UNIVERSITI PUTRA MALAYSIA

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By

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

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May 2013

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

Faculty: Biotechnology and Biomolecular Sciences

T1 lipase from *Geobacillus zalihae* is a thermophilic enzyme with an optimum temperature of 70 ^{o}C at pH 9 and stable up to 65 ^{o}C. By introducing an additional ion pair, T1 lipase was believed to be stable at high temperature. Thus, further study of D311E lipase variant are required to determine the effect of ion pairs on T1 stability. The aims of this work are to analyse the kinetic properties and characterise the D311E lipase variant, to optimise crystallisation conditions and to elucidate the 3-D structure of crystallised D311E lipase. This involved a few purification steps, characterisation, kinetic analysis, crystallisation and structural elucidation processes.

Wild-type T1 and variant D311E lipases were successfully purified in a series of purification steps. The recovery of wild-type T1 was 22.37 % with the purification fold of 5.16. Meanwhile, the final yield and fold of D311E lipase was 15.71 % and 10.56, respectively. The purification fold of D311E lipase variant was higher than

wild-type T1 lipase due to higher specific activity of purified D311E lipase. Purified lipases were used for characterisation and crystallisation.

The wild-type T1 and D311E lipase variant had an optimum temperature of 70 ^{o}C. The D311E lipase variant was stable and remaining activity at 70 % with pre-incubation at 60 ^{o}C for 75 min while the wild-type T1 lipase was stable only for 15 min. When the lipases were pre-incubated at 70 ^{o}C, the D311E lipase variant was stable and remaining activity at 70 % for 10 h while the wild-type T1 lipase was stable for 6 h. Kinetic studies of T1 and D311E lipase using *p*-nitrophenyl laurate as substrate was conducted by using Hanes-Woolf plot. For T1, the K_m, V_{max} and V_{max}/K_m were 39.03 mM, 5.75 mM s^{-1} and 0.147 mM s^{-1} mM^{-1}, respectively while for D311E lipase, the K_m, V_{max} and V_{max}/K_m were 14.08 mM, 0.23 mM s^{-1} and 0.016 mM s^{-1} mM^{-1}, respectively which shows that the variant has high substrate affinity compared to its wild-type. The circular dichroism spectra of T1 and D311E lipase were analysed as a function of temperature at 220 nm. T_m for T1 and D311E lipase were approximately 68.52 °C and 70.59 °C, respectively. It showed that mutation at D311 increases the stability of T1 lipase and exhibited higher T_m. The D311E also exhibited higher activity and stability compared to T1 lipase. Thus, crystallisation and X-ray diffraction were carried out to ensure the consistency of the results at atomic level.

Crystallisation of purified D311E lipase was carried out using sitting drop vapour diffusion and capillary gel tube counter diffusion method. Optimisation of D311E lipase crystallisation was performed in order to see the crystal growth effect by using

different buffer pH, protein concentration, salt concentration, and temperature using sitting drop vapour diffusion method. For sitting drop vapour diffusion method, the optimum crystallisation formulation was 1.5 M of NaCl, 0.1 M, MES pH 5.5, 1.35 mg/mL of D311E lipase and 20 ^{o}C for growth. The size of the crystal obtained was 0.2 mm x 0.1 mm x 0.1 mm. While in the capillary gel tube counter diffusion, optimum conditions to grow D311E lipase crystal were using 3.0 mg/mL protein with formulation C2-21 (Crystal Screen, Hampton Research) at 1 mm of gel tube length. However, the size of crystal grown was approximately 50 µm x 50 µm x 30 µm which is smaller than sitting drop vapour diffusion crystallisation method and therefore, insufficient for in-house X-ray diffraction. Thus, the crystal with optimum condition obtained in sitting drop vapour diffusion method was used for X-ray diffraction.

