UNIVERSITI PUTRA MALAYSIA

SITE-DIRECTED MUTAGENESIS OF N-TERMINAL ALPHA α-HELIX OF J15 LIPASE

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FBSB 2015 150

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By

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Thesis Submitted to the Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Bachelor of Science (HONS.)

June 2015

Abstract of thesis presented to the Department of Cell and Molecular Biology
in fulfilment of the requirement for the degree of
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Chair: Dr. Adam Leow Thean Chor, PhD.

Faculty: Faculty of Biotechnology and Biomolecular Sciences

The intrinsic propensity of each amino acid residue forming α-helices in a protein determines the stability of a protein. The purpose of this study is to improve the stability of the protein by stabilizing the N-terminal α-helix. Therefore, mutations were focused on N-terminal helix of J15 lipase derived from *Photobacterium* sp. strain J15. The structure of J15 lipase was modeled based on M37 lipase crystal structure as a template. Asp6 of N-terminal helix was chosen as competition of Asp6 with the adjoining main chain hydrogen bonding interaction could destabilize the N-terminal helical structure. *In silico* analysis using SDM and I-mutant 2.0 revealed that D6L and D6I enhanced the

stability of mutated J15 lipases with ΔΔG of 5.12 kcal/mol and 0.82 kcal/mol respectively. In order to validate the *in-silico* prediction, the gene encoding J15 lipase was cloned into *pEasy*-E2 expression vector and transformed into *Escherichia coli* BL21(De3). Point mutation was generated using Fast Mutagenesis System to increase the mutation efficiency. The Aspartate residue at the sixth position of the N-terminal α-helix was substituted with Isoleucine and Leucine. The wild-type and mutants were induced with 0.015 mM IPTG at 30°C for 16 h. The expected size of the protein was approximately 40.11kDa. Both the wild-type and mutants showed low expression level and most of the proteins were present as insoluble bodies. The specific activity of the crude protein calculated for J15 lipase is 1.953 U/mg, D6I is 1.55 U/mg and D6L is 1.429 U/mg. Overall, the specific activity was low. Due to time constraint, further experimental work could not be performed on time and it was not able to validate the result with the *in-silico* protein stability predictions.

Keywords : mutagenesis, lipase, *Photobacterium*, stability

Abstrak tesis yang dikemukakan kepada Jabatan Biologi Sel dan Molekul
Sebagai memenuhi keperluan untuk ijazah
Sarjana Muda Sains (K)

TAPAK BERARAH MUTAGENESIS DARIPADA N-TERMINI HELIX LIPASE J15

Oleh

LEE MEI YING

Jun 2015

Pengerusi: Dr. Adam Leow Thean Chor, PhD.

Fakulti: Fakulti Bioteknologi dan Sains Biomolekul

Kecenderungan intrinsic daripada setiap residu asid amino yang membentuk heliks alfa dalam protein menentukan kestabilan protein. Oleh itu, mutasi difokuskan kepada heliks N-termini lipase J15 yang didapati daripada spesies *Photobacterium* strain J15. Struktur lipase J15 dimodelkan berdasarkan struktur kristal lipase M37 sebagai templat. Asp6 daripada heliks N-termini dipilih sebagai tapak mutasi kerana persaingan di antara Asp6 dan rantaian ikatan hydrogen utama boleh menjejaskan kestabilan struktur heliks N-termini. Dalam analisis silico menggunaka SDM dan I-mutant 2.0, ia mendedahkan bahawa D6L dan D6I meningkatkan kestabilan lipase J15 bermutasi dengan bacaan $\Delta\Delta G$ 5.12 kcal/mol dan 0.82 kcal/mol. Dalam usaha untuk

