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COMPUTATIONAL STUDY OF T1 LIPASE ACTIVATION AND METALLOLIPASE ENGINEERING USING MODIFIED T1 LIPASE AS SCAFFOLD

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

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July 2013

Chairman: **Adam Leow Thean Chor, PhD**

Faculty: **Biotechnology and Biomolecular Sciences**

Biocatalysts play an important role in white biotechnology, but the features of the enzymes, such as the thermal stability, pH optimum, and the need for a co-factor for catalytic activity may not be compatible with the industrial processes. Screening of biocatalysts that can withstand harsh industrial processes is time consuming and may not readily available in nature. Through protein engineering, enzyme with desired characteristics could be designed and synthesized. However, in order to achieve such goals, detailed mechanism underlying the mode of action of the enzyme must be fully understood. The emergence of great interest in the field of biomimicry and synthetic biology in recent years, has led to a rapid development in the construction of artificial metalloenzyme. Artificial metalloenzyme which was claimed to be the chemical synthesis of the future is a combination of active organometallic moiety with a macromolecular host.

In this study, the activation process of T1 lipase at the molecular level was investigated via computational approach and a functional artificial metalloenzyme was engineered as a novel metallolipase by using the modified scaffold of T1 lipase.

Molecular Dynamics (MD) simulations of native T1 lipase in different solvent environments (water and water-octane interface) and temperatures (20˚C, 50˚C, 70˚C, 80˚C and 100˚C) were performed in order to investigate the enzyme activation process and the importance of the lid structure in activating the enzyme. Based on the structural analysis of the lipases in the family I.5, the lid domain was proposed to comprise α6 and α7 helices connected by a loop, thus forming a helix-loop-helix motif. Throughout the MD simulations experiments, lid displacements were only observed in the water-octane interface, not in the aqueous environment, and they were observed in respect to the temperature effect, suggesting that the activation process is governed by interfacial activation coupled with temperature switch activation. Examining the activation process in detail revealed that the large structural rearrangement of the lid domain was caused by the interaction between the hydrophobic residues of the lid with octane, a non-polar solvent, and this conformation was found to be thermodynamically favorable. These findings on T1 lipase activation process are very important and crucial as it will aid in the next step which is the redesigning of T1 lipase structure.

In order to investigate the importance of the lid domain toward the behavior of lipase, four new constructs (D1, D2, D3, and D4) were successfully designed and engineered, conferring deletion or modification within the lid domain of T1 lipase. Among those constructs, the D4 lipase was chosen for enzyme characterization since it possessed a completely exposed active site while retaining the catalytic efficiency compared to other

constructs. In order to study the effect on T1 lipase characteristics upon lid removal, D4 lipase was subjected to enzyme purification and characterization. The optimum temperature was shifted to a lower temperature (50˚C) and showed a higher preference toward substrate with a longer chain length.

By utilizing the solvent exposed structure of D4 lipase as the protein scaffold, a new zinc binding site was engineered for the attachment of the metal ion that was used as the nucleophile in the catalysis replacing the existing catalytic Ser113. The newly engineered enzyme was identified to be catalytically active and able to hydrolyze *p*-nitrophenyl decanoate with a specific activity of 0.435 U/mg. Although the catalytic efficiency of the artificial metallolipase was less than the D4 lipase, the catalytic effiency can be further enhanced by employing directed evolution in the future study. Furthermore, D4 metallolipase was the only metallolipase reported so far.

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

KAJIAN PENGAKTIFAN T1 LIPASE SECARA BERKOMPUTER DAN KEJURUTERAAN METALLOLIPASE MENGGUNAKAN T1 LIPASE YANG DIUBAHSUAI SEBAGAI PERANCAH

