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SITE-DIRECTED MUTAGENESIS TO DETERMINE THE ROLE OF SURFACE EXPOSED LYSINE ON THE STABILITY OF STAPHYLOCOCCAL LIPASE

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By

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Protein stability is governed mainly by the intrinsic characteristics of the protein such as the number and strength of intramolecular interactions and prevalence of specific amino acids in the sequence. Surface residue is one of the factors that defines protein stability, however little is known about their roles in relative to other factors. In this study, *Staphylococcus epidermidis* AT2 lipase which exhibits stability at low temperature and in the presence of organic solvents was subjected to surface lysine mutation to examine the effect of surface charged residue to the enzyme stability. As lysine denotes 7% out of the total amino acid composition, surface exposed and mutable lysine was identified to produce AT2 lipase mutants via *in silico* and analysed by Molecular Dynamics simulation. The mutant model structures were built using YASARA version 12.10.3 using *S. hyicus* lipase (PDB id: 2HIH) as template. The structures were validated by means of PROCHECK (Ramachandran plots), ERRAT2, Verify3D and QMEAN. The refined protein models were subjected to MD simulation in water environment using AMBER03 force field. Out of six mutant lipases, two mutants (K325G and K91A/K325G) showed improvement in structural stability by *in silico* analysis thus were selected for biochemical and biophysical characterizations. Both mutants exhibited a shift of 5°C in optimal temperature compared to the wild-type which optimum at 25°C. K325G and K91A/K325G showed optimum at 30°C and 20°C, respectively. K91A/K325G and the wild-type displayed similar pH profiles, pH 8, while mutant K325G exhibited slight changes of pH profile, pH 9. Meanwhile, no significant changes in substrate specificity were observed where the mutants showed similar preference towards long chain *p*-nitrophenyl esters. On the other hand, each mutant demonstrated slight alteration of the organic solvent stability profile upon mutation. A strong preference towards polar organic solvents and several other apolar solvents was observed in the mutants. Mutant K325G, generally, displayed enhancement and stability in DMSO, methanol, acetonitrile, ethanol, acetone, 1-propanol, diethyl ether and chloroform. While, K91A/K325G is

stable in methanol, acetonitrile, ethanol and acetone. Analysis of melting temperature measured by circular dichroism showed that mutant K325G exhibited the highest melting temperature, 62.95°C which positively correlated with a 5°C shift in its optimal temperature compared to the wild-type, 53.25°C. In addition, K91A/K325G composed the highest percentage of α-helices (25.4%) meanwhile K325G with highest β-sheets; 52.9% compared to the wild-type. Further MD simulation studies were carried out in two solvents to investigate the activation and inactivation effect on the mutants and wild-type. In general, both mutants showed greater conformational stability compared to the wild-type in the presence of methanol. Methanol showed a profound local dynamic alteration to mutant K325G where the values of RMSF observed were between the range of 1 to 7 Å with the highest value at 7 Å. The apparent change was observed at the lid region (lid 1) suggesting a larger displacement of the lid. Such observation was not seen in the wild-type and the double mutant which could explain the enhancement of lipase activity in methanol where the large opening of the lid might increase the accessibility of substrate to the catalytic pocket. The inactivation effect of *n*-hexane however could not be concluded as there was no significant event observed throughout the trajectories. As conclusion, this study highlights the strategy of replacing surface lysine with smaller residue to observe the effect of lysine residue to properties of enzyme. This approach can be considered as one of the parameters in protein stability engineering.

