UNIVERSITI PUTRA MALAYSIA

EXPRESSION, PURIFICATION AND CHARACTERIZATION OF ORGANIC SOLVENT TOLERANT LIPASE FROM BACILLUS SPHAERICUS 205Y

MOOHAMAD ROPANING SULONG.

EXPRESSION, PURIFICATION AND CHARACTERIZATION OF ORGANIC SOLVENT TOLERANT LIPASE FROM *BACILLUS SPHAERICUS* 205Y

By

MOOHAMAD ROPANING SULONG

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

November 2005

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

Expression, Purification and Characterization of Organic Solvent Tolerant Lipase from *Bacillus sphaericus* 205y

By

MOOHAMAD ROPANING SULONG

November 2005

Chairman: **Associate Professor Raja Noor Zaliha R. A. Rahman, PhD**

Faculty: **Biotechnology and Biomolecular Sciences**

One thousand and two hundred base pairs (bp) of open reading frame (ORF) encoding for an organic solvent tolerant lipase gene was cloned and expressed intra- and extracellularly. The intracellular expression was done using pBAD TOPO TA expression vector with 0.02% (v/v) of L-arabinose as optimum inducer after 4 h of incubation at 37°C with an optimum lipase activity of 0.5 U/ml. The extracellular expression was obtained by co-transforming pJL3 expression vector encoding bacteriocin release protein (BRP) into *E. coli* TOP10 harbouring the recombinant pBAD TOPO TA. The secretory expression of recombinant organic solvent tolerant 205y lipase increased the lipase activity tremendously to 2.5 U/ml.

The 205y lipase was purified to 8-fold and 32% recovery using two steps purification, ultrafiltration and hydrophobic interaction chromatography (HIC).

The molecular mass of the purified 205y lipase revealed homogeneity on SDS-PAGE at approximately 30 kDa.

The optimum pH for the purified 205y lipase was found at 7.0-8.0 and its stability showed a broad range of pH value between pH 5.0 to pH 13.0 at 37 °C. The purified 205y lipase exhibited an optimum temperature of 55°C. The lipase activity of the purified 205y lipase was enhanced in the presence of alkaline metal such as (Na) and alkaline earth metal such as (Mg^{2+}, Ca^{2+} and Ba^{2+}). However, the 205y lipase activity was inhibited in the presence of transition metal ions, Zn^{2+}, Cu^{2+} and Fe^{3+}. The chelating agent, ethylenediaminetetraacetic acid (EDTA), did not affect the purified 205y lipase activity while serine hydrolase inhibitor, phenylmethane sulfonoyl fluoride (PMSF), inhibited the lipase activity.

The activity of the purified 205y lipase demonstrated good stability in the presence of methanol, *p*-xylene and *n*-decane with Dimethylsulfoxide (DMSO) being the most stabilizing. The purified 205y lipase showed a preference toward hydrolysing medium carbon chain length of triglycerides, tricaprin (C10). The purified 205y lipase also exhibited 1,3- regiospecific nature of the enzyme.

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

PENGEKPRESAN, PENULINAN DAN PENCIRIAN LIPASE YANG TOLERAN TERHADAP PELARUT ORGANIK DARI *BACILLUS SPHAERICUS* 205Y

Oleh

MOOHAMAD ROPANING SULONG

November 2005

Pengerusi: **Profesor Madya Raja Noor Zaliha R. A. Rahman, PhD**

Fakulti: **Bioteknologi dan Sains Biomolekul**

Satu ribu dua ratus pasangan bes daripada rangka bacaan terbuka yang mengkodkan gen lipase toleran kepada pelarut organik telah diklonkan dan diekspeskan secara intrasel dan juga ekstrasel. Pengekspresan secara intrasel dijalankan dengan menggunakan vektor pengekspresan pBAD TOPO TA dengan 0.02% (v/v) L-arabinosa sebagai penggalak optimum selepas 4 jam pengeraman pada suhu 37°C dengan aktiviti optimum lipase sebanyak 0.5 U/ml. Pengekspresan ekstrasel diperolehi dengan kotransformasi vektor pengekspresan pJL3 yang mengkodkan protein bebas bakteriosin (BRP) ke dalam *E. coli* TOP10 yang mengandungi pBAD TOPO TA rekombinan. Pengekspresan ekstrasel bagi lipase toleran kepada pelarut organik telah meningkatkan aktiviti lipase secara mendadak kepada 2.5 U/ml.

