UNIVERSITI PUTRA MALAYSIA

PROTEIN-SOLVENT INTERACTION OF THERMOSTABLE LIPASE FOR BIOCATALYSIS IN ORGANIC SOLVENTS

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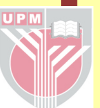

PROTEIN-SOLVENT INTERACTION OF THERMOSTABLE LIPASE FOR BIOCATALYSIS IN ORGANIC SOLVENTS

By

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April 2009

Chairman: Prof. Raja Noor Zaliha Raja Abdul Rahman, D. Eng.

Institute: Bioscience

A high expression of a solvent stable enzyme enables characterization of it solvent stability profiles which could aid in many aspects of structural studies in understanding protein-solvent interaction. A thermostable and solvent stable lipase (Lip 42) gene previously isolated from *Bacillus* sp. strain 42 was subcloned into pET-51b. High expression was achieved using this vector which employs T7 promoter, using *E. coli* host strain BL21(DE3)pLysS. Expression was achieved at 160 U/mg protein, after 25 hour incubation at 37˚C using 0.5 mM isopropyl *β*-d-thiogalactopyranoside (IPTG). Lip 42 was purified using Strep-tag affinity chromatography and it molecular weight size is 43 kDa by SDS-PAGE. Studies on purified lipase in solvent with different polarities (log P values) showed that it was generally stable in water miscible solvents such as DMSO, DMF, ethanol, and methanol; and in apolar solvent such as *n*-hexane. The enzyme retained high residual

activities in 45 to 60% v/v hydrophilic solvents, and also some enhancements (> 100%) were observed in low DMSO compositions (15-30% v/v). Stability preference in water miscible solvents would make Lip 42 lipase a suitable enzyme to be employed in bio-diesel production.

Structural characterizations of the enzyme in different solvent compositions were carried out using fluorescence spectroscopy, and also by using both far- and near- UV circular dichroisms (CD). Both secondary and tertiary structures were retained in low solvent (<45% v/v), but in > 60% v/v, the tertiary structure was perturbed accompanied by the formation of molten globule (MG), or an expanded helical structure state. Far-UV CD studies in methanol indicated the conserved secondary structure with an increase in α-helices, and decreased in β-sheets. Near UV-CD spectra in low methanol and DMSO compositions (30 - 45% v/v) resembled the native protein. At solvent > 60% v/v, the distinct tertiary structure perturbation was observed each in methanol and DMSO. Intrinsic fluorescence spectra in both solvents showed blue shifts at 0-45% v/v indicating a buried Tryptophan, and at > 60% v/v showed red shift indicating an exposed Tryptophan. Extrinsic fluorescence studies showed the possible formation of inactive molten globule (MG) at > 60% v/v solvent. In this state, the collapse of tertiary structure with an intact secondary structure was manifested by the loss in biological function.

Based on solvent stability profiles, molecular dynamic (MD) simulations were run in the presence of water, 60% v/v DMSO + 40% v/v water, and 100% v/v DMSO.

Structural (RMSD) and flexibility (RMSF) changes indicated that the major changes in the lid involving two helix-loop-helix motif loops. In 60% v/v DMSO, the gap between the loops was narrower and there was a collapse of a nearby hydrophobic cluster. However, the cluster was still seen in water and neat (100% v/v) DMSO. Consequently, the H-bond interaction and hydrophobic cluster region are important elements in protein solvent interaction. A site-directed mutation on the lid region (V171S) with residue Ser 171 replacing Val (hydrophobic to polar) impaired the enhancement in low solvent compositions. This effect was more pronounced at higher pre-incubation temperature (50˚C), showing 120% reduction from the amount achievable by the native enzyme. This indicated the crucial role of the hydrophobic residue on helix-loop-helix motif in providing the hydrophobic effect pre-requisite for interfacial activation mechanism. In conclusions, these studies provide better understanding in protein solvent interaction and suggest a suitable parameter in rational design strategy for a better non-aqueous catalysis.

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

SALING TINDAK PROTEIN-PELARUT BAGI ENZIM LIPASE TERMOSTABIL UNTUK BIOKATALISIS DI DALAM PELARUT ORGANIK

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Pengekpresan tinggi enzim yang stabil pelarut membolehkan corak kestabilan enzim dalam pelarut dikenalpasti, dan ini dapat membantu dalam pelbagai aspek kajian struktur bagi memahami saling tindak protein-pelarut. Gen lipase yang termostabil dan stabil dalam pelarut (Lip 42), yang dipencilkan daripada *Bacillus sp*. strain 42, telah pun disubklon ke dalam vektor pET-51b. Pengekpresan yang tinggi dicapai menggunakan vektor yang memiliki sistem promoter T7 melalui hos *E. coli* strain BL21(DE3)pLysS. Pengekpresan sebanyak 160 U/mg protein dicapai selepas pengeraman selama 25 jam pada suhu 37˚C, menggunakan 0.5 mM isopropil *β*-d-tiogalaktopiranosida (IPTG). Penulenan Lip 42 dicapai menggunakan kromatografi berafiniti Strep-tag dan saiznya adalah 43 kDa, secara kaedah SDS-PAGE. Kajian dalam pelarut-pelarut dari berbagai polariti (nilai Log P) menunjukkan kestabilan

lebih dalam pelarut-pelarut yang hidrofilik seperti DMSO, DMF, etanol, metanol, dan juga dalam pelarut yang hidrofobik seperti as *n*-heksana. Enzim mengekalkan aktiviti sisa (*residual activity*) yang tinggi di dalam 45 ke 60% v/v pelarut-pelarut hidrofilik, manakala terdapatnya peningkatan aktiviti (*enhancement* >100%) pada komposisi DMSO yang rendah (15-30% v/v). Kestabilan lebih dalam pelarut hidrofilik menjadikan enzim Lip 42 begitu sesuai digunakan dalam penghasilan bio-disel.

