UNIVERSITI PUTRA MALAYSIA

CONFORMATIONAL DESIGN OF MINILIPASE

HAFIDZA BT BAHARUM

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CONFORMATIONAL DESIGN OF MINILIPASE

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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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

CONFORMATIONAL DESIGN OF FUNCTIONAL MINILIPASE

By

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July 2013

Chairman: Adam Leow Thean Chor, PhD

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Protein of small size, mini protein, containing 30 to 80 amino acid residues are particularly attractive scaffold for protein design. They possess a great potential in overcoming difficulties associated with synthesis, or unfavorable physical properties. Therefore, a small zinc binding domain of T1 lipase was used as scaffold protein for grafting functional motifs coupled with disulphide bonds to form a stable miniprotein. Several constructs of increasing solubility were made by substituting charged and hydrophilic amino acids to the engineered scaffold. From the predicted structure, this newly designed miniprotein contains two short α-helix connected by loops. Several substrates with different carbon length were used to recognize (docking) the interaction between ligand and receptor where acetate, butyrate and caprylate fitted perfectly within the binding pocket of the ligand. Applying molecular approach the optimized sequenced was synthesized and cloned in the pET32b vector. The minilipase was purified 4.97-fold with 78.4% yield using affinity chromatography and the molecular weight of minilipase was determined to be 17 kDa by SDS-PAGE. The minilipase exhibited maximum activity at 35°C and was stable at temperature below 50°C. This mini protein favored substrates containing

short (pNP acetate, C2) and medium (pNP butyrate C4, pNP caprylate C8, pNP decanoate C10) carbon chain of acyl group, which correlated with docking result. The kinetic assay of minilipase for pNP-butyrate produced K_m of 0.3 mM and V_{max} 0.9 µmole/min/mL indicated the high binding affinity than T1 lipase on the same substrate. The lipolytic activity of purified lipase was slightly increased with 105.72% by the addition of 0.1% SDS, while Tween 60 and Tween 80 were the most effective inhibitors against the minilipase. Interestingly, the minilipase activities were increased by 130% and 115% in the presence of 1 mM Mg^{2+} and Mn^{2+}, respectively. However when Ca^{2+} concentration was increased from 1 mM to 10 mM, the activity of purified minilipase was completely inhibited. Based on the finding of the present study, the designed minilipase has potential as an alkaline lipase and a candidate for industrial and pharmaceutical applications.

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

MEREKA BENTUK MINILIPASE YANG BERFUNGSI

Oleh

HAFIDZA BAHARUM

July 2013

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

Fakulti: **Institut Biosains**

Mini protein merupakan protein kecil yang mempunyai 30 hingga 80 amino asid, mempunyai potensi sebagai perumah dalam pengubahsuaian protein. Dengan memiliki saiz yang kecil ia mampu mangatasi masalah dalam sintesis protein kerana bahagian yang tidak diperlukan dalam struktur protein dibuang. Oleh itu, zink domain yang terdapat dalam T1 lipase boleh digunakan sebagai 'scaffold' untuk menghasilkan protein yang kecil yang berfungsi dan distabilkan oleh ikatan sulfida. Selain dari itu, untuk meningkatkan kadar kebolehlarutan protein mini ini, beberapa amino acid yang bercas dan hidrofilik ditambah dalam jujukan protein mini ini. Daripada ramalan struktur didapati, protein mini yang terhasil ini mengandungi α-heliks yang disambungkan oleh gegelung. Interaksi antara substrat dan ligan diuji untuk mengenalpasti kecenderungan protein mini ini terhadap jenis substrat, dan di dapati acetat, butyrat dan kaprylat berada pada kedudukan yang terbaik didalam poket ikatan protein mini.

