IN SILICO SITE-DIRECTED MUTAGENESIS OF *Acinetobacter haemolyticus* LIPASE KV1 FOR IMPROVED ALKALINE STABILITY

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DEDICATION

Specially dedicated to my beloved parents and siblings, who have been with me every step of the way, through good times and bad. Most especially to The Almighty Allah SWT, who has been the source of my strength throughout this journey and on His plans only have I trusted.

Thank you for everything.

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ABSTRACT

The interest on alkaline-stable lipases by the scientific community is increasing due to its great potential use. As most industrial processes are performed under highly basic conditions, alkaline-stable lipases become hugely valued biocatalysts. In this study, three aspartic acid residues at positions 51, 122 and 247 in the outer loop of LipKV1 from *Acinetobacter haemolyticus* was computationally mutated into lysine using the SWISS-MODEL program, followed by energy minimization of the protein models. PROCHECK, ERRAT and Verify3D refined models of LipKV1 and Mut-LipKV1 indicated that the Mut-LipKV1 protein conformation is in a good condition. The study found that the overall electrostatic surface potentials and charge distributions of the Mut-LipKV1 model was more stable and better adapted to conditions of elevated pHs (pH 8.0 −10.0). Molecular dynamics (MD) simulation of Lip-KV1 and Mut-LipKV1 protein models under different alkaline pHs using GROMACS version 2018.6 revealed that Mut-LipKV1 was more stable at the high pH 9.0 (RMSD ~0.3 nm, RMSF ~0.05 – 0.2 nm), compared the optimal pH 8.0 of LipKV1 (RMSD 0.3 nm, RMSF 0.05 – 0.20 nm). Molecular docking using AutoDock Vina with tributyrin as the substrate identified detailed changes that occurred post mutation. The highest binding affinity (−4.1 kcal/mol) with Mut-LipKV1 which occurred at pH 9.0 was from a single hydrogen bond with His289. MD simulations showed that configurations which formed between Mut-LipKV1-tributyrin (RMSD 0.3 nm; RMSF 0.05 − 0.3 nm) and the LipKV1-tributyrin complexes (RMSD 0.35 nm; RMSF 0.05 − 0.4 nm) were comparably stable at pH 8.0. Furthermore, MM-PBSA calculation validated that the Mut-LipKV1-tributyrin complex at pH 8.0 (-44.01 kcal/mol) showed comparable binding free energy to LipKV1-tributyrin complex (−43.83 kcal/mol). Whereas the lowest binding free energy for Mut-LipKV1-tributyrin complex was simulated at pH 12.0 (−44.04 kcal/mol). Thus, adaptive strategy of replacing the outer loop surface aspartic acid to lysine in LipKV1 successfully broadened pH stability of Mut-LipKV1 towards higher pH, raising it from pH 8.0 − 11.0 to pH 8.0 − 12.0 in the mutant lipase. In a nutshell, this research offered a considerable insight for further improving the alkaline tolerance of lipases.

