UNIVERSITI PUTRA MALAYSIA

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By

NOR HAFIZAH AHMAD KAMARUDIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

January 2014

DEDICATION

To my beloved family and husband

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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Chair: Professor Raja Noor Zaliha Raja Abd Rahman, D. (Eng)

Faculty: Biotechnology and Biomolecular Sciences

Lipase which constitutes one of the most important industrial enzymes is undeniably a versatile biocatalyst, capable of catalyzing both hydrolysis and synthesis reactions in aqueous and non-aqueous media, respectively. The search of novel lipases with unique properties is a continuous effort for decades. Locally isolated *Staphylococcus epidermidis* AT2 was previously identified as an organic solvent tolerant lipase producer. The mature active form of lipase gene was cloned and expressed in *E. coli*. Two modes of expression were investigated, intra- and extracellular expression of AT2 lipase. Intracellular expression showed an optimal specific activity of 22.16 U/mg.

To improve the expression level, Bacteriocin Release Protein (BRP) system was employed using two types of BRP vectors (pJL3 and pSW1) which were distinguished by their promoter systems. The aim was to probe the effect of dependent and independent inducible promoter (between BRP vector and recombinant AT2 lipase) in mediating the extracellular expression of AT2 lipase. For both cases, an increment by 1.5-fold and 3.5-fold in the enzymatic activity compared to that of intracellular expression was detected in *E. coli* harbouring pJL3 and pSW1, respectively. This suggested that independent inducible promoter system permitted better regulation of BRP and AT2 lipase expression which consequently promoted higher secretion of soluble proteins into the culture medium.

The enzyme was purified by two-step chromatographic method; hydrophobic interaction chromatography (HIC) and ion exchange chromatography (IEX) with 47.1% recovery and 3.5-fold purification factor. The estimated molecular weight as observed on SDS-PAGE was 43 kDa. The optimal temperature and pH were 25 °C and pH 8, respectively. The lipolytic activity was significantly enhanced by Ca^{2+} ions,

Tween 60 and Tween 80. On the downside, EDTA, pepstatin A, Zn^{2+} and Fe^{3+} ions, showed strong inhibition on the enzymatic activity. AT2 lipase exhibited high preference towards long chain triglycerides, natural oils and was found stable in various organic solvents namely; 25% (v/v) of DMSO, methanol, ethanol, acetone, diethyl ether, toluene, and n-hexane.

Following biochemical properties determination, crystallization of AT2 lipase was attempted. Crystallization screenings were conducted over purified protein at concentrations 1-5 mg/mL which resulted in several crystal hits but of low diffraction quality. To promote AT2 lipase crystallizability, large scale expression were performed and yielded a marked improvement in total purified protein thus rendering screening trials to be carried out at protein concentration of 5-10 mg/mL. Construct optimization using three fusion tags (His, GST, MBP) were prepared in which $GSTHis_6$-tagged AT2 lipase construct was selected for purification and high-throughput crystal screenings. Yet, none of the trials yielded positive outcomes.

Alternatively, AT2 lipase was subjected to structural modification to endow higher propensity of producing crystal. A homology model was built using YASARA and *S. hyicus* lipase as template. Aided with the knowledge on enzyme structure and crystallization prediction tools, flexible C-terminal region and surface exposed lysine were targeted for mutation. Subsequently, three mutants of AT2 lipase were constructed; M386, K214A and K325A.

M386, a truncated C-terminal mutant had yielded two crystal leads. The needle-like crystals which were obtained from Formulation 46 of PEG/Ion Screen and Formulation 87 of Index Screen appeared after approximately 40 days of incubation at 10 °C albeit not suitable for structure determination. Synergistically, swapping of the tail-like region resulted in a slight shift of the thermostability profile. The lipolytic activity of M386 retained by 43% compared to its wild-type with 18% remaining activity at 45 °C. *In silico* analysis conducted at 25 °C and 45 °C was found to be in accordance to the experimental findings in which RMSD values of M386 was more stable and less fluctuated throughout the total trajectory in comparison to its wild-type. Terminal moieties were also observed to exhibit large movement and flexibility as denoted by high RMSF values at both dynamics. Variation in organic solvent stability property was also detected where lipolytic activity of M386 was stimulated in the presence of 25% (v/v) of DMSO, isopropanol and diethyl ether.