Dengan menggunakan pendekatan molekul, jujukkan protein mini yang telah dioptimumkan telah disintesis dan diklon dalam vektor pET32b. Protein mini ini

telah ditulenkan kepada 4.97 kali ganda, dengan penghasilan sebanyak 78.4% dengan menggunakan kromatografi affiniti dan berat molekul protein mini ini adalah 17 kDa seperti yang terdapat dalam SDS-PAGE. Maksimum aktiviti bagi protein mini ini adalah 35^0C dan stabil pada suhu di bawah 50^0C. Protein mini ini lebih memilih untuk menghidrolisiskan substrat berantai pendek (pNP acetat, C2) dan sederhana (pNP butirat C4, pNP kaprilat C8, pNP decanoat C10). Daripada ujian kinetik, menunjukkan K_m protein mini terhadap substrat pNP butyrat ini adalah 0.3 mM dan V_{max} 0,9 μmole/min/mL dan ini menunjukkan bahawa protein mini mempunyai ikatan affiniti yang tinggi terhadap substrat berbanding dengan T1 lipase. Aktiviti lipolytic bagi protein mini yang telah ditulenkan ini meningkat sebanyak 105.72% dengan pertambahan 0.1% SDS, manakala Tween 60 dan 80 merupakan perencat paling berkesan terhadap protein mini. Menariknya, aktiviti minilipase telah meningkat sebanyak 130% dan 115% dalam kehadiran 1 mM Mg^{2+} dan Mn^{2+}. Walau bagaimanapun, apabila kepekatan Ca^{2+} telah meningkat dari 1 mM hingga 10 mM, aktiviti protein mini ini terencat sama sekali. Berdasarkan dapatan kajian ini, protein mini yang direka berpotensi sebagai lipase alkali dan sesuai untuk aplikasi dalam bidang industri dan farmaseutikal.

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APPROVAL

I certify that an Examination Committee has met on 24 July 2013 to conduct the final examination of Hafidza Baharum on her Master of Sciences thesis entitle 'Conformational design of functional minilipase' in accordance with 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 candidate be awarded the Master of Sciences.

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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 other instituition.

HAFIDZA BAHARUM

Date: 24 July 2013

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LIST OF ABBREVIATIONS

REM	Replica exchange molecular dynamics
PME	Particle Mesh-Ewald
RMSD	Root-mean-square deviation
MD	Molecular dynamics
OG	Lone pair electron of oxygen
CT	Carbonyl carbon of substrate
cm	Centimeter
Da	Dalton
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
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

Why design novel proteins? Nature has provided a large number of natural proteins, which fold into varieties of different tertiary structures and functions. However, direct screening of natural biocatalysts is time consuming and the desired biocatalysts may not readily available in nature. Furthermore, the enzymes isolated from nature do not fulfill the specific demands they have to meet for industrial and medical application. Therefore, protein engineering concepts have emerged to achieve both requirements. The design of enzymes with new functions and properties has long been a goal in protein engineering.

Engineering a biocatalyst for a specific reaction is not trivial. However, the tool in the relatively new field of protein engineering is becoming more available (Agarwal *et al*., 1970; Leung *et al*., 1989; Joyce *et al*., 1994; Stemmer *et al.*, 1994; Alberghina *et al.,* 2005 and Van den Brulle *et al.,* 2008). Protein engineering approaches are now at hand in designing artificial enzymes or novel enzymes particularly polymers or oligomers with enzyme-like activities (Bolon *et al.,* 2001; Kaplan *et al*., 2004 and Rothlisberger *et al.,* 2008) and also used as a tool when investigating protein stability (Eijsink *et al.,* 2004), altering or improving the catalytic properties of enzymes (Jestin *et al.,* 2004), and understands functionality (Schliebs *et al.,* 1997; Russell *et al.,* 2000; Nardella *et al*, 2004).

Modification on proteins can be done by a combination of several techniques and knowledge. For example, the protein of interest need to be first purified and characterized with regards to its functional properties before cloned and overexpressed in a suitable host organism, and then modified to improve its performances. Nowadays, varieties of analytical as well as structural techniques such as X-ray crystallography and nuclear magnetic resonance (NMR), can be employed for its characterization. The above technique represented in the protein engineering cycle as shown in Figure 1.