ABSTRAK

Enzim lipase yang bersifat tahan alkali semakin mendapat perhatian ahli sains kerana potensi aplikasinya yang sangat besar. Oleh kerana kebanyakan proses industri berlaku di bawah keadaan beralkali tinggi, lipase stabil-alkali menjadi pemangkin yang amat bernilai. Dalam kajian ini, tiga residu asid aspartik pada kedudukan 51, 122 dan 247 di gelung luar LipKV1 dari Acinetobacter haemolyticus telah dimutasi secara komputer menjadi lisin menggunakan program SWISS-MODEL, diikuti dengan peminimuman tenaga model protein. Model LipKV1 dan Mut-LipKV1 yang diperhalusi oleh PROCHECK, ERRAT dan Verify3D menunjukkan bahawa konformasi protein Mut-LipKV1 berada pada tahap yang baik. Kajian ini menunjukkan bahawa keseluruhan keupayaan elektrostatik dan taburan cas pada Mut- LipKV1 adalah lebih stabil dan lebih beradaptasi pada keadaan pH tinggi (pH 8.0 − 10.0). Simulasi dinamik molekul (MD) ke atas model protein LipKV1 dan Mut- LipKV1 pada pH alkali yang berbeza menggunakan GROMACS versi 2018.6 menunjukkan Mut-LipKV1 lebih stabil pada pH 9.0 (RMSD ~0.3 nm, RMSF ~0.05 − 0.2 nm), berbanding LipKV1 yang optimum pada pH 8.0 (RMSD 0.3 nm, RMSF 0.05 − 0.20 nm). Pengedokan molekul menggunakan Autodock Vina dengan tributirin sebagai substrat menunjukkan perubahan terperinci yang berlaku pasca mutasi. Keafinan pengikatan tertinggi (−4.1 kcal/mol) dengan Mut-LipKV1 yang berlaku pada pH 9.0 telah membentuk satu ikatan hidrogen tunggal dengan His289. Simulasi MD menunjukkan bahawa konfigurasi komplek Mut-LipKV1-tributirin (RMSD = 0.3 nm; RMSF = 0.05 − 0.3 nm) dan kompleks LipKV1-tributyrin (RMSD 0.35 nm; RMSF 0.05 − 0.4 nm) adalah setara kestabilannya pada pH 8.0. Tambahan lagi, pengiraan MM-PBSA membuktikan bahawa kompleks Mut-LipKV1-tributirin pada pH 8.0 (-44.01 kcal/mol) mempunyai tenaga bebas pengikatan yang setara dengan kompleks LipKV1-tributirin (-43.83 kcal/mol). Manakala, tenaga bebas terendah adalah daripada kompleks Mut-LipKV1-tributirin yang disimulasi pada pH 12.0 (-44.04 kcal/mol). Oleh itu, strategi penyesuaian dengan menggantikan asid aspartik di gelung permukaan LipKV1 kepada lisin berjaya meluaskan kestabilan pH Mut- LipKV1 kepada pH yang lebih tinggi, meningkatkannya daripada pH 8.0 − 11.0 ke pH 8.0 − 12.0 dalam lipase mutan. Secara keseluruhannya, kajian ini menawarkan lebih kefahaman dalam meningkatkan daya tahan kealkalian lipase.

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LIST OF ABBREVIATIONS

APBS	-	Adaptive Poisson-Boltzmann Solver
ATB	-	Automated Topology Builder
BLAST	-	Basic Local Alignment Search Tool
DNA	-	Deoxyribonucleic Acid
EIE	-	Enzymatic Interesterification
FAME	-	Fatty Acid Methyl Esters
GROMACS	-	GROningen Machine for Chemical Simulations
LipKV1	-	Lipase from *Acinetobacter haemolyticus* strain KV1
MD	-	Molecular Dynamics
MM-PBSA	-	Molecular Mechanics Poisson-Boltzmann Surface Area
Mut-LipKV1	-	Mutant lipase KV1
PET	-	Polyethylene terephtalate
PUFA	-	Polyunsaturated Fatty Acids
PB	-	Poisson-Boltzmann
PDB	-	Protein Data Bank
RMSD	-	Root Mean Square Deviation
RMSF	-	Root Mean Square Fluctuation
Rg	-	Radius of gyration
SOPMA	-	Self-Optimized Prediction method with Alignment
SAV	-	Solvent Accessible Volume
3-D	-	Three-dimensional
WCA	-	Weeks-Chandler Andersen

LIST OF AMINO ACIDS

A or Ala	-	Alanine
R or Arg	-	Arginine
N or Asn	-	Asparagine
D or Asp	-	Aspartic Acid
C or Cys	-	Cysteine
E or Glu	-	Glutamic Acid
Q or Gln	-	Glutamine
G or Gly	-	Glycine
H or His	-	Histidine
I or Ile	-	Isoleucine
L or Leu	-	Leucine
K or Lys	-	Lysine
M or Met	-	Methionine
F or Phe	-	Phenylalanine
P or Pro	-	Proline
S or Ser	-	Serine
T or Thr	-	Threonine
W or Trp	-	Tryptophan
Y or Tyr	-	Tyrosine
V or Val	-	Valine

LIST OF SYMBOLS

Å	-	Armstrong
α	-	Alpha
β	-	Beta
kT	-	Boltzmann constant
Cm	-	Centimeter
°C	-	Degree Celsius
e	-	Electron
γ	-	Gamma
pI	-	Isoelectric Point
K	-	Kelvin
Kcal	-	Kilocalorie
kDa	-	Kilodalton
%	-	Percent
φ	-	Phi
ψ	-	Psi
mol	-	Molar
M	-	Molarity
nm	-	Nanometre
ns	-	Nanosecond
ps	-	Picosecond

