UNIVERSITI PUTRA MALAYSIA

STRUCTURAL STUDIES OF A CHEMICALLY MODIFIED THERMOSTABLE LIPASE FROM *Geobacillus* SP. STRAIN T1

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By

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

STRUCTURAL STUDIES OF A CHEMICALLY MODIFIED THERMOSTABLE LIPASE FROM *Geobacillus* SP. STRAIN T1

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September 2007

Chair : Professor Abu Bakar Salleh, PhD

Faculty: Biotechnology and Biomolecular Sciences

Alkylation has been successfully performed using propionaldehyde on four batches of T1 thermostable lipase (M1, M2, M3 and M4) with different degrees of modification (27% to 55%) to represent the different levels of hydrophobicity. Based on the crystal structure, T1 possessed 11 lysine residues, of which four of the lysine residues, Lys84, 102, 138 and 251 have scores between 53.7% and 95.8% exposure ratio, were totally exposed. Another four residues, Lys185, 329, 344 and 345 have a ratio between 20% and 50% (moderately exposed) and three of the lysine residues, Lys28, 207 and 229 are buried. The hydrolytic activity of the modified enzymes dropped drastically by 10 to 40-fold upon chemical modification, despite both the native and modified form showed distinctive α-helical bands at 208 and 222 nm by Far Ultra-Violet Circular Dichroism (CD) spectropolarimetry. As cooperative unfolding transitions were observed, the modified lipases were distinguished from the native state, which the former

possessed a T_m in lower temperature range, 60-64 ºC whilst the latter at 68 ºC. Consequently, this has led us to the hypothesis of formation of a molten globule (MG)-like structure.

Subsequent analysis of both native and modified lipases by Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) study was carried out to ascertain the modifications and the location of these modifications. Four lysine residues, Lys28, 84, 207 and 329 were clearly identified from the native spectrum. As expected, Lys84 and Lys102 were clearly modified. Surprisingly, Lys185 which has a very low exposure ratio (27.5%) was also identified to be one of the modified residues. To further support the hypothesis of the formation of a molten globule, intrinsic and extrinsic fluorescence were performed. A decrease of fluorescence intensity was observed for modified lipase M1, which was modified using 0.5% of propionaldehyde. However, subsequent addition of propionaldehyde enhanced the fluorescence intensity of M2, M3 and M4, which indicated an inversion of placement for tryptophans to a more hydrophobic environment. As for extrinsic fluorescence, the alkylated lipases showed a clear enhancement of fluorescence intensity as compared to the native lipase due to the exposure of the hydrophobic interior of the enzymes.

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

KAJIAN STRUKTUR THERMOSTABIL LIPASE DARI *Geobacillus* SP. JENIS T1 YANG DIMODIFIKASI MELALUI TINDAK BALAS KIMIA

Oleh

CHEONG KOK WHYE

September 2007

Pengerusi: Profesor Abu Bakar Salleh, PhD

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Pengalkilan telah berjaya dilaksanakan ke atas empat kelompok enzim thermostabil lipase T1 (M1, M2, M3 dan M4) dengan menggunakan propil aldehid pada beberapa peringkat modifikasi yang berbeza (27% sehingga 55%) untuk menggambarkan perbezaan hidrofobia. Berdasarkan struktur kristal, T1 mempunyai 11 lysine, yang mana empat lysine iaitu Lys84, 102, 138 dan 251 mempunyai skor di antara 53.7% dan 95.8% nisbah pendedahan, adalah dalam kategori pendedahan lengkap. Empat lysine yang lain, Lys185, 329, 344 dan 345 mempunyai skor di antara 20% dan 50% (pendedahan separa) dan tiga lysine yang terakhir, Lys28, 207 dan 229 adalah tertimbus. Sungguhpun begitu, selepas menjalani modifikasi secara kimia, aktiviti hidrolisis lipase yang telah diubahsuai didapati turun secara mendadak (10 hingga 40-kali ganda), walaupun spektrum spektropolarimetri 'Circular Dichroism' (CD) ultraungu jauh bagi kedua-dua lipase asal dan yang terubah menunjukkan struktur α-helix pada 208 dan 222 nm.

Disebabkan kedua-dua lipase asal dan yang terubah menunjukkan graf transisi pembukaan lipatan yang lancar, lipase yang terubah suai hanya dapat dibezakan dengan lipase asal kerana lipase terubah suai mempunyai T_m pada julat suhu yang lebih rendah, 60-64 ºC manakala lipase asal pada 68 ºC. Susulan bukti ini, ia membawa kami kepada hipotesis pembentukan struktur seakan 'molten globule' (MG).

