



UNIVERSITI PUTRA MALAYSIA

***STRUCTURE ELUCIDATION OF A THERMOSTABLE ORGANIC
SOLVENT-TOLERANT RECOMBINANT HZ LIPASE AND
DETERMINATION OF ITS THERMOSTABILITY***

MALIHE MASOMIAN

FBSB 2013 37

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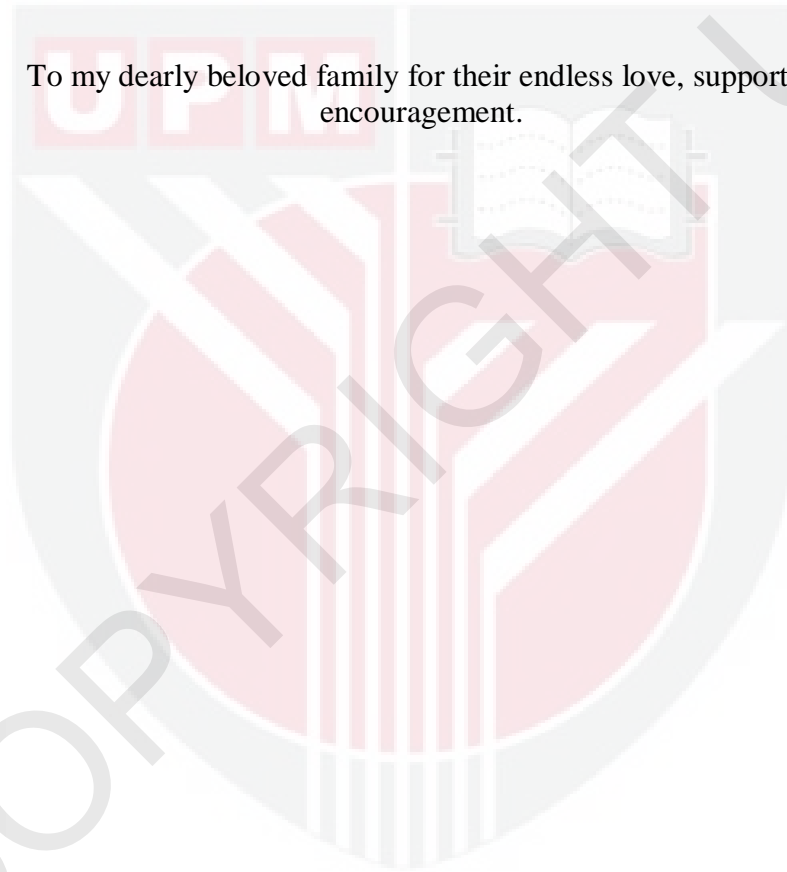
MALIHE MASOMIAN

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfillment of the Requirement for the degree of Doctor of Philosophy**

July 2013

DEDICATION

To my dearly beloved family for their endless love, support, care and encouragement.



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

STRUCTURE ELUCIDATION OF A THERMOSTABLE ORGANIC SOLVENT-TOLERANT RECOMBINANT HZ LIPASE AND DETERMINATION OF ITS THERMOSTABILITY

By

MALIHE MASOMIAN

July 2013

Chairman: Professor Raja Noor Zaliha Raja Abd Rahman, D.Eng.

Faculty: Biotechnology and Biomolecular Sciences

The principles of enzyme activity and stability have become a growing interest for researchers in exploring how enzymes adapt in the presence of organic solvent and high temperatures. The structural studies and X-ray crystallography of enzymes could provide clues towards understanding of their properties and function. The atomic details of molecular structures could offer accurate models of molecular interactions that promote protein engineering to improve the application of protein. To realize the goal, thermostable and organic solvent tolerant HZ lipase from newly isolated local *Aneurinibacillus thermoaerophilus* strain HZ was crystallized to elucidate the molecular structure of the enzyme. Owing to the unique properties of HZ lipase, to study the functional genomics of this potentially important bacterium requires not only overexpressing the corresponding genes but also detailing understanding of molecular protein structure.