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CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Microbial lipases are versatile and for the most part, preferred due to their wide-ranging catalytic activity and currently commercially mass-produced with moderate cost (Liu *et al.*, 2017b). Lipases (EC 3.1.1.3) fall under the α/β-hydrolase-fold superfamily which mediate reactions, for instance, hydrolysis of water insoluble or poorly-water soluble substrates i.e. fatty acid esters, as well as synthetic reactions involving esterification and transesterification to yield alkyl esters (Gurung *et al.*, 2013; Kumar *et al.*, 2016a; Mohammadi *et al.*, 2016). Lipases are highly regarded as industrial biocatalysts with no cofactors needed, have broad substrate specificity and stability over a wide pH and temperature range (Kumari *et al.*, 2019), also showing reasonable stability in the presence of chemicals (Rabbani *et al.*, 2013). The ability of lipases to competently catalyze enantioselective reactions with high enantiomeric purity, have made them invaluable in the fine chemicals industry (Kumar *et al.*, 2016a; Kumar *et al.*, 2016b).

Most industrial biotechnological processes are performed under highly basic conditions, thereby warrant the use of alkaline-stable lipases for carrying out reactions (Batumalaie *et al.*, 2018d; Shamim *et al.*, 2018). However, demands for bio-based reagents remains low due to low acceptance of manufacturers on lipases, believing the enzymes are not sufficiently robust, along with their traditional reliance on using familial and well-proven active ingredients, i.e. phosphate-based chemicals (Boran, 2018). This issue may be solved by improving the robustness of lipases for industrial settings through protein engineering and recombinant technology. Such approaches have allowed tailoring next-level lipases for improved stability and catalytic properties, *inter alia* thermotolerance, wide pH tolerance, stable enzyme

activity over a wide range of temperature and in harsh reaction conditions (Nigam, 2013; Khan *et al.*, 2017; Soleymani *et al.*, 2017).

The use of bioinformatics has gradually taken centre stage with regard to modulating properties of a variety of proteins, in hopes of reducing empirical works (Wahab *et al.*, 2012; Kamarudin *et al.*, 2014; Hamid *et al.*, 2015; Wahab *et al.*, 2016). While there are a number of popularly used protein engineering software which include FoldX (Schymkowitz *et al.*, 2005), AMBER (Aronica *et al.*, 2016), YASARA (Wijma *et al.*, 2014), GROMACS which developed in Herman Berendsen's group of University of Groningen in the version of 2018 series (Lemkul, 2018), is the choice of this study. GROMACS has widely reported with good success for dynamical simulations to assess the conformational stability of a protein in various settings i.e. pH, temperature and presence of salts. (Abraham *et al.*, 2015). Molecular dynamic simulation identifies the most stable state of the protein in relation to its functions and can adequately demonstrate the impact of mutation of proteins and the structural changes brought about by the mutation(s) (Ishak *et al.*, 2019). Moreover, the software has successfully performed *in silico* mutagenesis and predicted the newly introduced or improved catalytic features of the mutated enzyme (Elengoe *et al.*, 2014; Peng *et al.*, 2017; Imani *et al.*, 2018).

This study explored a recently isolated bacterium, *Acinetobacter haemolyticus* which produced an alkaline-stable lipase, lipase KV1, and was consequently cloned and overexpressed in *Escherichia coli*. The recombinant lipase KV1 (LipKV1) exhibits an optimal temperature and pH of 40°C and pH 8.0, and shows reasonable alkaline stability up to 24 hours over a wide-ranging pH values from pH 7.0 − 11.0 while retaining relative hydrolytic activities at >80% (Batumalaie *et al.*, 2018a; Batumalaie *et al.*, 2018b; Batumalaie *et al.*, 2018f). The insufficient optimal pH of LipKV1, nonetheless renders the enzyme unsuitable as a bioactive ingredient in cleaning reagents. For this application, the enzyme must have an optimal pH that lies between pH 9.0 – 11.0 (Boran, 2018). The redesigning of the LipKV1 protein to increase its optimal pH and improve alkaline-stability is therefore required. Thus, a firm understanding of the structural architecture as well as structure-function relationship of LipKV1 is valuable to study protein features of the

lipase that may be exploited and mutated to improve its alkaline-stability. The study noted that it is amongst the few crucial considerations to be taken into thought, if the alkaline-stability of LipKV1 was to be further tailored to match the extreme conditions of industrial processes.

