UNIVERSITI PUTRA MALAYSIA

EXPRESSION, CHARACTERIZATION AND STRUCTURE ELUCIDATION OF THERMOTOLERANT LIPASE WITH BROAD PH ISOLATED FROM ANTARCTIC Pseudomonas sp. STRAIN AMS3

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By

WAHHIDA BT LATIP

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

JANUARY 2018

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DEDICATION

To my beloved family and husband

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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

EXPRESSION, CHARACTERIZATION AND STRUCTURE ELUCIDATION OF THERMOTOLERANT LIPASE WITH BROAD pH ISOLATED FROM ANTARCTIC *Pseudomonas* sp. STRAIN AMS3

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January 2018

Chairman: Professor Raja Noor Zaliha Raja Abd Rahman, PhD
Faculty: Biotechnology and Biomolecular Sciences

Lipase is an enzyme that plays an important role in detergent, cosmetic and pharmaceutical industries. Lipase from Antarctic region has gained huge interest for industrial applications but was impaired due to the limited crystallographic and structural information. To maximize its full biocatalytic potentials, the understanding on the biochemical and structural features of this class of enzyme are imperative. Therefore, this research was conducted to isolate, express, characterize and determine the structure of lipase from an Antarctic bacteria via *in silico* and crystallographic approaches. In this study, the best lipolytic producing bacterium was identified as *Pseudomonas* sp. AMS3. The lipase gene was isolated from the reconstructed of genomic library. The positive colonies were verified as recombinant lipase producer using triolein and Rhodamine B agar plates. Recombinant plasmid analysis revealed an open reading frame (ORF) of 1353 nucleotides encoding 450 amino acids. The protein sequence has two domains, namely GST C and lipase domains at the N and C terminal, respectively. The gene encoding the AMS3 lipase was cloned into a pET 51b vector and heterologously expressed in *E. coli* BL21 (De3). The best expression condition for AMS3 lipase was at 0.5 mM IPTG, incubated at 20 °C and 12 h of induction time. The recombinant AMS3 lipase was purified using single step affinity chromatography with a 50% recovery and 1.52 fold purity. The molecular weight of the recombinant AMS3 lipase was estimated at ~60 kDa. Biochemical characterization of the lipase showed the enzyme work at a broad temperature profile of 10-70 °C and stable at 10-60 °C. The AMS3 lipase activity also exhibited a broad pH range of 5 to 10 and was activated in the presence of metal ions. The lipase was able to hydrolyze long chain lipid substrate with and without organic solvents. The AMS3 lipase was successfully crystallized using sitting drop method. The X-ray diffraction of the AMS3 lipase crystal was very poor, thus no crystal structure was able to be determined. In order to obtain the crystal structure, the AMS3 lipase was truncated at the N terminal domain based in the computational analysis.

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Molecular dynamic simulation revealed a flexible N-terminal domain possibly interfering with the crystallization process. The truncated AMS3 lipase was subcloned and express in *E.coli* BL21 (De3*)* .This truncated enzyme has an approximate molecular weight of ~45 kDa. The truncated AMS3 lipase has similar features with native except for pH profile whereby the lipase was most active at alkaline condition (pH 8-pH 10). The truncated AMS3 lipase was successfully crystallized using sitting drop method and the crystal was diffracted to 2.7 Å. The 3D structure was refined, validated and deposited to Protein Data Bank (PDB no. 5XPX). The refined truncated AMS3 lipase structure revealed a common α/β hydrolase fold with bound calcium and zinc ions. The protein active site contained serine hydrolase catalytic triad, namely serine, aspartic acid and histidine. Superposition of the lipase thermostable homologs showed structural differences that could be potentially important towards the lipase temperature adaptation. It was generally concluded, the reduction of hydrogen bond and changes to the secondary structure content of the lipase allowed the enzyme to be active at broad temperatures. The structural and biochemical information will be beneficial towards rational design of lipase suitable for industrial application.

