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IDENTIFICATION AND FUNCTIONAL ANALYSIS OF CYOTOCHROME P450 FROM Bacillus lehensis G1

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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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

IDENTIFICATION AND FUNCTIONAL ANALYSIS OF CYTOCHROME P450 FROM *Bacillus lehensis* G1

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June 2016

Chairman : Prof. Dato' Abu Bakar Salleh, PhD
Institute : Institute of Bioscience

Cytochrome P450s (CYPs) are a superfamily of heme monooxygenases which catalyze a wide range of biochemical reactions. The reactions involve the introduction of an oxygen atom into an inactivated carbon of a compound which is essential to produce an intermediate of a hydroxylated product. Vitamin D 25-hydroxylase catalyses the first step in vitamin D biosynthetic pathway, essential in the activation of vitamin D. Several types of CYPs had been found as potential 25-hydroxylases. However, most of them originate from eukaryotes and are membrane associated proteins. A putative gene sequence encoding a CYP, termed *CYP107CB2* was found in the genome of a new isolate *Bacillus lehensis* G1, and this gene shared sequence identity with the bacterial vitamin D hydroxylase (Vdh) from *Pseudonocardia autotrophica*. In order to deepen the understanding on the properties and biological function of CYP in *B. lehensis* G1, the objective of this study was to mine for a novel CYP from *B. lehensis* G1 with hydroxylase activity on vitamin D metabolites. Computational methods to search for the novel CYP from CYP structural databases were employed to identify the conserved pattern, functional domain and sequence properties of the uncharacterized CYP from *B. lehensis* G1. The *CYP107CB2* gene was isolated and amplified using PCR and the CYP107CB2 protein was over-expressed in *E. coli* Rosetta-gami (DE3) followed by enzyme purification via single step affinity chromatography. The biological properties and possible functions of CYP107CB2 were characterized through absorption spectral analysis and were assayed for vitamin D hydroxylation activity. Optimization and CYP characterization were conducted to increase the turnover of hydroxylated products with an NADPH-regenerating system. Crystallization trials on CYP107CB2 protein were conducted via preliminary screening with Crystal Screen I and II through vapour-diffusion sitting drop method. Sequence analysis studies indicated that CYP107CB2 contained the fingerprint heme binding sequence motif FxxGxxxCxG at amino acid position 336-345 as well as other highly conserved motifs characteristic of CYP proteins. Docking studies showed several potential substrates, including vitamin D_3, 25-hydroxyvitamin D_3 and 1α-hydroxyvitamin D_3, were located proximally to the enzyme's heme center. The

over-expressed CYP107CB2 protein was dominantly in cytosolic and the purified fraction showed a protein band at approximately 62 kDa on SDS-PAGE, representative for CYP107CB2. Spectral analysis demonstrated that the protein was properly folded and it was in its active form. HPLC and MS analysis on the product from a reconstituted enzymatic reaction confirmed that CYP107CB2 converted vitamin D_3 and 1α-hydroxyvitamin D_3 into 25-hydroxyitamin D_3 and 1α,25-dihydroxyvitamin D_3, respectively. CYP107CB2 formed crystal in formulation No. 38 from Crystal Screen II comprising 20% (v/v) PEG 10 000 and 0.1 mM HEPES buffer pH 7.5. In conclusion, a novel CYP107CB2 was identified from *B. lehensis* G1 and these findings proved that CYP107CB2 is a biologically relevant vitamin D_3 25-hydroxylase.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PENGENALPASTIAN DAN ANALISIS FUNGSIAN BAGI CYTOCHROME P450 DARIPADA *Bacillus lehensis* G1

