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REDESIGNING OF Geobacillus zalihae T1 LIPASE BASED ON SPACEGROWN CRYSTAL STRUCTURE

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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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REDESIGNING OF *Geobacillus zalihae* T1 LIPASE BASED ON SPACE-GROWN CRYSTAL STRUCTURE

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Chair : Professor Raja Noor Zaliha Raja Abd Rahman, D. Engr.
Faculty : Biotechnology and Biomolecular Sciences

A microgravity environment is a favorable condition meant for growing a protein crystal, due to less sedimentation and convection. These factors would have benefited the protein crystal in terms of morphologies, crystal quality and appearance, which are important in producing a high quality electron density map. Nevertheless, the differences of structural architecture and features in protein related to the formation of hydrogen bonds and ion interactions remains unclear. In order to understand the relative contributions of a space atomic model in protein structure stability, it was necessary to compare the structure with the one grown on earth condition. There are existing limitations about manipulation of structural information for production of new enzyme due to insufficient analysis of both structures. Therefore, an earth and space condition crystal structures from a thermostable T1 lipase of *Geobacillus zalihae* were analyzed and compared. It was anticipated that the differences in hydrogen bonds and ion interactions are the main contributing factors towards protein stabilization. A molecular dynamics simulations approach was used to study differences of atomic fluctuations and conformational changes of both T1 lipase structures. From here, the structures stability was determined by a set of parameters comprising root mean square deviation (RMSD), radius of gyration, and root mean square fluctuation (RMSF) in which the results showed a more stable space-grown structure compared to the earth-grown structure due to the presence of more hydrogen bonds. According to the *in silico* data, hydrogen bond interactions at position Asp43, Thr118, Glu250 and Asn304 and ion interaction

at position Glu226 were chosen to imitate the space-grown crystal structure. Following that, the impact of combined interactions in mutated structure of T1 lipase was studied. The molecular interactions of five single mutants and the one that combined all five mutations, 5M were predicted based on structural changes and energy landscape by GROMACS simulation package. Site directed mutagenesis was applied on wild-type HT1 (wt-HT1) lipase to generate five single mutants (D43E, T118N, E226D, E250L and N304E), in which these sites were further combined by a gene synthesis to generate a new mutant showing five mutation points (D43E/T118N/E226D/E250L/N304E). The native lipase wt-HT1, single mutants and 5M mutant lipases were purified by affinity chromatography showing a recovery between 49.6 to 59.9% and a purification fold of 2.5 to 3.3. All lipases exhibited high activity at 60 to 80 °C. Mutants E250L and N304E shifted in optimum temperature to 80 °C as compared to wt-HT1 lipase. All lipases showed high activity at alkaline conditions of pH 6.0 to 9.0. The thermostability study indicates the mutant E226D as the most stable lipase having prolonged half-life ($T_{1/2}$) values and melting temperature. A $T_{1/2}$ value of E226D was found at 28 hours, 165 minutes and 47 minutes at 60 °C, 70 °C and 80 °C, respectively where the mutant reportedly showing a melting temperature (T_m) of 77.4 ± 2.6 °C. In contrast, mutation of all five positions in the 5M mutant failed to increase the stability of lipase as the half-life at 60 °C exhibited a decline from 9 hours to 6 hours. At 70 °C and 80 °C, the half-life was found to be 23 minutes and 8 minutes, respectively. The melting temperature decreased 3.3 °C to 67.6 ± 0.8 °C. The presence of metal ions, especially calcium ion, had a positive effect on the stability of D43E, T118N, E250L and 5M lipases, which increased as more calcium was added. Meanwhile, Zn^{2+}, Cu^{2+}, Mg^{2+} and Fe^{3+} ions inhibited the activity of lipases. In addition, the activities of D43E, T118N and 5M lipases increased in the presence of DMSO. All lipases showed a good hydrolysis rate in natural oil, except for coconut oil. All lipases shown to have loss in activities in the presence of surfactants and sodium dodecyl sulfate (SDS). In the presence of calcium ion, the stability of 5M mutant and wt-HT1 lipases were increased towards high temperatures and organic solvents. The presence of calcium prolonged the half-life of 5M and wt-HT1, and increased the T_m at 8.4 and 12.1 °C, respectively. The combination of substituted amino acid had produced a highly stable mutant hydrolyzing oil in selected organic solvents such as DMSO, n-hexane and n-heptane. To correlate mutations in 5M mutant with its structural transition, 5M mutant lipase was subjected to crystallization in 0.5 M sodium cacodylate trihydrate, 0.4 M sodium citrate tribasic pH 6.5 supplemented with 0.2 M sodium chloride (NaCl). The protein structure was elucidated at resolution 2.64 Å with 90.9% completeness. The crystal structure of 5M mutant consists of two asymmetric units that are similar to each other, with RMSD value of 0.7789 Å after superimpositions of chains A and B. The structure analysis revealed that 5M failed to introduce hydrogen bonds and ionic interaction at the intended positions. The cumulative mutations also resulted in decreasing in molecular