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

PENGHASILAN, PENCIRIAN DAN ELUSIDASI STRUKTUR LIPASE TAHAN HABA DENGAN PH LUAS DARI ANTARTIKA *Pseudomonas* SP. STRAIN AMS3

Oleh

WAHHIDA LATIP

Januari 2018

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Lipase adalah enzim yang memainkan peranan penting dalam industri pencuci, kosmetik dan farmaseutikal. Lipase dari rantau Antartika telah mendapat perhatian yang tinggi untuk aplikasi perindustrian tetapi terdapat halangan disebabkan oleh maklumat kristal dan struktur terhad. Untuk memaksimumkan potensi biokatalitik penuhnya, pemahaman tentang ciri-ciri biokimia dan struktur enzim kelas ini adalah penting. Oleh itu, kajian ini dijalankan untuk mengasingkan, menyatakan, mencirikan dan menentukan struktur lipase dari bakteria Antartika melalui pendekatan *silico* dan kristalografik. Dalam kajian ini, bakteria penghasil lipolitik yang terbaik dikenalpasti sebagai *Pseudomonas* sp. AMS3. Gen lipase telah diasingkan daripada dibina semula dari perpustakaan genomik. Koloni positif telah diverifikasi sebagai pengeluar lipase rekombinan menggunakan agar triolein dan Rhodamine B agar. Analisis plasmid rekombinan mendedahkan bingkai bacaan terbuka (ORF) daripada 1353 nukleotida pengekodan untuk protein adalah 450 asid amino. Urutan protein mempunyai dua domain, iaitu GST C dan lipase domain pada terminal N dan C masing-masing. Pengekodan gen lipase AMS3 ini diklon ke dalam vektor pET 51b dan dinyatakan secara heterolog dalam *E. coli* BL21 (De3). Keadaan optimum terbaik untuk lipase AMS3 adalah pada 0.5 mM IPTG, diinkubasi pada 20 ° C dan 12 jam masa inkubasi. Lipase AMS3 rekombinan telah ditulenkan menggunakan kaedah kromatografi afiniti langkah tunggal dengan penulinan sebanyak 50% dan hasil penulenan mempunyai gandaan 1.52 kali. Berat molekul lipase AMS3 rekombinan dianggarkan pada ~60 kDa. Pencirian biokimia lipase menunjukkan enzim aktif pada profil suhu 10-70 ° C dan stabil pada 10-60 ° C. Aktiviti lipase AMS3 juga mempamerkan julat pH luas, dari pH 5 hingga 10 dan kekal aktif dengan kehadiran ion logam. Lipase mampu menghidrolisis substrat lipid berantai panjang didalam pelarut organik atau tanpa pelarut organik. Lipase AMS3 berjaya dikristalkan dengan menggunakan kaedah

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titisan duduk. Pembelauan sinar-X dari kristal lipase AMS3 sangat rendah, oleh itu tiada struktur kristal dapat ditentukan. Untuk mendapatkan struktur kristal, lipase AMS3 dipotong pada domain terminal N berdasarkan analisis pengkomputeran. Simulasi dinamik molekul mendedahkan domain N-terminal yang fleksibel yang mungkin mengganggu proses penghabluran kristal. Lipase AMS3 dipotong dan dihasilkan dalam *E.coli* BL21 (De3). Enzim ini mempunyai berat molekul kira-kira ~ 45 kDa. Lipase AMS3 yang dipotong ini mempunyai ciri-ciri yang sama seperti yang asli kecuali profil pH di mana lipase paling aktif pada keadaan alkali (pH 8-pH 10). Lipase AMS3 yang dipotong berjaya dikristalkan menggunakan kaedah titisan duduk dan kristal dibelau kepada 2.7 Å. Struktur 3D disempurnakan, disahkan dan didepositkan ke Protein Data Bank (PBD no. 5XPX). Struktur AMS3 lipase mendedahkan hidrolase lipatan α/β dan mempunyai ion kalsium dan zink. Tapak aktif protein serine hidrolase, tapak pemangkin bertiga iaitu serina, asid aspartik dan histidina. Pertindihan homolog dengan struktur suhu panas menunjukkan perbezaan struktur yang mungkin berpotensi penting ke arah penyesuaian suhu lipase. Secara amnya disimpulkan, pengurangan ikatan hidrogen dan perubahan kandungan struktur sekunder lipase membolehkan enzim aktif pada suhu yang luas. Maklumat struktur dan biokimia akan memberi manfaat kepada reka bentuk lipase yang sesuai untuk aplikasi perindustrian.

