UNIVERSITI PUTRA MALAYSIA

MUTATIONAL EFFECTS ON ENHANCING THE STABILITY OF Geobacillus zalihae T1 LIPASE IN NON-AQUEOUS ORGANIC SOLVENTS

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By

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Chairman : Associate Professor Adam Leow Thean Chor, PhD
Faculty : Biotechnology and Biomolecular Sciences

Lipases are one of nature`s most endowed group of proteins when considering their broad functional biotechnological and industrial relevance. The fundamental and technological conditions requirements for enzymes hampers the application of lipases as biocatalysts. Central to these challenges are the space and time in prospecting for natural enzymes with biocatalytic properties. In this respect, naturally obtained lipases are engineered and designed into biocatalysts that can efficiently be used. Inspired by the proven thermostability and diminished solvent stability of a lipase from *Geobacillus zalihae*, this dissertation addresses the impediment of solvent stability by way of directed evolutionary construction of mutant variants capable of maintaining important structural elements, protein folding and stability in high concentrations of organic solvents. Firstly, the behavior of T1 lipase was investigated in hydrophilic chain length organic solvents by molecular dynamic simulations. For this purpose, the dynamics, and the conformational changes folding transitions, stability and structural dynamics which alters interactions between solvent molecules and amino acid residues was investigated. The RMSD revealed the effects, decreasing solvent polarity had on the protein`s simulation dynamics and equilibrium state. Residue motions were influenced greatly in butanol and pentanol water mixtures. Comparatively the residue RMSF and SASA was correspondingly higher to flexibility and *vice-versa*. More hydrogen bonds in methanol, ethanol, and propanol water mixtures were formed and thus, it is assumed that correlated increase in intraprotein hydrogen bond is linked to stability of the protein. Solvent accessibility analysis revealed an exposure of hydrophobic residues in all solvent mixtures with polar residues buried away from the solvent. Furthermore, it was observed that the active site pocket was not conserved in organic solvent mixtures. This attribute was proposed to be responsible for the weakened strength in the catalytic H-bond network and most likely a drop in catalytic activity. Altogether, the data obtained suggests that the solvent-induced lid domain conformational opening was gradual. The additional formation of cooperative network

of hydrogen bonds and hydrophobic interactions could render stability to the protein in some solvent system. Dynamic cross-correlated atomic motions between the atoms from atomic coordinates was in concerted functional network with regions of residues of the lid domain. *Geobacillus zalihae* T1 lipase was used as a parent lipase for random mutagenesis and mutant variants with stability in polar organic solvents were constructed. The solvent stability of the mutant variants in a broad range of 50, 60 and 70 % of methanol, ethanol, propanol, butanol, and pentanol at a temperature of 60 ºC was retained in six (6) mutants A83D/K251E, R21C, G35D/S195N, K84R/R103C/M121I/T272M, R106H/G327S. Mutant A83/K251E acquired enhanced organic solvent stability with higher stability in methanol as compared to other mutants. The models of these mutants as well as each mutation residue built *in silico* and analyzed for their conformational stability, showed significant stable conformational fold of mutants. Structural analysis of various networks of covalent interactions of the mutant models was found to reveal further formation of hydrogen bonds and hydrophobic networks which stimulated folding and stability. Site-directed mutagenesis constructs of beneficial single mutants G35D, A83D, M121I, S195N, K251E, T272M and G327S was further resolved. Significantly, butanol and pentanol diminished stability of mutants whereas about 60 % of residual stability was maintained particularly for methanol in mutants M121I, S195N and T272M. Furthermore, stable single mutants assembled in a combinative approach via site-directed mutagenesis, yielded mutants A83D/M121I/K251E/G327S and A83D/M121I/S195N/T272M with improved stability towards 50, 60, and 70 % methanol, ethanol, and propanol. Kinetic investigation showed higher k_m and V_{max} ranging from 0.003529 µM and 588 µmoles/min/mL respectively, for best mutants. The half-life was significantly higher for all mutant proteins in methanol, although the mutants had better exponential decay constant. Visible circular dichroism (CD) on the possible changes of the secondary structure of selected improved mutants in 50 % and 60 % methanol, showed overall, thermally-induced unfolding of mutants accompanied with some loss of secondary structure content at relative methanol solvent conditions. Perturbations on the protein matrix, including a significant net loss of secondary structure triggered a secondary structure reorganization that led to an increase or decrease in the structural elements content. The spectral differences in 50 % methanol suggests a considerable peak shifts in all mutant proteins as compared to that in buffer. The secondary structure formation of the α-helix, β-sheets, β-turns and random coil were preserved among all mutant proteins with observed changes in the β-turn. This illustrates how changes in the structural organization are intertwined with conformational interplay of the protein backbone in organic solvents. The relative contribution of various structural interactions in respect to the overall protein stability of the best mutants via molecular dynamics simulations revealed the interplay between structural features and the conformational stability of the protein. Changes in residue motions leads to the proposition that the higher stability in some mutants may not appear to be directly correlated to the hydrophobicity of residues. Protonation of some residues also affected both the stability and the conformational dynamics of the protein fold. Evidence of gain in both hydrogen bonds and hydrophobic interactions indiscriminately contributed to overall stability. Observations derived from MD simulations, suggests that hydrophilic polar organic solvents play important role in the dynamical conformational diversity of proteins. Short distances of radial distribution function provided the required distance of interaction between atoms which enables

