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EFFECTS OF ONE AMINO ACID SUBSTITUTIONS AT THE C-TERMINAL REGION OF THERMOSTABLE L2 LIPASE FROM Bacillus sp. L2

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By

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EFFECTS OF ONE AMINO ACID SUBSTITUTIONS AT THE C-TERMINAL REGION OF THERMOSTABLE L2 LIPASE FROM *Bacillus* sp. L2

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June 2017

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The substitutions of the amino acid residue at the predetermined critical point of the C-terminal of L2 lipase may increase its thermostability and lipase activity. N and C-terminal regions in most proteins are often disordered and flexible. However, some protein function was closely related to flexibility as well as play a role in the enzyme reaction. The critical point of the stability of L2 lipase structure was predicted at position 385 (wild type residue Serine) of the L2 sequence based on I-Mutant2.0 software. The effects of substitution of the amino acids at the critical point with Glutamic acid, Isoleucine, and Valine were analyzed with Molecular Dynamics (MD) simulation by using Yet Another Scientific Artificial Reality Application (YASARA) software and it showed that the best predicted mutant L2 lipases had lower RMSD value as compared to L2 lipase. It indicated that the three mutants had higher compactness in the structure consequently enhancing the stability. From RMSF analysis, mutations had reduced the flexibility of the enzyme. The best predicted mutants (S385E, S385I, and S385V) were produced in the experimental lab by site-directed mutagenesis. The mutant L2 lipases (60.4 kDa) were purified to homogeneity by a single chromatography step before proceeding with characterization. There were high lipase activities produced by purified mutant L2 lipases at a temperature range of 60-85 ℃ with the optimum temperature of 80 ℃, 75 ℃ and 70 ℃ for S385E, S385V, and S385I lipases respectively. The optimum temperature for recombinant L2 lipase was at 70 ℃. Mutant L2 lipases (S385E and S385V) had higher optimum temperature compared to recombinant L2 lipase. The optimum pH for mutant L2 lipases (S385E and S385V) was found to be at pH 8 and for S385I was at pH 9, whereas the optimum pH for recombinant L2 lipase was at pH 9. S385I lipase was more thermostable as compared to recombinant L2 lipase and other mutants at temperature 60 ℃ within 16 hours preincubation. The stability of S385V lipase in varies organic solvents was higher as compared to recombinant L2 lipase. S385V lipase had relative activities higher than 100% which 111% in DMSO, 105% in acetone, 123% in diethyl ether and 124% in n-hexane. *T*m values for S385V and S385E lipases were at 85.96 ℃ and 84.85 ℃ and the values were higher as compared to recombinant L2 lipase which is only 66.73 ℃. This

showed the higher thermal stability of S385E and S38V lipases as compared to recombinant L2 lipase. Thus, the substitutions at the predetermined critical point of the C-terminal (Ser385) changed the functionality of the protein structure towards the activity, stability, and flexibility of L2 lipase. The critical point mutation towards the structure of L2 lipase provided a very advantageous strategy for the improvement of enzyme with better function to adapt with harsh environment.

Keywords: L2 lipase, thermostability, site-directed mutagenesis, Molecular Dynamics (MD) simulation.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KESAN PENGGANTIAN SATU ASID AMINO DI KAWASAN C-TERMINAL TERMOSTABIL L2 LIPASE DARI BAKTERIA *Bacillus* L2