HZ lipase gene was isolated and cloned into plasmid pET32b and expressed into *E.*

coli BL21 (DE3). The optimum lipase expression was obtained after 8 h incubation at 30 °C post induction with 0.025 mM IPTG, where the lipase activity was approximately 145 times higher than wild type HZ lipase. The presence of His tag was confirmed by western blot analysis. To purify the enzyme, affinity chromatography was used as the first step following the treatment of the fusion protein at 20 °C to get mature HZ lipase without using any protease treatment step. A high purification yield of 78.9% with 1.3-fold purification and 21.8 mg total purified protein was obtained from 50 mL culture. The mature lipase was highly active at 65 °C and pH 7 with a half-life of 45 min at 60 °C and 2 h 15 min at 55 °C. HZ lipase showed an enhancement effect in the presence of Li⁺, Na⁺, K⁺, Rb⁺, Cs⁺, Mg²⁺, Ca²⁺, Sr²⁺ and Mn²⁺ after 30 min treatment. HZ lipase activity was increased by glycerol and dimethyl sulfoxide, very polar organic solvents, however its activity was inhibited in the presence of alcohols.

A structural model of the HZ lipase was built using YASARA structure (Yet Another Scientific Artificial Reality Application) from a known three-dimensional structure (T1 lipase, PDB ID: 2DSN) which it had the highest sequence identity (57%) with the HZ lipase. The predicted 3D structure of HZ lipase revealed the topological organization of α/β -hydrolase fold consisting of 10 α -helices and 13 β -strands. Ser113, His308 and Asp350 were assigned as catalytic triad residues. In order to get a more accurate structure, HZ mature lipase was successfully crystallized via vapor-diffusion and counter diffusion techniques. However, the crystal formed had poor quality to diffract X-ray in a house X8 Proteum, BRUKER. Therefore, crystallization was performed under microgravity conditions in space as well as in a ground control. The ground control crystal had poor quality for diffraction of synchrotron X-rays.

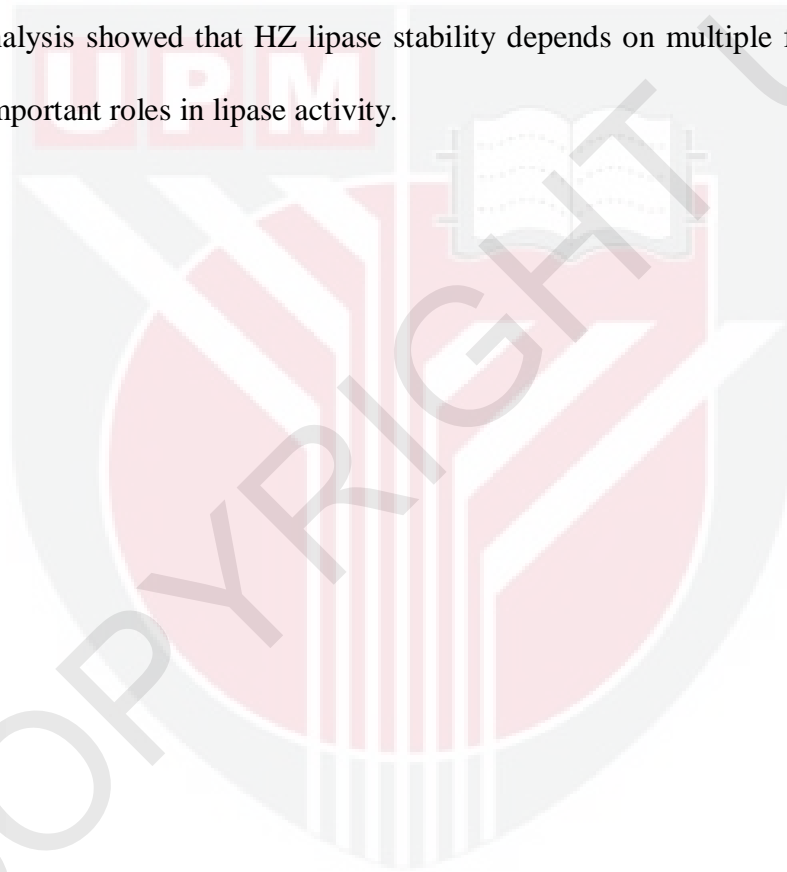
However, the microgravity HZ lipase crystal diffracted to 1.58 Å resolution and belonged to the primitive monoclinic space group $P1\ 2_1\ 1$. The phase problem was solved by the Balbes method and T1 lipase crystal structure (PDB entry: 2DSN) was used as the search model. Based on Balbes calculation, the crystal contained eight molecules per asymmetric unit, with a Matthews's coefficient of $2.14\ \text{Å}\ \text{Da}^{-1}$ and solvent content of 42.60%. The generated model was further built and refined up to 1.8 Å with final R factor and R free of 0.2905 and 0.3579, respectively. The crystal structure of HZ lipase showed the presence of two metal ions, Zn^{2+} and Ca^{2+} .