interactions such as hydrogen bonds and interactions. The impacts of the mutations resulted in decreasing in stability and half-life of lipase against high temperature. As a conclusion, it is difficult to emulate the cumulative interactions happened in the space-grown T1 lipase as shown by mutant 5M. Nonetheless, lipases containing a single mutant of D43E and E226D were found to be successful in introducing and increasing the mutant stability, where the stability of protein structure was highly dependent on the role of hydrogen bonds and ion interactions.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
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REKA BENTUK SEMULA *Geobacillus zalihae* T1 LIPASE BERDASARKAN STRUKTUR HABLUR ANGKASA LEPAS

Oleh

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Kekurangan daya tarikan graviti di angkasa lepas yang mempengaruhi pemendapan dan daya perolakan menjadi persekitaran yang sesuai untuk pembentukan hablur protein. Factor-faktor ini memberi manfaat kepada pembentukan protein dari segi morfologi, kualiti dan rupa bentuk hablur yang merupakan faktor penting untuk penghasilan peta kepadatan elektron berkualiti tinggi. Walau bagaimanapun, ciri-ciri struktur yang berkait rapat dengan pembentukan ikatan hidrogen dan interaksi ion masih belum jelas sepenuhnya. Untuk memahami dengan lebih mendalam tentang sumbangan struktur tersebut yang dihasilkan di bawah persekitaran mikrograviti terhadap kestabilan protein, perbandingan struktur angkasa dengan struktur yang terhasil di bumi perlu dijalankan. Selain itu, manipulasi data yang diperolehi dari struktur enzim dari mikrograviti untuk penghasilan enzim baru yang lebih berkualiti amat terbatas kerana analisa yang tidak mencukupi. Oleh itu, struktur hablur T1 lipase *Geobacillus zalihae* yang diperolehi dari mikrograviti dan bumi dibandingkan dan dianalisa dengan lebih mendalam. Dijangkakan bahawa perbezaan dalam pembentukan ikatan hidrogen dan interaksi ion memberi sumbangan yang besar terhadap kestabilan protein. Pendekatan simulasi dinamik molekul digunakan untuk mengkaji perbezaan pergerakan atom dan perubahan konformasi kedua-dua struktur T1 lipase. Seterusnya, kestabilan protein ditentukan dengan menggunakan parameter seperti ralat punca min kuasa dua (RMSD), jejari legaran, dan ralat purata turun naik (RMSF) di mana hasilnya menunjukkan struktur mikrograviti lebih stabil berbanding dengan

