STRUCTURAL DETERMINATION OF IMPROVED METHANOL-TOLERANT MUTANT FROM *Geobacillus zalihae* T1 LIPASE BY X-RAY CRYSTALLOGRAPHY

By

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Chair : Assoc. Prof Adam Leow Thean Chor, PhD
Faculty : Biotechnology and Biomolecular Sciences

Lipases are versatile enzymes that have been altered through various modification methods to improve their enzymatic properties to meet biotechnology industry requirement. Since decades ago, lipases from many sources have been study widely as potential biocatalyst to assist synthesis of biodiesel. Some of them have been altered via protein engineering approach to enhance their enzymatic performance and stability. The thermostable T1 lipase from *Geobacillus zalihae* can also be a great biocatalyst candidate in biodiesel production. However, inactivation of T1 lipase when the enzyme is surrounded by high concentration of methanol solvent is limiting its uses in industrial applications. Since introduction of non-bonded interactions hardly improved their stability of T1 lipase in methanol, the introduction of disulphide bond could be the best proposition to retain protein conformation and the enzyme stability in the presence of methanol. Hence, current study aims to engineer a methanol-tolerant lipase by site-directed mutagenesis and divulge the interaction that stabilizes the mutant by X-ray crystallography. The preliminary study on enzyme stability was conducted by using online software ERIS, FoldX and MAESTRO. The stability of the mutant 2DC lipase was tested virtually and molecular dynamic simulation was performed in water and methanol solvent. Experimentally, the purified protein of mutant 2DC lipase was used to screen protein crystal for diffraction to elucidate and validate the mutant's structure. It showed that the substitution of amino acid S2 and A384 with cysteine could enhance the stability of the enzyme by promoting the formation of disulphide bond to tighten the both terminal ends of the protein structure. The substitution of amino acid cysteine showed the changes on the active site distance (S113, D317, and H358), however, it was not affected the lipase activity and folding of protein structure. The 2DC mutant was successfully constructed and cloned into pET32-b and transformed into Origami B (DE3) expression host. The expression and purification using Ni^{2+}-Sepharose affinity chromatography and gel filtration

chromatography S-200 of the protein yielding 4.0 mg/ml mutant 2DC lipase suitable for protein crystallization. The mutant 2DC lipase was crystallized after 24-hour incubation at 20°C and diffracted by X-ray crystallography for deeper evaluation in term of stability and rigidity. The crystal was diffracted at 2.04 Å using in-house X-ray beam and the crystal belongs to monoclinic space group C2, with unit cell parameter of a = 118.17, b = 81.5, c = 100.05. Details information on structural elucidation of the mutant 2DC lipase has disclosed the changes within the mutant structure which associated with the alteration of enzyme activity and stability posed by the mutant. The increased in rigidity of the structure as well as changes of interaction within the catalytic region of the mutant 2DC lipase were suggested to be the factor influenced its enzymatic activity, stability and tolerance towards methanol. Hence, this newly improved mutant 2DC lipase could be the next potential biocatalyst in biodiesel production industry.

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

PENENTUAN STRUKTUR KEATAS MUTAN TOLERANSI METANOL YANG DIPERBAIK DARI *Geobacillus zalihae* T1 LIPASE MELALUI KRISTALOGRAFI X-RAY