The effect of stabilization factors on HZ lipase thermostability was studied through a rational design strategy. The critical point residues were identified in order to increase the number of ion pairs in HZ lipase structure. The substitutions of Trp225 with Lys and Val361 with Arg were done using YASARA software and the new 3D structure was designated as mutant lipases (W225K and V361R lipases). The analysis of mutated lipases showed one and two new additional ion pairs in W225K and V361R lipases, respectively. Meanwhile, both mutants showed reduction in the solvent accessible surface area calculated by the YASARA software. The HZ lipase mutants W225K and V361R were constructed by site-directed mutagenesis. Mutant V361R lipase showed a decrease in optimum activity temperature from 65 to 55 °C, but the pH stability profile of the mutant was improved compared to the HZ lipase.

Mutant W225K lipase showed a half-life of 3 h 45 min at 55 °C and 1 h 20 min at 60 °C, which was higher than HZ lipase thermostability. Superposition analysis of V361R with HZ lipase showed that α -helices became shorter. Hence, the α -helix stabilization is important for HZ lipase stability. However, both computational and experimental analysis of W225K lipase indicated that presence of additional ion pair

could increase the stability of enzyme. In addition, the activity of HZ lipase strictly depends on the length and hydrophobicity of its lid.

In conclusion, crystallization analysis of HZ lipase indicated that the crystals grown under microgravity are superior to the control crystals grown on earth under normal gravity. The structural elucidation of HZ lipase revealed exclusive features that made this enzyme unique in structure and valuable in fundamental study. In addition, overall analysis showed that HZ lipase stability depends on multiple factors and its lid play important roles in lipase activity.



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

**PENJELASAN STRUKTURSTABIL HABA TAHAN-PELARUT
ORGANIKREKOMBINANHZLIPASEDAN PENENTUAN KESTABILAN
HABA**

Oleh

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Julai 2013

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Prinsip aktiviti dan kestabilan enzim ini telah menarik minat penyelidik dalam meneroka bagaimana enzim ini menyesuaikan diri dalam kehadiran pelarut organik dan persekitaran suhu yang tinggi. Kajian struktur enzim dan kaedah kristalografi sinar $-X$ dapat digunakan sebagai petunjuk bagi pemahaman tentang sifat-sifat dan fungsi mereka. Maklumat terperinci atom dalam interaksi protein boleh dijadikan sebagai model yang mempunyai struktur molekul yang tepat bagi tujuan penggunaan protein melalui kejuruteraan protein. Untuk merealisasikan matlamat ini, lipase stabil haba dan tahan pelarut organik yang didapati daripada *Aneurinibacillus thermoaerophilus* strain HZ telah dikristalkan untuk menjelaskan struktur molekul enzim. Oleh kerana HZ lipase mempunyai sifat-sifat yang unik, penyelidikan genomik dalam bakteria ini bukan sahaja penting dalam pengekspresan gen yang sama tetapi juga dalam pemahaman terperinci bagi struktur protein molekul.