struktur bumi dengan penghasilan lebih banyak ikatan hydrogen. Berpandukan kepada data *in silico* yang diperolehi, interaksi ikatan hidrogen pada kedudukan asid amino Asp43, Thr118, Glu250 dan Asn304 dan interaksi ikatan ion pada kedudukan Glu226 dipilih untuk meniru struktur hablur angkasa lepas. Seterusnya, kesan daripada interaksi gabungan kelima-lima kedudukan ikatan tersebut kepada struktur T1 lipase dikaji. Interaksi dan struktur mutan lipase yang terhasil iaitu lima mutasi tunggal dan satu mutasi gabungan (5M) diramalkan berdasarkan perubahan struktur dan landskap tenaga menggunakan kaedah simulasi menggunakan program GROMACS. Kaedah mutasi terarah digunakan untuk menghasilkan mutasi tunggal (D43E, T118N, E226D, E250L dan N304E) dengan menggunakan lipase asli (T1) sebagai templat, dan kaedah sintesis gen dilakukan untuk menggabungkan lima mutasi tunggal dalam satu protein (D43E/T118N/E226D/E250L/N304E). Lipase asli (wt-HT1), mutasi tunggal dan 5M (D43E/T118N/E226D/E250L/N304E) ditulinkan dengan kaedah kromatografi afiniti menunjukkan kadar pemulihan antara 49.6 hingga 59.9% dan lipatan penulinan sebanyak 2.5 hingga 3.3. Kesemua lipase menunjukkan aktiviti yang tinggi pada suhu 60 hingga 80 °C. Mutan E250L dan N304E menunjukkan perubahan dalam suhu optimum dengan mencatatkan aktiviti yang lebih tinggi pada suhu 80 °C berbanding lipase asli. Semua lipase menunjukkan kestabilan aktiviti pada keadaan beralkali pH 6.0 hingga 9.0. Kajian kerintangan haba menunjukkan bahawa mutan E226D adalah lipase yang paling stabil terhadap suhu tinggi dengan peningkatan nilai separuh hayat dan takat lebur. Ia mempamerkan nilai separuh hayat ($T_{1/2}$) sebanyak 28 jam, 165 minit dan 47 minit pada suhu 60 °C, 70 °C dan 80 °C dan mencatatkan suhu takat lebur pada 77.4 ± 2.6 °C. Walau bagaimanapun, gabungan lima mutasi asid amino (5M mutan) menghasilkan lipase yang kurang stabil berbanding lipase asli dengan penurunan nilai separuh hayat sehingga 6 jam pada 60 °C, 23 minit pada 70 °C dan 8 minit pada 80 °C dan penurunan suhu takat lebur dengan nilai 67.6 ± 0.8 °C iaitu 3.3 °C lebih rendah berbanding lipase asli (wt-HT1). Penambatan enzim dalam pelbagai ion logam menunjukkan bahawa ion kalsium mempunyai kesan yang luar biasa terhadap kestabilan lipase D43E, T118N, E250L dan 5M. Penambahan ion kalsium meningkatkan aktiviti mutan ini. Walau bagaimanapun, ion Zn^{2+}, Cu^{2+}, Mg^{2+} dan Fe^{3+} menghalang aktiviti lipase. Di samping itu, aktiviti lipase mutan D43E, T118N dan 5M telah meningkat secara mendadak dengan kehadiran DMSO. Lipase menunjukkan kadar hidrolisis yang baik dalam kebanyakan minyak semulajadi yang diuji. Walau bagaimanapun, semua lipase menunjukkan penurunan aktiviti apabila minyak kelapa digunakan. Kehadiran pelbagai jenis surfaktan didapati merencat aktiviti lipase terutamanya di dalam larutan SDS dengan hampir tiada aktiviti di kesan. Analisis struktur sekunder mendapati kesemua lipase menunjukkan perubahan yang tidak ketara. Oleh kerana aktiviti lipase meningkat dengan kehadiran ion kalsium, kestabilan mutan 5M dan wt-HT1 terhadap pelarut organik dan suhu dikaji dengan kehadiran kalsium. Hasil