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MUTASI KHUSUS UNTUK MENGKAJI PERANAN RESIDU LISINA DI PERMUKAAN KEPADA KESTABILAN STAPHYLOCOCCAL LIPASE

Oleh

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Kestabilan protein dipengaruhi oleh ciri-ciri intrinsik protein seperti nombor dan kekuatan interaksi intermolekul dan kelaziman khusus asid amino di dalam urutan protein. Residu permukaan adalah salah satu faktor untuk mendefinisikan kestabilan protein, namun peranan residu permukaan yang berkaitan dengan faktor-faktor lain masih belum dikaji secara mendalam. Residu ini dianggap kurang releven kepada kestabilan protein sehingga penemuan terbaru mendapati residu permukaan bercas berupaya untuk mengubah ciri-ciri protein. Dalam kajian ini, *Staphylococcus epidermidis* AT2 lipase yang stabil pada suhu yang rendah dan terhadap pelarut organik dimutasikan dengan menukarkan residu lisina yang berada di permukaan lipase untuk menyiasat kepentingan residu permukaan bercas kepada kestabilan enzim. Terdapat sebanyak 7% lisina residu daripada sejumlah asid amino yang terdedah di permukaan dan lisina yang boleh dimutasikan telah dikenalpastikan untuk menghasilkan mutan AT2 lipase melalui cara '*in silico*' dan simulasi 'Molecular Dynamics'. Model mutan telah dibina menggunakan YASARA versi 12.10.3 dengan lipase daripade *S. hyicus* (PDB id: 2HIH) sebagai templat. Struktur mutan disahkan dengan menggunakan 'PROCHECK (Ramachandran plots), ERRAT2, Verify3D dan QMEAN'. Simulasi MD dengan medan kuasa 'AMBER03' di dalam persekitaran air dijalankan menggunakan model protein yang elok. Daripada enam mutan, dua mutan (K325G dan K91A/K325G) menunjukkan penambahbaikan dari segi kestabilan struktur seterusnya terpilih untuk dikaji ciri-ciri biokimia dan biofizik. Kedua-dua mutan menunjukkan sedikit perubahan dalam suhu optimum dan profil pH berbanding konstruk tanpa modifikasi. Sementara itu, tiada perubahan yang signifikan dapat dilihat pada subtrat khusus di mana mutan menunjukkan kecenderungan yang sama terhadap p-nitrofenil ester berantai panjang. Sebaliknya, profil kestabilan pelarut organik untuk setiap mutan memperlihatkan sedikit perubahan selepas mutasi. Kecenderungan yang kuat terhadap pelarut organik 'polar' seperti DMSO, metanol, asetonitril, etanol, aseton, dan beberapa

daripada pelarut organik 'nonpolar' seperti dietil eter dan klorofom. Analisis suhu lebur dinilai dengan menggunakan 'dichroism' pekeliling dimana mutan K325G menunjukkan suhu lebur paling tinggi, 62.95°C, yang berhubung kait dengan peralihan suhu optimum 5°C berbanding konstruk tanpa modifikasi dengan 53.25°C. Tambahan lagi, peratusan α-lingkar mutan K91A/K325G adalah paling tinggi (25.4%) manakala, peratusan 'β-sheets' mutan K325G paling tinggi, 52.9% berbanding dengan konstruk tanpa modikasi. Simulasi MD lanjut telah dijalankan di dalam dua jenis pelarut untuk menyelidik kesan pengaktifan dan penyahaktifan terhadap mutan dan konstruk tanpa modifikasi. Secara umum, kedua-dua mutan menunjukkan lebih stabil daripada konstruk tanpa modifikasi dalam metanol. Metanol memberikan pengubahan kepada dinamik setempat mutan K325G di mana nilai lingkungan antara 1 hingga 7 Å dengan nilai paling tinggi, 7 Å. Perubahan yang jelas dilihat di kawasan penutup (penutup 1) pada mutant K325G, mencadangkan berlakunya anjakan yang besar di kawasan tersebut. Pemerhatian berikut tidak dilihat berlaku pada mutan K91A/K325G dan konstruk tanpa modifikasi. Pemerhatian ini boleh menjelaskan peningkatan lipolitik activiti dimana anjakan yang besar tersebut mungkin meningkatkan substrat aksesibiliti terhadap poket pemangkin. Kesan penyahaktifan oleh heksana tidak dapat disimpulkan kerana tiada pengubahan signifikan sepanjang trajektori. Berdasarkan pelbagai data daripada uji kaji dan *'in silico*' dalam kajian ini, residu permukaan bercas telah menunjukkan dapat mempengaruhi kestabilan protein. Kesimpulannya, kajian ini menunjukkan yang mutase permukaan lisina memberikan kesan kepada aktiviti dan kestabilan enzim. Penggantian permukaan lisina yang terdedah dengan residu yang lebih kecil boleh dijadikan sebagai salah satu kaedah dalam kejuruteraan kestabilan protein.