GenHZlipasetelah dipencilkandandiklonkedalamplasmidpET32b dan

diekspreskan ke dalam *E. coli* BL21 (DE3). Pengekspresan optima lipase telah diperoleh selepas tempoh 8 jam induksi penderaman pada suhu 30 °C dengan induksi IPTG sebanyak 0.025mM, di mana aktiviti HZ lipase didapati menghampiri 145 kali lebih tinggi daripada lipase asal. Enzim mentah adalah diperoleh daripada pengekspressan secara intrasel. Kehadiran tag-His dikenalpasti melalui analisis western blot. Kromatografi affiniti telah digunakan sebagai langkah pertama diikuti rawatan pada suhu 20 °C bagi protein masih bertag untuk memperolehi protein matang tanpa menggunakan sebarang langkah rawatan protease. Langkah penjujukan terminal-N telah dilakukan untuk memastikan tempat yang betul untuk pemisahan tag-tag pada lipase HZ selepas rawatan penderaman pada 20 °C. Hasil penulenan tinggi telah berjaya diperoleh melalui strategi ini di mana peratus penghasilan sebanyak 78.9% dengan 1.3 kali ganda penulenan dan 21.8 mg jumlah protein tulen berjaya diperolehi daripada 50 mL kultur. Lipase matang didapati sangat aktif pada 65 °C dan pH 7 dengan separuh hayat pada adalah selepas 45 min pada 60 °C dan 2 jam 15 minit pada 55 °C. Aktiviti lipase HZ juga telah menunjukkan kesan peningkatan dengan kehadiran ion Li^+ , Na^+ , K^+ , Rb^+ , Cs^+ , Mg^{2+} , Ca^{2+} , Sr^{2+} dan Mn^{2+} selepas 30 minit penderaman. Aktiviti lipase HZ juga telah diaktifkan oleh gliserol dan DMSO yang merupakan pelarut yang sangat polar organik.

Model struktur lipase HZ telah dibina menggunakan struktur YASARA berdasarkan daripada struktur 3 dimensi yang telah diketahui yang terdapat di dalam simpanan data (T1 lipase, PDB ID: 2DSN) yang mempunyai persamaan jujukan tertinggi (57%) dengan gen lipase HZ. 3D struktur yang diramalkan telah mendedahkan organisasi topologi lipatan α/β -hidrolase yang mengandungi 10 α -heliks dan 13 β -helaian. Ser113, His308 dan Asp350 telah dikenalpasti sebagai tiga tempat katalisis

berlaku.

Bagi mendapatkan struktur protein yang tepat, lipase matang HZ telah berjaya dihablurkan secara optima melalui kaedah titis gantung dan titis duduk pada kepekatan protein sebanyak 5.0 mg/mL, dengan menggunakan 30% w/v Polyethylene glycol 3350 sebagai agen pemendakan pada suhu 20 °C. Bagi kaedah resapan balas yang menggunakan kapilari 'C-Tube', lipase HZ telah dihablurkan menggunakan formulasi Index 1, bernombor 44 yang mengandungi 0.1M HEPES pH 7.5, 25% w/v Polyethylene glycol 3350 dengan menggunakan gel 6 mm di mana hablur kristal dilihat terhasil selepas 30 hari. Dalam penyediaan eksperimen mikrograviti, formulasi kristal yang digunakan dalam kapilari 'C-Tube' telah digunakan untuk penghabluran kristal dalam 'JCB Crystal Tube' bagi tujuan eksperimen pengkristalan di angkasa dan juga sebagai eksperimen kawalan di bumi. Protein kristal yang terbentuk di dalam kapilari 'C-Tube' dan juga di dalam 'JCB Crystal Tube' (kawalan di bumi) didapati menghasilkan kualiti Kristal yang rendah untuk dibelaukan dengan menggunakan sinar-X 'In-house' ataupun belauan sinar-X synchrotron. Walau bagaimanapun, pengkristalan lipase HZ di angkasa telah dibelaukan pada resolusi 1.58Å dan kristal ini didapati tergolong dalam kumpulan ruang $P_1 2_1 1$ dengan parameter-parameter sel $a= 112.6 \text{ \AA}$, $b= 84.26\text{\AA}$, $c= 155.46\text{\AA}$, $\alpha=\gamma= 90.0^\circ$, $\beta= 91.3^\circ$. Set data pembelauan ini mempunyai lingkungan resolusi 50.000 – 1.58 Å dengan kesempurnaan sebanyak 67.3%. Permasalahan fasa telah diselesaikan menggunakan kaedah Balbes dan dengan menggunakan struktur kristal lipase T1 (PDB:2DSN) sebagai templat. Berdasarkan pengiraan Balbes, kristal ini mengandungi lapan molekul dalam setiap unit simetri, dengan pekali Matthew 2.14 Å Da dengan kandungan pelarut sebanyak 42.60%. Struktur kristal yang dihasilkan

telah diperhaluskan sehingga 1.8 \AA dengan pengurangan R-factor sebanyak 29%. Struktur Kristal lipase HZ menunjukkan kewujudan dua ion logam, Zn^{2+} dan Ca^{2+} . Tidak seperti lipase tahan suhu yang lain, ikatan Ca^{2+} dalam lipase HZ adalah berbentuk lima (pentahedral).