Oleh

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Penggantian daripada sisa asid amino pada titik kritikal yang telah ditetapkan pada C-terminal lipase L2 boleh meningkatkan termostabiliti dan aktiviti lipase. Kawasan N dan C-terminal di kebanyakan protein adalah sering tidak tersusun dan fleksibel. Walau bagaimanapun, sesetengah fungsi protein berkaitan rapat dengan fleksibiliti serta memainkan peranan dalam tindak balas enzim. Titik kitikal untuk kestabilan struktur lipase L2 telah dijangkakan pada kedudukan 385 (sisa jenis liar Serine) jujukan L2 yang berdasarkan perisian I-Mutant2.0. Kesan penggantian asid amino di titik kritikal dengan Asid glutamik, Isoleucine dan Valine dianalisis dengan Molekul Dinamik (MD) simulasi dengan menggunakan perisian 'Yet Another Scientific Artificial Reality Application' (YASARA) dan ia telah menunjukkan bahawa mutan lipase L2 yang terbaik diramalkan mempunyai nilai RMSD yang lebih rendah berbanding lipase L2. Ia menunjukkan bahawa tiga mutan tersebut mempunyai kepadatan yang lebih tinggi di dalam struktur yang seterusnya meningkatkan kestabilan. Daripada RMSF analisis, mutasi telah mengurangkan fleksibiliti enzim tersebut. Mutan yang terbaik diramalkan (S385E, S385I dan S385V) telah dihasilkan di makmal eksperimen oleh mutagenesis mengarah lokasi. Mutan lipase L2 tersebut (60.4 kDa) telah ditulenkan menjadi kehomogenan oleh satu langkah kromotografi sebelum meneruskan dengan pencirian. Terdapat aktiviti-aktiviti lipase yang tinggi dihasilkan oleh mutan lipase L2 yang ditulenkan pada julat suhu 60-85 ℃ dengan suhu optimum masing-masing adalah 80 ℃, 75 ℃ dan 70 ℃ untuk lipase S385E, S385V dan S385I. Suhu yang optimum untuk lipase L2 rekombinan adalah pada 70 ℃. Lipase L2 mutan (S385E dan S385V) mempunyai suhu optimum yang lebih tinggi berbanding dengan lipase L2 rekombinan. PH yang optimum bagi lipase L2 mutan (S385E dan S385V) didapati pada pH 8 dan untuk S385I adalah pada pH 9, manakala pH yang optimum bagi lipase L2 rekombinan adalah pada pH 9. Lipase S385I adalah lebih termostabil berbanding dengan lipase L2 rekombinan dan mutan yang lain pada suhu 60 ℃ dalam tempoh 16 jam pra-inkubasi. Kestabilan S385V lipase dalam organik pelarut yang berbeza-beza adalah lebih tinggi berbanding lipase L2 rekombinan. Lipase S385V mempunyai aktiviti-aktiviti relatif yang lebih tinggi daripada 100% dimana 111% dalam

DMSO, 105% dalam aseton, 123% dalam dietil eter dan 124% pada n-heksana. Nilai-nilai *T*m untuk lipase S385V dan S385E berada pada 85.96 ℃ dan 84.85 ℃ dan nilainya adalah lebih tinggi berbanding lipase L2 rekombinan yang hanya 66.73 ℃. Ini menunjukkan kestabilan haba lebih tinggi untuk lipase S385E dan S385V berbanding lipase L2 rekombinan. Oleh yang demikian, penggantian pada titik kritikal yang telah ditetapkan pada C-terminal (Ser385) telah menukar fungsi struktur protein terhadap aktiviti, kestabilan, dan fleksibiliti lipase L2. Mutasi titik kritikal terhadap struktur lipase L2 menyediakan satu strategi yang sangat berfaedah untuk peningkatan enzim dengan fungsi yang lebih baik untuk menyesuaikan diri dengan persekitaran yang sukar.

Kata kunci: lipase L2, termostabiliti, mutagenesis mengarah lokasi, simulasi molekul dinamik (MD).

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I certify that a Thesis Examination Committee has met on 13 June 2017 to conduct the final examination of Hartini binti Ahmad Sani on her thesis entitled "Effects of One Amino Acid Substitutions at the C-Terminal Region of Thermostable L2 Lipase from *Bacillus* sp. L2" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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LIST OF ABBREVIATIONS

α	Alpha
Å	Angstrom
β	Beta
β-ME	β-Mercaptoethanol
℃	Degree celsius
%	Percentage
µg	Microgram
µL	Microlitre
µm	Micrometre
µM	Micromolar
Abs	Absorbance
APS	Ammonium persulfate
bp	Base pair
$CaCl_2$	Calcium chloride
CD	Circular Dichroism
CV	Column volume
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DDG	Free Energy change
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	double stranded DNA
E. coli	*Escherichia coli*
ESBRI	Evaluating the Salt BRIdges
g	Gram
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
h	Hour
HCl	Hydrochloric acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobase
kcal	Kilocalories
kDA	Kilodaltons
L	Litre
LB	Luria-Bertani
M	Molar
mA	Milliamps
mM	Millimolar
MD	Molecular Dynamics
mg	Milligram
mL	Millilitre
N	Molar
ng	Nanogram
nm	Nanometre
ns	Nanoseconds
NaOH	Sodium hydroxide

OD	Optical density
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
ps	Picoseconds
RI	Reliability index
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuations
RNA	Ribonucleic acid
RPM	Rate per minute
SASA	Solvent Accessible Surface Area
SDS	Sodium Dodecyl Sulphate
SVM	Support vector machines
TEMED	TetramethylEthylenediamine
T opt	Optimal temperature
U/mL	Units per millilitre
v/v	Volume/volume
xg	Times gravity

CHAPTER 1

INTRODUCTION

Protein function may be controlled by protein structure. Protein is composed of a precise sequence of amino acids that allow it to fold up into a particular three- dimensional shape, or conformation (Alberts *et al*., 2002). Besides that, protein contains regions that are directly involved in protein function, such as active sites or binding sites, as well as regions that are less critical to the protein function and where mutations are likely to have less effect (Betts and Russell 2003). Protein sequences are classically considered as consisting of the whole information for their three-dimensional (3D) structure (Bornot *et al*., 2007). Each type of protein has a unique sequence of amino acids and has a particular three-dimensional structure, which governed by the order of the amino acids in its chain (Alberts *et al*., 2002). Changing of critical amino acid can cause changes in protein conformation. However, some studies have shown that changing of less hydrophobic residue (Arg) to a more hydrophobic residue (Ser) at the position 157 of ARM lipase increased the internal hydrophobicity to maintain the structural stability at a high temperature (Salleh *et al*., 2012).

It is long known that the N and C-terminal regions in most proteins are often disordered and flexible (Kamarudin *et al*, 2014). Flexibility may be closely related to protein function, as well as play a role in enzyme catalysis (Karshikoff *et al*., 2015). C-terminal of a protein is known as the residue that has free carboxyl group or at least does not acylate to another residue of amino acid, means that this residue is the end residue of the protein (Hardy *et al*., 1985). Gudiukaite *et al*., (2014) reported that 10 and 20 C-terminal amino acids of GD-95 lipase from *Geobacillus* sp. 95 crucially affect other physiochemical characteristics and the stability of this enzyme.

This research explored the roles of the critical amino acid at the C-terminal towards the structure of L2 lipase. The lipase was isolated from bacteria known as *Bacillus* sp. L2, one of the thermophilic bacteria from a hot spring in Perak. Recombinant L2 lipase was successfully overexpressed with a 178-fold increase in activity compared to crude native L2 lipase. The recombinant L2 lipase (43.2 kDa) was purified to homogeneity in a single chromatography step. The purified lipase was found to be reactive at a temperature range of 55–80 ℃ and at a pH of 6–10 and the optimum activity was found to be at 70 ℃ and pH 9. The melting temperature (*T*m) of L2 lipase was 59.04 ℃ when analysed by circular dichroism (CD) spectroscopy studies. (Shariff *et al*., 2011).

The substitution of the amino acid residues at the predetermined critical point at the C-terminal of the L2 lipase may cause an increase in protein stability and lipase activity or may speed up the unfolding of the protein structure. The prediction of the critical point of the L2 lipase was done by using one of the software from internet known as I-Mutant2.0 where 20 amino acids from the C-terminal were analysed in terms of stability. Then, the effects of substitution of the amino acids at the critical point were proceeded

by Molecular Dynamics (MD) simulation by using other software known as Yet Another Scientific Artificial Reality Application (YASARA) and followed by mutagenesis in the experimental lab. The mutant L2 lipases were analysed in terms of lipase activity and other physiochemical properties before can proceed with investigating the secondary structure of the protein. Therefore, the main objective of this research is to investigate the effects of critical amino acid at the C-terminal towards the activity, stability, and flexibility o L2 lipase.

There are two sub-objectives of the research**:**

- To predict the critical point and analyze the effects of amino acid substitutions at the C-terminal by *in silico* study.
- To validate the effects caused by the substitution of selected amino acids at the target residue of the C-terminal of L2 lipase experimentally.

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