Lipase 205y telah ditulenkan sebanyak 8 kali dengan 32% perolehan menggunakan dua langkah penulinan, ultrafiltrasi dan kromatografi interaksi

hidrofobik. Jisim molekular bagi lipase 205y yang tulen telah menunjukkan kehomogenan melalui SDS-PAGE dengan anggaran sebanyak 30 kDa.

pH optimum lipase 205y yang tulen adalah di antara 7.0-8.0 dan menunjukkan kestabilan pada nilai pH yang luas di antara pH 5.0 hingga pH 13.0 pada suhu 37ºC. Suhu optimum bagi lipase 205y yang tulen adalah pada suhu 55ºC. Aktiviti lipase 205y yang tulen ini ditingkatkan dengan kehadiran logam alkali seperti (Na) dan logam alkali bumi seperti (Mg^{2+}, Ca^{2+} dan Ba^{2+}). Walau bagaimanapun, aktiviti lipase 205y yang tulen ini direncat dengan kehadiran ion logam peralihan, Zn^{2+}, Cu^{2+} and Fe^{3+}. Agen pengkelatan, ethylendiamintetraacetat (EDTA), tidak memberi sebarang kesan ke atas aktiviti lipase 205y ini, manakala perencat serine hydrolase, phenylmethylsulfonyl fluoride (PMSF), merencatkan aktiviti lipase.

Aktiviti lipase 205y yang tulen ini menunjukkan kestabilan dengan kehadiran metanol, *p*-xylena dan *n*-dekana dangan Dimetilsulfoksida (DMSO) menunjukkan lebih stabil. Lipase 205y yang tulen ini menunjukkan kecenderungan menghidrolisiskan rantaian tengah trigliserida, tricaprin (C10). Lipase 205y rekombinan yang toleran terhadap pelarut organik yang telah ditulenkan ini juga telah menunjukkan 1,3-regiospesifik.

ACKNOWLEDGEMENTS

Thanks to Allah the Most Merciful and Gracious for blessing me and giving me strength to complete my study.

I would like to express my deepest gratitude and appreciation to the chairperson, Assoc. Professor Dr. Raja Noor Zaliha Raja Abd. Rahman for her consistence guidance and valuable advises throughout my study. I am also grateful to supervisory committee members, Professor Dr. Abu Bakar Salleh and Professor Dr. Mahiran Basri for their supports and guidance. My appreciation also forwarded to supervisory meeting committee members, Assoc. Professor Dr. Mohd. Basyaruddin Abdul Rahman and Dr. Norazizah Shafee.

Also special thanks to all my labmates- Leow, Ain, Ina, Bimo, Bro. Mohamad, Azira, Fairol, Aiman, Sha, Ayub, Shuk, Ghani, Su, Tengku, Kok Whye, Amalia, Ada, Chee Fah and Elly for their friendship and help.

Last but not least, I am grateful and indebted to my parents, brothers, lovely wife, Azah binti Abdul Aziz, for her everlasting support and her understanding; for being patient and uncomplaining, also my children, Ahmad Faris and Yasmin, for cheering my life.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A	adenine
bp	base pair
BRP	bacteriocine release protein
C	cytosine
°C	degree centigrade
CCMB	calcium/manganese-based buffer
DNA	deoxyribonucleic acid
dH_2O	distilled water
G	guanine
g	gram
Xg	gravity
h	hour
HIC	hydrophobic interaction chromatography
IPTG	isopropyl-ß-thio-D-galactopyranoside
IEC	ion exchange chromatography
kb	kilobase pair
kDa	kilo Dalton
M	molar
mg	milligram
min	minute
ml	millilitre
mM	millimolar

MW	molecular weight
ng	nanogram
OD	optical density
ORF	open reading frame
PBS	phosphate buffer
PCR	polymerase chain reaction
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED N,N,N,N	tetramethyl-ethylene diamine
T	thymine
U	unit of activity
uv	ultra violet
µM	micro molar
µg	microgram
µl	micro liter
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Enzymes or biological catalysts are complex proteins produced by all living cells. They are responsible for supporting almost all of the chemical reactions meaning that they speed up the chemical reactions in living things. Currently enzymes are grouped into six functional classes by the International Union of Biochemists (I.U.B), where lipases (E.C.3.1.1.3) belong to the class of hydrolases enzymes and the family of carboxylic ester. Lipases have the ability to hydrolyse long-chain acylglycerols ($\geq C_{10}$), whereas esterases hydrolyse ester substrate with short-chain fatty acids ($\leq C_{10}$) (Rahman, *et al.,* 2003).

According to Bornscheuer *et al.* (2002) lipase catalysis occurs at the lipid-water interface and most of lipases show the phenomenon of a so-called interfacial activation whereby high catalytic activity is observed only in the presence of a hydrophobic phase, a lipid droplet dispersed in water or an organic solvent. This phenomenon has been related to the presence of a hydrophobic oligopeptide (the lid or flap) covering the entrance to the active site.