Ciri stabil haba bagi HZ lipase telah dikaji menerusi kaedah ciptaan rasional berdasarkan kaedah kesamaan serta perbandingan struktur kristal HZ lipase dengan struktur lipase stabil haba yang lain. Untuk tujuan itu, titik residu kiritikal telah dikenalpasti bagi meningkatkan jumlah pasangan ion didalam struktur HZ lipase di mana penukaran dan Yrp225 dan Lys kepada Lys dan Val361 kepada Arg telah dilakukan menggunakan program komputer YASARA dan struktur 3D baru telah direka sebagai mutan lipase (W225K dan V361R lipase). Analisis lipase yang telah termutasi dijalankan dengan menggunakan perisian Evaluating the Salt Bridges in Protein (ESBR) di mana perisian ini menunjukkan tambahan dua pasangan ion di dalam W225K dan V361R lipase. Kedua-dua mutan ini juga menunjukkan penurunan pada nilai bahagian permukaan kebolehmasukkan pelarut dengan menggunakan perisian YASARA.

Dalam analisa eksperimen, mutan W225K dan V361R telah dibina menggunakan mutagenesis tapak-terarah, dan mutasi telah disahkan oleh penjujukan amino asid.

HZ, W225K dan lipases V361R yang tulen telah disahkan menjadi homogen seperti yang ditunjukkan oleh baris pada SDS-PAGE dan Native-PAGE. Aktiviti mutant V361R lipase telah menunjukkan penurunan suhu aktiviti optimum dari 65 kepada 55 °C. Walau bagaimanapun, profil kestabilan pH mutan menunjukkan profil yang lebih baik berbanding dengan lipase HZ. Mutant W225K lipase menunjukkan jangka

setengah-hayat selama 3 jam 45 min pada 55 ° C dan 1 jam 20 menit pada 60 ° C, di mana kestabilan terhadap suhu adalah lebih tinggi berbanding lipase HZ. Analisis pertindihan antara V361R dengan HZ lipase menunjukkan bahawa α -helix telah menjadi lebih pendek. Oleh itu, penstabilan α helix adalah penting untuk kestabilan lipase HZ. Walau bagaimanapun, kedua-dua analisis komputer dan eksperimental W225K lipase menunjukkan bahawa kehadiran pasangan ion tambahan boleh meningkatkan kestabilan enzim. Di samping itu, aktiviti HZ lipase sepenuhnya bergantung kepada panjang dan hidrofobisiti bahagian 'lid' pada lipase.

Kesimpulannya, analisis penghabluran HZ lipase mendedahkan bahawa kristal yang ditanam di bawah mikrograviti yang unggul kristal kawal yang ditanam di bawah graviti biasa di bumi. Penentuan struktur HZ lipase mendedahkan ciri-ciri eksklusif yang menjadikan enzim ini unik dalam struktur dan ia adalah amat penting dalam kajian asas. Di samping itu, analisis keseluruhan menunjukkan bahawa kestabilan HZ lipase bergantung kepada pelbagai faktor dan bahagian 'lid' pada lipase memainkan peranan penting dalam aktiviti lipase.

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APPROVAL

I certify that an Examination Committee has met on date of viva to conduct the final examination of Malihe Masomian on her degree of Doctor of Philosophy thesis entitled “Structure elucidation of a thermostable organic solvent-tolerant recombinant HZ lipase and understanding the enzyme thermostability through rational design” in accordance with Universiti Putra Malaysia (Higher Degree) Act 1980 and Universiti Putra Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree.

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

MALIHE MASOMIAN

Date: 12 July 2013



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