



**UNIVERSITI PUTRA MALAYSIA**

**SIGNIFICANCE OF L2 LIPASE N-TERMINAL RESIDUE IN  
MAINTAINING PROTEIN INTEGRITY**

**NUR HANA MD JELAS**

**FBSB 2010 20**



**SIGNIFICANCE OF L2 LIPASE N-TERMINAL  
RESIDUE IN MAINTAINING PROTEIN  
INTEGRITY**

**NUR HANA MD JELAS**

**MASTER OF SCIENCE**

**UNIVERSITI PUTRA MALAYSIA**

**2010**



**SIGNIFICANCE OF L2 LIPASE N-TERMINAL RESIDUE IN  
MAINTAINING PROTEIN INTEGRITY**

**By**

**NUR HANA MD JELAS**

**Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia,  
in Fulfilment of the Requirements for the Degree of Master of Science**

**December 2010**



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

**SIGNIFICANCE OF L2 LIPASE N-TERMINAL RESIDUE IN  
MAINTAINING PROTEIN INTEGRITY**

By

**NUR HANA MD JELAS**

**December 2010**

**Chairman : Professor Abu Bakar Salleh, PhD**

**Faculty : Faculty of Biotechnology and Biomolecular Sciences**

To understand the function of amino acid residue at lipase N-terminal end, substitution of the second amino acid in L2 lipase, Serine, to Phenylalanine was carried out. Phenylalanine is a very hydrophobic amino acid compared to Serine. Substituting amino acid of a protein with other amino acids that have opposite properties are known to cause major changes towards the characteristics of the protein if the residue holds an important function in the protein. General biochemical characteristics of mutated L2 lipase were studied and compared to the recombinant wild-type to determine the role of L2 lipase N-terminal residue.

Mutated L2 lipase was created by extracting and purifying genomic DNA from thermophilic lipolytic *Bacillus* sp. L2. The purified genomic DNA was used as the template to amplify L2 lipase gene. Two degenerate primers, FT and RT were designed for the gene amplification. During the process, site-directed mutation of S2F was



introduced at the N-terminal of the protein. The mutated gene was ligated into pTrcHis vector and cloned into *Escherichia coli* DH5 alpha.

The recombinant gene was expressed using 0.05 mM IPTG at the late log phase of cell growth stage,  $OD_{600nm} \sim 0.75$ . The cell was harvested and lysed to obtain the expressed protein. The protein was purified using one step affinity chromatography. Recombinant wild-type L2 lipase worked optimally at 70°C while mutated L2 lipase showed only minute activity when assayed at 70°C. Instead, the mutant was found to be working optimally at 55°C. In addition, thermostability of mutated L2 lipase was also reduced to 45°C, compared to 60°C for wild-type when incubation was carried out for more than 60 min.

The optimum pH for activity and stability of L2 lipase were also changed. Wild-type L2 lipase worked optimally and possessed highest stability at pH 9 while mutated L2 lipase worked optimally at pH 7 and maintained the best stability in solution with pH 10. Despite the decrease in thermostability and changes in optimum pH of the mutated L2 lipase, specific activity of the purified protein was found to be at 2043.14 U/mg. This is much higher compared to the recombinant wild-type which has a specific activity of 458 U/mg.

Based on the nucleotide sequence of the wild-type L2 lipase, no other unintentional mutation had occurred within the newly cloned gene. Hereafter, any difference observed in the characteristics of the mutated L2 lipase which are different compared to the wild-type, it is deemed to be caused by the site-directed mutation of S2F. The effect of S2F



mutation towards the biochemical characteristics of L2 lipase such as characteristics of the cofactor of the enzyme, substrate preferences, positional specificity, and the effect of surfactant and inhibitor towards the activity of the enzyme were studied to determine the potency of the mutation towards the general characteristics of the L2 lipase. It was later found that these properties were the same as the wild-type.

Due to the similarity of the above properties, protein thermal melting analysis was carried out to verify the mutated L2 lipase structure integrity. The analysis confirmed the changes in L2 lipase thermostability. This study supports the theory that the point where the mutation happened has caused alteration of the surface of the proteins. The protein lost the integrity of its surface structure which caused the thermostability of the protein to change. The effect is also confirmed by the altered pH for optimum activity and stability of L2 lipase which occur due to the changes in the surface ionic charges of the protein. The loss however is seen to be compensated by the increase in the specific activity of the protein. Increase in L2 lipase specific activity by the mutation is however inconclusive.

From this study, it was found that the 'tail' structure at the N-terminal residues of L2 lipase actually played a very important role in maintaining the integrity of the protein. Site-directed mutation at one site of the amino acids in the N-terminal residues results in a loss of protein integrity which is seen as a decrease in the protein thermostability. Interestingly, the loose protein structure seemed to allow the substrates to have an easier access to the active site of the protein which resulted in an increase in the specific activity of the protein.