mengesahkan ramalan dalam silico, kajian eksperimen telah dijalankan. Gen yang mengandungi lipase J15 telah diklonkan ke dalam *pEasy*-E2 vektor ekspresi dan ditransformasikan ke dalam *Escherichia coli* BL21(DE3). Titik mutasi telah dijanakan dengan menggunakan Sistem Mutagenesis cekap untuk meningkatkan kecekapan mutasi. Residu Aspartate di kedudukan keenam N-termini telah α-heliks digantikan dengan Isoleucine dan Leucine. Lipase J15 jenis liar dan mutan telah diaruhkan dengan 0.015 mM IPTG pada 30°C selama 16 jam. Jangkaan saiz protein adalah lebih kurang 40.11kDa. Lipase J15 jenis liar dan mutan mempunyai tahap ekspresi yang rendah dan sebahagian besar daripada protein adalah tidak larut. Aktiviti spesifik protein mentah dikira untuk jenis liar J15 lipase adalah 1,953 U/mg, D6I adalah 1.55 U/mg dan D6L adalah 1.429 U/mg. Secara keseluruhannya, aktiviti khusus adalah rendah. Oleh kerana kekangan masa, kerja eksperimen lanjut tidak dapat dilakukan pada masa ini dan ia tidak dapat mengesahkan keputusan dengan ramalan dalam silico.

Kata kunci : mutagenesis, lipase, Photobacterium, kestabilan

APPROVAL LETTER

This thesis was submitted to the Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences and has been accepted as fulfilment of the requirement for the degree of Bachelor of Science (HONS.). The member of the Supervisory Committee was as follows:

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Date:

DECLARATION

Declaration by undergraduate student

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This is to confirm that:

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ACKNOWLEDGEMENTS

First and foremost, I am very grateful to my supervisor, where no words could describe how thankful I am except to express my sincerest gratitude to Dr. Adam Leow Thean Chor for his support, guidance and patience. He has always been there to help and guide me in every possible way.

Special thanks to all my seniors in Enzyme and Microbial Technology Research group for their contributions, knowledge and friendship. I would like to specially thank my lab mentor, Ang Thiau Fu, for his guidance and help throughout the whole experimental process. Not forgetting my coursemates who are always there to discuss and see through my problems.

I wish to express my deepest gratitude to my parents for they are always there to support me in every possible way and they will always keep me in their prayers. Without my parents' support and encouragement, I would never be here.

Sincerest thanks to everyone who have helped me throughout my final year project. Thank You.

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LIST OF ABBREVIATIONS

PCR	Polymerase Chain Reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
IPTG	Isopropyl-β-D-tiogalactopyranoside
Å	Armstrong
RMSD	Root Mean Square Deviation
rpm	Rotation per minute
TEMED	Tetramethylethylenediamine
U/mg	Unit per milligram
U	Unit of enzyme activity
w/v	Weight per volume
v/v	Volume per volume
sp.	Species

CHAPTER 1

INTRODUCTION

Lipases catalyze the hydrolysis of triacylglycerol to glycerol and fatty acids (Sharma *et al.*, 2011). Lipase will only be activated when interface between the aggregated substrates and aqueous solution is formed. This is known as the interfacial activation. The active sites of the enzyme are shielded from the solvent (Brady *et al.,* 1990). Some lipases are stable in organic solvents, which in turn giving them a chance for their exploitation in organic synthesis such as esterification, transesterification and aminolysis (Drauz and Waldman, 1995). Lipases are being utilized as cheap and versatile biocatalysts as the enzymes are widely spread in nature such as plants, animals and microorganisms (Gandhi, 1997). Besides, lipases are also widely used in many industrial applications such as in the production of fats and oils, detergents, food processing and production of cosmetics (Rubin and Dennis, 1997a,b).

In an α-helix, the first four amine group and the last four carboxyl group lacks internal hydrogen bonds. Instead, they are often capped by an alternative hydrogen bond partner (Aurora and Rose, 1998). Presta and Rose (1998) hypothesized that helix end capping plays an important role in helix stabilization. Basically, by substituting one or several amino acids at the N- or C- terminal will help to improve the protein stability by increasing the thermal stability of the protein.

Most of the enzymes are marginally stable under cellular environments and are hardly stable under harsh condition such as high temperature. Mutation was introduced at the

N-terminus so that enzymes with improved properties and desired characteristics can be obtained. Point mutation was modeled first and protein stability was analysed *in-silico.* Experimental studies were then carried out to validate the *in-silico* protein stability prediction results.