Although lipases are of widespread occurrence throughout the Earth's flora and fauna, they are found more abundantly in microbial flora comprising bacteria, fungi and yeast (Pandey *et al.,* 1999). Microbial lipases have

potential in industrial value due to their substrate specificity and ability to remain active in organic solvent (Sharon *et al.,* 1998). They had been widely used in chemical reactions, especially in optical resolution (Fukusaki *et al.,* 1991; Matsumae *et al.,* 1994).

Bacillus represents a genus of Gram-positive bacteria, which are ubiquitous in nature (soil, water, and airborne dust) capable of forming heat-resistant endospores and producing a variety of degradative extracellular enzymes. Recently, several extracellular lipases have been reported from the genus *Bacillus* such as *B. subtilis* (Dartois *et al.,* 1992; Eggert *et al.,* 2003), *B. liqueniformis* (Khyami, 1996), *B. catenulatus* (Schmidt *et al.,* 1996; 1994), *B. megaterium* (Ruiz *et al.,* 2002) and *B. stearothermophilus* (Hwang *et al.,* 2004).

Organic solvent tolerance has become a desirable characteristic for many lipases due to their functionality in the presence of organic solvent used in many industrial processes. The synthetic potential of lipases in organic solvent has been widely recognized and documented in several publications. Substrates and products of lipase are often insoluble in aqueous solutions, and the enzyme is usually insoluble in organic solvents. Some reactions catalysed by lipase were carried out in organic aqueous two-phase media, which are favourable because the separation of enzyme from substrates or products is easy. However, in general, enzymes are easily denatured and their catalytic activities disappear in the presence of organic solvents (Ogino *et al.,* 1999) unless the enzymes or lipases are organic solvent tolerant.

Other advantageous of using lipases in organic solvent is at low water activity many side-reactions that are water dependent can be prevented, including the denaturation of enzymes (Secundo and Carrea, 2002). The use of organic solvents as reaction media can thus greatly expand the repertoire of enzyme-catalysed transformations. Consequently, a number of potential applications of enzymes that are either impossible or marginal in water become quite feasible and commercially attractive in other solvents (Klibanov, 2001).

The recently acquired ability to incorporate exogenous DNA into bacteria, and to have that DNA replicated as part of the bacterial genetic complement, is of considerable scientific interest. But commercial applications of this new technology demand that foreign genes implanted into bacteria be expressed into protein encoded by that DNA (Levin *et al.*, 1983). On the other hand, microbial lipases are very diverse in their enzymatic properties as well as characteristics that make them attractive for industrial applications. Therefore, the objectives of this research are:

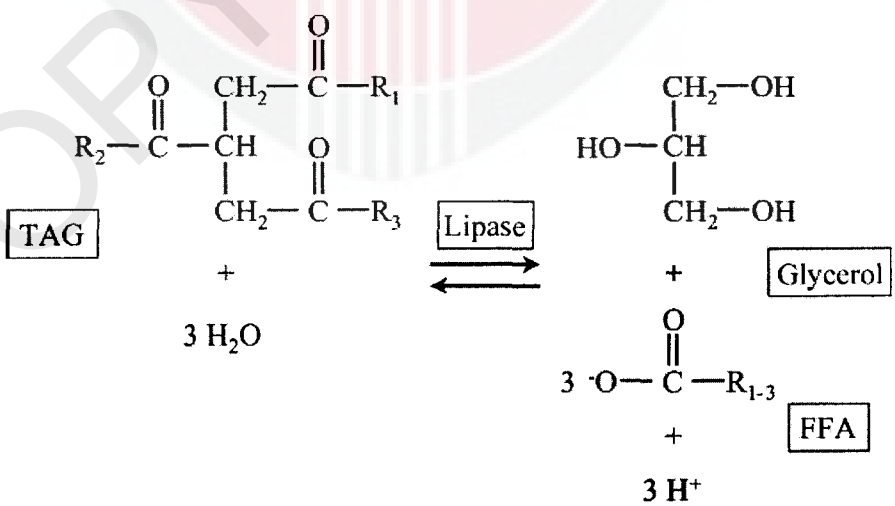
1. To express the organic solvent tolerant lipase gene from *Bacillus sphaericus* 205y.
2. To purify the organic solvent tolerant recombinant lipase from *Bacillus sphaericus* 205y.
3. To characterize the organic solvent tolerant recombinant lipase from *Bacillus sphaericus* 205y.

