REGIOSPECIFICITY MODIFICATION OF *Geobacillus zalihae* T1 LIPASE VIA MOLECULAR ENGINEERING APPROACHES

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 Doctor of Philosophy

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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

To date, only a few lipases have been reported to have *sn*-1, *sn*-2, or *sn*-3-regioselectivity. This single selectivity offers various important applications, especially the most valuable is producing chiral products for the drug and food industries. T1 lipase, which was isolated from *Geobacillus zalihae* with specific regioselectivity at *sn*-1,3, was used in this study. This work aims to study the potential of implementing rational and semi-rational protein design methods to target the lid and binding site area of *sn*-1,3-regiospecific T1 lipase. Three strategies were applied for modifying T1 lipase regiospecificity, and this modification is essential to improve the T1 lipase industrial application, especially in the production of chiral molecules. The first strategy targeted the lid area by a semi-rational protein design method and resulted in 7 variants (F180C, F180G, F180S, F180L, F180I, F180N, and F180Y). The resulting variants showed an increased and decreased in their optimum temperature and thermal denaturation point ranging from 60 to 75 °C, and 63 to 78 °C, respectively, compared to wt-T1, which has an optimum temperature of 70 °C and thermal denaturation point at a temperature of 73 °C. The resulting variants from this strategy have optimum pH as wt-T1 lipase and displayed a modified selectivity toward long-chain *p*NP-ester (C10-C18) compared to wt-T1 lipase, which has a preference towards C10-C14. All resulting variants displayed different catalytic efficiencies ranging from 309 to 604 $\times 10^{-6}$ s^{-1} /mM compared to wt-T1 catalytic efficiency of 518.4 $\times 10^{-6}$ s^{-1} /mM. However, this strategy didn't show any regioselectivity modification. The second strategy applied was rational design around the binding site, and this approach resulted in only one variant with five (5) mutation sites (1M/F25L/I262V/E189K/V247I). This resulting variant did not show any changes at optimum temperature and pH but enhanced the selectivity toward long-chain *p*NP-ester (C14-C18) compared to wt-T1 lipase. The resulting variant showed improved catalytic efficiency around 5472 $\times 10^{-6}$ s^{-1} /mM compared to wt-T1 lipase but did not result in any regiospecific modifications. The third strategy targeted both the lid by rational design (F180G/F181S) and the oxyanion hole by semi-rational design strategy, which resulted in twelve (12) variants (F16X, X=C, G, V, Y, D, H, S, L, I, W, F, N). The newly generated variants conserved the optimum temperature of 70 °C. However, thermal

denaturation was negatively affected, and this was represented by a decline in the denaturation temperature ranging from 5 to 7 °C. However, these targeted mutations shifted the optimum pH to 10 for some variants compared to pH 9 for wt-T1. Regarding the selectivity study, the resulting variant showed improved selectivity toward *p*NP-ester long-chain fatty acids from C12-C18 compared to wt-T1 lipase. In addition, the variants of this strategy displayed different catalytic efficiencies, ranging from 86.4 to 777 $\times 10^{-6}$ s^{-1}/mM compared to wt-T1 lipase. Furthermore, six variants, F16I, F16V, F16W, F16S, F16G, and F16C, displayed a regioselectivity modification from *sn*-1,3 regioselectivity of wt-T1 to only *sn*-3. Gas- chromatography (GC) with flame ionization detection of these six variants confirmed the regioselectivity modification. The modified regiospecific variants were shown to have a varied preference toward different palm stearin fatty acids lengths ranging from C16 to C20:1 compared to wt-T1 lipase with the specificity of C16 to C18:1. The *sn*-3 modified regiospecific structure (F16W) and wt-T1 lipase structures were remodelled and predicted within open conformation, then subjected to docking and molecular simulation (MD) study complexed with an acylglycerol analogue as a substrate. The docking study showed that *sn*-3 modified regiospecific structure has a higher affinity toward *sn*-3 acylglycerol chain than wt-T1, which displayed binding affinity toward *sn*-1,3 acylglycerol chain. Whereas the MD simulation study showed conformational changes that occurred were approximately on the lid domain and distant from the oxyanion hole mutation site (Zn^{2+} coordination domain), consisting of helices α3 and α5. The conformational changes resulting from altering bulky side-chain residues of the lid and oxyanion hole have increased binding site flexibility and affected the hydrogen networking of the Zn^{2+} coordination domain. In conclusion, the substitution of lid and oxyanion hole residues (strategy three) successfully modified regioselectivity and shifted lipase specificity and activity. Therefore, targeting both the lid and binding site (strategy three) is sufficient to create a novel regiospecificity of an enzyme. Thus, *sn*-3 lipase is essential in producing pure fatty acids with high specificity, which can be applied to obtain high-value chemicals for drug and food industries.