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

**PERANAN RESIDU N-TERMINAL LIPASE L2 DALAM MENGEKALKAN INTEGRITI PROTIN**

Oleh

**NUR HANA MD JELAS**

**Disember 2010**

**Pengerusi: Profesor Abu Bakar Salleh, PhD**

**Fakulti : Fakulti Bioteknologi dan Sains Biomolekul**

Bagi memahami fungsi residu asid amino di hujung N-terminal lipase, penggantian amino asid yang kedua dalam lipase L2, Serine, kepada Phenylalanine dilakukan. Phenylalanine adalah asid amino yang sangat hidrofobik berbanding Serine. Penggantian asid amino yang bertentangan sifat diketahui dapat menyebabkan perubahan ketara terhadap ciri-ciri protin sekiranya residu tersebut memainkan peranan yang penting dalam struktur tersebut. Ciri-ciri biokimia lipase L2 termutasi dikaji dan dibandingkan dengan lipase L2 rekombinan yang asal untuk menentukan peranan residu pada N-terminal lipase L2.

Lipase L2 ternutasi dihasilkan melalui pengekstrakan dan penulinan DNA genomik daripada *Bacillus* sp. L2 yang mempunyai sifat termofilik dan lipolitik. DNA genomik yang ditulinkan itu digunakan sebagai acuan untuk penggandaan gen lipase L2. Dua primer degenerate, FT dan RT direka untuk membantu proses penggandaan gen. Mutasi terarah S2F pada N-terminal terhasil semasa proses penggandaan gen. Gen termutasi lipase L2 disatukan dengan vektor pTrcHis dan diklonkan ke dalam *Escherichia coli* DH5 alpha.

Ekspresi gen dilakukan menggunakan 0.05 mM IPTG pada fasa log akhir perkembangan sel dengan bacaan  $OD_{600nm} \sim 0.75$ . Sel dituai dan dilisiskan untuk memperoleh protein yang diekspreskan. Proses penulinan protein dilakukan menggunakan afiniti kromatografi. Lipase L2 yang asal bekerja pada tahap optima pada suhu  $70^{\circ}C$  sementara lipase L2 termutasi hanya menunjukkan sedikit aktiviti apabila diuji pada suhu  $70^{\circ}C$ . Sebaliknya, lipase L2 termutasi mempunyai suhu optima pada  $55^{\circ}C$ . Selain itu, ketahanan terhadap haba lipase L2 termutasi juga berkurangan sehingga  $45^{\circ}C$ , berbanding  $60^{\circ}C$  bagi lipase L2 yang asal apabila enzim dibiarkan selama 60 min pada suhu tersebut.

pH optimum untuk aktiviti dan kestabilan protein juga berubah. Lipase L2 yang asal aktif dan stabil pada pH 9 sementara lipase L2 termutasi aktif pada pH 7 dan stabil pada pH 10. Walaupun pengurangan kestabilan terhadap haba dan perubahan pH optimum berlaku selepas lipase L2 dimutasi, aktiviti spesifik protein yang dituliskan adalah 2043.14 U/mg. Ini adalah sangat tinggi berbanding lipase L2 yang asal yang mempunyai aktiviti spesifik sebanyak 458 U/mg.

Berdasarkan jujukan nukleotida lipase L2 yang asal, tiada perubahan selain yang dikehendaki telah berlaku dalam gen yang baru diklonkan itu. Oleh itu, setiap perubahan yang dilihat dalam ciri-ciri lipase L2 termutasi berbanding lipase L2 yang asal, dianggap adalah disebabkan oleh mutasi S2F. Kesan mutasi S2F terhadap ciri-ciri biokimia lipase L2 seperti kofaktor enzim, substrat pilihan, kedudukan khusus ikatan ester dalam substrat, dan kesan kehadiran surfaktant dan inhibitor terhadap aktiviti enzim dikaji bagi menentukan potensi mutasi tersebut terhadap ciri-ciri lipase L2. Sifat-sifat ini kemudiannya didapati sama dengan lipase yang asal.



Disebabkan persamaan sifat-sifat di atas, analisis pencairan haba protin dilakukan bagi mengkaji integriti struktur lipase L2 termutasi. Analisis tersebut mengesahkan perubahan yang berlaku terhadap kestabilan haba yang dimiliki lipase L2. Kajian ini menyokong teori bahawa titik di mana mutasi tersebut berlaku telah menyebabkan perubahan struktur permukaan protin. Protin termutasi itu telah kehilangan integriti struktur permukaan yang menyebabkan perubahan terhadap kestabilan haba protin. Walaubagaimanapun, kehilangan tersebut dilihat sebagai diganti dengan kenaikan dalam aktiviti spesifik protin. Tetapi penggandaan aktiviti tersebut adalah tidak begitu meyakinkan tanpa kajian kinetik enzim untuk mengesahkan kesahihannya.