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LIST OF ABBREVIATIONS

Å	Angstrom
A600/O.D600	Absorbance/optical density at 600 nm
bp	Base pair
Da	Dalton
DMSO	Dimethyl sulfoxide
FFA	Free fatty acid
g	Relative centrifugal force
g	Gram
h	hour
IPTG	isopropyl β-D- Thiogalactopyranoside
kDa	kilo Dalton
L	Liter
M	Molar
mM	Milimolar
mg	Miligram
mg/mL	Miligram per mililiter
min	Minute
nm	Nanometer
ns	nanosecond
PCR	Polymerase chain reaction
ps	Picosecond
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
rpm	Rotation per minute
s	Second
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
U	Unit
U/mL	Unit per mililiter
µL	microlitre
µmol	micromole
w/v	Weight per volume
v/v	Volume per volume
V	Voltage

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CHAPTER 1

INTRODUCTION

More than three-quarters of the Earth's surface is occupied by cold ecosystems which comprised of the depth oceans, polar and alpine regions. These permanently cold environments have been successfully colonized by a class of extremophilic microorganisms that are known as psychrophiles (from the Greek 'psychro'=cold). The ability of these organisms to thrive well at temperatures that are close to or below the freezing point of water requires a vast array of adaptations to maintain the metabolic rates and sustained growth compatible with life in these severe environmental conditions. Enzymes have evolved to maintain the threshold of metabolic activities (Hirokoshi *et al.,* 2011).

Psychrophiles mostly produced cold active enzymes that are more active at low and moderate temperatures compared to homologous mesophilic enzymes. The enzymes are able to retain their flexibility and dynamics of their active site at low temperatures. They are highly useful in biotechnological applications such as saving energy cost, preventing undesired chemical transformation, loss of volatile compounds and do not require expensive heating or cooling system (Margesin *et al.,* 2003). Very limited number of enzymes that can catalyse reactions and at the same time to remain stable at high temperature was reported from psychrophilic bacteria such as *Oleispira* Antarctica lipase (60 °C) and esterase EML28 (55 °C) (Park *et al.,* 2007)

Lipases or triacylglycerol acylhydrolase (EC 3.1.1.3) are long chain fatty acid ester hydrolases that can break down lipids into free fatty acids and glycerol. The enzymes occur widely in nature and largely produced by animals, plants and microbes. Microbial lipases have gained huge attention from industry because of their temperature, pH and organic solvent flexibility (Verma *et al.,* 2012). The enzymes have been widely used for biotechnological and industrial applications such as food industry, oil processing, surfactants, detergents, pesticides, environmental management and leather industries. The temperature and pH stability of lipases has been regarded as the most important characteristics required by the industries. Most of the industrial lipases are currently from thermophilic bacteria with thermophilic properties (Turner *et al.,* 2007). Thermotolerant lipases have become attractive due to their catalytic activity at broader temperatures ranging from cold to hot temperatures. The thermotolerant lipases offer huge economic benefits of energy saving as they negate requirement for expensive heating steps, function in hot and cold environment, provide increase reaction yield and accommodate a high level of stereo specificity. To better understand the potential applications of these lipases in industry and biotechnology the structural information of thermotolerant lipase is very crucial.

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Protein engineering is an excellent and powerful method used to design a stable protein with high efficiency. In other words, it is a tool used to understand a protein structure and function (Kapoor and Travesset, 2015). There are many protein engineering methods such as recombinant technology, homology modelling and X-ray crystallography. The recombinant technology is a conventional technique in which two or more foreign genes are combined together. High level expression of a protein allows for its biochemical characterization. The development of excellent expression systems such as *E. coli* expression system offer rapid production and can be easily manipulated (Rosano *et al*., 2014).

Functional information of lipases is obtained from their 3D structures usually derived from X-ray crystallography studies. The X-ray crystallography is the most perfect method used to describe enzymes behaviours. The three-dimensional structure of a protein at atomic level is important for several reasons such as to gather information on the protein biology and function. Chemical processes like biomass conversion, bioremediation and fermentation are highly performed at lower temperature as it reduces the risk of contamination by mesophiles and saves energy (Tsai *et al*., 2009). Therefore, enzymes with such unique properties (i.e. broad temperature and pH) have gained an increasing interest. Currently, there are only a few reports on the structure of thermotolerant Antarctic lipase that is active at a broad temperature and pH ranges. Understanding the relationship of the thermotolerant structure with the enzyme features are still new and vague. To answer this problem a few objective have been designed.

The objectives of this study were to isolate a lipase gene from Antarctic bacteria and to determine the enzymes features via biochemical, biophysical and structural biology. The specific objectives of this study are as follows:

1) To indentify and isolate lipase gene from psychrophilic bacteria
2) To optimize the recombinant AMS3 lipase expression
3) To purify and characterize the recombinant AMS3 lipase via biochemical and biophysical approaches
4) To predict 3D structure and molecular dynamic simulation of the AMS3 lipase
5) To crystallize and elucidate the crystal structure of truncated AMS3 lipase

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