Surface lysine mutants, K214A and K325A, on the other hand, could not be crystallized. Single mutation by substitution of high entropy residue, lysine to alanine on high entropy patches did not likely to engage any intermolecular crystal contacts required for crystal packing. However, changes in organic solvent stability profile were observed. Both K214A and K325A displayed activation of lipolytic activity in polar organic solvents. Enhancement of lipolytic activity in K214A was detected in ethanol, acetone and isopropanol while K325A was stimulated by ethanol, but inhibited by acetone.

Overall, AT2 lipase displayed attractive biochemical properties exclusively as a cold-adapted, organic solvent stable enzyme isolated from a mesophilic *Staphylococcus epidermidis* AT2. The functional properties of the enzyme in correlation to its structural architecture were investigated through homology modeling and structural modification. This enzyme was also identified to be recalcitrant to crystallization in its native form due to its inherent flexibility and molecular surface property. Nonetheless, AT2 lipase shows a huge potential to be employed in various industrial applications.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENCIRIAN DAN MODIFIKASI STRUKTUR LIPASE TAHAN SEJUK DAN STABIL TERHADAP PELARUT ORGANIK DARIPADA *Staphylococcus epidermidis* AT2 YANG DIHASILKAN DALAM *Escherichia coli*

Oleh

NOR HAFIZAH AHMAD KAMARUDIN

Januari 2014

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Lipase yang merupakan salah satu daripada enzim industri terpenting, adalah biokatalis yang serba boleh, berkemampuan untuk memangkin kedua-dua tindak balas hidrolisis dan sintesis dalam keadaan akues dan bukan akues. Pencarian lipase baru yang mempunyai ciri-ciri unik adalah usaha yang berterusan sejak beberapa dekad lalu. Terdahulu, mikroorganism yang telah dipencilkan dari kawasan tempatan, *Staphylococcus epidermidis* telah dikenal pasti sebagai penghasil lipase yang toleran terhadap pelarut organik. Gen yang mengekod bahagian aktif lipase tersebut telah diklon and diekspres di dalam sistem *E. coli*. Dua mod pengekspresan telah dikaji iaitu secara intrasel dan ekstrasel. Pengekspresan secara intrasel telah menunjukkan aktiviti spesifik optimum sebanyak 22.16 U/mg.

Untuk meningkatkan tahap pengekspresan, sistem 'Bacteriocin Release Protein' (BRP) telah diaplikasi dengan menggunakan dua jenis vektor BRP iaitu pJL3 dan pSW1, di mana keduanya adalah berbeza dari segi sistem 'promoter' yang dikodkan. Tujuannya adalah untuk mengkaji kebergantungan antara dua 'promoter' yang boleh diinduksi (di antara vektor BRP dan rekombinan AT2 lipase) dalam mengantara pengekspresan secara ekstrasel. Bagi kedua-dua kes, peningkatan aktiviti sebanyak 1.5 dan 3.5 kali ganda berbanding pengekspresan intrasel telah dikesan, masing-masing bagi *E. coli* yang mengandungi pJL3 dan pSW1. Ini mencadangkan, bagi sistem induksi promoter yang tidak bergantung, kawalan pengekspresan vektor BRP dan rekombinan AT2 adalah lebih baik, seterusnya menggalakkan pengeluaran protein larut ke dalam medium kultur.

Enzim ini telah ditulenkan melalui dua teknik kromatografi iaitu interaksi hidrofobik dan pertukaran ion kromatrografi dengan perolehan protein sebanyak 47.1% dan 3.5 gandaan penulenan. Anggaran jisim molekular seperti yang diperoleh berdasarkan SDS-PAGE adalah 43 kDa. Suhu dan pH optimum adalah 25 °C dan pH 8, masing-masing. Aktiviti lipolitik telah dimangkin secara signifikan oleh ion Ca^{2+}, Tween 60

dan Tween 80. Sebaliknya, EDTA, pepstatin A, ion Zn^{2+} dan Fe^{3+} menunjukkan kesan perencatan terhadap aktiviti enzim. AT2 lipase mempunyai kecenderungan yang tinggi terhadap trigliserid berantai panjang, minyak semulajadi dan stabil terhadap pelbagai pelarut organik iaitu 25% (v/v) DMSO, metanol, etanol, aseton, dietil eter, toluena dan n-heksana.