Oleh

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Cytochrome P450 (CYP) merupakan sejenis hem monooksigenase daripada satu keluarga besar yang memangkinkan pelbagai jenis tindak balas biokimia. Tindak balas tersebut melibatkan penyatuan satu atom oksigen ke dalam komponen yang mengandungi karbon yang tidak aktif di mana tindak balas ini adalah penting untuk menghasilkan bahan perantaran bagi produk yang dihidroksilkan. Vitamin D 25-hidroksilase memangkinkan langkah yang pertama dalam laluan bio-sintetik vitamin D yang penting dalam pengaktifan vitamin D. Beberapa jenis CYP telah dijumpai sebagai 25-hidroksilase yang berpotensi. Namun demikian, kebanyakan daripada mereka berasal daripada eukariot dan merupakan protein sekutu membran. Satu urutan gen yang mengekodkan CYP digelar sebagai *CYP102CB2* telah dijumpai dalam genom isolasi baru iaitu *Bacillus lehensis* G1, dan gen ini berkongsi struktur kesamaan dengan vitamin D hidroksilase (Vdh) dari *Pseudonocardia autotrophica*. Dalam usaha untuk memperdalam pemahaman yang berkaitan dengan sifat dan fungsi biologi CYP dalam *B. lehensis* G1, objektif kajian ini adalah untuk mengenalpasti CYP yang novel dari *B. lehensis* G1, di mana CYP tersebut dapat menghidroksilkan metabolit vitamin D. Kajian ini menggunakan kaedah pengkomputeran untuk mencari CYP yang novel dari pangkalan data struktur CYP untuk mengenalpasti corak terabadi, domain fungsi dan sifat urutan CYP yang masih belum diklasifikasikan dari *B. lehensis* G1. Gene rekombinan *CYP107CB2* kemudian diperoleh daripada strain dengan menggunakan PCR (reaksi rantai polimerase) dan protein dilebih-ekspresikan dalam *E. coli* Rosetta-gami (DE3) diikuti dengan penulenan enzim melalui afiniti kromatografi langkah tunggal. Sifat-sifat biologi dan fungsi CYP107CB2 dicirikan melalui analisis penyerapan spektrum dan dengan itu, enzim dicerakinkan untuk aktiviti vitamin D penghidroksilan. Pengoptimuman dan pencirian CYP telah dijalankan untuk meninggikan perolehan bagi penghasilan produk hidroksil dalam sistem regenerasi-NADPH. Percubaan untuk penghabluran protein CYP107CB2 telah dijalankan melalui penyaringan awal dengan Crystal Screen I and II melalui kaedah "vapour diffusion sitting drop". Analisis urutan menunjukkan bahawa CYP107CB2 mengandungi urutan motif ikatan hem FxxGxxxCxG pada kedudukan 336-345

serta ciri-ciri motif terabadi yang boleh didapati dalam CYP protein. Dengan menggunakan kajian dok, beberapa substrat yang berpotensi termasuk vitamin D_3, 25-hidroksivitamin D_3 dan 1α-hidroksivitamin D_3, terletak berhampiran di pusat hem enzim. Kebanyakan protein CYP107CB2 yang dilebih-ekpresikan berada dalam bentuk sitosol dan pecahan penulenan menunjukkan jalur protein adalah bersaiz lebih kurang 62 kDa dalam SDS-PAGE di mana jalur tersebut mewakili CYP107CB2. Analisis spektrum menunjukkan bahawa protein tersebut dilipat dengan betul dan dalam betuk aktif. Analisis HPLC dan LCMS ke atas produk hidroksil yang dihasilkan selepas tindak balas enzim mengesahkan bahawa CYP107CB2 menukarkan vitamin D_3 dan 1α-hidroksivitamin D_3 kepada 25-hidroksivitamin D_3 dan 1α,25-dua-hidroksivitamin D_3, masing-masing. CYP107CB2 membentuk hablur dalam formulasi No. 38 daripda Crystal Screen II dimana formulasi tersebut mengandungi 20% (v/v) PEG 10 000 dan 0.1 mM bufer HEPES pH 7.5. Kesimpulannya, CYP107CB2 yang novel telah dikenalpasti daripada *B. lehensis* G1 dan penemuan ini membuktikan bahawa CYP107CB2 merupakan sejenis vitamin D_3 25-hidroksilase.

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I certify that a Thesis Examination Committee has met on 06 June 2016 to conduct the final examination of Ang Swi See on her thesis entitled "Identification and Functional Analysis of Cytochrome P450 from *Bacillus lehensis* G1" 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 Doctor of Philosophy.

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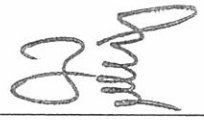

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

α	alpha
Å	Angstrom
ß	beta
BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
bp	base pair
CD	circular dichroism
CDD	conserved domain database
cm	centimeter
CO	carbon monoxide
CV	column volume
CYP or P450	cytochrome P450
°C	degree Celsius
δ	delta
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DIMS	direct infuse mass spectrometry
EC	enzyme classification
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization source
ε	extinction coefficient
Fe^{2+}	ferrous
FAD	flavin adenine dinucleotide
Fdr	ferredoxin $NADP^+$ reductase

Fdx	ferredoxin
g	gram
x g	gravitational force
h	hour
HPLC	high performance liquid chromatography
IPTG	isopropyl β-D-thiogalactoside
kb	kilo base pair
kDa	kilo Dalton
kV	kilovolts
L	liter
LB	Luria-Bertani
LC	liquid chromatography
LC-MS	liquid chromatography-mass spectroscopy
M	Molar
MS	mass spectroscopy
mg	miligram
min	minute
mL	milliliter
mM	milimolar
mm	millimeter
m/z	mass to charge ratio
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information

ng	nanogram
nm	nanometer
NMR	nuclear magnetic resonance
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
PDB	Protein Data Bank
PEG	polyethylene glycol
ppm	part per million
psi	per square inch
Q-TOF	quadrupole time-of-flight
RMSD	root mean square deviation
RPM	revolutions per minute
RT	retention time
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SQD	single quadrupole detector
TEMED	N, N, N, N-tetramethyllenediamide
T_m	melting temperature
Tris-HCl	tris hydrochloric acid
UV-Vis	ultraviolet-visible
V	voltage
v/v	volume per volume
w/v	weight per volume
µg/mL	microgram/mililiter
µL	microliter