Homology modelling of LipKV1 revealed the protein structure is made up of a single compact domain consisting of seven parallel and one anti-parallel β-strands surrounded by nine α-helices with the conserved active-site residues being Ser165, Asp259 and His289. Surface potentials of LipKV1 yielded a predominant population of acidic surface or negative charge residues (70%) at optimal pH 8.0. (Batumalaie *et al.*, 2018a). The protein architecture of LipKV1 showed the characteristic of an alkaline-stable enzyme due to a higher number of negatively charged exposed amino acids on the protein surface. This feature is pertinent for a better stabilization of the protein under basic extreme environment. This is consistent with bacteria-producing enzymes adapting to surrounding alkaline conditions, by producing enzymes specially that cater to changes in their subcellular environments (Talley and Alexov, 2010). Alkaline-stable enzymes with improved pH stability have been documented for α-amylase from *Bacillus* sp. (pH 6.0 – 10.0) (Dahiya and Rathi, 2015), lipase from *Bacillus* sp. (pH 8.0 – 10.0) (Bora and Bora, 2012) and lipase from *Geobacillus* sp. (pH 5.0 – 11.0) (Ishak *et al.*, 2019). A previous mutagenesis study by Zheng *et al.* (2014) reported on the profound improvement in pH-activity profile of a mutant enzyme from *Paenibacillus* bacteria. The researchers successfully elevated the alkaline-stability of a xylanase whereby the pH optimal was elevated from pH 7.0 to 9.0 using site-directed mutagenesis approach. Another study successfully improved the alkaline stability of a *Bacillus* sp. from the optimal pH 7.5 to 8.5 by site-directed mutagenesis of aspartic acid or glutamic acid on the outer loop of protein surface of xylanase with lysine, arginine or histidine (Bai *et al.*, 2015; Bai *et al.*, 2016). The computational approach to redesign a protein appears to be the way forward in expediting creation of proteins with specific catalytic repertoire, as well as for enhancing catalytic properties of enzymes.

1.2 Problem Statement

Considering the ever-increasing demand for better and more robust enzymes with the ability to withstand extreme industrial conditions i.e. highly alkaline environment in the manufacturing processes, it is apparent that the moderately alkaline-stable LipKV1 falls short of this industrial requirement and limits its further application. A plausible way to profoundly improve the applicability of LipKV1 is to tailor the protein folds of the enzyme, which can be expediently, done by an *in silico* approach using bioinformatics.

In light of this, the study proposed the *in silico* mutation on LipKV1 using PyMOL, to replace the outer most three Asp residues with Lys, in order to improve the alkaline-stability of the lipase and bring it closer to industrial requirements as bioactive ingredient in cleaning products. It is hypothesized that replacing the three Asp residues with Lys on the outer loop regions of LipKV1 protein surface can have a profound impact on improving the pH-activity profile of the mutant LipKV1 (Mut-LipKV1). This approach suggested in this study was possible as information on the structure of LipKV1, previously obtained by homology modelling, is available (Batumalaie *et al.*, 2018a). Moreover, this is the first reported mutational work on LipKV1 that replaces outer Asp residues of lipase KV1 with Lys, to modulate its alkaline stability. The *in silico* study can offer in-depth insights for future empirical mutagenesis work with better understanding and improve stable conformation of lipase KV1 at increasing basic pH conditions.

1.3 Objectives of the Study

The objectives of the research are as follows:

(a) To perform *in silico* site-directed mutagenesis on the outer loop regions of the *A. haemolyticus* LipKV1 model protein and compare stability of proteins of Mut-LipKV1 to LipKV1 by molecular dynamics (MD) simulations under varying alkaline pH.

(b) To conduct molecular docking and molecular dynamics (MD) simulations of the enzyme-substrate complex and compare the stability Mut-LipKV1- and LipKV1-substrate complexes under varying alkaline pH.

(c) To validate the results of MD simulations using molecular-mechanics Poisson-Boltzmann analysis (MM-PBSA).

1.4 Scopes of the Study

The study was divided into three parts, each of which to meet the aforementioned three objectives. The *in silico* site-directed mutagenesis of LipKV1 protein was done on the previous homology modelled lipase at its pH optimal (pH 8.0). Homology modelling of LipKV1 and Mut-LipKV1 was performed using SWISS-MODEL server to construct a three-dimensional protein by using carboxylesterase from *Archaeglobus fulgidus* as a template. Mutagenesis was done using PyMOL and the quality of each protein model was assessed using ProtParam tool on the ExPASy server and Self-Optimized Prediction method with Alignment (SOPMA). Mut-LipKV1 models were subjected to an automated H++ server to deprotonate the mutant protein under different alkaline pH (pH 8.0 – 12.0) for subsequent simulations. Adding hydrogen atoms using PDB2PQR server allowed the LipKV1 and Mut-LipKV1 models for electrostatic calculations using Adaptive Poisson-Boltzmann Solver (APBS) plugin embedded in PyMOL software. The generated protein models were further ascertained through molecular dynamics (MD) simulations at 100 ns to assess changes in the alkaline-stability of LipKV1 and Mut-LipKV1 protein under varying basic conditions. Structural validation was undertaken to predict the proposed 3-D protein models through several evaluation tools such as PROCHECK, ERRAT and Verify-3D.

Next step of the study involved the molecular docking to predict the binding affinity (kcal/mol) and intermolecular interactions when substrate is docked into the active sites of LipKV1 and Mut-LipKV1. The substrate used for the docking was tributyrin which was previously empirically proven to be the preferential substrate of

LipKV1 (Batumalaie *et al.*, 2018a). This docking method implemented AutoDock Vina to generate the docking, and PyMOL to visualize the binding poses. Molecular dynamics (MD) simulations of the enzyme-substrate complex was run again in triplicates at 100 ns to analyze the molecular motion of LipKV1 and Mut-LipKV1 in the presence of substrate tributyrin. The MD simulations was carried out using Gromos53a7 force-field under GROMACS 2018.6 to assess the dynamical behaviors of the lipase-tributyrin complexes and compare the conformational stabilities of the LipKV1- and Mut-LipKV1-substrate at different simulated pH values. The trajectory analyses included root-mean square deviation (RMSD), root-mean square fluctuation (RMSF), radius of gyration (Rg) and hydrogen bond, generated after MD simulations.

Finally, the enzyme-substrate complex was subjected to MM-PBSA calculations to predict the free binding energy by taking into account the MD trajectories. The MM-PBSA calculated the van der Waals, electrostatic, polar solvation, and nonpolar solvation energies of each lipase-tributyrin complex and the resulting energies were converted into kcal/mol. The findings of MM-PBSA are valuable for validating the results obtained from earlier MD simulations on the LipKV1- and Mut-LipKV1-substrate complexes under the tested elevated alkaline conditions (pH 8.0 – 12.0).

1.5 Significance of the Study

The findings from the *in silico* mutation on LipKV1 will be useful to identify crucial residues that governs the alkaline-stability of the lipase. The same information may also be useful for further improvement on the alkaline-stability of the lipase or any other type of lipases, for that matter. The findings would greatly add to the body of knowledge in better predicting mutation sites that profoundly broaden the pH-activity profile of a lipase.

CHAPTER 2

LITERATURE REVIEW

2.1 Lipases from Acinetobacter species

Acinetobacter are known as Gram-negative, oxidase-negative and strictly aerobic bacteria which belongs to *Pseudomonadales* order. *Acinetobacter* has pathogenic and non-pathogenic species with various bacterial strains which grow optimally at 33 – 45°C and survives at a pH range of 5.0 – 9.0 (Jung and Park, 2015). It was reported in the literature that *Acinetobacter* sp. producing lipase also included *Acinetobacter calcoaceticus* and *Acinetobacter johnsonii* (Wang *et al.*, 2012a). So far, alkaline-stable lipases remained unreported except for the *Acinetobacter haemolyticus* (Batumalaie *et al.*, 2018a; Batumalaie *et al.*, 2018b; Batumalaie *et al.*, 2018c; Batumalaie *et al.*, 2018d; Batumalaie *et al.*, 2018e).

Batumalaie *et al.* (2018d) discovered lipase-producing bacterium, *Acinetobacter haemolyticus* strain KV1 isolated from oil palm mill effluent. Lipase produced by this bacterium exhibited quite remarkable stability of pH values (pH 8.0 – 11.0) and optimum activity at 40°C, while retaining hydrolytic activities >80% for up to 24 hours. These unique properties discovered by the earlier study suggested LipKV1 as an alkaline-stable lipase. Sarac and Ugur (2016) described *A. haemolyticus* isolated from soil and olive pomace samples persisted over a wide range of temperature and pHs, as well as retained stability at 4°C and activity of 90% for the treatment of lipid-rich wastewater. Lipases from *Acinetobacter* sp. are useful for the bioremediation of oil-contaminated wastewater by converting lipids into carbon dioxide, water and biomass (Iqbal and Rehman, 2015; Gururaj *et al.*, 2016).

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