Objectives

1.1 To clone J15 lipase gene into *pEasy*-E2 expression vector

1.2 To model point mutation *in-silico*

1.3 To analyse the protein stability *in-silico*

1.4 To generate point mutation through site-directed mutagenesis

1.5 To study the effect of mutation on the stability of J15 lipase

REFERENCES

Anderson, D. E., Becktel, W. J. and Dahlquist, F. W. (1990) pH-induced denaturation of proteins: a single salt bridge contributes 3–5 kcal/mol to the free energy of folding of T4 lysozyme *Biochemistry.* 29: 2403–240

Aurora, R. and Rose, G.D. (1998) Helix Capping. *Protein Science* 7(1):21-38

Baneyx, F. (1999) Recombinant protein expression in *Escherichia coli*. *Curr. Opin. Biotechnol* 10: 411-421

Bhardwaj, A., Leelavathi, S., Mazumdar-Leighton, S., Ghosh, A., Ramakumar, S., (2010) The Critical Role of N- and C Terminal Contact in Protein Stability and Folding of a Family10 Xylanase under extreme Conditions. *PLoS ONE* 5(6): l1347

Bosshard, H.R., Marti, D.N., Jelesarov, I. (2004) Protein stabilization by salt bridges: concepts, experimental approaches and clarification of some misunderstandings. *Journal of Molecular Recognition* 17(1): 1-16

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochemistry* 72: 248–254.

Carugo, O. and Pongor, S. (2001) A normalized root-mean-square distance for comparing protein three-dimensional structures. *Protein Science* 10(7):1470-3.

Charles Ogugua Nwuche, James Chukwuma Ogbonna (2011) Isolation of lipase producing fungi from palm oil Mill effluent (POME) dump sites at Nsukka. *Brazilian Archeology of Technology* 54(1)

Cheetham, P. S. J. (1995) The application of enzymes industry, *In A. Wiseman (ed.) Hand Book of Enzyme Biotechnology. Ellis Horwood, UK.* 419-522.

Dao-pin, S., Sauer, U., Nicholson, H., and Matthews, B.W. (1991) Contributions of engineered surface salt bridges to the stability of T4 lysozyme determined by directed mutagenesis. *Biochemistry.* 30:7142–7153

Forood. B., Feliciano, E.J,, Nambiar, K,P., (1993) Stabilization of alpha-helical structures in short peptides via end capping. *Proceedings of the National Academy of Sciences USA.* 90(3): 838–842.

Furutani, M., Ideno, A., Iida, T., Maruyama, T. (2000) FK506 binding protein from a thermophilic archaeon, *Methanococcus thermolithotrophicus* has chaperone-like activity in vitro. *Biochemistry.* 39: 453–462

Gandhi, N.N. (1997) Application of lipase. *J. Am. Oil Chem. Soc*. 74: 621-634

Hecht, M. H., Sturtevant, J. M., and Sauer, R. T. (1986), Stabilization of λ repressor against thermal denaturation by site-directed Gly→Ala changes in α-helix 3. *Proteins*. 1: 43–46.

Ibrik A., Chahinian, H., Rugani, N., Sarda, L., and Comeau, L.C. (1998). Biochemical and structural characterization of triacylglycerol lipase from *Penicillium cyclopium*. *Lipids.* 33: 377 -384.

Ideno, A., Furutani, M., Iwabuchi, T., Lida, T., Iba, Y., Kurosawa, Y., Sakuraba, H., Ohshima, T., Kawarabayashi, Y. and Maruyama, T. (2004). Expression of foreign proteins in *Escherichia coli* by fusing with an archaeal FK506 binding protein. *Applied Microbiology and Biotechnology* 64: 99–105

Ionita, A., Mosovici, M., Dragolici. A., Eremia, M., Buca, C. Albulescu, R., Pavel, I. M. and Vamanu, C. (2001). Lipase production in discontinuous operation system using *Candida lipolytica* strain. *Biotechnology Letters* 7 (1): 547-552.