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PENGUBAHSUAIAN KESPESIFIKAN REGIO LIPASE T1 DARI *Geobacillus zalihae* OLEH PENDEKATAN KEJURUTERAAN MOLEKUL

Oleh

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Sehingga kini, hanya beberapa lipase terasing telah dilaporkan mempunyai kepilihan regio *sn*-1, atau *sn*-2, atau *sn*-3. Kepilihan tunggal ini menawarkan pelbagai aplikasi penting, terutamanya yang paling berharga adalah menghasilkan produk khi bagi industri dadah dan makanan. Lipase T1, yang mana telah diasing daripada *Geobacillus zalihae* dengan kepilihan spesifik regio pada *sn*-1,3 telah digunakan dalam kajian ini. Kerja ini bertujuan untuk mengkaji potensi pelaksanaan kedua-dua kaedah reka bentuk rasional dan semi-rasional bagi mensasarkan lapik tiang dan kawasan tapak pengikat lipase T1 *sn*-1,3-spesifikregio. Tiga strategi telah digunakan untuk mengubahsuai spesifik regio lipase T1 dan pengubahsuaian ini penting untuk penambahbaikan ciri-ciri lipase untuk digunakan dalam industri terutamanya dalam penghasilan molekul khi. Strategi yang pertama mensasarkan kawasan lapik tiang oleh kaedah reka bentuk semi-rasional dan telah menghasilkan 7 varian (F180C, F180G, F180S, F180L, F180I, F180N, F180Y). Varian terhasil menunjukkan peningkatan dan penurunan suhu optimum dan titik denaturasi terma 60 kepada 75 °C, dan 63 kepada 78 °C, masing-masing, berbanding wt-T1, yang mempunyai suhu optimum 70 °C dan titik denaturasi terma pada 73 °C. Varian yang terhasil daripada strategi ini mempunyai optimum pH yang sama seperti lipase wt-T1 dan memaparkan kepilihan terubah suai kepada asid lemak ester *p*NP berantai panjang C10-C18, berbanding lipase wt-T1, yang mempunyai pemilihan terhadap C10-C14. Kesemua varian terhasil memaparkan kecekapan mangkinan yang berbeza dari 309.6 kepada 604.8 s -1 /mM $\times 10^{-6}$ berbanding kecekapan mangkinan wt-T1 518.4 s^{-1} /mM $\times 10^{-6}$. Walau bagaimanapun, strategi ini tidak menunjukkan sebarang pengubahsuaian kepilihan regio. Strategi kedua adalah reka bentuk rasional di sekeliling tapak ikatan, dan pendekatan ini menghasilkan hanya satu varian dengan lima (5) tapak mutasi (1M/ F25L/I262V/E189K/ V247I). Varian terhasil ini tidak menunjukkan sebarang perubahan pada suhu optimum dan pH, akan tetapi menggalakkan kepilihan terhadap asid lemak ester *p*NP berantai panjang daripada C14-C18 berbanding lipase wt-T1. Varian terhasil menunjukkan kecepakan mangkinan yang lebih baik hampir 5472 s^{-1} /mM $\times 10^{-6}$ berbanding lipase wt-T1 tetapi tidak menghasilkan sebarang pengubahsuaian spesifik regio. Strategi yang ketiga mensasarkan kedua-dua lapik tiang

