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OVEREXPRESSION AND CRYSTALLIZATION OF 205Y LIPASE FROM Bacillus sphaericus 205Y

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By

SAYYEDEH HODA HAMOONI

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

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DEDICATION

To my dearly beloved parents (Jalil and Fatemeh) for their endless love, support, care and encouragement.

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

OVEREXPRESSION AND CRYSTALLIZATION OF 205Y LIPASE FROM *Bacillus sphaericus* 205Y

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Chairman : Professor Raja Noor Zaliha Raja Abd Rahman, D.Eng.
Faculty : Biotechnology and Biomolecular Sciences

Lipases are ubiquitous in nature and produced by different plants, animals and microorganisms. They catalyse hydrolysis as well as the reverse reaction, esterification, transesterification and interesterification. Structural studies and X-ray crystallography of lipases can provide clues towards understanding their properties and function. Previously, the gene encoding 205y lipase was isolated from Bacillus sphaericus 205y, cloned into the pUC19 vector and expressed extracellularly in Escherichia coli TOP10 host. The gene encoding mature lipase (without signal peptide and transmembrane) was cloned into the pBAD vector and expressed intracellularly in E. coli TOP10 host. This enzyme was purified and characterized as an organic solvent tolerant lipase. However, the recombinant cells harbouring pUC19/205y and pBAD/205y-SP-TM showed low expression of target protein that was not sufficient for crystallization. Therefore, high levels of protein expression and obtaining the best quality crystal suitable for X-ray diffraction were the desired goals of this research. Comparative sequence analysis of 205y lipase gene along with phylogenetic tree explored a new family in lipases classification, family IX. The 205y lipase gene was resynthesized to remove 16 rare codons in the gene sequence. The resynthesized gene was cloned into pET-16b vector and transferred into E. coli Top10. In order to overexpress the 205y lipase gene, different parameters were optimized. The recombinant pET-16b/205y vector was subtransformed into three different expression hosts and the highest expression was achieved by E. coli Roseta-gami pLysS (DE3). The highest 205y lipase activity was 118 U/mL using p-NP decanoate as substrate. The 205y enzyme was purified using two steps of hydrophobic interaction chromatography (HIC) and gel filtration (GF) chromatography to 55% recovery with 3.3 fold purification. To screen for crystallization, different formulations from three screening kits were used. Formulation of crystal screen I No 16 composed of 150 mM sodium citrate tribasic dihydrate, (pH 5.6), 20% 2-propanol, 20% polyethylene showed the best result. Subsequently, to obtain a high quality crystal, the formulation was optimized using different parameters such as protein concentration, buffer,

precipitant and temperature in setting drop vapour diffusion technique. Izit Crystal Dye (Hampton Research, USA) was used to distinguish the protein crystal from salt crystal. The attempt to diffract the 205y lipase crystal using in-house X-ray diffractometer system (Bruker AXS) was able to be processed. The diffraction images were collected and they showed good intensity and regular reflection spots at 2.25Å resolution. In conclusion, the 205y lipase gene with a few closed isolated genes from GenBank databased was represented as a new family of lipases (family IX). The expression of lipase under the control of chemically inducible T7 promoter was higher than other previously tested promoters. In addition, the high level expression led to appropriate amount of pure protein for crystal optimization. Ultimately, the proper size and quality crystal was formed and successfully X-ray diffracted.

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

PENGEKSPRESAN LAMPAU DAN PENGHABLURAN LIPASE 205Y DARIPADA *Bacillus sphaericus* 205Y

