STRUCTURAL IMPLICATIONS OF Pseudomonas fluorescence STRAIN AMS8 COLD-ACTIVE LIPASE IN ORGANIC SOLVENT

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By

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Chairman : Mohd Shukuri Mohamad Ali, PhD
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Cold-active lipases from bacterial sources could benefit the enzyme industry significantly due to its adaptive structural features which makes it active at low temperature and flexible at low water medium. Unfortunately, there was lack of understanding regarding the structure adaptation of cold-active lipase in organic solvent. This study embarks on studying structure-function relationship of family I.3 cold-active AMS8 lipase in selected organic solvents. Cold-active AMS8 lipase was catalytically active at 25 – 45 °C and has two alpha-helix lids, a pentapeptide motif with nucleophilic-serine and repeat-toxin sequence motifs distribution at C-terminus.

The experiment begins with structure prediction of AMS8 lipase by Small Angle X-ray Scatterings (SAXS) and homology modeling. AMS8 lipase *ab-initio* model from SAXS was found to be similar with the homology model and MIS38 *Pseudomonas* lipase structure. Following this, molecular dynamics (MD) simulations and docking analyses were performed with homology-modelled lipase where ethanol, toluene, dimethyl sulfoxide and 2-propanol have shown interactions with active site residues. Toluene achieved the highest energy binding (4.92 kcal/mol) with AMS8 lipase and strongly interacts with Ser-204, Gly-205 and His-206. Based on simulations in toluene, a strong hydrogen bond was formed at catalytic site between Gly-210 and Ser-238, but weaker hydrogen bond was found at lid 2 between Gly-156 and Ser-160. An increase in α-helices structure could be subjected to enzyme surface interference by toluene. AMS8 lipase also exhibited higher number of disallowed region when simulated in toluene (1.9 %) and hexane (3.2 %). Methanol and toluene showed improvements in AMS8 lipase substrate binding (p-nitrophenol palmitate) but slows down the catalytic rate, k_{cat}. Based on these characterizations, site-directed mutagenesis was applied on regions with high accessibility to toluene.

Leu-208 located next to polar Ser-207 was chosen as a mutation site because of the high solvent-accessible surface area and was located within aggregation-prone sites. Substitution of leucine to alanine ruined hydrogen bond that formed between Ser-238 and Gly-210 causing the tunnel to collapse. Following this, a reduction in substrate affinity for *p*NPP and *p*NPL was observed in 0.5 % (v/v) toluene. The enhanced stability of L208A was contributed by increase in aggregation and denaturation points which made it easy to adapt at slightly high temperature, 45 °C. Mutating Thr-52 and Gly-55 to tyrosine on lid 1 area stabilizes the protein conformation and improves the surface recognition of toluene due to the presence of aromatic side chain. Presence of tyrosine at lid 1 did not draw electrostatic interactions on the protein surface for substrate binding but being there, the local flexibility on both surface and catalytic site embraced positive changes to lipase activity. Mutant T52Y favours *p*NPP (C16) but G55Y hydrolysed smaller *p*NPC (C8) in aqueous solvent. Both lid 1 mutants favoured *p*NPC when reacted in 0.5 % (v/v) toluene. Although binding improvements of long-chain substrate was evident in T52Y, its activity in toluene remained low in comparison to the recombinant.

In characterisation study, lid 1 mutants have lower optimal temperature compared to its recombinant and L208A. Mutant T52Y has the longest half-life in aqueous medium at 25 and 37 °C while exhibited longer half-life in 0.5 % (v/v) toluene at 25 °C. Both lid 1 mutants were stable in toluene up to 3 % (v/v) concentration. In 0.5 % (v/v) toluene, all lipases aggregated at higher temperature but denaturation happened at lower temperature for lid 1 mutants. Unlike others, mutant T52Y displayed increased values of enthalpy and entropy from 0 to 5 % (v/v) toluene showing improvements of protein stability and decline in catalytic rate. All lipases exhibited no structure loss due to the unchanged and minimal increase of its entropy value in toluene at 25 to 35 °C. In conclusion, adaptations of cold-active lipase AMS8 in toluene (0.5 - 5 %, v/v) at temperatures 20 - 35 °C was factorised by lid 2 flexibility, formations of substrate-tunnel, hydrogen bond in catalytic area, alpha-helix reduction, higher aggregation points, low enthalpy and a slight increase in entropy.

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

IMPLIKASI STRUKTUR LIPASE AKTIF-SEJUK *Pseudomonas fluorescens* STRAIN AMS8 DALAM PELARUT ORGANIK

Oleh

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Lipase aktif-sejuk dari sumber bakteria dapat memberi manfaat secara langsung kepada industri enzim disebabkan oleh ciri-ciri penyesuaian struktural yang menjadikannya aktif pada suhu rendah dan melentur di medium rendah air. Malangnya, terdapat kurang pemahaman berkait dengan penyesuaian struktur lipase aktif-sejuk di dalam pelarut organik. Kajian ini bermula dengan meneliti hubungan struktur-fungsi pada keluarga lipase I.3 AMS8 aktif-sejuk di dalam pelarut organik terpilih. Lipase AMS8 aktif-sejuk aktif memangkin pada 25- 45 ºC dan mempunyai dua penutup alfa helix, motif pentapeptida berserta serina-nukleofilik dan kedudukan jujukan motif toksin-berulang di terminal-C.

Experimen bermula dengan ramalan struktur lipase AMS8 menerusi Serakan X-ray Sudut Kecil (SAXS) dan pemodelan homologi. Model *ab-initio* SAXS lipase AMS8 mempunyai persamaan dengan model homologi dan struktur lipase *Pseudomonas* MIS38. Berikutan ini, simulasi dinamik molekular (MD) dan analisis mengedok dilakukan dengan model homologi lipase di mana etanol, toluena, dimetil sulfoksida dan 2-propanol telah menunjukkan interaksi dengan residu-residu tapak aktif. Toluena mencapai tenaga ikatan yang tertinggi (4.92 kcal/mol) dengan lipase AMS8 dan kukuh berinteraksi dengan Ser-204, Gly-205 dan His-206. Berdasarkan simulasi dalam toluena, ikatan hidrogen yang kuat telah terbentuk di tapak mangkin antara Gly-210 dan Ser-238, tetapi ikatan hidrogen yang lemah telah dijumpai pada penutup 2 di antara Gly-156 dan Ser-160. Peningkatan dalam struktur alfa-helix boleh merujuk kepada gangguan permukaan enzim oleh toluena. Lipase AMS8 juga menunjukkan pertambahan bilangan bagi kawasan yang tidak dibenarkan apabila disimulasi di dalam toluena (1.9 %) dan heksana (3.2 %). Metanol dan toluena menunjukkan penambahbaikan ke atas substrat mengikat lipase AMS8 (p-nitrofenol palmitat) tetapi rendah bagi kadar memangkin, k_{cat}.

Berdasarkan pencirian tersebut, mutagenesis kawasan terarah digunakan pada bahagian yang mempunyai ketercapaian tinggi kepada toluena.

Leu-208 yang terletak bersebelahan Ser-207 yang polar dipilih sebagai kawasan mutasi disebabkan tingginya keluasan permukaan pelarut mudah capai dan terletak dalam kawasan cenderung pengagregatan. Pertukaran leusina kepada alanina merosakkan ikatan hidrogen terbentuk antara Ser-238 dan Gly-210 menyebabkan keruntuhan pada terowong. Berikutan ini, pengurangan dalam afiniti substrat untuk *p*NPP dan *p*NPL dilihat pada 0.5 % (v/v) toluena. Peningkatan kestabilan oleh L208A disumbangkan melalui kenaikan takat pengagregatan dan penyahaslian yang menjadikan ia mudah menyesuai pada suhu sedikit tinggi, 45 ºC. Memutasi Thr-52 dan Gly-55 kepada tirosina pada bahagian penutup 1 menstabilkan konformasi protein dan menambahbaik pengecaman permukaan oleh toluena disebabkan kehadiran rantai sisi aromatik. Kewujudan tirosina pada penutup 1 tidak mendorong interaksi-interaksi elektrostatik di atas permukaan protein untuk mengikati substrat, namun keberadaannya di sana, membawa kepada kelenturan setempat di kedua permukaan dan kawasan pemangkin yang memberi perubahan positif kepada aktiviti lipase. Mutan T52Y menyukai *p*NPP (C16) tetapi G55Y menghidrolisis *p*NPC (C8) yang bersaiz kecil di dalam larutan akueus. Kedua-dua mutan penutup 1 memilih *p*NPC apabila bereaksi di dalam 0.5 % (v/v) toluene. Walaupun peningkatan mengikat bagi substrat rantai-panjang adalah jelas dalam T52Y, aktiviti di dalam toluena kekal rendah jika dibandingkan dengan rekombinan.

Dalam kajian pencirian, mutan-mutan penutup 1 mempunyai suhu optima yang lebih rendah berbanding dengan rekombinan dan L208A. Mutan T52Y mempunyai separuh hayat yang terpanjang di dalam medium akueus pada 25 dan 37 ºC sementara menyaksikan separuh-hayat yang panjang di dalam 0.5 % (v/v) toluena pada 25 ºC. Kedua mutan penutup 1 adalah stabil di dalam toluena sehingga kepekatan 3 % (v/v). Dalam 0.5 % (v/v) toluena, kesemua lipase mengagregat pada suhu yang meninggi tetapi penyahaslian berlaku pada suhu yang rendah untuk mutan-mutan penutup 1. Tidak seperti yang lain, mutan T52Y memperlihatkan peningkatan nilai-nilai entalpi dan entropi dari 0 sehingga 5 % (v/v) toluena sekaligus menunjukkan penambahbaikan ke atas penstabilan protein dan penurunan dalam kadar memangkin. Semua lipase menunjukkan tiada struktur yang hilang berdasarkan pada nilai entropi yang tidak berubah dan sedikit tinggi di dalam toluena pada 25 hingga 35 ºC. Secara kesimpulan, penyesuaian lipase aktif-sejuk AMS8 di dalam toluena (0.5 – 5 %, v/v) pada suhu 20 – 35 ºC dipuncakan pada pelenturan penutup 2, pembentukan terowong substrat, ikatan hidrogen dalam kawasan pemangkin, pengurangan alfa helix, kenaikan takat pengagregatan, penurunan entalpi dan sedikit peningkatan dalam entropi.

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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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α	Alpha
β	Beta
°C	Degree celsius
%	Percentage
Å	Angstrom
A_{600nm}	Optical density at wavelength 600 nanometer
µL	Microlitre
µm	Micrometer
µmole	Micromole
bp	Base pair
$CaCl_2$	Calcium chloride
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
g	Gram
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobase
kDa	Kilodalton
k_{cat}	Catalytic efficiency
K_{fr}	Folding rate constant
K_m	Michaelis-Menten constant
L	Litre
LB	Luria-Bertani
log *P*	logarithm of the partition coefficient between 1- octanol and water H_2O
M	Molar
mA	MilliAmps
µmole	Micromole
mg	milligram
pNP	*p*-nitrophenol
porod	particles macromolecular volume
RMSD	Root-mean-square deviations
RMSF	Root-mean-square fluctuations
RTX	Repeat-in-toxin motifs
SASA	Solvent-accessible surface area
SAXS	Small Angle X-ray Scatterings
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
T_m	Melting temperature/Denaturation point
U/ml	Unit per millilitre
U/mg	Unit per milligram
v/v	volume per volume
ΔG‡	Gibbs free energy activation
ΔH‡	enthalpy of activation
ΔS‡	entropy of activation