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Lipase merupakan sejenis enzim yang serba boleh dan telah diubahsuai melalui pelbagai kaedah untuk meningkatkan kekuatan pada sifat enzimnya. Termostabil enzim, T1 lipase daripada *Geobacillus zalihae* mampu menjadi bio-pemangkin dalam industry penghasilan biodiesel. Sejak dulu, lipase dari pelbagai sumber telah dikaji untuk dijadikan sebagai biopemangkin dalam penghasilan biodiesel. Ada diantaranya telah ditambah baik prestasi dan kestabilannya melalui kaedah kejuruteraasan protein. Namun demikian, pentakaktifan yang berlaku pada T1 lipase semasa berada dalam persekitaran pelarut organik metanol yang pekat, telah mengehadkan fungsi enzim ini untuk kegunaan industri. Kehadiran interaksi tanpa ikatan dikatakan tidak mampu menambah baik kestabilan enzim. Oleh itu, dengan mewujudkan ikatan disulfida dalam struktur enzim, ia berkemungkin akan membantu mengekalkan kestabilan enzyme dalam pelarut organik metanol. Justeru, penyelidikan ini adalah bertujuan untuk menghasilkan lipase yang mempunyai toleransi terhadap metanol melalui mutagenesis terarah tapak serta merungkai interaksi yang menstabilkan mutan tersebut melalui proses kristalografi sinar-X. Penyelidikan preliminari ke atas kestabilan enzim telah diramal melalui perisian atas talian seperti ERIS, FoldX and MAESTRO. Kestabilan mutan 2DC lipase telah diuji secara maya dan simulasi dinamik dengan kehadiran air dan pelarut metanol. Seterusnya, protein tulen mutan ini telah disaring bagi menghasilkan protein kristal untuk merungkai dan mengenalpasti strukturnya. Berdasarkan ramalan, penggantian asid amino S2 dan A384 kepada sisteina mampu menambah baik kestabilan enzim serta menyumbang kepada penghasilan ikatan disulfida untuk menguatkan ikatan diantara penghujung kedua-dua terminal struktur protein. Penggantian asid amino sisteina telah mengubah jarak di antara tapak aktif (S113, D317, dan H358), namun ia tidak menggangu aktiviti lipase dan struktur lipatan protein. Mutan lipase 2DC telah berjaya dikonstruk dan diklon ke dalam vektor pET32-b dan ditransform ke dalam perumah pengekspresan Origami B (DE3). Pengekspresan dan penulenan protein melalui kromatografi keafinan dan

kromatografi gel telah menghasilkan 4.0mg/ml protein mutan tulen untuk proses kristalografi. Kristal mutant 2DC berjaya dihasilkan selepas pengeraman selama 24 jam pada suhu 20°C. Kristal tersebut telah dibelau untuk kajian stabiliti dan ketegaran. Kristal mutan lipase 2DC telah dibelau pada resolusi 2.04 Å dan ia telah dikenalpasti tergolong dalam kumpulan monoklinik C2 dengan unit sel parameter a = 118.17, b = 81.5, c = 100.05. Maklumat yang diperoleh telah merungkaikan struktur mutan lipase 2DC yang berkait dengan tindak balas aktiviti, kestabilanserta toleransi terhadap larutan metanol. Berdasarkan kajian, penghasilan mutan baru 2DC lipase ini mampu menjadi biopemangkin yang baik dalam industri penghasilan biodiesel.

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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

α	Alpha
Å	Angstrom
A_{600nm}	Optical density at wavelength 600 nanometer
ΔΔG	delta-delta energy
Amp	ampicillin
APS	Ammonium persulfate
APIs	Active Pharmaceutical Ingredients
β	Beta
bp	Base pair
CaCl	Calcium chloride
CD	Circular Dichroism
C_e	equilibrium concentration
COOT	Crystallographic Object-Oriented Toolkit
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylene-diamine-tetra acetic acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kan	kanamycin
kb	Kilobase
kDA	kilodaltons
LB	Luria-Bertani
M	Molar

MDS	Molecular Dynamic Simulation
MetOH	Methanol
Na	Sodium
NGS	Next-Generation Sequencing
NJ	Neighbor-Joining
NMR	Nuclear Magnetic Resonance
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PEGs	Polyethylene glycols
PHENIX	Python-based Hierarchical Environment for Integrated Xtrallography
pI	isoelectric point
Rgyr	Radius of Gyration
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation
rpm	revolutions per minute
SASA	Solvent Accesible Surface Area
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
tet	tetracycline
YASARA	Yet Another Scientific Artificial Reality Application
Zn	zinc