µm	micrometer
µM	micromolar
µmol	micromole
YASARA	Yet Another Scientific Artificial Reality Application

CHAPTER 1

INTRODUCTION

1.1 Hypothesis and problem statement

Cytochromes P450s are a superfamily of heme monooxygenases which are present in a wide variety of organisms in nature (Bernhardt, 2006; Zurek et al., 2006). These proteins are involved in monooxygenation, activates dioxygen to incorporate a single oxygen atom into a wide range of substrates (Jin et al., 2004; Zurek et al., 2006). They catalyze a broad range of biochemical reactions and play an essential role in the assimilation of carbon source. The reactions include hydroxylations, epoxidations, sulfoxidations, and so on as stated by Bernhardt, (2006). The typical reaction catalyzed by CYP is the hydroxylation of organic substrates on carbon atoms (Munro et al., 2007). Prototypical reactions catalyzed by CYPs include fatty acid hydroxylation, secondary metabolite biosynthesis and drug metabolism (Schallmey et al., 2011; Sono et al., 1996). They oxidize non-activated carbon at either carbon ring or lateral alkyl chain which is difficult to be achieved via chemical reactions, making CYP versatile and attractive in various fields (Schlichting et al., 2000; Lombard et al., 2011).

In biotechnology point of view, CYPs are known as interesting potential 'green' catalysts (Bernhard and Urlacher, 2014; Farinas et al*.,* 2004) and gained much attention for the production of useful fine chemicals (Yun et al*.,* 2007). The practical goal in CYPs research is to search for the enzymes which are able to produce chemicals that are difficult to be prepared by traditional organic synthetic (Bhattarai et al., 2012). This is due to its exclusive spectroscopic properties, catalytic diversity, and broad substrate range variety of useful chemical transformations that are essential in both biological function and chemical synthesis (Sigman et al*.,* 1999). The enzymes produce the fine chemical in regio and stereo-selective way making CYP a versatile and powerful oxidant. Therefore, CYP had gained much attention in producing many useful fine chemicals of hydroxylated products which are essential in both biological function and chemical synthesis (Sigman et al., 1999; Urlacher and Girhard, 2012; Yun et al., 2007).

CYPs cover a wide range of applications in the production of drugs, drug metabolites and work as catalysts in various chemical (Gillam 2008; Jung et al*.,* 2011). Despite their exclusive catalytic capabilities, only a limited number have been exploited in preparative of chemical reactions or industrial chemical processes (Julsing et al*.,* 2008). As reported by Jung et al*.* (2011), many natural CYPs are insoluble, expressed at low levels, and exhibit activity insufficient for practical biocatalysis.

The biotransformation of vitamin D_3 to 1α,25-dihydroxyvitamin D_3 is one of the most successful applications of CYP in biotechnology. The product has been used to treat numerous diseases such as osteoporosis, chronic renal failure and hypothyroidism (Sakaki, 2012; Yasutake et al., 2009). Vitamin D_3 is a biologically inactive compound that requires one or more CYPs to catalyze the formation of the most active form of vitamin D hormone, 1α,25-dihydroxyvitamin D_3 or calcitriol. The major function of calcitriol is to maintain safe levels of calcium and phosphorus in the blood, by regulating the absorption of these ions in the intestine, bone and kidney. In addition, calcitriol is also recognized as an important anti- proliferative factor for dividing cells and tissues, as vitamin D deficiency is now linked to over 20 forms of cancer (Guyton et al., 2003). The chemical synthesis of 1α,25-dihydroxyvitamin D_3 requires complex procedures, including almost 20 reactions steps with low production yields. Therefore, development of an efficient and simplified production process for calcitriol remains an important area of investigation (Sakaki et al., 2011; Zhu and Okamura, 1995).

In the process of vitamin D_3 conversion to 1α,25-dihydroxyvitamin D_3, the hydroxylation of the side chain at C-25 or C-24 is a prerequisite. The enzyme-catalyzed vitamin D_3 25-hydroxylation is an essential step for the activation of vitamin D_3 and important for understanding the entire activation process of vitamin D_3 (Aiba et al., 2006). Knowledge on vitamin D 25-hydroxylation by bacterial CYP is limited, although their involvement in several essential bioconversions has been reported. Further exploration of bacterial CYP enzymes which could metabolize vitamin D_3 is necessary.