CHAPTER 1

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are part of hydrolases that hydrolyzes carboxylic ester bonds and triglycerides into diglycerides, monoglycerides, fatty acids and glycerol (Houde *et al*., 2004). These enzymes are gaining high importance as a biocatalyst for several novel reactions in aqueous and non-aqueous medium. Different lipases behave differently in different organic solvents with different level of resistance in different reaction systems. *Pseudomonas* lipases are very interesting not only that they are stable in organic solvents but also because they display special biochemical characteristics not common among the lipases produced by other microorganisms, such as the thermoresistance and activity at alkaline pH (Kumar *et al*., 2016, Soberón-Chávez and Palmeros. 2008). *Pseudomonas* lipases can be classified into three subfamilies: I.1, I.2 and I.3 (Jaeger and Eggert. 2002). Family I.3 lipases are distinguished from other lipases by their amino acid sequences and secretion mechanism via type I secretion system (TISS). It is through the secretion of TISS that produced the up-stream of secretion signal termed repeat in toxin motifs (RTX) attracting Ca^{2+} to bind. Eventually, Ca^{2+} is important for lipase activity and folding. The family I.3 lipases are composed of two domains with distinct yet related functions (Angkawidjaja and Kanaya. 2006). Among the family I.3 lipases secreted from cold-adapted *Pseudomonas sp*. are KB-Lip from *Pseudomonas fluorescens* strain KB700A (Rashid *et al*., 2001), Lip TK3 from *Pseudomonas fluorescens* strain TK3 (Tanaka *et al*., 2012) and LipS from *Pseudomonas mandelii* JR1 (Kim *et al*., 2013). Unlike other cold-active lipases, LipS is active and stable at 40-50 °C showing a relatively high in thermal stability which is unique to cold-adapted enzymes. Cold-active lipases from psychotropic microorganisms showing high catalytic activity at low temperatures can be highly expressed through recombinant methods, making them useful for biotechnological applications.

In spite of these advantages, enzymes do not always meet desired levels of activity, productivity and, most importantly, stability in organic solvents. Inactivation by organic solvents is most likely due to conformational changes of the protein structure cause by changes in medium hydrophobicity and non-covalent interactions between the enzyme and solvent molecules, which leads to protein unfolding and activity loss (Dror *et al*., 2014, Stepankova *et al*., 2013). Hydrophilic polar solvents such as DMSO, ethanol and methanol can create homogenous systems with water, but they can easily penetrate the enzyme surface or strip off essential water molecules from the enzyme. In contrast with the hydrophobic solvents such as hexane and toluene (methylbenzene), the lack of water stripping from the enzyme surface is due to the partitioning of hydration water between enzyme and the bulk solvent, which benefits in active site hydration through mobile and weakly bound water. The presence of water around the enzyme could maintain the three-dimensional structure needed for the enzyme activity. Hence, active site hydration is dependent to the enzyme activity contributed by organic solvent polarity (Yang *et al*., 2004). In a reaction system with low hydration, organic solvent replaces molecular functions of water at the protein surface. This is an alternative way to provide a medium for diffusion of substrate and product. Because enzyme activity is possible at very low

hydration level, enzymatic modification should be introduced to minimize the structure-linked disorder upon contact with organic solvent (Kurkal *et al*., 2005).