Daripada kajian ini, struktur seperti 'ekor' pada residu N-terminal lipase L2 sebenarnya memainkan peranan yang sangat penting dalam mengekalkan integriti protin. Mutasi pada titik tertentu pada salah satu asid amino pada residu di N-terminal menyebabkan kehilangan integriti protin yang dapat dilihat sebagai penurunan dalam kestabilan termal protin. Sungguhpun begitu, struktur protin yang lemah dilihat telah membenarkan lebih banyak substrat dapat memasuki tapak aktif protin. Ini dilihat sebagai kenaikan dalam aktiviti spesifik protin.

## ACKNOWLEDGEMENTS

With humble feeling, I am so grateful to Allah, Alhamdulillah that I am able to finally end my Master's study. I would like to express my gratitude to my parents for their never ending love and care, support and nagging which had helped me to keep going when I was lost. The patience they always exhibited in leading me back on track to finish my study. I would have gained nothing if not for of them.

I would like to express my biggest appreciation to the leaders in my study, Professor Dr. Raja Noor Zaliha Raja Abd Rahman and Professor Dr. Abu Bakar Salleh. They have been just like my parents with respect to my study; besides the guidance, the love, the care, and the patience they gave had kept me moving. My sincere appreciation also goes to Professor Dr. Mahiran Basri, and Professor Dr Basyaruddin Abd Rahman who always support me from behind. Thank you for always being there and giving me your best.

My biggest gratitude is to Kak Ferrol who is a teacher, a sister and a shoulder to rely on. You have been the cursor in my study. Fairalniza Shariff has kindly assisted me in doing my research for this study. I also would like to express my sincere gratitude to Dr Adam, the person I would always turn to when I faced any major lab work problems. My gratitude also goes to Kak Su who had been the angel and had helped me from the very beginning of my study until I managed to gain, which I believe, pretty much everything I had now. I would also like to express my recognition to my lab mates in Lab 140 and IBS. Thank you, all.



I certify that an Examination Committee has met on **8 December 2010** to conduct the final examination of **Nur Hana Md Jelas** on her **Master of Science** thesis entitled “**Significance of L2 lipase N-terminal residue in maintaining protein integrity**” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree.

Members of the Examination Committee are as follows:

**Shuhaimi Mustafa, PhD**

Associate Professor

Department of Microbiology

Faculty of Biotechnology and Biomolecular Sciences

Universiti Putra Malaysia

(Chairman)

**Mohd Yunus Abd Shukor. PhD**

Associate Professor

Department of Biochemistry

Faculty of Biotechnology and Biomolecular Sciences

Universiti Putra Malaysia

(Internal Examiner)

**Norazizah Shafee, PhD**

Department of Microbiology

Faculty of Biotechnology and Biomolecular Sciences

Universiti Putra Malaysia

(Internal Examiner)

**Sheila Nathan, PhD**

Professor

Bioinformatics Unit

National Institute for Genomics and Molecular Biology

(External Examiner)

---

**Shamsuddin Sulaiman, PhD.**

Professor/Deputy Dean

School of Graduate Studies

Universiti Putra Malaysia

Date:



This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

**Abu Bakar Salleh, PhD**

Professor

Faculty of Biotechnology and Biomolecular Sciences

Universiti Putra Malaysia

Serdang Selangor

(Chairman)

**Raja Noor Zaliha Raja Abd. Rahman, PhD**

Professor

Faculty of Biotechnology and Biomolecular Sciences

Universiti Putra Malaysia

Serdang Selangor

(Member)

**Mahiran Basri, PhD**

Professor

Faculty of Science

Universiti Putra Malaysia

Serdang Selangor

(Member)

---

**HASANAH MOHD GHAZALI, PhD**

Professor and Dean

School of Graduate Studies

University Putra Malaysia

Date:



## **DECLARATION**

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or other institutions.