kajian menunjukkan bahawa ion kalsium dapat meningkatkan kerintangan lipase terhadap pelarut organik dan suhu tinggi. Kehadiran kalsium telah memanjangkan tempoh separuh hayat mutan 5M dan wt-HT1, dan pada masa yang sama meningkatkan suhu takat lebur (T_m) masing-masing dengan peningkatan 8.4 dan 12.1 °C dibandingkan dengan suhu takat lebut tanpa ion kalsium. Kajian menunjukkan bahawa mutasi asid amino secara berkumpulan telah menghasilkan mutan yang sangat stabil ketika menghidrolisis minyak dalam pelarut organik tertentu seperti DMSO, n-heksana, dan n-heptane. Untuk mengkaji korelasi antara penggantian asid amino dalam 5M mutant dan perubahan struktur, mutan 5M telah dihablurkan dalam formulasi 0.5 M natrium cacodylate trihydrate, 0.4 M sodium sitrat tribasic pH 6.5 ditambah dengan 0.2 M natrium klorida dan telah dibelau pada resolusi 2.64 Å dengan kesempurnaan 90.9%. Struktur hablur mutan 5M terdiri daripada dua unit asimetri yang sangat serupa antara satu sama lain dengan nilai RMSD, 0.7789 Å apabila proses penindihan molekul A dan B dilakukan. Analisis pada struktur tersebut mendedahkan bahawa mutasi kumulatif gagal untuk menyerupai ikatan hidrogen dan interaksi ion pada kedudukan yang dikenalpasti seperti struktur mikrograviti. Keadaan ini menjelaskan penurunan kestabilan mutan 5M dibandingkan dengan mutan tunggal dan lipase asli. Secara ringkasnya, dapat disimpulkan bahawa matlamat untuk menyerupai interaksi yang terdapat pada struktur mikrograviti tidak tercapai seperti yang ditunjukkan dalam struktur mutasi kumulatif 5M. Walau bagaimanapun, interaksi dalam mutan tunggal seperti D43E dan E226D telah berjaya diperkenalkan dan telah meningkatkan kestabilan mutan. Oleh itu, dapat disimpulkan bahawa ikatan hidrogen dan interaksi ikatan ion sangat penting dan memainkan peranan yang besar dalam kestabilan struktur protein.

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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 Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

µL	Microliter
µmol	Micromole
Å	Angstrom
Bp	Base pair
DMSO	Dimetyl sulfoxide
G	Gram
H	Hour
IPTG	Isopropyl β-D-Thiogalactopyranoside
kDa	Kilo Dalton
L	Liter
M	Molar
mM	Milimolar
Mg	Milligram
mg/mL	Milligram per milliliter
Min	Minute
Ns	Nano second
PCR	Polymerase chain reaction
PDB	Protein Data Bank
Ps	Pico second
RMSD	Radius mean square deviation
Rpm	Rotation per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
$T_{1/2}$	Half-life
T_m	Melting temperature
U/mL	Unit per milliliter
v/v	Volume per volume
w/v	Weight per volume
X*g*	Relative centrifugal force

CHAPTER 1

INTRODUCTION

Microbial lipases are the most flexible and versatile enzymes compared to the other lipases derived from plants and fungi. Lipases derived from bacteria are commercially applied in various industries due to their feasible extraction process and having favorable properties and applications (Chouhan and Dawande, 2010). Lipase (triacylglycerol acylhydrolase E.C.3.1.1.3) is a class of enzymes belonging to the serine hydrolases responsible to hydrolyze carboxylic ester bonds and triacylglyceride esters into diglycerides, monoglycerides, free fatty acid and glycerols (Houde *et al*., 2004). These enzymes are known as important biocatalysts which are involved in various catalytic reactions such as interesterification, hydrolysis, esterification, acidolysis, aminolysis and alcoholysis (Kumari *et al*., 2009; Omar *et al*., 2016; Duarte *et al*., 2016; Abed *et al*., 2018; Tirunagari *et al*., 2018; Abdullah *et al*., 2019). As a leading biocatalyst in various bio-industries, it is common to observe improvements on lipases exhibiting special characteristic such as ability to withstand the presence of various organic solvents and high temperature. Lipases having different kinds of stability and flexibility displays resilience towards reactions which effectively take place under an extreme condition, whereby some reactions may require the presence of organic solvents, extremely high temperatures and salinity. Most of the time, enzymes are denatured under these unfavorable conditions. From a commercial perspective, a thermostable and organic solvent tolerant lipase is important for the application of fatty acid ester synthesis, such as biodiesel production as well as food and detergent industries (Sharma and Kanwar, 2014). Therefore, in order to fulfil demands for thermostable and organic solvent tolerant lipase, improvements on the structure and characteristic of such enzymes are needed to increase tolerance and stability for broad applications. Protein engineering is one of the leading methods to use for altering the enzyme characteristic following the changes of enzyme specificity, enzyme stability, regioselectivity and catalytic activity (Tiwari *et al*., 2012; Zhang *et al*., 2003; Wang *et al*., 2014).