Pencirian struktur enzim dalam pelbagai komposisi pelarut dilakukan melalui kaedah spektroskopi berpendaflor, dan juga melalui UV-CD (*Ultra Violet Circular Dichroisms*) dalam kedua-dua jarak jauh dan dekat. Didapati, struktur tertiari dan sekunder protein masih kekal pada komposisi rendah pelarut (<45% v/v), manakala pada komposisi > 60% v/v, struktur tertiari terganggu, diiringi pembentukan 'globul lebur' (*molten globule, MG)*, atau pembentukan struktur helik lampau (*highly expanded helix*). Kajian UV-CD jarak jauh dalam metanol menunjukkan kandungan struktur sekunder adalah masih kekal dengan penambahan helik-α dan pengurangan plat β. Spektra UV-CD jarak dekat pada komposisi rendah (30 - 45% v/v) DMSO dan metanol adalah menyerupai spektra enzim yang asli. Pada komposisi > 60% v/v, spektra UV-CD dekat menunjukkan struktur tertiari terganggu dengan sedikit perbezaaan pada spektra metanol berbanding DMSO. Spektra berpendaflour intrinsik bagi kedua-dua pelarut menunjukkan anjakan biru pada komposisi 0-45% v/v yang menandakan keadaan triptofan (Trp) yang terlindung, diikuti oleh ajakan merah pada > 60% v/v yang menandakan kumpulan Trp yang terdedah. Kajian berpendaflor ekstrinsik menunjukkan pembentukkan globul lebur yang tidak aktif, pada komposisi

> 60% v/v pelarut. Dalam keadaan ini, kemusnahan struktur tertiari dimanifestasikan dengan hilangnya fungsi biologi, di mana struktur sekunder adalah masih kekal.

Berdasarkan profil kestabilan dalam pelarut, simulasi dinamik molekul (*Molecular Dynamic, MD*) dilakukan dalam keadaan bersama air, 60% v/v DMSO + 40% v/v air dan 100% DMSO. Perubahan struktur (RMSD) dan fleksibiliti (RMSF) menunjukkan perubahan ketara pada penutup (*lid*) yang melibatkan dua lengkuk yang bermotif helik-lingkar-helik. Dalam 60% v/v pelarut, jurang di antara dua lengkuk ini menyempit di samping runtuhnya satu kelompok hidrofobik yang berhampiran. Bagaimanapun, di dalam air dan 100% v/v DMSO, kelompok hidrofobik ini masih kelihatan. Oleh yang demikian, interaksi ikatan-ikatan hidrogen dan kelompok hidrofobik merupakan elemen-elemen penting dalam saling tindak di antara protein dan pelarut. Mutasi secara tapak-terarah (*site-directed mutagenesis*) pada penutup (V171S), di mana residu Valina ditukarkan ke Serina yang lebih polar telah melemahkan peningkatan aktiviti pada komposisi rendah pelarut. Kesan ini didapati lebih ketara pada suhu pre-inkubasi yang lebih tinggi (50˚C), yang menunjukkan pengurangan peningkatan aktiviti sebanyak ~120% berbanding yang dicapai enzim asli. Ini menunjukkan peranan penting residu hidrofobik pada motif helik-lingkar-helik dalam memberikan kesan hidrofobik yang menjadi prasyarat dalam pengaktifan antara fasa (*interfacial activation*) dalam lipase. Sebagai kesimpulan, kajian-kajian ini telah dapat membantu dalam memahami saling tindak protein dan pelarut, dan mencadangkan satu parameter sesuai bagi strategi dalam rekabentuk rasional untuk memperbaiki pemangkinan dalam keadaan tanpa akuas.

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

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DECLARATION

I declare the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

____

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LIST OF ABBREVIATIONS

a.a.	amino acids
ANS	8-anilino-1-napthalenesulphonic acid
CD	circular dichroism
CV	column volume
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylene diamine trichloro acetic acid
Glu	Glutamate
IPTG	isopropyl β-d-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
LB	Luria-bertani
Lip 42	lipase 42
LPS	lipo-polysacharride
MD	molecular dynamic
MG	molten globule
M_r	molecular weight
NCBI	National Centre of Biotechnology
NMR	nuclear magnetic resonance

NPT	ensemble at constant pressure and temperature
NSAIDs	non-steroid anti inflammatory drugs
OD_{600}	optical density at 600 nm
ORD	optical rotatory dispersion
P.F.	purification factor
PDB	Protein Data Bank
Phe	Phenylalanine
PUFA	polyunsaturated fatty acids
RBS	ribosomal binding site
RE	restriction enzyme
R_g	radius of gyration
RMSD	Root mean square of displacement
RMSF	Root mean square of fluctuation
rpm	round per minute
S. A.	specific activity
SDS-PAGE	Sodium dodecyl sulphate polyachrylamide gel electrophoresis
TFB	transformation formulation buffer
TFE	2-2-2 trifluoroethanol
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine