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EFFECTS OF SINGLE RESIDUE SUBSTITUTION AT N-TERMINAL REGION OF L2 LIPASE TOWARDS ITS TEMPERATURE STABILITY AND ACTIVITY

NORAMIRAH BINTI BUKHARI @ ALBUKHRI

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By

NORAMIRAH BINTI BUKHARI @ ALBUKHRI

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Chair : Fairolniza binti Mohd Shariff, PhD
Faculty : Biotechnology and Biomolecular Sciences

Enzymes as biocatalyst have been engineered to suit the extreme conditions of industrial processes. In a previous C-terminal region study to improve temperature stability, a single residue substitution enhanced the stability and activity of L2 lipase. However, the role of the N-terminal region of L2 towards stability and activity remained unexplored. Thus, this study aimed to determine the effects of single residue substitution at a critical point of the N-terminal region of L2 lipase towards its temperature stability and activity through *in silico* approach and experimental characterisations. Prediction software was employed to predict the critical point and stability changes upon residue substitution. Position Ala8 was chosen as the critical point and substituted with valine (V), proline (P) and glutamic acid (E). Molecular dynamics simulation was used to analyse the stability changes in the mutant lipases. The results showed mutant lipase A8E was the most stable, followed by lipases A8P, wild-type L2 (wt-L2) and A8V. Substrate docking of wt-L2 and mutant lipases showed only slight differences in binding affinity. Site-directed mutagenesis was then employed to construct the mutant lipases, which expressed the enzymes, subsequently purified for characterisation. The optimum temperature of the mutant lipases remained the same as wt-L2 at 70 °C, but A8V showed higher activity compared to wt-L2 lipase. All mutant lipases showed an improvement in thermostability, especially A8V that was able to retain 84 % residual activity after 30 min pre-incubation at 70 °C compared to 55 % by that of wt-L2. A8P showed half-life at 12 h compared to wt-L2 at 8 h at 60 °C. A8E (73.59 °C) showed the highest thermal denaturation point followed by A8V (70.68 °C) and A8P (70.19 °C). Secondary structure analysis showed wt-L2 had a higher composition of *α*-helix compared to mutant lipases. The optimum pH had shifted from pH 9.0 in wt-L2 to pH 8.0 in A8V and A8P. A8E was optimal at pH 7.0. Similarly, the pH stability of mutants has broadened in range (pH 6.0 to 10.0) compared to wt-L2 (pH 8.0 to 10.0). All mutant and wt-L2 lipases showed a preference in substrate *p*-nitrophenol decanoate, but with different catalytic efficiency. A8V (260.57 s^{-1}/mM) was most efficient, followed

by wt-L2 (162.43 s^{-1}/mM), A8P (94.93 s^{-1}/mM) and A8E (27.23 s^{-1}/mM). In conclusion, substitution at the N-terminal region enhanced the activity of A8V and improved the stability of all mutants compared to wt-L2, suggesting that the N-terminal region influences the characteristics of L2 lipase.
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KESAN PENGGANTIAN RESIDU TUNGGAL DI KAWASAN TERMINAL N L2 LIPASE TERHADAP KESTABILAN SUHU DAN AKTIVITI

Oleh

NORAMIRAH BINTI BUKHARI @ ALBUKHRI

November 2020

Pengerusi : Fairolniza binti Mohd Shariff, PhD
Fakulti : Bioteknologi dan Sains Biomolekul

Enzim sebagai biomangkin telah direka bentuk agar sesuai dengan kondisi ekstrem proses industri. Melalui kajian terdahulu di terminal C lipase L2 untuk meningkatkan kestabilan suhu, penggantian residu di kawasan tersebut berjaya meningkatkan kestabilan dan aktiviti. Walau bagaimanapun, peranan kawasan terminal N dalam kestabilan dan aktiviti L2 masih tidak diterokai. Oleh itu, kajian ini bertujuan untuk mengkaji kesan penggantian residu tunggal pada titik kritikal di kawasan terminal N terhadap kestabilan suhu dan aktiviti lipase L2 melalui pendekatan in siliko dan pelbagai pencirian uji kaji. Perisian ramalan digunakan untuk meramal titik kritikal dan perubahan kestabilan lipase L2 selepas peggantian tunggal residu. Posisi Ala8 dipilih sebagai titik kritikal dan digantikan dengan residu valina (V), proline (P) dan asid glutamik (E). Molekular dinamik telah dijalankan untuk mengkaji perubahan kestabilan lipase L2. Keputusan menunjukkan lipase mutan A8E paling stabil, diikuti lipase mutan A8P, jenis liar (wt-L2) dan A8V. Dok substrat menunjukkan perbezaan minimal antara wt-L2 dan lipase mutan dalam keafinan dengan substrat. Mutagenesis terarah tapak digunakan untuk membuat konstruk yang kemudianya diekspreskan dan ditulen untuk pencirian. Suhu optimum semua lipase mutan adalah sama dengan wt-L2 iaitu pada 70 °C tetapi, A8V menunjukkan aktiviti yang lebih tinggi berbanding lipase wt-L2. Semua lipase mutan menunjukkan peningkatan thermostabiliti, terutamanya A8V, mampu mengekalkan 84 % aktiviti selepas pre-inkubasi selama 30 min pada 70 °C berbanding 55 % untuk wt-L2. A8P menunjukkan setengah hayat, 12 jam apabila di inkubasi pada 60 °C berbanding wt-L2 yang hanya bertahan selama 8 jam. A8E (73.59 °C) menunjukkan terma denaturasi tertinggi diikuti A8V (70.68 °C) dan A8P (70.19 °C). Analisis struktur sekunder menunjukan komposisi *α*-heliks wt-L2 lebih tinggi berbanding semua lipase mutan. pH optimum lipase mutan berubah daripada pH 9.0 (wt-L2) ke pH 8.0 (A8V dan A8P). Manakala pH optimum A8E adalah di pH 7.0. Julat kestabilan pH lipase mutan menjadi lebih luas (pH 6.0 hingga 10.0) berbanding wt-L2 (pH 8.0 hingga 10.0). Substrat *p*-nitrophenol decanoate menjadi pilihan semua lipase

mutan dan wt-L2 tetapi dengan kecekapan pemangkin yang berbeza. A8V (260.57 s^{-1}/mM) menunjukkan kecekapan pemangkin paling tinggi diikuti oleh wt-L2 (162.43 s^{-1}/mM), A8P (94.93 s^{-1}/mM) dan A8E (27.23 s^{-1}/mM). Kesimpulanya, penggantian residu tunggal di kawasan terminal N mempengaruhi kestabilan suhu dan aktiviti lipase, menunjukkan bahawa kawasan terminal N memberi pengaruh menyeluruh kepada ciri-ciri lipase L2.

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Fairolniza binti Mohd Shariff, PhD
Senior Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Raja Noor Zaliha bt Raja Abd. Rahman, D.Eng
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Adam Thean Chor Leow, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

ZALILAH MOHD SHARIFF, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 8 April 2021

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LIST OF ABBREVIATIONS

Å	Angstrom
α	Alpha
β	Beta
°C	Degree celsius
DNA	Deoxyribonucleic acid
g	Gravity
h	Hour
kDa	Kilo dalton
L	Litre
M	Molar
mM	Millimolar
mL	Millilitre
mg	Milligram
mg/mL	Milligram per millilitre
min	Minute
µm	Micrometre
µg	Microgram
µL	Microlitre
N	Normality
nm	Nanometre
OD_{280nm}	Optical density at wavelength 280 nanometre
OD_{410nm}	Optical density at wavelength 410 nanometre
OD_{595nm}	Optical density at wavelength 595 nanometre
OD_{600nm}	Optical density at wavelength 600 nanometre
OD_{715nm}	Optical density at wavelength 715 nanometre
rpm	Revolutions per minute
s	Seconds
sp.	Species
U	Unit
U/mL	Unit per millilitre
U/mg	Unit per milligram

V	Volt
v/v	Volume over volume
wt	Wild type
w/v	Weight over volume
Zn	Zinc
%	Percentage