Analisis seterusnya, Spektrometri Jisim Matrik Laser Nyahserapan/Pengionan Penerbangan Masa (MALDI-TOF MS) dilakukan bagi memastikan proses modifikasi telah berlaku dan menjejak lokasi-lokasi modifikasi tersebut. Empat lysine, Lys28, 84, 207 dan 329 dapat dikesan dalam spektrum lipase asal. Seperti yang telah diduga, Lys84 dan Lys102 telah dimodifikasi. Walau bagaimanapun, Lys185 yang mempunyai pendedahan yang sangat rendah (27.5%) juga telah dimodifikasi. Bagi menyokong hipotesis pembentukan struktur seakan MG, pendarfluor dalaman dan luaran dijalankan. Penurunan keamatan pendarfluor dapat diperhatikan bagi lipase terubah suai M1 yang diubahsuai menggunakan 0.5% propil aldehid. Walau bagaimanapun, apabila lebih banyak propil aldehid ditambah, keamatan pendarfluor lipase terubah suai M2, M3 dan M4 didapati meningkat, menunjukkan asid amino tryptofan mengubah kedudukan kepada persekitaran yang lebih hidrofobia. Bagi pendarfluor luaran pula, keamatan pendarfluor bagi lipase terubah suai adalah lebih tinggi berbanding lipase yang asal kerana bahagian dalaman enzim yang hidrofobia didedahkan.

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I certify that an Examination Committee has met on 11th September 2007 to conduct the final examination of Cheong Kok Whye on his Master of Science thesis entitled "Structural Studies of a Chemically Modified Thermostable Lipase from *Geobacillus* Sp. Strain T1" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the Master of Science.

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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:

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Date: 15 November 2007

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 at any other institutions.

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CHEONG KOK WHYE

Date: 1 October 2007

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LIST OF ABBREVIATIONS

ANS	8-anilino-1-naphthalenesulfonic acid
BPTI	Bovine pancreatic trypsin inhibitor
CD	Circular dichroism
DTT	Dithiothreitol
FT-IR	Fourier transform – infra red
GST	Glutathione S-transferase
IPTG	Isopropylthio-β-D-galactoside
kDa	Kilo Dalton
LB	Luria Bertani
MALDI-TOF MS	Matrix assisted laser desorption ionization time of flight mass spectrometry
MG	Molten globule
NMR	Nuclear magnetic resonance
PBS	Phosphate buffer saline
PTH	Phenylthiohydantoin
SASA	Solvent accessible surface area
SDS PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
T_m	Midpoint of sigmoidal melting curves
TNBS	2,4,6-trinitrobenzenesulphonic acid

CHAPTER 1

INTRODUCTION

Since the booming development in enzymology, the manipulation of the enzyme structure was seen as a new way to enhance its biochemical properties, be it chemical modifications (Mine *et al.*, 2001; Basri *et al.*, 1998), protein engineering (Bornscheuer, 2002; Bornscheuer and Pohl, 2001; Harris and Craik, 1998) or by changing the reaction medium rather than the enzyme itself (Klibanov, 1989). Native enzymes are limited and not specific in its application. In contrast, modified enzymes with specific purpose are useful in certain fields. Therefore, modifications have been carried out in order to increase their activities, selectivity and stability. Chemically modified enzymes was seen as a fruitful research in Basri and co-workers' work (1998) and Ampon *et al.* (1991), as these lipases' enantioselectivity and specificity were successfully enhanced.

Protein chemists have long been interested in altering the chemical, physical and biological properties of proteins by chemically changing their structure. One of the first things discovered about proteins was how easily they were changed upon treatment with chemical reagents. Their liability to chemical reagents and reaction conditions has been a serious problem for them to be used for many purposes. The application of modern knowledge of proteins, new chemical reagents and more sophisticated analytical techniques, however, has made chemical modification of protein as one of the most useful approaches to the study many of their properties.

Numerous efforts are being made in the improvement of enzyme efficiency to exploit their environmental friendly processes more extensively. The alteration of protein surface characteristics by chemical modification is a good strategy to improve biocatalyst performance in nonaqueous conditions (Longo and Combes, 1999). Attachment of hydrophobic groups using aldehydes to the enzyme surface has been considered to increase enzyme solubility in organic solvent (Inada *et al.*, 1986). Yet, the essential of chemical modification of proteins lie beneath the opportunities for studying localization of individual amino acids in proteins, their participation in the maintenance of the native conformation (Torchilin *et al.*, 1979), their stabilization (Ryan *et al.*, 1994), conversion to molten globule structures (Dolginova *et al.*, 1992), tailoring of enzyme specificities and their structure-function relationship (Wong and Wong, 1992).

Reductive alkylation is a convenient method to convert surface exposed amino groups in proteins into their alkylamino derivatives. The functional residues are located on or near the surface of proteins, and thus are reactive to chemical reagents (Alberts *et al.*, 1983). Unlike other procedures for the modification of amino groups of proteins, reductive alkylation has little effect on the physicochemical properties of protein (Inada *et al.*, 1986). Alkyl substitution of hydrogen will increase both the bulkiness and hydrophobicity of the amino groups and reduce its ability to form hydrogen bonds (Ampon *et al.*, 1993; Means and Feeney, 1971).

