermostability, cold activity, gene expression, solubility, enantio-selectivity, substrate specificity, and solvent tolerance (Goomber, Kumar, Singh, Mishra, & Kaur, 2016; Sukumaran et al., 2015).

Previously, an organic tolerant AT2 lipase from *Staphylococcus epidermidis* had been truncated and designated as rT-M386 and in this study rT-M386 lipase was randomly mutated to enhance its thermal property. Therefore, this research aimed:

a) To evolve rT-M386 lipase by directed evolution and identify the mutation point.
b) To determine the biochemical and biophysical characteristics of selected purified mutant lipase (G210C).
c) To analyze the effect of mutation on the structure of G210C lipase via *in silico* studies.

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