oleh reka bentuk rasional (F180G/F181S), dan lubang oxyanion oleh strategi semi-rasional, yang menghasilkan dua belas (12) varian (F16X, X=C, G, V, Y, D, H, S, L, I, W, F, N). Varian janaan baru ini memulihara suhu optimum 70 ℃, tetapi titik denaturasi terma telah terkesan secara negatif dan ini digambarkan oleh pengurangan suhu dari 5 kepada 7 °C. Walau bagaimanapun, mutasi sasaran ini menganjakkan pH optimum beberapa varian kepada 10 berbanding 9 bagi wt-T1. Berkenaan kepilihan, varian terhasil menunjukkan penambahbaikan kepilihan terhadap asid lemak ester *p*NP berantai panjang daripada C12-C18 berbanding lipase wt-T1. Sebagai tambahan, varian terhasil daripada strategi ini memaparkan kecekapan mangkinan yang berbeza dari 86.4 sehingga 777.6 s^{-1} /mM × 10^{-6} berbanding lipase wt-T1. Tambahan lagi, enam varian, F16I, F16V, F16W, F16S, F16G dan F16C memaparkan pengubahsuaian kepilihan regio wt-T1 *sn*-1,3-kepilihan regio kepada hanya *sn*-3. Kromatografi gas (GC) bersama pengesanan pengionan nyala ke atas enam varian ini mengesahkan pengubahsuaian kepilihan regio. Varian yang diubah suai spesifik regio menunjukkan pemilihan terhadap panjang asid lemak stearin sawit yang berbeza dari C16-C20:1 berbanding lipase wt-T1 dengan kespesifikan C16 to C18:1. Struktur kespesifisikan regio *sn*-3 yang terubah suai (F16W) dan lipase wt-T1 telah dimodel dan diramal dalam konformasi terbuka, dan tertakluk pada dok dan simulasi dinamik molekul (MD) bersama analog acylglycerol sebagai substrat. Kajian dok menunjukkan struktur kespesifisikan regio *sn*-3 yang terubah suai mempunyai afiniti yang lebih tinggi terhadap rantai *sn*-3 acylglycerol berbanding wt-T1 yang memaparkan afiniti pengikat pada rantai *sn*-1,3 acylglycerol. Sementara itu, kajian simulasi dinamik molekul menunjukkan perubahan konformasi pada lapik tiang domain dan jauh daripada tapak mutasi lubang oxyanion (domain koordinasi Zn^{2+}), yang terdiri daripada heliks α3 dan α5. Perubahan konformasi yang disebabkan oleh pengubahsuaian sisi residu besar pada lapik tiang dan lubang oxyanion telah meningkatkan flesibiliti tapak pengikat dan mempengaruhi rangkaian hydrogen pada domain koordinasi Zn^{2+}. Kesimpulannya, penggantian lapik tiang dan residu lubang oxyanion berjaya mengubahsuai kepilihan regio dan juga berjaya menganjakkan kespesifisikan dan aktiviti lipase. Oleh itu, penyasaran kedua-dua lapik tiang dan tapak ikatan (strategi ketiga) adalah mencukupi untuk menghasilkan spesifik regio enzim yang novel. Oleh itu, lipase sn3 adalah penting dalam penghasilan asid lemak tulin dan boleh digunakan untuk mendapatkan bahan kimia bernilai tinggi yang mempunyai kegunaan penting dalam industri perubatan dan makanan.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Å	Angstrom
α	Alpha
β	Beta
Bp	Base pair
℃	Degree Celsius
CBD	Cellulose binding domain tag
CBP	Calmodulin-binding peptide
CD	Circular dichroism
DNA	Deoxyribonucleic acid
EtOH	Ethanol
epPCR	Error-prone Polymerase Chain Reaction
FA	Fatty acids
g	Gravity
G	Gram
GC FID	Gas chromatography flame ionization detector
GTase	Glucosyltransferase
h	Hour
HCl	Hydrochloric acid
HNO_2	Nitrous acid
IPTG	Isopropyl-L-D-thiogalactopyranoside
ISM	Iterative saturation mutagenesis
kDa	Kilo Dalton
L	Liter
LB	Luria Bertani
M	Molar

MBP	Maltose-binding protein
MD	Molecular dynamics
mM	Millimolar
mL	Millilitre
mg	Milligram
mg/ml	Milligram per millilitre
min	Minute
um	Micrometre
µg	Microgram
µL	Microlitre
MtOH	Methanol
N	Normality
NaCl	Sodium chloride
NaOH	Sodium hydroxide
nm	Nanometre
NMR	Nuclear magnetic resonance
ns	Nano second
OD 280nm	Optical density at wavelength 280 nanometre
OD 410nm	Optical density at wavelength 410 nanometre
OD 595mm	Optical density at wavelength 595 nanometre
OD 600nm	Optical density at wavelength 600 nanometre
OD 715nm	Optical density at wavelength 715 nanometre
PDB	Protein Data Bank
PCR	Polymerase Chain Reaction
*p*NP	*p*-nitrophenyl
PrOH	Isopropyl alcohol
QM	Quantum mechanics

QM/MM	Quantum mechanics / molecular mechanics
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
rpm	Revolutions per minute Seconds
SASA	Solvent Accessible Surface Area
SBP	Streptavidin-binding peptide
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sp.	Seconds Species
SSM	Saturation mutagenesis strategy
TAG	Triacylglycerol
TLC	Thin Layer Chromatography
T_m	Melting Temperature
U/mL	Unit per millilitre
U/mg	Unit per milligram
V	Volt
v/v	Volume over volume
V_{max}	Maximum velocity
wt	Wild type
w/v	Weight over volume
Zn	Zinc
%	Percentage
NKK	codon degeneracy (32 codons/20 aa) where N = A/C/G/T and K = G/T
NDT	codon degeneracy (12 codons/12 aa)