One of the most interesting and well-investigated class of enzymes in this particular field is lipase or triacylglycerol hydrolases (E.C. 3.1.1.3). Lipases also

catalyze various useful reactions, for instance hydrolysis, esterification, transesterification and polyesterification reactions, and act as chiral catalysts in the production of various fine-chemicals and intermediates (Miyawaki and Nakamura, 2002; Berglund, 2001; Jääskeläinen *et al.*, 1997). The diverse functions and the enzyme specificity, both stereospecificity and regiospecificity, make lipase one of the most important biocatalyst in biotechnological applications (Shaw, 2002). The stringent requirements associated with the use of enzyme in industry reflect the need of lipases which have the desired characteristics such as thermostability and solvent stability. Thermostable lipases are expected to play a significant role in industrial processing because running bioprocess at elevated temperature lead to higher diffusion rate, increased solubility of lipids and hydrophobic substrates in water and reduced risk of contamination (Becker *et al.*, 1997).

The lipase used in the present study, from *Geobacillus* sp. strain T1 was isolated together with 28 other putative lipase producers from palm oil mill effluent in Malaysia by Leow and co-workers in 2004 (Leow *et al.*, 2004). T1 was selected for further studies as T1 has the highest lipase production rate, 0.15 Uml^{-1}. Later, it was identified as *Geobacillus* sp. strain T1, which has a maximum activity at 70 °C and pH optimum of 9.0. It was cloned, over-expressed and purified to homogeneity before the protein was used for chemical modification purposes (Rahman *et al.*, 2005). The fact this is a thermostable enzyme added new perspective to this study as this is the first studies carried out on a thermostable enzyme. The crystal structure of this 43 kDa protein has been resolved at 1.8 Å resolutions (Matsumura *et al.*, 2007).

Since the late 1960s, the molecular mechanisms of thermostability of enzymes from thermophilic microorganisms have attracted much interest from scientists from the fundamental point of view and from engineers engaging in biotechnology from the applied point of view (Mozhaev and Martinek, 1984; Mozhaev *et al.*, 1988a). With the rapid development of chemical reagents and methods, chemical modification coupled with spectroscopy has become a handy tool with the growing needs for identifying and modifying the functional amino acid residues in proteins.

Over the past 20 years, there have been major developments in the application of spectroscopic methods to systems of biological interest. Physical scientists, biological scientists and engineers have an impressive array of powerful and elegant tools for gathering qualitative and quantitative information about composition and structure of matter. With the increase in interdisciplinary research in recent years, the need for accurate and sensitive methods for the analysis of biomolecules has been increasingly important for both the chemist and biologist. The three-dimensional structure of a protein is governed by its primary structure (sequence of amino acids) and its environment. Changes in either of these can have important effects on its properties. Modifying any of its amino acid residues necessarily changes the primary structure of a protein. Thus, sophisticated analytical methods such as circular dichroism spectropolarimetry (CD) (Choi *et al.*, 2005; Kelly *et al.*, 2005; Hosseinkhani *et al.*, 2004; Greenfield, 1999), matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) (Wa *et al.*, 2006; Henzel *et al.*, 2003) and

fluorescence spectroscopy (Mossavarali *et al.*, 2006; Hosseinkhani *et al.*, 2004) have become standard tools to probe these changes.

Thus, the principal objective of this thesis is to probe the effect of modification on lipase via reductive alkylation by means of spectroscopy methods. Hence, the research was undertaken with the following specific objectives:

- To modify T1 lipase using propionaldehyde through reductive alkylation with different degree of modification.
- To investigate the effect of modification on T1 lipase's structure by using CD, MALDI-TOF MS and fluorescence spectroscopy.
- To evaluate the extent of modification on T1 lipase's structure using both spectroscopy data and structural analysis.

CHAPTER 2

LITERATURE REVIEW

Lipases

Versatile Biocatalysts

The demand for industrial enzymes is ever increasing owing to their applications in a wide variety of processes. Enzyme-mediated reactions are attractive alternatives to tedious and expensive chemical methods. Enzymes find great use in a large number of fields such as food, dairy, pharmaceutical, detergent, textile, fine chemicals and cosmetic industries (van Beilen and Zhi Li, 2002). With the realization of the biocatalytic potential of lipases in both aqueous and nonaqueous media in the last one and a half decades, industry has shifted towards utilizing this enzyme for a variety of reactions of immense importance. Enzymes are used quite extensively as industrial catalysts as they offer the following advantages in comparison with chemical catalysts (Humphrey and Lee, 1983):

1. They are specific, thus minimizing the undesirable side reactions and by-products.
2. They are relatively cheap when used in crude form.
3. They are effective catalysts for chemical conversion, offering mild conditions for reaction; low temperatures and pressures.
4. They are relatively non-toxic and thus, acceptable for applications in food processes and medicinal purposes.
5. They are effective within a wide range of substrate concentrations.