A good quality crystal of D311E lipase was diffracted at 2.1 Å using an in-house X-ray beam and this crystal belonged to the monoclinic space group *C2* with the unit cell parameters a = 117.32 Å, b = 81.16 Å and c = 100.14 Å. The generated model was further built and refined. The phase problem was solved by using molecular replacement method. T1 lipase crystal structure (PDB ID: 2DSN) was used as the template. The quality of lipase structure was validated using Ramachandran plot and Errat. Hence, the coordinates of D311E crystal structure was deposited to RCSB Protein Data Bank under PDB ID code 3UMJ.

Structural analysis showed the existence of an additional ion pair around E311 in D311E structure which led to the formation of an ion pair network comprising of five

amino acid residues (Arg274, Thr278, Gly279, Arg303 and Glu311) connected by seven ion pairs. In conclusion, the presence of the additional ion pair stabilised the formation of inter-connection in D311E and regulates stability of lipase at high temperatures.

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

KINETIK, PENGKRISTALAN, DAN ELUSIDASI STRUKTUR BAGI THERMOSTABIL VARIAN LIPASE D311E T1 DARIPADA *Geobacillus zalihae*

Oleh

RUDZANNA RUSLAN

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Pengerusi: Professor Raja Noor Zaliha Raja Abd. Rahman, D.Eng.

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T1 lipase dari *Geobacillus zalihae* adalah enzim termofilik dengan suhu optimum 70 ^{o}C pada pH 9 dan stabil sehingga ke 65 ^{o}C. Dengan memperkenalkan pasangan ion tambahan, T1 lipase telah dipercayai menjadi stabil pada suhu tinggi. Oleh itu, D311E lipase telah dibina untuk menentukan kesan pasangan ion terhadap kestabilan T1. Objektif kerja ini adalah untuk menganalisis sifat-sifat kinetik dan mencirikan lipase varian D311E, untuk mengoptimumkan keadaan pengkristalan dan untuk mengilusidasi struktur 3-D bagi D311E lipase. Ini melibatkan beberapa langkah penulenan, pencirian, analisis kinetik, pengkristalan dan proses elusidasi struktur.

T1 jenis liar dan varian lipase D311E telah berjaya ditulenkan dalam satu siri langkah penulenan. Hasil akhir lipase jenis liar T1 adalah 22.37 % dengan lipatan penulenan sebanyak 5.16. Sementara itu, hasil akhir dan lipatan penulenan untuk

varian lipase D311E masing-masing adalah 15.71 % dan 10.56. Lipatan penulenan bagi varian D311E lipase adalah lebih tinggi daripada T1 lipase jenis liar disebabkan aktiviti khusus yang lebih tinggi oleh D311E lipase. Lipase yang telah ditulenkan digunakan untuk pencirian dan pengkristalan.

T1 lipase jenis liar dan D311E varian mempunyai suhu yang optimum pada suhu 70 ^{o}C. D311E varian stabil dan mengekalkan aktiviti sebanyak 70 % dengan pra-pengeraman pada suhu 60 ^{o}C selama 75 min manakala T1 lipase liar jenis stabil hanya selama 15 minit. Apabila lipases telah pra-dieram pada suhu 70 ^{o}C, D311E lipase varian adalah stabil dan mengekalkan aktiviti sebanyak 70 % selama 10 jam manakala T1 lipase jenis liar stabil selama 6 jam. Kajian kinetik untuk lipase T1 dan D311E dilakukan melalui plot Hanes-Woolf. K_m, V_{max} dan V_{max}/K_m untuk T1 masing-masing adalah 39.03 mM, 5.75 mM s^{-1} dan 0.147 mM s^{-1} mM^{-1} manakala bagi lipase D311E, *Km*, V_{max} dan V_{max}/K_m masing-masing adalah 14.08 mM, 0.23 mM s^{-1} dan 0.016 mM s^{-1} mM^{-1}. Ini menunjukkan bahawa varian D311E mempunyai afiniti substrat yang tinggi berbanding dengan lipase jenis liar. Spektrum dikroism bulat bagi lipase T1 dan D311E dianalisis sebagai fungsi suhu pada 220 nm. T_m untuk lipase T1 adalah 68.52 °C dan D311E adalah 70.59 °C. Ini menunjukkan bahawa mutasi pada D311 telah meningkatkan kestabilan dan nilai T_m lipase T1. D311E juga menunjukkan aktiviti dan kestabilan yang lebih tinggi berbanding dengan lipase T1. Oleh itu, penghabluran dan pembelauan oleh sinar-X telah dijalankan bagi memastikan ketekalan keputusan di peringkat atom.