Over the past decade, crystallization of proteins and biological components such as nucleic acids, nucleosomes, viruses, ribosomal subunits or nucleo-protein complexes under microgravity environment (~10^{-3} – 10^{-6} x g) has received attention among researchers. A condition where microgravity turns ideal for

macromolecular crystal growth concerns an environment of less convection and lack of sedimentation. Under these factors, the formation of high quality protein crystals with better internal orders will be obtained. More particularly, the crystallization of macromolecules under the said microgravity condition has proven to improve the size, perfection, morphology and internal orders of the protein crystal (Snell and Helliwell, 2005). Such microgravity-grown crystals can diffract to high resolution and have a lower mosaic spread which defines the quality of the crystals. High quality protein crystals are essential to determine their structure via diffraction analysis. Hence, the improvement of protein crystals via microgravity leads to better understanding of structural mechanism and biological activity for the production of high quality enzymes with good characteristics.

Over time, the number of enzymes being crystallized in space had increased and elaborately discussed by scientists surrounding the findings of morphologies, crystal quality and the appearances of enzymes crystal. The productions of good quality crystals in the microgravity condition and their characteristics of crystal formed are well discussed. However, there is inadequacy towards exposure on the discrepancy of structural architecture and features that surrounds hydrogen bonds formation and ion pair interactions of the atomic model derived from microgravity or space and earth crystal structure. The purpose of enzyme crystallization is to elucidate the structure to understand their mechanism and function. Until now, the manipulation of structural information obtained from space grown crystal structure for the production of new enzyme are difficult due to the elusive characteristics of enzyme crystallized in space condition.

In this experiment, the detailed structural architecture of the space- and earth-grown crystal structure of T1 lipase produced under the same purification and crystallization method were investigated. The novelty of this experiment underlies modifications or different gravity conditions of previously reported T1 lipase crystallization process. The structural architecture of the microgravity as well as gravity-grown crystal were addressed in this study. Hence, T1 lipase structure obtained from space was applied as model to reengineer the structure of enzyme and thus, giving unique characteristics on the mutated T1 enzymes which highlights on their disparity to the wild-type.

1.1 Research Statement

Producing high quality and diffractable T1 crystal with better morphology under space environment was discussed. However, there are limited information to

address on the differences of structural architecture and features such as hydrogen bonds formation and ion pair interactions of the atomic models derived from the space and earth crystallization conditions. The manipulation of structural information for better performing enzymes and production are limited due to insufficient analysis on structure of the space- and earth-grown crystals.

1.2 Hypothesis

The addition of hydrogen bond and ion interaction in the protein structure will increase the protein thermostability. The new lipase enzymes obtained will be different in characteristics.

1.3 Objectives

The main objective of this research was to produce a new mutant lipase based on the outcomes generated from a comparison between the earth- and space-grown.

Specific objectives:

1. To compare structural features of the space- and earth-grown T1 lipase crystal structure by using *in silico* analysis and molecular dynamics simulation.
2. To determine major points of mutation based on the differences of the earth- and space-grown T1 lipase crystal structures.
3. To genetically redesign the lipase for the production of new lipase mutants.
4. To determine the characteristics of new lipase mutants.
5. To crystallize and elucidate the structure of new lipase mutant.

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