UNIVERSITI PUTRA MALAYSIA

ENHANCEMENT OF ENZYMATIC PROPERTIES OF T1 LIPASE BY SATURATION MUTAGENESIS AT GLUTAMINE 114

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By

ROSWANIRA BINTI AB. WAHAB

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

July 2012

To my loving husband, Fahrul Zaman bin Huyop and my four girls, Farhanah, Farhah, Fathiah and Fatini and also my parents, Roslina binti Nik Musa and Abdul Wahab bin Saad who have always been there for me.

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

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July 2012

Chairman: Professor Mahiran binti Basri, PhD

Faculty: Science

Rational design of the recombinant T1 lipase gene by saturation mutagenesis on the oxyanion Gln114 was successfully carried out to afford a library of twenty lipase variants. The objective of the study was to investigate the impact of a single point saturation mutagenesis at the oxyanion Gln114 on the enzymatic behavior and enantioselectivity of T1 lipase. Furthermore, the effect of such mutation in the active site of T1 lipase has never been studied. The selection of the mutation site was based on the close proximity of Gln114 residue to the catalytic machinery and is intimately involved in the substrate-enzyme interaction. It was hypothesized that the mutation could invoke substantial changes in the catalytic efficiency and enantioselectivity of T1 lipase.

Computational assessment using YASARA, FoldX and Voronoia 1.0, found significant variations in potential energy, total cavity and protein compactness. It was anticipated

that Q114L and Q114M are more stable than T1 lipase. Profiling of the seven enzyme behaviors found Q114L to show a 10^{o}C increase in optimum temperature as compared to 70^{o}C in T1 lipase. Amino acids of small side-chains, polar, acidic and basic, generally, caused reduction in the optimum temperatures. A downward shift in pH optimum from pH 9 to pH 8 was observed when non-polar and basic amino acids were replaced into Gln114. pH optimum of lipases were unchanged upon substitution with polar amino acids. Specificities towards hydrolysis of natural oils were also very different from one lipase to another, with an overall reduction in lipase activity.

Various chemical reagents affected the lipase variants, namely, organic solvents, surfactants, metal ions and inhibitors/chelating agents. Lipases substituted with non-polar amino acids exhibited higher frequency of improved enzyme properties. Their non-polar nature that does not interact strongly with charged molecules in the active site could be the contributing factor. Less enzyme deactivation was observed in hydrophobic solvents as compared to hydrophilic ones. This is due to the nature of hydrophilic solvents that tend to partition preferentially into the cytoplasmic membrane, while hydrophobic solvents tend to partition away. The enzymes showed preference towards surfactant Span 20, KI and SC, as well as catalysis activation by Sr(II). Substitution of Leu and Met into Gln114 resulted in variants Q114L and Q114M that showed the most number of cumulative enhancements and were more competent than T1 lipase.

In the esterification of menthol with butyric anhydride, T1 lipase afforded a higher yield of 99.3% menthyl butyrate as compared to 75.1% in Q114L. The former gave

optimum conditions at 60^{o}C, 2.53 mg enzyme, molar ratio of butyric anhydride:menthol at 1:1 to 1.5:1 and incubated for 13 h and 15 min, while the latter were at 67.5^{o}C, 3 mg enzyme, substrate molar ratio at 3.5:1 and incubated for 13 h and 21 min. The higher yield could be attributed to the hydrophilic nature of the substrates that could interact better with the hydrophilic Gln114 in T1 lipase. Also, the higher temperature in Q114L-catalyzed reactions could have increased evaporation of menthol, hence, lowering its concentration in the reaction. In the enantioselectivity study for the enzymatic resolution of racemic ibuprofen using oleyl alcohol, variants Q114L and Q114M were selected for this purpose. Mutation at oxyanion Gln114 had changed enantioselectivity, whereby Q114M was the most enantioselective followed by T1 lipase and Q114L.

Hence, it can be concluded that saturation mutagenesis of oxyanion Gln114 had significantly changed the enzymatic behavior and enantioselectivity of T1 lipase. The observed cumulative improvement in lipase properties mostly came from substitutions with mainly medium-sized non-polar hydrophobic amino acids. The most catalytically competent variants were found to be Q114L and Q114M, respectively. On the other hand, substitution with hydrophilic, basic, acidic and bulky side-chain amino acids affected T1 lipase negatively. Hence, it is recommended that future mutational studies to avoid using these amino acids as it would only lead to inferior enzyme qualities.

Abstrak thesis dikemukakan ini kepada Senat Universiti Putra Malaysia bagi memenuhi syarat untuk mendapatkan ijazah Doktor Falsafah

PENAMBAHBAIKAN CIRI-CIRI ENZIM T1 LIPASE MELALUI MUTAGENESIS TEPU KE ATAS GLUTAMINA 114

Oleh

ROSWANIRA BINTI AB WAHAB

Julai 2012

Pengerusi: Profesor Mahiran binti Basri, PhD

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Rekabentuk rasional ke atas gen rekombinan lipase T1 melalui mutagenesis tepu ke atas oksianion Gln114 telah dilaksanakan untuk menghasilkan satu koleksi sebanyak dua puluh varian lipase. Objektif kajian ini adalah untuk mengkaji kesan mutagenesis tepu satu titik pada oksianion Gln114 terhadap sifat enzim dan enantioselektiviti lipase T1. Tambahan pula, kajian ke atas kesan mutasi di tapak aktif lipase T1 masih belum pernah dilakukan. Pemilihan lokasi mutasi adalah berdasarkan Gln114 yang terletak berhampiran dengan tapak aktif dan terlibat dengan interaksi substrat-enzim. Hipotesisnya adalah mutasi ini akan mencetuskan perubahan yang besar pada kecekapan pemangkinan dan enantioselektiviti lipase T1.