Oleh

MOHD ZULHILMI BIN ABDUL RAHMAN

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Pemangkin biologi memainkan peranan yang penting dalam bioteknologi putih, tetapi ciri-ciri enzim, seperti kestabilan haba atau pH optimum serta keperluan untuk faktor bersama bagi aktiviti pemangkin mungkin tidak serasi dengan proses perindustrian. Saringan pemangkin biologi yang boleh bertahan pada proses perindustrian yang kasar bukan sahaja memakan masa tetapi tidak mudah didapati dalam alam semula jadi. Dengan kemajuan dalam kejuruteraan protein, enzim dengan ciri-ciri yang dikehendaki boleh direka dan disintesis. Walau bagaimanapun, untuk mencapai matlamat tersebut, mekanisme terperinci di sebalik mod tindakan enzim mesti difahami sepenuhnya, kerana cabaran utama dalam bidang kejuruteraan protein adalah untuk menguji pengetahuan kita melalui reka bentuk. Kemunculan minat yang besar dalam bidang biomimikri dan biologi sintetik dalam tahun-tahun kebelakangan ini, telah membawa kepada pembangunan yang pesat dalam pembinaan “metalloenzyme” tiruan. “Metalloenzyme” tiruan yang didakwa

menjadi sintesis kimia masa depan adalah gabungan ‘‘moiety’’ organologam aktif dengan pelbagai makromolekul.

Dalam kajian ini, proses pengaktifan T1 lipase pada peringkat molekul telah disiasat melalui pendekatan pengiraan berkomputer dan satu ‘‘metalloenzyme’’ tiruan yang berfungsi telah dihasilkan sebagai ‘‘metallolipase’’ dengan menggunakan perancah yang diubahsuai daripada T1 lipase.

Simulasi Molekular Dinamik (MD) untuk T1 lipase dalam persekitaran pelarut yang berbeza (air dan antara muka air-oktana) dan suhu (20˚C, 50˚C, 70˚C, 80˚C dan 100˚C) telah dilakukan dalam usaha untuk menyiasat proses pengaktifan enzim dan kepentingan struktur penutup dalam mengaktifkan enzim. Berdasarkan analisis struktur lipase dalam keluarga I.5, ‘‘domain’’ penutup dicadangkan terdiri daripada helik α6 dan α7 dihubungkan oleh gegelung, sekali gus membentuk motif helik-gelung-helik. Sepanjang ujikaji simulasi MD, anjakan penutup hanya diperhatikan antara muka air-oktana, dan bukannya di dalam persekitaran berair, dan melalui pemerhatian terhadap kesan suhu, telah dicadangkan bahawa proses pengaktifan dikawal oleh pengaktifan antara muka yang disertai dengan pengaktifan suis suhu. Penelitian terhadap proses pengaktifan secara terperinci mendedahkan bahawa penyusunan semula struktur domain penutup adalah disebabkan oleh interaksi antara residu hidrofobik penutup dengan oktana, pelarut tidak berkutub, dan pengesahan ini telah ditemui untuk menjadikannya lebih termodinamik. Penemuan proses pengaktifan T1 lipase ini adalah sangat penting kerana ia akan membantu dalam langkah seterusnya, iaitu mereka bentuk semula struktur T1 lipase.

Dalam usaha untuk menyerlahkan kepentingan domain penutup ke arah tingkah laku lipase, empat konstruk baru (D1, D2, D3, dan D4) telah berjaya direka dan dihasilkan,

mengandungi penghapusan atau pengubahsuaian dalam domain penutup T1 lipase. Diantara kesemua konstruk, D4 lipase telah dipilih untuk kajian selanjutnya melalui pencirian enzim kerana ia memiliki tapak aktif yang terdedah sepenuhnya dan pada masa yang sama mengekalkan kecekapan sebagai pemangkin berbanding konstruk lain. Dalam usaha untuk mengkaji kesan ke atas ciri-ciri T1 lipase selepas pembuangan penutup, D4 lipase telah melalui proses penulenan dan pencirian enzim, dan suhu optimum telah beralih ke 50˚C serta menunjukkan kecenderungan terhadap rantaian substrat yang lebih panjang.

Dengan menggunakan struktur pelarut yang terdedah iaitu D4 lipase sebagai perancah protein, tapak pengikat zink yang baru telah dihasilkan untuk memegang ion logam yang kemudiannya digunakan sebagai nukleofil dalam pemangkinan dengan menggantikan Ser113 yang sedia ada pada pemangkin. Enzim yang baru dihasilkan telah dikenalpasti sebagai aktif dan dapat mengurai *p*-nitrophenyl decanoate dengan aktiviti spesifik sebanyak 0.435 U/mg. Walaupun kecekapan pemangkin metallolipase tiruan adalah kurang daripada lipase D4, kadar kecekapan sebagai pemangkin boleh dipertingkatkan dengan menggunakan evolusi yang diarahkan dalam kajian masa depan. Tambahan pula, D4 metallolipase adalah metallolipase yang satu-satunya diketahui setakat ini dan adalah yang pertama seumpamanya.