---

**NUR HANA MD JELAS**

Date: 8 December 2010



## TABLE OF CONTENTS

	<b>Page</b>
<b>ABSTRACT</b>	ii
<b>ABSTRAK</b>	v
<b>ACKNOWLEDGEMENT</b>	viii
<b>APPROVAL</b>	ix
<b>DECLARATION</b>	xi
<b>LIST OF TABLES</b>	xvi
<b>LIST OF FIGURES</b>	xvii
<b>LIST OF ABBREVIATIONS</b>	xix
<b>CHAPTER</b>	
<b>1 INTRODUCTION</b>	<b>1</b>
<b>2 LITERATURE REVIEW</b>	<b>4</b>
2.1 Lipase	4
2.2 Lipases application in industry	7
2.3 Thermostable lipase	8
2.4 Lipase cloning and gene modification	9
2.4.1 Site-directed mutagenesis	11
2.4.2 Directed evolution	12
2.5 Bacterial lipase classification	13
2.6 Lipase conserved residues	15
2.6.1 Lipase lid	17
2.6.2 Lipase active site	18
2.6.3 Amino acids of lipase protein core residues	20
2.6.4 Amino acids of protein N-terminal	21
<b>3 MATERIALS AND METHODS</b>	<b>22</b>
3.1 Materials	22
3.2 Lipase gene isolation and modification	22



3.2.1	Growth of <i>Bacillus</i> sp. L2	22
3.2.2	<i>Bacillus</i> sp. L2 genomic DNA isolation	23
3.2.3	Quantification of genomic DNA	24
3.2.4	Lipase gene amplification and gene cloning	25
3.2.5	Plasmid extraction	27
3.2.6	Double Digestion of Vector and Mutated L2 Lipase Gene	28
3.2.7	Ligation of pTrcHis with L2 lipase	29
3.2.8	<i>Escherichia coli</i> DH5 $\alpha$ competent cells preparation	29
3.2.9	Transformation of cells with ligated recombinant gene	30
3.2.10	Screening of positive recombinant colonies	30
3.2.11	Confirmation for presence of inserted gene	31
3.3	Optimization on expression of recombinant L2 lipase	31
3.3.1	Effect of different inducer concentration on lipase expression	32
3.3.2	Effect of lipase expression when induced at different growth stage	32
3.3.3	Time-course studies of lipase expression	32
3.3.4	Assay of lipase activity	32
3.4	Purification of recombinant L2 lipase	33
3.4.1	Production of crude lipase	34
3.4.2	His-tag affinity chromatography	34
3.4.3	Analysis of purified protein through SDS-PAGE	35
3.4.4	Protein content determination	36
3.5	Characterization of purified L2 lipase	36
3.5.1	Effect of pH towards lipase activity	37
3.5.2	Effect of pH towards lipase stability	37
3.5.3	Effect of temperature towards lipase activity	37
3.5.4	Effect of temperature towards lipase stability	37
3.5.5	Effect of metal ions towards lipase activity	38
3.5.6	Effect of triglycerides with different chain length towards lipase activity	38
3.5.7	Effect of different natural oils towards lipase activity	39
3.5.8	Lipase positional specificity test	39



3.5.9	Effect of surfactant towards lipase activity	40
3.5.10	Effect of inhibitors towards lipase activity	40
3.5.11	Thermal melting analysis of L2 lipase	41
3.6	Characteristics comparison between mutated L2 lipase and recombinant wild-type L2 lipase	42
3.7	Statistical analysis	42
<b>4</b>	<b>RESULTS AND DISCUSSION</b>	<b>44</b>
4.0	Background of L2 lipase	44
4.1	Construction of L2 lipase recombinant gene	45
4.1.1	Isolation of <i>Bacillus</i> sp. L2 genomic DNA	45
4.1.2	Lipase gene amplification and gene cloning	47
4.2	Screening of positive clones	53
4.2.1	Conformation of S2F mutation	58
4.3	Optimization of high-level expression	61
4.3.1	Optimization of inducer concentration	61
4.3.2	Optimization of growth stage for protein expression	63
4.3.3	Optimization of time-course for protein expression	65
4.4	Purification of recombinant L2 lipase	65
4.5	Study of mutated lipase through general characterization	69
4.5.1	Effect of pH towards lipase activity and stability	69
4.5.2	Effect of temperature towards lipase activity and activity	72
4.5.3	Effect of metal ion towards lipase activity	77
4.5.4	Effect of triglycerides with different chain length towards lipase activity	80
4.5.5	Effect of different natural oils towards lipase activity	83
4.5.6	Lipase positional specificity test	85
4.5.7	Effect of surfactant towards lipase activity	87
4.5.8	Effect of inhibitors towards lipase activity	91
4.5.9	Thermostability study using circular dichroism (CD) spectra analysis	93
4.6	Comparison between mutated L2 lipase and recombinant wild-type L2 lipase	101





5	<b>CONCLUSION AND RECOMMENDATIONS</b>	105
	<b>REFERENCES</b>	108
	<b>APPENDICES</b>	116
	<b>BIODATA OF STUDENT</b>	129