CHAPTER 1

INTRODUCTION

1.1 Background of study

Lipases have diverse applications in industrial processes that demand enzymes that are highly stable and tolerant at extreme conditions. Thermostability and catalytic efficiency are favourable characteristics of enzymes, as many industrial processes occur at high temperatures to facilitate the processes and reduce the risk of biological contamination (Sangeetha et al., 2011). The stability and robustness of thermostable lipases make the enzyme appealing to industries such as leather tanning and in the processes of removing pitch in pulp and paper industry (Kapoor and Gupta, 2012; Verma et al., 2012a; Jaeger and Reetz, 1998; Hardwood, 1989). Lipases are also known to catalyse reactions such as hydrolysis and esterification, making them valuable in the oleochemical industry and fats and oil modifications (Javed et al., 2018; Verma et al., 2012a). The ability of lipases to have a broad range of preference toward fatty acid chain length makes them advantageous in wastewater treatment and detergent formulation (Javed et al., 2018). Therefore, significant interest exists in using thermostable lipases as these enzymes are more resistant towards high temperatures in addition to a broad catalytic capacity.

Thermostability in enzymes is contributed by several factors such as atomic packing of the protein core, hydrophobic residues that encourage compactness and rigidity of structure, and inclination of *α*-helix forming residues. A high number of ion pair interaction and hydrogen bonds contribute to the globular compactness of the structure thus increasing stability at high temperature (Verma et al., 2012a; Shih and Pan, 2011; Bhardwaj et al., 2010; Kumar et al., 2000; Jaeger and Reetz, 1998). These factors can be engineered through rational design to satisfy the increasing need for producing more stable lipases. Typical target regions are the ones directly involved in the catalytic function and stability, but some studies have shown that the terminal region may also have an influence. Despite many studies performed to understand the relationship between the characteristics of a thermophilic lipase and the impacts on stability and activity, the contribution of the terminal region remained mostly unexplored.

In this study, thermostable lipase, namely L2 lipase was used. L2 lipase from thermophilic *Bacillus* sp. L2 isolated from a hot spring in Slim River, Perak was discovered able to remain active between 55 to 80 °C with pH stability range from pH 6.0 to 10.0 (Shariff et al., 2011). Structure of L2 lipase was solved using X-ray crystallography and showed a globular *α*/*β* hydrolase fold. Temperature stability of L2 lipase could be further improved. However, the

relationship between the lipase structure and its characteristics with temperature and substrate must be taken into consideration.

To explore the relationship between the lipase characteristics and thermostability, a study by Sani et al. (2017) substituted a single residue at a critical point in the C-terminal region of L2 lipase. It was shown that the substitution increased the optimum temperature of L2 lipase by 10 °C, elongated the half-life at 60 °C and increased the thermal denaturation point by 19 °C. To date, little is known about the effect of residue substitution in the N-terminal region of L2 lipase. Hence, the study on characteristics of single residue substitution at a critical point in the N-terminal region of L2 lipase can be the starting point to understand the role of the terminal region towards improving stability and activity.

1.2 Problem statement

The temperature stability of L2 lipase could potentially be improved without the cost of its catalytic efficiency. In a previous study, it was reported that the C-terminal region of L2 lipase has an influence on the overall stability of the lipase (Sani et al., 2017). Single residue substitution at the C-terminal region improved activity and stability of L2 lipase at elevated temperatures.

A separate study on SML lipase conducted a single residue substitution at the N-terminal region which resulted in improvement of half-life without affecting its catalytic activity (Mohammadi et al., 2016). However, the impact of residue substitution at the N-terminal region of L2 lipase towards temperature stability and catalytic activity remained unexplored.

1.3 Objectives

The main objective of this study is to determine the effect of single amino acid residue substitution at a critical point of the N-terminal region of L2 lipase towards its temperature stability and activity.

The specific objectives of this research are:

1. To predict the critical point and analyse the effects of the single amino acid residue substitution via *in silico* studies.

2. To validate the effects of single amino acid residue substitution at the predicted critical point through site-directed mutagenesis and characterisation of the temperature stability and activity.

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