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I certify that a Thesis Examination Committee has met on 8 July 2013 to conduct the final examination of Mohd Zulhilmi B. Abdul Rahman on his thesis entitled "Computational Study of T1 Lipase Activation and Metallolipase Engineering Using Modified T1 Lipase as Scaffold" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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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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DECLARATION

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

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MOHD ZULHILMI B. ABDUL RAHMAN

Date: 8 July 2013

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LIST OF ABBREVIATIONS

C	Cytosine
cm	Centimeter
Da	Dalton
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
G	Guanine
g	Gram
g/L	Gram per liter
GST	Glutathione-*S*-Transferase
h	Hour
IPTG	Isopropyl β-D Thiogalactoside
kDa	Kilo Dalton
L	Liter
M	Molar
m	Minute
mg	Milligram
ml	Milliliter
mM	Millimolar
nm	Nanometer
OD	Optical density
PCR	Polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

TEMED	N, N, N, N-Tetramethyllenediamide
U/mg	Unit per milligram
U/ml	Unit per milliliter
v/v	Volume per volume
w/v	Weight per volume
µg	Microgram
µl	Microliter
µm	Micrometer

CHAPTER 1

INTRODUCTION

Lipases are classified under serine hydrolase's family which are able to hydrolyze triacylglycerides into free fatty acids and glycerol. In contrast to carboxyl esterases, most lipases show a phenomenon of a so-called interfacial activation (Brzozowski *et al*., 1991), in which the lipase activity was greatly enhanced at the lipid water interface. This phenomenon was later found out to be related with the presence of a amphipathic structure (the lid or flap) covering the entrance of the active site. Since the discovery of interfacial activation, many researches had been conducted in order to understand the detail mechanism governing the event and the parameters that would affect directly or indirectly the enzyme activation processes.

The deposition of thousands of protein crystal structures in the Protein Data Bank (PDB) facilitates better understand of protein structures and functions. Previously, the protein structure of a locally isolated lipase known as T1 lipase was successfully determined in closed conformation. However, limited information could be gained in such conformation. Several computational studies exploring the relationship between the structural features and functions of the enzyme were reported, as *in silico* experiments help in studying the dynamic of the protein at atomic level (Hagiwara *et al*., 2009; Karjiban *et al*., 2010; Wang *et al*., 2010).

Advance in protein engineering and accumulated knowledge of protein structure, stability, function and mechanism had led to the rapid development of artificial enzyme with new properties. The rational and *de novo* design of novel proteins offers a brand-new method of studying structure and function, and makes possible the

construction of new biomaterials (Hellinga, 1996). An enzyme may be useful in industrial process but the features of the protein, such as temperature stability or pH optimum for the catalytic activity or the need for a co-factor may not be compatible with the harsh industrial processes. Thus, industrial processes require enzyme with high conversion efficiency, high enantioselectivity, high stability (thermostable) and specificity. The interesting characteristics of metalloenzyme in nature attract scientists' attention in engineering bio-inspired catalysts. Bio-mimicking of naturally occurring metalloenzymes in the creation of novel artificial metalloenzymes need a great understanding about the structure, function and mechanism of the protein as well as the basic knowledge of bio-inorganic chemistry.

The main objective of this study is therefore, to investigate the activation process of T1 lipase at the molecular level *via* computational approach and to engineer a functional artificial metalloenzyme using the modified scaffold of T1 lipase as a novel metallolipase. This study entails several specific objectives as follows:

1. To study the effects of temperature and solvent environment on the activation of T1 lipase and to propose the mechanism regarding the activation process.
2. To investigate the effect of lid removal on T1 lipase and to design a new protein scaffold from T1 lipase structure.
3. To engineer a new metal binding site in the designed scaffold

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