hydrogen bond formation and hydrophobic interactions for stability. The solvent stability and secondary structural characteristics of mutants A83D/M121I/S195N/T272M and A83D/M121I/K251E/G375S indicates robust and improved variants that can act as biocatalyst for industrial applications. Newly formed structural interactions between mutant residues and other surrounding residues will enhance the native conformation flexibility in non-aqueous reaction media and hence promoting stability.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KESAN MUTASAN UNTUK MENINGKATKAN KESTABILAN *Geobacillus zalihae* T1 LIPASE PADA PENYELESAIAN ORGANIK NON-AQUEOUS

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Disember 2017

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Lipase adalah salah satu protein semulajadi yang mana ia banyak menyumbang kepada kepelbagaian fungsi dalam bidang bioteknologi dan perindustrian. Keperluan asas dan syarat teknologi untuk enzim telah menghalang penggunaan *lipase* sebagai biopemangkin. Ruang dan masa adalah cabaran utama dalam pencarian enzim semulajadi yang mempunyai ciri ciri biopemangkin. Oleh yang demikian, enzim semulajadi lipase telah digubah dan direka bentuk menjadi biopemangkin yang boleh digunakan dengan berkesan. Diilhamkan dari bukti kajian kestabilan suhu dan pengurangan kestabilan pelarut oleh enzim *lipase* dari *Geobacillus zalihae*, disertasi ini membincangkan halangan kestabilan pelarut melalui pembentukan varian mutan dengan cara evolusi terarah yang berpotensi dapat mengekalkan unsur-unsur struktur yang penting, lipatan protein, dan kestabilan dalam pelarut organik yang berpekatan tinggi. Pertama, tindakbalas enzim T1 *lipase* telah dikaji dalam rantai panjang pelarut organik hidrofilik melalui simulasi dinamik molekul. Melalui kaedah ini, dinamik, peralihan perubahan konformasi lipatan, kestabilan, dan struktur dinamik yang mengubah interaksi antara molekul pelarut dengan sisa-sisa asid amino dapat dikaji. Punca min persegi sisihan (RMSD) telah menunjukkan kesan pengurangan polariti pelarut terhadap dinamik simulasi protein dan keadaan keseimbangan. Gerakan-gerakan sisa telah banyak berlaku di dalam campuran air butanol dan pentanol. Secara relatif, sisa RMSF dan SASA adalah lebih tinggi dari fleksibiliti dan sebaliknya. Lebih banyak ikatan hydrogen dalam campuran air dengan methanol, etanol, dan propanol telah terbentuk yang mana ia menunjukkan bahawa kenaikan dalam ikatan hidrogen intraprotein berkait rapat dengan kestabilan protein. Analisis kebolehcapaian pelarut menunjukkan pendedahan sisa-sisa hidrofobik dalam semua campuran pelarut dengan sisa polar telah tertimbus jauh dari pelarut. Selain itu, poket tapak aktif didapati tidak abadi di dalam campuran pelarut organik. Atribut ini menunjukkan ia mempengaruhi kelemahan dalam rangkaian katalitik ikatan hidrogen dan berkemungkinan dalam