Selepas mengenalpasti ciri-ciri biokimia, pengkristalan AT2 lipase telah dijalankan. Pemeriksaan terhadap pengkristalan dilakukan menggunakan protein pada kepekatan 1-5 mg/mL dan beberapa petunjuk kristal telah diperoleh, walaubagaimanapun pada tahap pembelauan yang rendah. Untuk meningkatkan kadar pengkristalan AT2 lipase, pengekspresan berkala besar telah dijalankan di mana hasilnya, peningkatan protein tulen secara total telah diperoleh dan cubaan pengkristalan dapat dilakukan pada kepekatan protein 5-10 mg/mL. Pengubahsuaian konstruk menggunakan tiga jenis tag gabungan telah dijalankan di mana gabungan $GSTHis_6$-AT2 lipase telah dipilih untuk penulenan dan percubaan pengkristalan. Walaubagaimanapun, tiada hasil positif didapati daripada percubaan ini.

Sebagai alternatif, modifikasi terhadap struktur AT2 lipase telah dikaji bagi menambah kebarangkaliannya untuk membentuk kristal. Model homologi telah dibina menggunakan YASARA dan *S. hyicus* lipase sebagai 'template'. Melalui informasi yang diperoleh berkaitan struktur enzim, bahagian terminal-C yang fleksibel dan lysin yang terdedah kepada persekitaran telah di target untuk modifikasi. Selanjutnya, tiga mutan AT2 lipase telah dihasilkan, M386, K214A dan K325A.

M386, mutan yang mempunyai bahagian terminal-C yang dipendekkan, telah menghasilkan dua petunjuk kristal. Dua petunjuk kristal berbentuk jarum (diperoleh daripada Formulasi 46, PEG/Ion Screen dan Formulasi 87, Index Screen) terhasil selepas hampir 40 hari pada suhu 10 °C, namun tidak sesuai untuk penentuan struktur. Pemendekkan bahagian yang membentuk ekor ini juga telah menyebabkan sedikit perubahan pada profil termostabiliti. Sebanyak 43% daripada lipolitik aktiviti M386 dikesan pada suhu 45 °C, berbanding 18% aktiviti diperoleh daripada konstruk tanpa modifikasi. Analisis 'in silico' yang dijalankan pada suhu 25 °C dan 45 °C juga menepati hasil eksperimen di mana nilai RMSD untuk M386 adalah lebih stabil sepanjang trajektori jika dibandingkan dengan konstruk tanpa modifikasi. Bahagian terminal juga diperhatikan mempunyai pergerakan yang besar dan fleksibel seperti berdasarkan nilai RMSF yang tinggi untuk kedua-dua dinamik. Perubahan dalam kestabilan terhadap pelarut organik juga dikesan di mana lipolitik aktiviti M386 dirangsang dengan kehadiran 25% (v/v) DMSO, isopropanon dan dietil eter.

Mutan yang telah dimodifikasi pada lysin yang terletak di permukaan protein, K214A dan K325A, sebaliknya tidak dapat dikristalkan. Modifikasi tidak dapat menghasilkan sebarang kontak intermolekul yang diperlukan untuk pembentukan kristal. Walaubagaimanapun, perubahan pada kestabilan terhadap pelarut organik dikesan. Lipolitik aktiviti kedua-dua K214A dan K325A diaktifkan oleh pelarut organik larut

air. Peningkatan aktiviti K214A didapati pada etanol, aseton dan isopropanon, sementara aktiviti K325A dirangsang oleh etanol, dan direncat oleh aseton.

Pada keseluruhannya, AT2 lipase mempamerkan ciri-ciri biokimia yang menarik terutama sebagai enzim tahan sejuk dan stabil terhadap pelarut organik yang telah dipencilkan dari bakteria mesofilik, *Staphylococcus epidermidis* AT2. Ciri-ciri fungsional enzim ini dengan kaitannya terhadap struktur enzim telah dikaji melalui model homologi dan modifikasi struktur. Enzim ini telah dikenalpasti tidak dapat membentuk kristal dalam keadaan struktur asalnya disebabkan oleh ciri fleksibiliti semulajadi dan permukaan molekulnya. Namun, AT2 lipase menunjukkan potensi yang tinggi untuk diaplikasi dalam pelbagai bidang industri.

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And for those who involved directly or indirectly in this project, I sincerely thank you.

I certify that a Thesis Examination Committee has met on 29 January 2014 to conduct the final examination of Nor Hafizah binti Ahmad Kamarudin on her thesis entitled "Characterization and Structural Modification of Cold-Adapted Organic Solvent Stable Lipase from *Staphylococcus epidermidis* AT2 Produced in *Escherichia coli*" 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 Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

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Date: 10 March 2014

The thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Date:

DECLARATION

Declaration by graduate student

I hereby confirm that:

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Declaration by Members of Supervisory Committee

This is to confirm that:

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Prof. Dr. Raja Noor Zaliha
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