DETERMINATION OF GDSL- SUBTRATE COMPLEX IN UNDERSTANDING THE SELECTIVITY OF NOVEL GDSL ESTERASE CATALYSIS

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 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

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Chairman : Adam Leow Thean Chor, PhD
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GDSL esterase is designated as a member of Family II of lipolytic enzymes known to catalyse the synthesis and hydrolysis of ester bonds. The enzyme possesses a highly conserved motif Ser-Gly-Asn-His (SGNH) in the four conserved blocks I, II, III, and V respectively. Esterases perform synthesis by using a two-step mechanism where the nucleophilic Ser initiate the synthesis by first attacking the ester's carbonyl carbon and produce an acyl enzyme intermediate and alcohol. Second, the catalytic His will facilitate the proton transfer from water which resulting in hydroxide attacks the carbonyl carbon and release of carboxylic acid. The enzyme's characteristics such as region-, chemo- and enantioselectivity help in resolving the racemic mixture of single-isomer chiral drugs. To date, esterase enzyme identified from microbial sources have been widely used in various biotechnological applications such as manufacturing of biofuels and fine chemicals whereas identification of esterase enzyme in plant contributed to the regulation of plant hormone. The side effects of racemic mixture of commercially available naproxen can be

overcome by resolving the racemic mixture of naproxen esters. In this study, determination of GDSL esterase and p-nitrophenyl butyrate which act as a substrate is identified to guide the protein engineering of GDSL esterase in resolving the racemic mixture of naproxen esters in the future. Recently, crystal structure of GDSL esterase from *Photobacterium* J15 has been reported (PDB ID: 5XTU) but not in complex with substrate. Therefore, GDSL in complex with substrate could provide insights into the binding mode of substrate toward inactive form of GDSL esterase (S12A) and identify the hot spot residues for the designing of a better binding pocket. Insight into molecular mechanisms is limited due to the lack of crystal structure of GDSL esterase-substrate complex. The crystallization of mutant GDSL esterase (S12A) and its complex with substrate are successfully reported. X-ray crystallography was performed in order to obtain the three-dimensional (3D) structure of the mutant GDSL esterase (S12A) and its complex with substrate. The recombinant *Escherichia coli* Rosetta-gami (DE3) pLysS harbouring mutant GDSL S12A was expressed at 20°C and induced by 0.1 mM IPTG. The expressed protein was then subjected to two-step of chromatography; affinity chromatography and ion-exchange chromatography. Prior to protein crystallisation, the affinity tags were removed of the purified protein and validated by SDS-PAGE analysis. The size of the mutant GDSL S12A is 36 kDa without affinity tags. The purified protein of the mutant GDSL S12A was crystallised in an optimized formulation containing 0.2 M ammonium acetate as salt, 30% (w/v) polyethylene glycol 4,000 as precipitant in 0.1 M sodium acetate trihydrate buffer at pH 4.6 with incubation at 15°C. For crystal of the mutant GDSL S12A, it was diffracted at 1.96 Å. As for mutant GDSL S12A-

substrate, the crystal was first soaked in pNP-butyrate solution prior to X-ray diffraction. The crystal of mutant GDSL S12A-substrate was diffracted at 1.73 Å. Both the crystals belong to orthorhombic space group of $P2_12_12_1$. Analysis of the 3D structure of both structures showed that Ser12 of the catalytic triad has been mutated to Ala12. A DMSO molecule was found in the catalytic triad instead of the substrate pNP-butyrate. Further, a hydrolysed product of pNP-butyrate (i.e. butyric acid) was found far from the catalytic site of the mutant GDSL S12A. The substrate-bound structure has become vital in determining the hot spot residue for GDSL esterase. The solved structures aid in unveiling the interactions between the mutant GDSL S12A with substrate. The information could guide in the rational design of GDSL esterase in overcoming the medical limitations associated with racemic mixture.