penurunan aktiviti pemangkin. Keseluruhannya, data yang diperolehi telah menunjukkan bahawa pembukaan konformasi tudung domain teraruh-pelarut adalah secara beransur-ansur. Pembentukan tambahan rangkaian ikatan-ikatan hidrogen dan interaksi hidrofobik dapat menstabilkan protein dalam beberapa system pelarut. Pergerakan atom korelasi-silang yang dinamik antara atom-atom dari koordinat atom adalah berada dalam rangkaian yang berfungsi dengan sisa-sisa kawasan tudung domain. Enzim *Geobacillus zalihae* T1 *lipase* telah digunakan sebagai enzim *lipase* induk untuk mutagenesis secara rawak dan varian mutan dengan pembentukkan kestabilan dalam pelarut organik polar. Kestabilan pelarut varian mutan dalam 50, 60 and 70 % kepekatan metanol, etanol, propanol, butanol and pentanol pada suhu 60 °C telah dikekalkan ke atas enam (6) mutan A83D/K251E, R21C, G35D/S195N, K84R/R103C, M121I/T272M, dan R106H/G327S. Mutant A83D/K251E menujukkan peningkatan kestabilan dalam pelarut organik methanol berbanding mutan-mutan lain. Model-model mutan ini dan setiap sisa mutasi yang dibentuk secara siliko dan kestabilan konformasi yang telah dianalisa menunjukkan kestabilan yang ketara pada lipatan konformasi mutan tersebut. Analisis struktur ke atas pelbagai jenis rangkaian interaksi kovalen model mutan telah mendedahkan banyak pembentukan ikatan hidrogen dan rangkaian hidrofobik yang mana ianya telah merangsang lipatan dan kestabilan. Mutasi tunggal yang bermanfaat, G35D, A83D, M121I, S195N, K251E, T272M dan G327S telah diselesaikan melalui mutagenesis tapak-terarah. Secara ketara, butanol dan pentanol telah menghalang kestabilan mutan manakala hampir 60 % kestabilan sisa telah dapat dikekalkan terutamanya dalam larutan methanol oleh mutan M121I, S195N dan T272M. Selain itu, mutant-mutan tunggal yang stabil telah dihimpun dalam satu pendekatan gabungan melalui mutagenesis tapak-terarah, yang mana telah menghasilkan mutan A38B/M121I/K251E/G327S dan A83D/M121I/S195N/T272M dengan kestabilan yang lebih baik terhadap 50, 60, dan 70 % kepekatan metanol, etanol, dan propanol. Kajian kinetik telah menunjukkan peningkatan tinggi km dan Vmx berjulat dari 0.003529 µM dan 588 µmoles/min/mL untuk mutan yang terbaik. Setengah hayat semua protein mutan adalah jauh lebih tinggi dalam larutan metanol walaupun ia mempunyai pemalar pereputan eksponen yang lebih baik. Dichroism lingkaran ketara (CD) ke atas struktur sekunder mutan terpilih yang berpotensi berubah dalam larutan methanol berpekatan 50 % dan 60 %, secara keseluruhannya menunjukkan pembentukkan teraruh secara terma oleh mutan-mutan serta kehilangan beberapa kandungan struktur sekunder pada keadaan pelarut methanol relatif. Pertubasi pada matriks protein serta kehilangan struktur sekunder yang ketara menyebabkan penyusunan semula struktur sekunder yang membawa kepada peningkatan dan pengurangan kandungan unsur-unsur sekunder. Perbezaan spektrum dalam kepekatan 50 % metanol menunjukkan anjakan puncak yang besar dalam semua mutan protein berbanding dalam penimbal. Pembentukan struktur sekunder, α-heliks, β-lembaran, β-lingkaran dan gegelung rawak adalah abadi dalam semua mutan protein dengan perubahan yang diperhatikan dalam β-lingkaran. Ini telah menunjukkan bagaimana perubahan-perubahan dalam penyusunan semula struktur saling berkaitan dengan interaksi konformasi tulang belakang protein dalam pelarut organik. Sumbangan relatif pelbagai interaksi struktur terhadap keseluruhan kestabilan protein mutan yang terbaik melalui simulasi dinamik molekul menunjukkan interaksi antara ciri-ciri struktur dan kestabilan konformasi protein tersebut. Perubahan pergerakan sisa menyumbang kepada penerangan mengenai kestabilan yang tinggi oleh mutan mungkin tidak berkaitan secara langsung dengan sisa-sisa