CHAPTER 2

LITERATURE REVIEW

2.1 Organic solvent tolerant lipases

Lipases (Glycerol ester hydrolases EC 3.1.1.3) are a group of enzymes related to the degradation or synthesis of lipids such as acylglycerols. The mode of actions of lipase are varied from an environment to another, in aqueous solutions with high water content, lipase can hydrolyse acylglycerols or fatty acids esters, while in those with low water content, such as organic solvents; the synthetic reaction of acylglycerols or exchange of fatty acids is catalysed. The lipase reaction can be represented as follows according to Stehr *et al.,* in (2003):



Lipase is believed to be able to perform the reverse reaction of synthesis of triacylglycerols from free fatty acid and glycerol as shown in the previous reaction. This property is extensively used in trans- and inter-esterification reactions in organic solvents to produce useful acylglycerols (Kamini *et al.,* 2000). Lipases can also act at the interface between a hydrophobic lipid substrate and hydrophilic aqueous medium. This phenomenon is due to the presence of a hydrophobic oligopeptide, the lid or flap, covering the entrance of the active site. In hydrophobic environment, the lid moves aside and the substrate can enter the binding pocket. Even so, not all lipases are considered to present this phenomenon (Bornscheuer *et al.,* 2002).

The use of lipase in organic solvents instead of in water provided numerous advantageous. These advantageous such as increasing solubility of hydrophobic substrates, shifting of thermodynamic equilibrium to favour the synthesis over hydrolysis and increasing thermostability of the enzymes (Persson *et al.,* 2002). Nevertheless, not all the lipases will exhibit the same degree of tolerances towards the organic solvents. The stability of a particular protein toward different organic solvents mainly depends on the native conformation of the proteins it self. The reason is due to the organic solvents may distort enzyme molecules or may become competitive inhibitors through specific interaction kinetics and substrate specificity (Ogino and Ishikawa, 2001).

Many questions have been raised among enzymologists regarding the use of enzymes in organic solvents. Perhaps the most obvious question is whether

the protein can conserve its native conformation when it is transferred from the tranquillity of an aqueous buffer to the harsh realities of a non-aqueous process environment (Yang and Russell, 1996). Several hypotheses were proposed by many enzymologists in order to answer the question. One of the current hypotheses is that when an appropriately prepared enzyme is placed in an anhydrous organic solvent, it is kinetically trapped in its native-like conformation (Zaks and Klibanov, 1988) and thus the protein is able to maintain its native structure. Nonetheless, the organic solvent tolerant enzymes have their own profitable as compared to those non-organic solvent tolerant enzymes.

2.2 Sources of lipases and their industrial applications

Lipases are widely distributed among microorganisms, animals, and plants. Different microorganisms have been known to produce a large number of lipases, including yeast, moulds, and bacterial. Microbial lipases are very diverse in their enzymatic properties and substrate specificity, which makes them attractive for industrial applications. The vast majority of wild type microbial lipases reported in the literature are extracellular enzymes, being excreted through the external membrane into the culture medium (Aires-Barros *et al.,* 1994).

Due to their extracellular nature, most of microbial lipases can be produced in large quantities and are stable under non-natural conditions such as high temperatures and non-aqueous organic solvents employed in many

applications (Schmidt-Dannert, 1999). However, the quantity of these extracellular lipases produced naturally, in terms of activity, is less as compared to the recombinant lipases. Therefore, recently many scientists have started to manipulate these lipase genes of wide variety seeking for greater level. For example, the *Bacillus thermocatenulatus* lipase gene, BTL2, was expressed in *Escherichia coli* in order to obtain large amounts of the active enzyme (Schlieben *et al.*, 2004).

In terms of industrial applications, according to Jaeger *et* al., in (1997), the estimated world-wide sales volume for industrial enzymes in 1995 was US$ 1 billion and this volume is definitely forecasted to double until 2005. Among these enzymes, lipases are considered to be the third largest group based on the total volume. The most commercially important field of application for hydrolytic lipases is their addition to detergents, which are used mainly in household dishwasher and industrial laundry, as well as in removing the pitch from the pulp produced in the paper industry and flavour development for dairy products, achieved by selective hydrolysis of fat triglycerides to release free fatty acids; these can act as either flavour or flavour precursors. Lipases have also been employed in catalysis reactions in organic chemistry such as the synthesis of chiral amines and the synthesis of the calcium antagonist Diltiazem ™ (Reetz and Jaeger, 1998). Biopolymers such as polyphenols, polysaccharides and polyesters are receiving increasing attention because they are biodegradable and produced from renewable natural resources. Lipases are used as catalysts for the polymeric synthesis with the major advantages being their high selectivity under mild reaction conditions.