CHAPTER 1

INTRODUCTION

1.1 Background

Lipases (E.C. 3.1.1.3) catalyze the hydrolysis of triacyl-glycerides into fee fatty acids at the water-oil interface, and transesterification reactions in a low-water environment (Ghosh et al., 1996). The hydrolysis of carboxylic acid ester linkages to free fatty acids (FFAs) and organic alcohols is catalyzed by lipases at the organic-aqueous interface when there is an excess of water present (Melani et al., 2020). Lipases display great tolerance towards various pH levels, temperatures, polar and non-polar solvents, and metal ions (Verma et al., 2012). Structurally, all lipase families possess an α/β hydrolase conserved fold, Gly-Xaa-Ser-Xaa-Gly motif and a conserved catalytic triad composed of serine, histidine and aspartate/glutamate residues (Messaoudi et al., 2011; Jo et al., 2021). The α/β hydrolase fold has a central β-sheet consisting of eight strands, with some variation in length among different species. The solvent accessible catalytic triad for substrate binding is protected by lid domains, which often expose the active sites during artificial activation in the presence of hydrophobic substances for enzymatic activity (Jaeger et al., 1999; Chen et al., 2022).

Structure modification via protein engineering is a useful approach to alter part of an enzyme, which could enhance its structure, function and stability (Kumar et al., 2016). Certain approaches are highly recommended for protein modification, such as rational design, directed evolution, semi-rational design, and *De novo design* (Liu et al., 2019). These approaches are implemented to enhance the properties and interactions of the enzymes such as the catalytic region, enzyme folding, and metal binding site, and non-covalent and covalent interactions.

The stability of most proteins is often perturbed by reaction conditions such as temperature and non-aqueous solvents. The substitution of hydrophilic residues on the protein surface plays an important role in thermostability by permitting interaction with the solvent molecules and reducing the entropic strain of the protein structure (Khurana et al., 2011). The replacement of some neutral amino acids with basic amino acids has been shown to increase organic solvent stability of LST-03 lipase from *Pseudomonas aeruginosa*. The replacement of amino acid at the enzyme surface protects the buried region towards the organic solvents molecules (Kawata & Ogino, 2009).

Recently, the effect of mutation points in creating disulphide bridge on *Geobacillus thermoglucosidans* STB02 has highlighted the role of disulphide bridge in thermostability without protein conformation disturbance (Li et al., 2020). In addition, the presence of disulphide bond could decrease entropic change, unfolding and increase in the structure rigidity. Even so, the location of disulphide bond, geometric structure (dihedral angles, and steric hindrance), and local flexibility (destabilizing energy, B-factor, and RMSD) should all be taken into consideration before modification (Xu et al., 2020). However, enhanced thermostability does not necessarily contribute to organic solvent stability. Korman & co-workers reported that the addition of disulphide bond does not influence the methanol tolerance for native PML (Korman et al., 2013).

Though, in this study, elucidating the structure of disulphide mutant lipase was proposed to provide a combined solvent and thermal stability and focusing on improving the performance of T1 lipase when using in harsh condition such as high concentration of methanol in biodiesel production as a biocatalyst. The polarity of increasing methanol concentration to predict stability in MetOH lead to the observation of intramolecular and protein-solvent interaction patterns validated experimentally and characterized by numerical indices. Therefore, by introducing newly bonded covalent interaction such as disulphide bond which connected between the N-termini and C-termini of the enzyme structure, it could help in enhancing the activity as well as the stability of the T1 lipase towards methanol solvent and water.

1.2 Objectives

The main aim of this project was to engineer a methanol-tolerant lipase from the wild-type T1 lipase and unveil the interactions that stabilizes and increase its performance in the presence of organic solvent, methanol. Therefore, the specific objectives are:

1. To characterize the effect of different methanol solvent concentration on the structural stability of the mutant 2DC lipase via *in-silico* analysis.

2. To further explore the effects of disulphide bonds on the properties of mutant 2DC lipase biochemically and biophysically.

3. To validate the structure of the newly construct, mutant 2DC lipase by X-ray crystallography technique.

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