hidrofobik. Protonasi oleh sesetengah sisa juga memberi kesan kepada kestabilan dan dinamik konformasi lipatan protein. Penambahan ikatan hidrogen dan interaksi hidrofobik membuktikan ianya secara langsung menyumbang kepada kestabilan secara keseluruhan. Pemerhatian berdasarkan simulasi dinamik molekul menunjukkan bahawa pelarut organik polar hidrofilik memainkan peranan yang penting dalam kepelbagaian konformasi protein. Jarak dekat fungsi taburan jejarian telah menunjukkan jarak interaksi antara atom yang diperlukan untuk pembentukkan ikatan hidrogen dan interaksi hidrofobik untuk kestabilan protein. Kestabilan pelarut dan ciri-ciri struktur sekunder mutan A83D/M121I/S195N/T272M dan A83D/M121I/K251E/G375S menunjukkan varian yang telah diperbaik dan kuat boleh bertindak sebagai biopemangkin untuk kegunaan industri. Interaksi struktur yang baru terbentuk di antara sisa-sisa mutan dan sisa-sisa persekitaran lain akan meningkatkan fleksibiliti konformasi asli dalam media reaksi yang bukan berair.

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

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LIST OF ABBREVIATIONS

cm	centimetre
dH_2O	distilled water
EDTA	ethylene diamine tetraacetic acid
g	gram
g/L	gram per litre
hr	hour
IPTG	isopropyl β-D Thiogalactoside
kDa	kilo Dalton
L	liter
M	molar
mM	millimolar
mg	milligram
mL	milliliter
min	minute
ORF	open reading frame
PCR	polymerase chain reaction
DNA	deoxyribonucleic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED	N, N, N, N-Tetramethyllenediamide
µg	microgram
µL	microliter

v/v	volume per volume
w/v	weight per volume
CD	Circular dichroism
FAMEs	fatty acid methyl esters
LB	Luria bertani
pNPP	p-nitrophenyl palmitate acid
rRNA	ribosomal ribonucleic acid
ALA	α-linolenic acid
ISM	iterative saturation mutagenesis
MSSM	multi-site saturation mutagenesis
NDT	nucleobase-guanine-thymine
ePCR	Error-prone polymerase chain reaction
PEG	Polyethylene glycol
DMSO	Dimethyl Sulphur oxide
DiFMU	Difluoro-4-methyl-lumlliferyl
BSA	Bovine serum albumin
MD	Molecular Dynamic simulations
RMSD	Root mean square deviation
RMSF	Root mean square fluctuations
SASA	Solvent accessible surface area
YASARA	Yet Another Scientific Artificial Reality Application
DSSP	Dictionary of secondary structure for protein.
DCCM	Dynamic cross correlation matrix
IDT	Integrated DNA technology

α	Alpha
β	Beta
°C	Degree Celsius
%	Percentage
A_{600nm}	Optical density at wavelength 600 nanometer
µm	Micrometer
µmoles	Micromoles
APS	Ammonium persulfate
bp	Base pair
$CaCl_2$	Calcium chloride
$MgCl_2$	Magnesium chloride
HCL	Hydrochloric acid
K	Kelvin
Rgyr	Radius of gyration
Å	Armstrong
ns	Nanosecond
pNP	p-Nitrophenols
UV	Ultraviolet
U	Unit
OD	Optical density
CV	Column volume
MtOH	Methanol
EtOH	Ethanol
PrOH	Propanol
BtOH	Butanol

PtOH	Pentanol
ΔΔG	Free energy
PDB	Protein Data Bank
RDF	Radial distribution function
Π	Pi
OH	Hydroxyl
O	Oxygen
H_2O	Water
rpm	Revolution per minute
$\times g$	Gravitational force

CHAPTER 1

INTRODUCTION

1.1 Introduction

Enzymes in the presence of organic solvents undergo denaturation and inactivation. Although they possess desirable qualities, their solvent instability in many process formulations has hampered their widespread industrial application. Most enzyme-based processes is in monophasic systems mixtures which allow increased solubility of substrates, thermodynamic equilibria shift in favour of synthesis over hydrolysis, and eliminates probable chances of microbial contamination (Gupta, 1992; Mattos and Ringe, 2001). However, distortion of enzymes by organic solvents may be in a competitive manner through specific interactions between enzymes and solvent molecules, creating changes in both reaction kinetics and substrate specificity. Monophasic Water-miscible organic solvent systems are often desirable to prevent diffusional resistance of substrates and products within the water and organic solvent interface. Interactive contact between organic solvent molecules and enzymes can result in distortion of enzyme structure, rapid denaturation, and inactivation (Jeong et al., 2002).