Oleh

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Lipase adalah bahan yang sedia ada dalam alam sekitar dan dihasilkan oleh pelbagai tumbuhan, haiwan dan mikroorganisma. Ia merupakan pemangkin bagi hidrolisis serta reaksi songsangnya, iaitu esterifikasi, transesterifikasi serta interesterifikasi. Penyelidikan struktural dan sinar-X kristalografi lipase boleh memberi petunjuk ke arah memahami sifat dan kegunaanya. Sebelum ini, gen pengekodan 205y lipase telah diisolasi dari Bacillus sphaericus 205y, diklonkan ke dalam vektor pUC19 dan diekspresi secara luaran sel dalam perumah Esterichia coli TOP10. Gen pengekodan matang lipase (tanpa isyarat peptida dan transmembran) telah diklonkan ke dalam vektor pBAD dan diekspresi secara dalaman sel dalam perumah E.coli TOP 10. Enzim ini telah ditulen dan dikategorikan sebagai pelarut organik yang toleran kepada lipase. Walau bagaimanapun, sel rekombinan yang mengandungi pUC19/205y dan pBAD/205y-SP-TM menunjukkan nilai ekspresi protin sasaran yang tidak mencukupi untuk pengkristalan. Oleh itu, tahap eksperesi protin yang tinggi dan memperoleh kualiti kristal yang terbaik serta sesuai untuk proses pembelauan menggunakan sinar-X adalah matlamat yang dikehendaki untuk penyelidikan ini. Analisis urutan perbandingan bagi gen 205y lipase beserta pokok filogenetik menemui sebuah keluarga baru dalam klasifikasi lipase, keluarga IX. Gen lipase 205y telah disintesis semula bagi mengeluarkan 16 kodon yang jarang dalam urutan gen. Gen yang disintesis semula telah diklonkan ke dalam vektor pET-16b dan dipindahkan ke dalam E.coli Top10. Bagi mendapatkan pengekspresian lebih gen 205y lipase, parameter berbeza telah dioptimumkan. Vektor pET-16b/205y rekombinan telah mengalami sub-transformasi, kepada tiga ekspresi berlainan dan pengekspresian tertinggi diperoleh pada E.coli Roseta-gami pLysS (DE3). Aktiviti lipase 205y tertinggi adalah 118U/mL menggunakan p-NP dekanoat sebagai substrat. Enzim 205y telah ditulen menggunakan dua langkah kromatografi interaksi hidrofobik (HIC) dan filtrasi gel (GF) kromatografi kepada 55% pengambilan semula dengan 3.3 lipatan purifikasi. Untuk melayar bagi kristalisasi, formulasi yang berbeza daripada tiga kit layaran telah digunakan. Formulasi layaran kristal I No 16 terdiri daripada 150mM natrium sitrat

tribasic dihidrat (pH 5.6), 20% 2-propanol, 20% polietilena menunjukkan keputusan yang terbaik. Kemudiannnya, bagi memperoleh kristal berkualiti tinggi, formulasi telah dioptimumkan menggunakan parameter berbeza seperti konsentrasi protin, penampan, serta suhu dalam menetapkan teknik penyebaran jatuhan wap air. Pewarna Kristal Izit (Hampton Research, USA) telah digunakan bagi membezakan antara protin kristal daripada garam kristal. Cubaan untuk membelau kristal lipase 205y menggunakan sistem meter pembelauan sinar-X yang sedia ada (Bruker AXS) telah dapat diproses. Imej pembelauan telah dikumpul dan memunjukkan keamatan yang baik dan titik bayangan yang kerap pada resolusi 2.25Å. Kesimpulannya, gen lipase 205y dengan beberapa gen isolasi tertutup daripada pangkalan data GenBank telah diwakili sebagai keluarga baru lipase (keluarga IX). Ekspresi lipase bawah kawalan penggalak T7 secara pembawaan kaedah kimia lebih tinggi berbanding penggalak-penggalak yang diuji sebelum ini. Sebagai tambahan, ekspresi tahap tinggi membawa kepada jumlah protin tulen yang mencukupi bagi pengoptimuman kristal. Akhirnya,saiz dan kualiti kristal yang sesuai telah dapat dibentuk dan berjaya melalui pembelauan secara kaedah sinar-X.