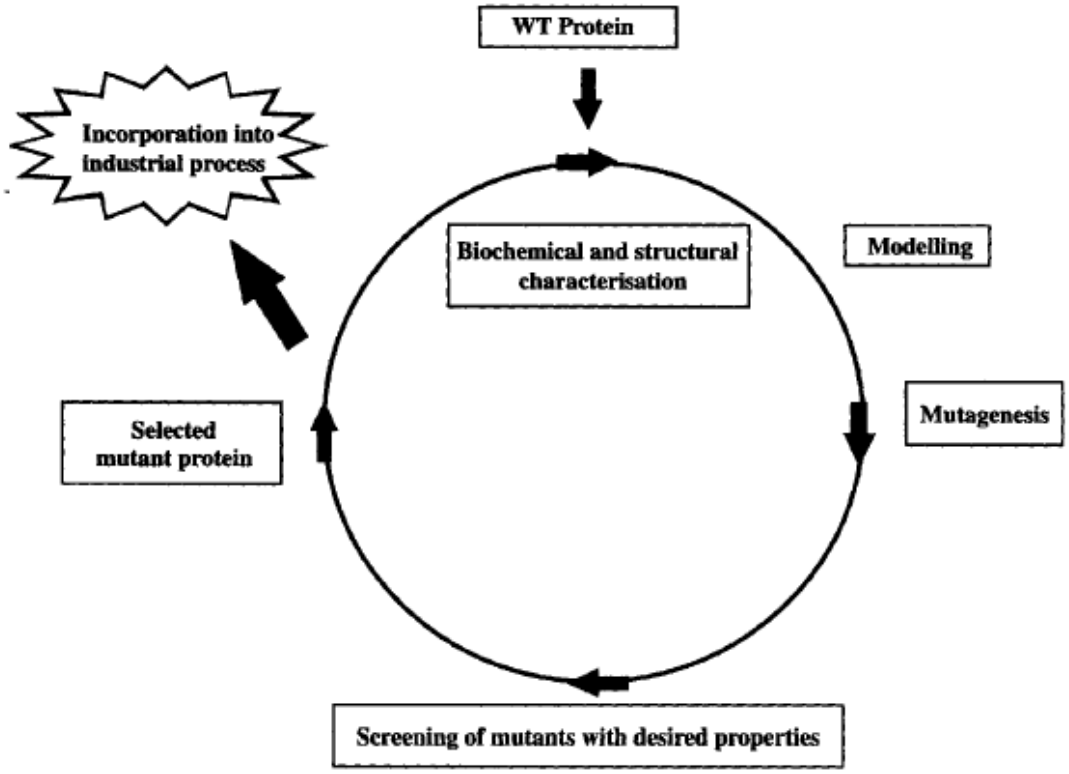

Figure 1: Protein engineering cycle (Alberghina *et al.*, 2005)

Basically, there are four basic techniques in engineering new protein functions. First is a rational design. Rational design is a technique requires a detailed information and knowledge of the protein structure and of structure-function relationships, in order to allow the introduction of mutations targeted to specific protein site. The main concept approaches in rational design such as Site Directed Mutagenesis (SDM) which is a substitution of nucleotide in correspondence of pre-selected positions in the polypeptide. This strategy is also known as 'rational mutagenesis'.

Secondly, the modification could be made by molecular evolution. This technique does not require any knowledge on the protein structure and mechanism of action. It is based on the random generation of a vast number of mutants followed by screening for the desired function. Thirdly, was generation of random libraries which produced a large number of proteins or peptide of region thereof. It is often coupled with surface display to ease screening of the mutants. Fourthly, was *De Novo* protein design. The aim of *de novo* design is to design new (not pre-existing) sequence endowed with predefined structure and function (Helingga *et al*., 1998)

With many natural proteins available to observe and manipulate, what can be gained by the design and characterization of novel protein? There are two main reasons for protein design. The first is based upon assumption that a complete understanding of any natural system can prove our ability to design similar artificial enzymes. Thus from understanding of natural proteins in term of their folding pathway, thermodynamic stabilities, and catalytic properties are enhanced by ability in designing novel proteins with predetermined structure and properties.

The second motivation for protein designed is designing a simple structure commonly peptide. This represents as a first step toward designing novel macromolecule that will solve the chemical, biochemical, industrial catalysis and biomedical problems.

In this study, a new approach in designing of new bioactive minilipase was proposed by grafting functional residues on a designed stable scaffold. This work clearly demonstrated that the designed scaffold can act as a template for minilipase engineering and a novel miniature biocatalyst could be designed by grafting appropriate active sites into a scaffold protein. In this case functional motif of lipase was grafted into a small domain of T1 lipase. This research was conducted with the following objectives:

1. Designing mini peptide scaffold.
2. Grafting the functional residue of lipase on designed scaffold.
3. Observing characteristic of the designed functional minilipase.

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