Keywords: GDSSL esterase, racemic mixture, substrate, hotspot

SDG: GOAL 3: Good Health and Well-being, GOAL 4: Quality Education

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

PENENTUAN KOMPLEKS GDSL- SUBTRATE DALAM MEMAHAMI PEMILIHAN PEMANGKIN GDSL NOVEL ESTERASE

Oleh

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GDSL esterase ditetapkan sebagai ahli Keluarga II enzim lipolitik yang diketahui memangkinkan sintesis dan hidrolisis ikatan ester. Enzim ini mempunyai motif Ser-Gly-Asn-His (SGNH) yang sangat terpelihara dalam empat blok terpelihara I, II, III, dan V masing-masing. Esterase melakukan sintesis dengan menggunakan mekanisme dua langkah di mana Ser nukleofilik memulakan sintesis dengan menyerang karbon karbonil ester dan menghasilkan perantaraan enzim asil dan alkohol. Kedua, pemangkin His akan memudahkan pemindahan proton daripada air yang mengakibatkan hidroksida menyerang karbon karbonil dan pembebasan asid karboksilik. Ciri-ciri enzim seperti rantau, kemo- dan enantioselektiviti membantu dalam menyelesaikan campuran rasemik ubat kiral isomer tunggal. Sehingga kini, enzim esterase yang dikenal pasti daripada sumber mikrob telah digunakan secara meluas dalam pelbagai aplikasi bioteknologi seperti pembuatan biofuel dan bahan kimia halus manakala pengenalpastian enzim esterase dalam tumbuhan menyumbang kepada pengawalseliaan hormon tumbuhan. Kesan

sampingan campuran rasemik naproxen yang tersedia secara komersial boleh diatasi dengan menyelesaikan campuran rasemik ester naproxen. Dalam kajian ini, penentuan GDSL esterase dan p-nitrophenyl butyrate yang bertindak sebagai substrat dikenal pasti untuk membimbing kejuruteraan protein GDSL esterase dalam menyelesaikan campuran rasemik ester naproxen pada masa hadapan. Baru-baru ini, struktur kristal GDSL esterase dari *Photobacterium* J15 telah dilaporkan (PDB ID: 5XTU) tetapi tidak dalam kompleks dengan substrat. Oleh itu, GDSL dalam kompleks dengan substrat boleh memberikan gambaran tentang mod pengikatan substrat ke arah bentuk tidak aktif GDSL esterase (S12A) dan mengenal pasti sisa-sisa titik panas untuk mereka bentuk poket pengikat yang lebih baik. Pengetahuan tentang mekanisme molekul adalah terhad kerana kekurangan struktur kristal kompleks substrat esterase GDSL. Penghabluran mutan GDSL esterase (S12A) dan kompleksnya dengan substrat berjaya dilaporkan. Penghabluran sinar-X dilakukan untuk mendapatkan struktur tiga dimensi (3D) esterase GDSL mutan (S12A) dan kompleksnya dengan substrat. Rekombinan *Escherichia coli* Rosetta-gami (DE3) pLysS yang melindungi mutan GDSL S12A telah dinyatakan pada 20°C dan diinduksi oleh 0.1 mM IPTG. Protein yang dinyatakan kemudiannya tertakluk kepada dua langkah kromatografi; kromatografi pertalian dan kromatografi pertukaran ion. Sebelum penghabluran protein, tag afiniti telah dikeluarkan daripada protein yang telah ditulenkan dan disahkan oleh analisis SDS-PAGE. Saiz mutan GDSL S12A ialah 36 kDa tanpa tag perkaitan. Protein yang telah ditulenkan bagi mutan GDSL S12A telah dihablurkan dalam formulasi optimum yang mengandungi 0.2 M ammonium asetat sebagai garam, 30% (w/v) polietilena glikol 4,000

sebagai pemendakan dalam penimbal natrium asetat trihidrat 0.1 M pada pH 4.6 dengan pengeraman pada 4.6°C. Untuk kristal mutan GDSL S12A, ia difraksi pada 1.96 Å. Bagi substrat GDSL S12A mutan, kristal pertama kali direndam dalam larutan pNP-butyrate sebelum pembelauan sinar-X. Hablur mutan GDSL S12A-substrat telah difraksi pada 1.73 Å. Kedua-dua kristal tersebut tergolong dalam kumpulan ruang ortorombik P212121. Analisis struktur 3D kedua-dua struktur menunjukkan bahawa Ser12 triad pemangkin telah bermutasi kepada Ala12. Molekul DMSO ditemui dalam triad pemangkin dan bukannya substrat pNP-butyrate. Selanjutnya, produk terhidrolisis pNP-butyrate (iaitu asid butirik) ditemui jauh dari tapak pemangkin GDSL S12A mutan. Struktur terikat substrat telah menjadi penting dalam menentukan sisa titik panas untuk esterase GDSL. Struktur yang diselesaikan membantu dalam mendedahkan interaksi antara mutan GDSL S12A dengan substrat. Maklumat ini boleh menjadi panduan dalam reka bentuk rasional GDSL esterase dalam mengatasi batasan perubatan yang berkaitan dengan campuran rasemik.