Advances in protein engineering and design has tremendously established enzymes as environmentally friendly alternative biocatalysts to traditional metal and organic dependent catalysts in industrial processes (Bornscheuer et al., 2012). Many product processes utilize enzymes in their manufacture, including chemical synthesis, production of agrochemical and pharmaceutical intermediates, and food ingredients (Schoemaker et al., 2003). Most industrial enzymes which find applications in the detergent, textile, leather, personal care and pulp and paper, and in animal feed supplements have been reported (Cherry & Fidantsef, 2003). Since enzymes are highly adaptable molecules and amenable to improvements, enhancing their properties through natural evolution is apparently the most significant breakthrough in obtaining variants with versatile adaptable functions. Efforts to enhance the functional integrity of proteins to carry out a given function requires far more sophistication (Rice 2008). In this light, protein engineering principles have made much progress in recreating and expanding enzyme natural functions.

Molecular dynamics simulations provide useful details of the interactions between enzymes and organic solvents whereby enzyme-solvent interaction can be understood and used to develop a stabilization strategy. Successful rational design of proteins is information intensive, and the limitations with this approach are that knowledge on how a protein's sequence of amino acids dictates its final shape, physical and biochemical properties is not fully predictable. Directed evolution over the last decade has been used to enhance and achieve the following breakthroughs, for examples; directed evolution and rational designs, as well as metabolic engineering, have been successfully used in enhancing biocatalytic properties and tolerance to alcohols

reaction conditions by lipases (Korman et al., 2013; Li, Zong, and Wu, 2009; Dusséaux et al., 2013); Thermostable lipase variants are obtained via a combination of rational design and saturation mutagenesis (Cesarini et al., 2012); Thermal adaptation of cold active and methanol tolerant lipase have also been modified via directed evolution (Korman et al., 2013; Gatti-Lafranconi et al., 2010); Organic solvent effect on enzyme hydration and solvent binding in the active site has also been investigated via MD simulations (Yang, Dordick, & Garde, 2004); site-directed mutagenesis was used to enhance the organic solvent stability by replacement of hydrophobic residues on the enzyme surface (Shokri et al., 2014; Martinez et al., 1992); Structure-guided consensus and random mutagenesis yielded beneficial mutations of residues which could have direct interactions with the solvent (Dror et al., 2014); Site saturation mutagenesis of the entire surface amino acids residues spanning the loop of a lipase resulted in increased surface polarity while enhancing stability in organic solvent (Yedavalli, & Madhusudhana Rao, 2013). The underlying reliability of this approach is the bias introduction of modifications in the amino acid sequence which also allows traversing of the local fitness landscapes in proteins. With a high throughput screening, the best variant of the active form of the enzyme is obtained. Therefore, in this study engineering, the weak spots associated with solvent stability of a *Geobacillus zalihae* T1 lipase, by computer-assisted protein simulations with lipase engineering can be a valuable approach towards enhancing stability and refining catalytic properties in an organic solvent in overcoming its industrial limitations.

1.2 Research hypothesis

1. The *in silico* evaluation of T1 lipase can provide insights in creating new hydrogen bonds, salt bridges and disulphide bond which will stabilize the protein in organic solvents.
2. The engineering of distinct amino acid motifs can enhance T1 lipase stability and functionality through a cluster network of electrostatic interactions.

1.3 Problem statement

The uses of chemical catalysts for the most industrial process have had several limitations especially in product recovery and contamination. Suitable enzyme biocatalysts are considered as preferred alternatives because they can easily be recovered, and production contamination does not occur. However, the organic solvent stability of these enzyme biocatalysts is a major drawback, which can be addressed by modifying their weak spots.

1.4 Research questions

1. Can the parental T1 lipase be stable in various chain length polar solvents?
2. What are the potential amino acids residues in the T1 lipase that can be mutated to improve organic solvent stability?
3. Can a functional and organic solvent stable T1 lipase be produced?

1.5 Objectives

The overall objective is to develop organic solvent stable lipase as potential biocatalysts by directed evolution.

1. To investigate the *in silico* effect of various polar hydrophilic solvents on structural stability and conformation of *Geobacillus zalihae* T1 lipase.
2. To develop organic solvent stable T1 lipase variants using random mutagenesis and activity screening.
3. To perform high-throughput screening and characterization of organic solvent stable T1 lipase variants.
4. To construct a combinatorial mutant library of solvent stable T1 lipase variant by site-directed mutagenesis.
5. To assess the structural basis of developed organic solvent stable T1 lipase variants *in silico*.

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