There are several types of CYP vitamin D 25-hydroxylases and most of them are of eukaryotic origin. They are mostly present in mitochondria and microsome of liver (Yamasaki et al., 2004). These include CYP27A1, CYP2R1, CYP3A4, CYP2C11, CYP2D25, CYP2J3 and CYP2J2. They have been found to be fairly important and critical compounds for the regulation of vitamin D metabolism as well as other hydroxylation processes in eukaryotic system. Additionally, some of these 25-hydroxylases were well identified and characterized (Aiba et al., 2006; Zhu and DeLuca, 2012).

Although eukaryotic CYPs are well studied, one of the common limitations of these proteins is that they are membrane-bound (Mclean et al., 2011; Werck-Reichhart and Feyereisen, 2000). The microsomal and mitochondrial 25-hydroxylases are associated in the endoplasmic reticulum and inner membrane of mitochondria, respectively (Zhu and Deluca, 2012). Contrastingly, bacterial CYP in particular is often soluble and targeting in cytoplasm as lacking of the N-terminal membrane anchor (Bernhardt, 2006; Mclean et al., 2011; Werck-Reichhart and Feyereisen, 2000). Its CYPs are found to be useful for oxidation of various compounds including drugs, steroids and fatty acids (Hilker et al., 2008; Lewis and Wiseman, 2005). From a practical point of view, bacterial CYPs can be easily isolated and manipulated and the proteins remain in soluble active

form as compared to insoluble eukaryote CYPs (Budde et al., 2004; Urlacher and Eiben, 2006).

Since, there is an increasing industrial demand to exploit CYP as a valuable biocatalyst; considerable attempts have been devoted to search for novel enzymes with unique metabolic properties. This strategy was attempted in this study, where a novel CYP was mined from the genome of alkaliphilic bacteria, *Bacillus lehensis* G1. This bacterium dwells in soil with the capability to thrive at high pH up to 11. A survey on the complete *B. lehensis* G1 genome revealed that the strain which is usually used for the production of cyclodextrin glucanotransferase (Noor et al., 2014) contained a single candidate gene that potentially coded for a functional CYP enzyme.

A single cDNA specifying soluble CYP enzyme termed as CYP107CB2 exhibited sequence identity of 44% with vitamin D_3 hydroxylase (Vdh) from *Pseudonocardia autotrophica* which had been grouped into the CYP107 family of enzymes. CYP Vdh is a vitamin D_3 hydroxylase (protein data bank ID: 3A4G; resolution: 1.75 Å) responsible in hydroxylating vitamin D_3 to 1α,25-dihydroxyvitamin D_3 via 25-hydroxylation and 1α-hydroxylation (Fujii et al., 2009; Yasutake et al., 2010). The lack of structural information for the putative CYP107CB2 from *B. lehensis* G1 prevented a more detailed characterization of its biological role. In this respect, this study seeks to address the hypothesis that an identified CYP biocatalyst from *B. lehensis* G1 is an active protein and could be used to hydroxylate vitamin D_3 metabolites.

The study was divided into four major experiments. The first was on bioinformatics analysis of CYP sequence from *B. lehensis* G1 followed by second experiment to clone, express, purify and confirm the presence of CYP107CB2 protein via molecular weight evaluation. The third experiment determined the characteristics and biological function of CYP107CB2 through UV-Vis spectroscopy analysis and hydroxylation assay. Optimization and characterization of CYP107CB2 to increase the production of hydroxylated products were also conducted. Last experiment was on crystallization trial of CYP107CB2 protein and X-ray diffraction on protein crystal.

1.2 Objectives

The main objective of this study was to mine, identify and characterize the putative CYP107CB2 from *B. lehensis* G1. The specific objectives were:

1. To conduct bioinformatics analysis on CYP sequence from the genome of *B. lehensis* G1
2. To clone, express, purify and determine the molecular weight of CYP protein
3. To characterize the spectral properties and biological function of CYP and to optimize the production of hydroxylated product

4. To crystallize the CYP protein and diffract the CYP crystal using in-house X-ray diffractometer.

1.3 Significance of study

Present study demonstrates vitamin D_3 25-hydroxylase derived from an environmental bacilli strain in identifying the metabolic function of a new CYP107CB2. The study provides useful insights into the nature of substrate selection for CYP107CB2, which can guide future docking studies, as well as functional experiments. The discovery of a CYP107CB2 that acts as a 25-hydroxylase, has suggested the contribution of CYP family to the metabolism of vitamin D_3. This study is important as CYP107CB2 can serve as a microbial model for eukaryotic's 25-hydroxylase and provide an alternative to study the metabolism of vitamin D_3 without the technical difficulties of handling insoluble membrane proteins.

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