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LIST OF ABBREVIATIONS

α	Alpha
Å	Angstrom
β	Beta
°C	Degree Celsius
%	Percentage
A_{280}	Absorbance at 280 nanometre
A_{410}	Absorbance at 410 nanometre
A_{595}	Absorbance at 595 nanometre
A_{600}	Optical density at wavelength 600 nanometre
µL	Microliter
µm	Micrometre
µmoles	Micromoles
µg	Microgram
Amp	Ampicillin
APS	Ammonium Persulfate Solution
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine Serum Albumin
cm	Centimetre
dH2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
g	Gram
g/L	Gram per Litre

g	Relative centrifugal force
h	Hour
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
K	Kelvin
kb	Kilo base
kDa	Kilo Dalton
L	Litre
LB	Luria Bertani
M	Molar
mAU	Mili absorbance unit
mdeg	Millidegree(s)
mg/mL	Milligram per millilitre
mL	Millilitre
mM	Millimolar
min	Minute(s)
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
nm	nanometre
ns	Nanosecond
OD	Optical density
PCR	Polymerase Chain Reaction
ps	Picosecond(s)
RE	Restriction Enzyme(s)
rpm	Revolution per minute
s	Second

SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
U	Enzyme unit
U/mg	Unit per milligram
U/mL	Unit per millilitre
µL	Microlitre
µmole	Micromole
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
XhoI	*Xanthomonas vasicola*

CHAPTER 1

INTRODUCTION

Proteins are built from different amino acids and unique in respect to its properties. Protein stability is the result from cooperative phenomenon of many interactions and delicate balance between the interactions such as hydrogen bond and weak intramolecular interactions. It is clear that hydrogen bonds and hydrophobic interactions make a favourable contribution to protein stability (Pace et al., 2014). Only recently that the importance of surface charged residue is acknowledged, however, whether the surface charged residue have contribution to protein stability is not clear. Surface charged residues have been presumed having no role in protein stability particularly because the interactions between the residues and solvents are similar in native and unfolded states (Strickler et al., 2006).

Enzymes are one of many proteins that are known as nature catalyst. Lipase, in particular, is classified as one of the enzymes that catalyse the hydrolysis of wide range of substrates especially long chain triglycerides to glycerol and fatty acids. Long chain fatty acids are naturally insoluble or poorly soluble as emulsion but lipase has the ability to identify insoluble or heavily aggregated substrates. Lipases are being produced in almost all kingdoms of life and have been identified in a wide range of organisms such as microbial enzymes (Hasan et al., 2006). Microbial lipases can be classified into several families and this study is focusing on staphylococcal lipase.

The role of surface charged residue has received much less attention in affecting protein stability particularly in staphylococcal lipase. It has been assumed that surface residues are insignificant to protein stability. To increase the understanding on the function of surface charged residue in protein stability, specifically surface lysine residue, organic stable *Staphylococcus epidermidis* AT2 lipase was subjected to site-directed mutagenesis. The principal objective of this study was to investigate the key role of surface charged lysine to protein stability of AT2 lipase, therefore, this project is aimed:

1. To construct AT2 lipase harbouring new surface lysine mutations.
2. To determine the effects of the lysine mutations on the activity and stability of AT2 lipase.

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