Penilaian komputer menggunakan YASARA, FoldX dan Voronoia 1.0, menunjukkan perubahan yang ketara dalam tenaga keupayaan, jumlah kaviti dan ketumpatan protein. Dijangkakan Q114L dan Q114M akan lebih stabil berbanding lipase T1. Pemprofilan

ke atas tujuh sifat enzim mendapati Q114L menunjukkan peningkatan sebanyak 10^{o}C suhu optimumnya berbanding 70^{o}C pada lipase T1. Asid amino berantai sisi kecil, berkutub, berasid dan berbes secara amnya, menurunkan suhu optimum. Peralihan menurun pH optimum dari pH 9 hingga pH 8 berlaku apabila Gln114 digantikan dengan asid amino tidak berkutub dan berbes. pH optimum lipase tidak berubah apabila digantikan dengan asid amino berkutub. Kespesifikan hidrolisis terhadap minyak semulajadi juga berubah dengan menunjukkan pengurangan secara keseluruhan ke atas aktiviti lipase.

Pelbagai reagen kimia didapati memberi kesan ke atas varian lipase, iaitu, pelarut organik, surfaktan, ion logam dan agen perencat/pengkelat. Lipase yang digantikan dengan asid amino tidak berkutub menunjukkan kekerapan penambahbaikan sifat enzim yang lebih `tinggi. Sifat asid amino tidak berkutub yang tidak berinteraksi kuat dengan molekul di tapak aktif mungkin adalah faktor penyebab. Penyahaktifkan enzim yang kurang di dalam pelarut hidrofobik berbanding hidrofilik mungkin disebabkan oleh sifat pelarut hidrofilik yang cenderung untuk masuk ke dalam membran sitoplasma, manakala pelarut hidrofobik pula berkecenderungan untuk berada di luar. Sesetengah enzim menunjukkan kespesifikan terhadap surfaktan Span 20, KI dan SC serta terhadap pengaktifan pemangkinan oleh Sr(II). Penggantian Leu dan Met ke atas Gln114 menyebabkan varian Q114L dan Q114M menunjukkan penambahbaikan terkumpul yang lebih tinggi dan pemangkinan yang lebih cekap daripada lipase T1.

Pengesteran mentol dengan butirik anhidrida yang dimangkinkan lipase T1, keadaan optimum adalah pada 60^{o}C dengan 2.53 mg enzim, nisbah molar butirik anhidrida:mentol adalah 1:1 kepada 1.5:1 dan 13 jam dan 15 minit pengeraman untuk menghasilkan 99.3% mentil butirat. Bagi Q114L, keadaan optimum adalah 67.5^{o}C, 3 mg enzim, nisbah molar butirik anhidrida:mentol sebanyak 3.5:1 dan dieram selama 13 jam dan 21 minit untuk hasil sebanyak 75.1%. Walaupun dalam keadaan optimum, lipase T1 menghasilkan lebih banyak mentil butirat berbanding Q114L. Ini mungkin disebabkan oleh sifat hidrofilik substrat yang berinteraksi lebih baik dengan Gln114 hidrofilik pada lipase T1. Suhu tindakbalas Q114L yang lebih tinggi juga mungkin telah meninggikan kadar sejatan mentol dan menurunkan kepekatannya di dalam tindak balas. Dalam kajian enantioselektiviti resolusi enzim ke atas ibuprofen rasemik menggunakan alkohol olel, varian Q114L dan Q114M telah dipilih untuk tujuan ini. Mutasi pada oksianion Gln114 telah mengubah enantioselektiviti lipase T1 di mana Q114M merupakan yang paling enantioselektif diikuti oleh lipase T1 dan Q114L.

Kesimpulannya, mutagenesis tepu pada oksianion Gln114 telah mengubah sifat enzim dan enantioselektiviti T1 lipase. Diperhatikan bahawa penambahbaikan terkumpul sifat enzim secara amnya disebabkan oleh penukaran dengan amino asid hidrofobik tidak berkutub dan yang bersaiz sederhana. Varian yang paling kompeten adalah Q114L dan Q114M. Namun begitu, penukaran dengan amino asid hidrofilik, berbes, berasid and berantai tepi yang besar memberi kesan negatif kepada T1 lipase. Dengan itu, untuk kajian mutasi pada masa hadapan adalah disarankan penggunaan asid amino ini dielakkan kerana ianya akan menghasilkan enzim yang berkualiti rendah.

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I certify that a Thesis Examination Committee has met on **13th July 2012** to conduct the final examination of Roswanira binti Ab. Wahab on his (or her) thesis entitled "**Enhancement of Enzymatic Properties of T1 lipase by Saturation Mutagenesis at Glutamine 114**" 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.

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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.

_____

ROSWANIRA BINTI AB. WAHAB
Date: 13 July 2012

I certify that a Thesis Examination Committee has met on **13th July 2012** to conduct the final examination of Roswanira binti Ab. Wahab on his (or her) thesis entitled "**Enhancement of Enzymatic Properties of T1 lipase by Saturation Mutagenesis at Glutamine 114**" 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.

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