CHAPTER 1

INTRODUCTION

Lipases exhibit unique stereoselectivity according to the chemical structure of triacylglycerols (TAGs), as they hydrolyze prochiral and chiral substrates, and this is the main lipases property which distinguishes them from other hydrolases, such as phospholipase, proteases, nucleases, phospholipases, which hydrolyse only one optical antipode of their substrates. The unique characteristics of lipases make them a suitable candidate to be extensively exploited in several industries such as animal feed, biofuel, dairy, food and beverage, cleaning, pharmaceuticals, textile, perfumery, cosmetic, flavor industry, fine chemical production, biocatalytic resolution, esters, and amino acid derivatives agrochemicals, bioremediation, and biosensor (Badgujar and Bhanage, 2016; Sadaf *et al.*, 2018; Vanleeuw *et al.*, 2019). Regiospecificity is considered one of the major advantages of implementing lipase technology in the oils and fats modification to synthesise high-value-added products, such as human milk fat substitutes, cocoa butter equivalents, and other specific-structured lipids. The main applications of lipases for normal oils and fats are still limited due to the high cost of biocatalysts. Therefore, positional specificity of lipases is important and will be targeted to exploit industrial and commercial industrial developments because of the lack of chemical method specificity. Thus, biocatalysts' regiospecificity is promising or possible for this task (Xu, 2000; Chaput *et al.*, 2008).

Protein engineering techniques are well-known as powerful tools to alter enzyme activity, stability, and specificity. In addition to modifying protein stability and activity, this approach is a novel method to enhance and regulate the stereoselectivity and specificity of the enzyme (Ali *et al.*, 2020). Advances in protein design and engineering strategies have remarkably established enzymes as alternative biocatalysts to traditional organic and metal-dependent catalysts in industrial processes (Bornscheuer *et al.*, 2012).

Besides the details of atomic interaction provided by docking analysis and molecular dynamics simulation (MD), it can also be used to predict the structures of biomolecular compounds. With current accumulated knowledge of protein sequences from DNA sequencing high-throughput, obtaining complete experimental of protein 3D structures is still much more difficult, as it helps in protein structure prediction (Geng *et al.*, 2019). Hence, molecular dynamics simulation was usually used to predict and provide insight into the protein structural flexibility of the docked complexes and protein−ligand stability, which helps improve protein specificity (Ravi *et al.*, 2021). By using the semi-rational design approach, many recent studies have improved lipase selectivity, specificity, stereoselectivity, and regioselectivity. It could combine rational design and direct evolution to overcome these approaches' limitations. The most targeted structural area in the enzyme's structure to improve their specificity toward specific molecules were the lid and binding site by utilizing both rational and semi rational design strategies (Sandström *et al.*, 2009; Damnjanović *et al.*, 2016; Willems *et al.*, 2018; Zorn *et al.*, 2018; Quaglia *et al.*, 2019; Moharana and Rao, 2020; Maldonado *et al.*, 2021). Undertaken site saturation mutagenesis (semi-rational design) method can modify

certain features of catalysts, such as enantioselectivity and substrate specificity that are usually controlled by active site steric factors (Reetz, 2004). Thus, structural visualization is the easiest method to guide a semi-rational design to identify target residues that are then altered by a site-saturation mutagenesis approach. Therefore, in this study, engineering the regioselectivity of *Geobacillus zalihae* T1 lipase by semi-rational design combined with computer-assisted protein docking can be a valuable approach toward improving and altering regiospecificity and selectivity and refining catalytic properties to overcome its industrial usage limitation. The success in producing the mutant lipase with special characteristics permitted high specificity, therefore can be used to obtain high-value chemicals products that are important in pharmaceuticals and other industries.

1.1 Problem statement

Natural lipases mostly are *sn*-1,3 or random-regioselectivity. However, special industrial applications require engineering lipases with a novel regiospecificity. Due to the scarcity of lipases with unique regioselectivity (*sn*-1, *sn*-2, or *sn*-3), there is a need for engineering new lipases with novel regioselectivity. Lipases with unique regioselectivity will offer various important applications, especially for chiral molecule production, biodiesel production and fat, and oils modification.

1.2 Research hypothesis

The thermostable T1 lipase regioselectivity can be altered by targeting bulky phenylalanine residues to create a suitable space that prevents steric hindrance. Targeting both the lid and binding site area can be used to modify the regioselectivity of T1 lipase.

1.3 Objectives

The main objective of this study was to produce a novel mutant *Geobacillus zalihae* T1 lipase with regiospecificity modifications.

Specific objectives:

1) To determine major points of the mutation using computational and docking analysis methods.
2) To genetically modify and characterize the lipase variants.
3) To evaluate the modified regiospecific T1 lipase structure through in silico analysis and compare it with wt-T1 lipase

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