Kammerer, R. A., Jaravine, V. A., Frank, S., Schulthess, T., Landwehr, R., Lustig, A., Garcia-Echeverria, C., Alexandrescu, A. T., Engel, J., and Steinmetz, M. O. (2001) An intrahelical salt bridge within the trigger site stabilizes the GCN4 leucine zipper. *Journal of Biology and Chemistry* 276: 13685–13688

Kamondi, S., Szilagyi, A., Barna, L., Zavodszky, P. (2008) Engineering the thermostability of a TIM-barrel enzyme by rational family shuffling. *Biochemical and Biophysical Research Communications*. 374: 725-730.

Kumar, D., Kumar, L., Nagar, S., Raina, C., Parshad, R., and Gupta, V. (2012). Isolation, production and application of lipase/esterase from *Bacillus sp.* strain DVL43. *Journal of Microbiology and Biotechnology*. 2(4): 521-528

Kunkel, T. A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proceedings of the National Academy of Science*. 82(2): 488-92

Leow, T. C., Abd Rahman R. N., Ali M. S., Basri M, Salleh A. B. (2004) High Level Expression of Thermostable Lipase from *Geobacillus sp*. Strain T1, *Bioscience, Biotechnology, and Biochemistry*. 68(1): 96-103,

Lesuisse, E., Schanck, K. and Colson, C. (1993). Purification and preliminary characterization of the extracellular lipase of *Bacillus subtilis* 168, an extremely basic pH-tolerant enzyme. *European Journal of Biochemistry* 216: 155-160

Prevost, M., Wodak, S. J., Tidor, B. and Karplus, M. (1991) Contribution of the hydrophobic effect to protein stability: Analysis based on simulations of the Ile-96- Ala mutation in Barnase. *Proceedings of the National Academy of Science USA* 88: 10880-10884

Martinelle M, Holmquist M, Hult K. (1995) On the interfacial activation of *Candida antarctica* lipase A and B as compared with *Humicola lanuginosa* lipase. *Biochimica at Biophysica Acta.* 1258:272–6.

Petsko, Gregory A, Ringe, Dagmar. (2004) Protein structure and function. *New Science Press.* ISBN : 978-0878936632

Presta, L.G., Rose, G.D. (1998) Helix signals in proteins. *Science.* 17. 240 (4859):1632–1641.

Ruslan, R., Abd Rahman, R.N., Leow, T. C., Ali, M. S., Basri, M., Salleh, A.B. (2012) Improvement of thermal stability via outer-loop ion pair interaction of mutated T1 lipase from *Geobacillus zalihae* strain T1. *International Journal of Molecular Science* 13(1):943-60.

Sharma, R., Chisti, Y., Banerjee, U. C., (2001). Production, purification, characterization, and applications of lipases. *Biotechnol*ogy *Advancement* 19: 627-662.

Shiraki, K., Nishikori, S., Fujiwara, S., Imanaka T. and Takagi, M., (2004) Contribution of protein-surface ion pairs of a hyperthermophilic protein on thermal and thermodynamic study. *Journal of Bioscience and Engineering.* 97(1): 75-55

Sugihara, A., Tani, T., Tominaga, Y. (1991) Purification and characterization of a novel thermostable lipase from *Bacillus* sp. *Journal of Biochemistry* 109:211–6

Svendsen, A. (2000). Lipase protein engineering. *Biochimica at Biophysica Acta* 1543 (2): 223–228

Sztajer, H., Maliszewska, I., Wieczorek, J. (1988) Production of exogenous lipase by bacteria, fungi and actinomycetes. *Enzyme Microbiology Technology* 10: 4927

Uppenberg, J., Hansen, M. T., Patkar, S., Jones, T. A. (1994) The sequence, crystal structure determination and refinement of two crystal forms of lipase B from *Candida antarctica*. *Structure.* 2: 293–308.

Uppenberg, J., Patkar, S., Bergfors, T., Jones, T. A. (1994) Crystallization and preliminary X-ray studies of lipase B from *Candida antarctica*. *Journal of Molecular Biology* 235:790–2

Whitford, D. (2005) *Proteins: structure and function*. New York *John Wiley & Sons.* ISBN : 978-0471498940

Winkler, F.K., D'Arcy, A., and Hunziker, W. (1990). Structure of human pancreatic lipase. *Nature 34.* 6260: 771–774.