Kata kunci: GDSL esterase, campuran rasemik, substrat, titik panas

SDG: MATLAMAT 3: Kesihatan dan Kesejahteraan Baik, MATLAMAT 4: Pendidikan Berkualiti

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

α	Alpha
Å	Angstrom
B	Beta
°C	Degree Celsius
%	Percentage
A_{600nm}	Absorbance at wavelength 600 nanometer
µL	Microliter
µM	Micrometer
DMSO	Dimethyl sulfoxide
E.coli	*Escherichia coli*
g	Gram
h	Hour
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDA	kilodalton
L	Litre
LB	Luria-Bertani
M	Molar
mM	Millimolar
mg/ml	Milligram/millilter
ml	Millilter
OD	Optical density
SDS-PAGE	Sodium dedocyl sulphate-polyacramide gel electrophoresis
U	Unit

CHAPTER 1

INTRODUCTION

1.1 Background of study

Biocatalysis offers an alternative approach to non-specific conventional chemical processes for the production of single-isomer chiral drugs in pharmaceutical (naproxen, ibuprofen, ketoprofen) and agrochemical (chrysanthemic) industries where the need for optically pure molecules is critical. The uses of enzymes are due to the characteristics of their region-, chemo- and enantioselectivity in the resolution process of racemates, without the use of cofactors (Caroline et al., 2015; Adamczak and Krishna, 2004).

Esterases and lipases are a class of hydrolytic enzymes that catalyse the hydrolysis and transesterification of fatty acid esters. They have potential use in hydrolysis and synthesis of important ester compounds of pharmaceutical, food, biochemical and biological interests (Akoh et al., 2004). A new subfamily of hydrolytic/lipolytic enzymes designated as GDSL-hydrolase superfamily have five consensus sequence (I-V) and invariant important catalytic residues Ser, Gly, Asn, and His in blocks I, II, III and V respectively. The oxyanion structure led to a new designation of these enzymes as SGNH-hydrolases. Interestingly, GDSL esterases have a flexible catalytic serine residue as nucleophile that is located near the N-terminus. GDSL esterases appear to change conformation with the presence and binding of different substrates,

much like induced fit mechanism, thus exhibiting multifunctional properties and regiospecificity (Wang et al., 2016).

Photobacterium sp. J15 was a locally isolated bacterium from seawater in Johor, Malaysia. The PacBio genome sequence analysis revealed its potential in producing enzymes with different catalytic functions (Roslan et al., 2016). Previously, the GDSL esterase derived from *Photobacterium* sp. was heterelogously expressed in *Escherichia coli* and biochemical properties characterized (Shakiba et al., 2016). This GDSL esterase selectively catalysed short acyl chain length substrates, particularly pNP-butyrate. It was discovered that the GDSL esterase shares low identity (23%) structurally to esterase EstA domain of two domains autotransporter from *Pseudomonas aeruginosa* (Berg, 2010). Mazlan et al., (2018) has successfully resolved the structure of wild-type GDSL EstJ15 by using X-ray crystallography whereby this enzyme exhibits α/β-hydrolase characteristic. The selectivity of EstJ15 towards short acyl chain (i.e. protein-ligand docking analysis) was also unveiled via a computational approach. The solved wild-type structure leads to determination of the active site of EstJ15 and its catalytic mechanism.

In this study, a GDSL hydrolase enzyme from Family II of lipolytic family was used. A novel cold-adapted GDSL family esterase from *Photobacterium* sp. J15 discovered having a catalytic triad composed of serine, aspartic and histidine residues with the role catalytic serine located at conserved pentapeptide Gly-X-Ser-X-Gly, positioned at the middle of the amino acid sequence (Chepyshko et al., 2012). This GDSL esterase selectively catalyzed short acyl chain length substrates, particularly p-nitrophenyl butyrate (C4).

Although there are reports on crystal structure of GDSL enzymes, there are less reported crystal structures in complex with substrate. Insights into GDSL-substrate interaction X-ray crystallographically can be a valuable strategy for determining hot spot that could resolve the better esters thereby overcoming their medical limitations.

1.2 Problem statement

A crystal structure of mutant GDSL and its complex structure could be resolved via X-ray crystallography and provides further understanding on the binding mode of substrate toward inactive form of GDSL esterase (S12A) thus determine the hot spot residues for the designing of a better binding pocket for substrate pNP-butyrate. Solving the structure of mutant GDSL EstJ15 and its substrate complex could provide functional information and demonstrate the enzyme behaviour at atomic level thus lead to the insights of the binding mode of mutant GDSL EstJ15 towards substrate.

1.3 Research objectives

The main objective of this study is to elucidate the structure of mutant GDSL esterase-substrate complex crystallographically. The specific objectives of this study are as follows:

i. To express and purify mutant GDSL esterase.

ii. To crystallise mutant GDSL esterase complexed with substrate pNP-butyrate.

iii. To solve the 3D structure of mutant GDSL esterase complexed with substrate pNP-butyrate.

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