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS AND SYMBOL

Å	Angstrom
bp	Base pair
cm	Centimeter
Da	Dalton
dH2O	Distilled water
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
g	Gram
h	Hour
IPTG	Isopropyl-β-D-thiogalactopyranoside
kDa	Kilo Dalton
L	Litre
µg	Microgram
µL	Microlitre
µmol	Micromole
mg	Milligram
mL	Millilitre
min	Minute
M	Molar
ORF	Open reading frame
%	Percentage
PMSF	Phenyl methyl sulphonyl fluoride
PCR	Polymerase chain reaction
rpm	Rotation per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
STDV	Standard deviation
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

Enzymes are biological catalysts and the product of all living cells. They are responsible for specific biochemical reactions. Their main analytical operations are to support almost all the chemical reactions by seeping up their metabolisms. Lipase and esterases are ubiquitous enzymes that can exist in all kinds of organisms such as humans, plants, animals and also microorganisms which are studied in this research. These enzymes have significant roles in physiological and biotechnological analyses systems. They are powerful enzymes for catalysing, hydrolysis, as well as esterification and transesterification reactions with water insoluble esters (Reis *et al.*, 2009). The enzyme conformation changes when it comes in contact with a water-insoluble substrate. This phenomenon is becoming more valuable and interesting for the understanding of structure-function of enzymes (Saxena, Jónsson*, et al.*, 2003). Lipases and esterases constitute a significant category of biotechnological enzymes for the purpose of synthesizing of biopolymers and biodiesel, resolution of racemic mixtures, region selective acylation of glycols and menthols, peptides synthesis, enantiopure pharmaceutical production, agrochemicals and flavor compounds (Jaeger and Eggert, 2002) as well as in the dairy, detergents and paper manufacturing industries (Miroliaei and Nemat-Gorgani, 2002).

Lipases and esterases also constitute enzymes that can recover activation in a variety of organic solvents, which is by the time they can manage various transformation and hydrolytic reactions (Tripathi *et al.*, 2004). The organic solvent tolerant lipases have been proven to be excellent biocatalysts for performing various synthetic reactions such as esterification, transesterification and interesterification in organic solvents, such as production of pharmaceuticals and biofuel, under a water-restricted environment (Tran and Chang, 2014).

Generally, enzyme functions can be well-understood through an extensive study of the molecular architecture of protein and it can be organized in four levels of structure: primary, secondary, tertiary and quaternary. Most proteins are not in biological functions unless they are in their native conformation, which comprises a three-dimensional structure (Boyer, 2003). The structure of a protein paves the way for some of the many details about its possible functions, as well as clues to find the critical residues and the location of functional sites (Aloy *et al.*, 2001; Reddy *et al.*, 2001; Yao *et al.*, 2003). In addition, X-ray crystallography visualizes protein structures at the atomic level and increases comprehension of the protein function. Particularly, study could provide detailed information toward an understanding of the interaction mechanism of proteins with other molecules, the factors affecting conformational changes of proteins and catalytic performance of enzymes. Enlightened, with this information, new drugs that target a particular protein could be designed and/or an enzyme could be rationally engineered for a specific industrial purpose (Rupp, 2009).

Hence, an attempt was made to exploit the potential of this technique for structure determination of proteins. Protein crystallization, an extensively researched process in the physical sciences, is nowadays a hindrance macromolecule structure (Pullara *et al.*, 2005). Therefore, a single crystal of high structural perfection, with minimum dimension of hundred microns is necessary to solve the structure of 205y lipase. Consequently, a wide range of optimization parameters is necessary to investigate the growth limit, quality and size of the crystal. Regarding the great applications of organic solvent tolerant lipases, when the representative gene clones into an appropriate expression system under the control of the *T7* promotor, the expression level will be increased (Wujak *et al.*, 2013). For further investigation on the structure of 205y lipase, the high quality of crystal will be required for X-ray diffraction. High level expression of 205y lipase can be obtained in proper expression system besides that optimizing some impressive parameters for overexpression. Also optimization of some impressive parameters for crystallization can lead to obtain a high quality crystal suitable for X-ray diffraction.

Objectives

The objectives of this study was improve the expression level of 205y lipase to ensure the proper amount of protein for crystallization.

Subsequence objectives are as follows:

- To analyses the 205y lipase gene sequence of 205y lipase and update the lipase classification.
- To purify and crystallization of 205y lipase gene from *Bacillus sphaericus* 205y.

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