Lipase D311E tulen berjaya dikristalkan melalui kaedah difusi wap cara titis duduk dan difusi kaunter cara kapilari tiub gel. Pengoptimuman pengkristalan lipase D311E dilakukan untuk melihat kesan pertumbuhan kristal dengan menggunakan penimbal pH yang berbeza, kepekatan protein, kepekatan garam, dan suhu menggunakan kaedah titis gantung duduk. Bagi kaedah difusi wap cara titis duduk, formulasi pengkristalan yang optimum adalah 1.5 M NaCl, 0.1 M, MES pH 5.5, 1.35 mg/mL lipase D311E dan suhu 20 ^{o}C untuk pertumbuahan. Saiz kristal yang diperolehi adalah 0.2 mm x 0.1 mm x 0.1 mm. Manakala, bagi kaedah penyebaran kaunter cara kapilari tiub gel, keadaan optimum untuk kristal lipase D311E berkembang adalah menggunakan 3.0 mg/mL protein dengan formulasi C2-21 (Crystal Screen Hampton Research,) pada seluruh kepanjangan tiub (1, 5, 8 dan 10 mm). Walau bagaimanapun, saiz kristal yang tumbuh menggunakan kaedah ini adalah lebih kecil iaitu pada 50 µm x 50 µm x 30 µm berbanding dengan kristal kaedah difusi wap cara titis duduk. Saiz ini tidak mencukupi untuk pembelauan sinar-X. Oleh itu, kristal daripada kaedah difusi wap cara titis duduk dalam keadaan yang optimum telah digunakan untuk tujuan pembelauan sinar-X.

Kristal lipase D311E yang berkualiti tinggi telah dibelaukan pada resolusi 2.1 Å menggunakan X-ray dan kristal ini mempunyai ruang monoklinik kumpulan C2 dengan parameter sel unit a = 117.32 Å, b = 81.16 Å dan c = 100.14 Å. Model yang dihasilkan terus dibina dan diperhalusi. Masalah fasa telah diselesaikan dengan menggunakan kaedah penggantian molekul. Struktur kristal lipase T1 (PDB ID: 2DSN) telah digunakan sebagai templat. Struktur lipase telah disahkan dengan menggunakan plot Ramachandran dan Errat. Kemudian, koordinat struktur kristal

bagi D311E telah didepositkan ke RCSB Protein Data Bank di bawah kod PDB ID 3UMJ.

Analisis struktur menunjukkan kewujudan satu pasangan ion tambahan di sekitar E311 pada struktur D311E. Pembentukan rangkaian sepasang ion terdiri daripada lima residue asid amino (Arg274, Thr278, Gly279, Arg303 dan Glu311) yang disambungkan oleh tujuh pasangan ion. Sebagai ringkasan, penambahan satu pasangan ion telah menstabilkan pembentukan sambungan antara dalam D311E serta mengawal kestabilan lipase pada suhu yang tinggi.

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I certify that an Examination Committee has met on date of viva to conduct the final examination of Rudzanna Ruslan on her degree of Masters of Science thesis entitled "Kinetics, Crystallization, and Structural Elucidation of Thermostable D311E T1 Lipase Variant from *Geobacillus zalihae*" in accordance with Universities and University Collages Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the candidate be awarded the Master of Science.

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This thesis 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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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 institution.

RUDZANNA RUSLAN

Date: 10 May 2013

TABLE OF CONTENTS