Stability of cold-active lipase in organic solvents has been regarded essential for various biotechnology applications that involved synthesis reactions such as esterification, interesterification and transesterification. The stability of cold-active lipase correlates to the distribution of surface charged residues and surface property of the enzyme (Samantha *et al*., 2006). Organic solvents preferentially localize the hydrophobic patch in the active-site vicinity and so it creates a hydration shell on residues that did not demonstrate solvents binding preference. Those set of residues that have showed change in chemical shifts/perturbation via [15N, 1H] - HSQC resonance in 6B lipase represents the same large hydrophobic regions on the protein surface having good affinity towards organic molecules (Kamal *et al*., 2013). The surface residues localizing protein surface determines the hydrophobicity profile of this lipase. Other prominent features were the extensive formation of new hydrogen bonds between surface residues directly or mediated by structural water molecules and the stabilization of Zn and Ca binding sites (Dror *et al*., 2015). Active site (Ser, Asp and His), lid region, tunnels and RTX motifs are important structural features for regulating enzymatic functions and stability. Some of these residues must be conserved while some of them are tandemly distributed. Site-directed mutagenesis is commonly used to reveal insights regarding structure-stability correlations influenced by organic solvent interactions. Generally, any alteration on this conserved motif will generate inactive protein. However, other areas that are associated with solvent stability often found in loops on the surface of protein and the regions near the substrate binding site. Those residues that are far from the active site region are predicted not to affect catalytic activity.

One experimental method that allows direct identification on these residues is theoretical analyses using molecular dynamics (MD) simulation. Following these approach, protein modification via site-directed mutagenesis can be done to alter protein structure, function or stability. To date, there are few reports highlighting the significance of active site and lid regions of cold-active lipase via mutations suggesting their potential stability in polar and non-polar organic solvents. Hence, a collective approach that includes molecular dynamics (MD) simulation, site-directed mutagenesis, biochemical and biophysical characterisation in the presence of selected organic solvents would facilitate the study related to the structure and function of this extraordinary lipase. As it is not easy to elucidate atomic details of cold-active lipase in the presence of organic solvent via X-ray protein crystallography, many researchers have turned to computational method and kinetics approaches in elucidating underlying mechanisms responsible for organic solvent effects on molecular level.

1.1 Research Statements

The influence of organic solvents on the activity of cold-active AMS8 lipase might be the results of the conformational changes which happened around the active site or other region. At the same time, the hydrophobicity properties of each organic solvent could play essential role starting from the process of solvent penetration to protein structure

via diffusion or interfacial activation to tertiary structure changes caused by the solvent molecules intrusion. However, the effects of organic solvent to alterations in lipase structure and function need to reflect the role of highly flexible cold-active lipase which is supposed to be different from its mesophilic and thermophilic type. Among general but relevant issues that deserved scholastic attentions to provide sufficient information about the characteristics of the special lipases in organic solvent are listed as follows:

1) The associations of organic solvent hydrophobicity with cold-active lipase destabilization.

2) Differences in lipase aggregation, folding and denaturation correspond with changes in medium hydrophobicity.

3) Concepts of protein hydration in organic solvent and its effects on protein dynamics and function. This topic focuses on the probability of hydration shell formed at active site region and small structural perturbation due to water molecules shifting.

1.2 Hypothesis

It is hypothesized that:

1. Lid regulates the access of substrate to the catalytic site in the presence of hydrophobic organic solvent due to stronger hydrophobic interactions.

2. Active site and lid region had higher solvent accessible surface area to organic solvents.

3. Lipases with more than one lid could have other roles than being there for surface activation and catalytic function.

1.3 Objectives

General Objective:

To determine the role of AMS8 lipase structure for better understanding of lipase reaction in the presence of organic solvents.

Specific Objectives:

1. To compare the tertiary structure of recombinant cold-active AMS8 lipase by biophysical (SAXS) and computational methods.

2. To examine the interactions of AMS8 lipase in the presence of organic solvents via molecular dynamics simulation and protein-solvent docking approaches.

3. To determine secondary structure changes and kinetic differences of recombinant AMS8 lipase in polar and non-polar organic solvents.

4. To determine the importance of lids and nucleophilic binding region for organic solvent stability of cold-active lipase AMS8 by using site-directed mutagenesis (SDM).

5. To associate the influence of toluene towards biophysicochemical and thermodynamics of recombinant AMS8 and other mutant lipases.

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