UNIVERSITI PUTRA MALAYSIA

A COLD ACTIVE BIFUNCTIONAL LIPASE WITH PROTEASE ACTIVITY FROM ANTARCTIC MICROORGANISM PI 12

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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 Doctor of Philosophy

A COLD ACTIVE BIFUNCTIONAL LIPASE WITH PROTEASE ACTIVITY FROM ANTARCTIC MICROORGANISM PI 12

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Chairman: Professor Raja Noor Zaliha Raja Abd Rahman, PhD

Faculty: Biotechnology and Biomolecular Sciences

A psychrophilic microorganism (cold loving) named PI 12 was isolated from the Antarctic sea ice near Casey station, Antarctica. This psychrophilic microorganism produced extracellular lipase and the activity was determined by using both qualitative and quantitative methods. Clearing zones was formed when it was grown at 4°C on top of tributyrin agar plates, indicating an extracellular cold active lipase with activity at 0.051U/ml. 16rRNA genes revealed an apparent homology of 99 % to *Janthibacterium* sp. However, further identification of isolate PI 12 confirmed that it was a yeast, *Leucosporodium antarcticum*. The lipase gene of isolate PI 12 was isolated via shotgun cloning. Gene analysis showed an open reading frame of 783 bp was found to encode a lipase. The lipase was assayed at 4°C with activity at 0.1 U/ml. The gene was

also successfully expressed extracellularly by co-transformation of pJL3 plasmid which encode Bacteriocin Release Protein (BRP)

The lipase gene did not show high similarity to other lipases as anticipated. Interestingly, the gene shared high homology to protease. Thus more investigations in trying to understand such novel finding were done. In order to visualize the enzyme, LipPI12 was modeled using the template of psychrophilic protease from *Pseudomonas* sp. TACII18. The putative 3D structure of the enzyme showed the typical properties of psychrophilic enzyme, which is increasing number of loops and a non compact structure to cater the lipase structural flexibility. Purification of recombinant intracellular and extracellular LipPI12 was done using Nickel Sepharose affinity chromatography. The purified intracellular LipPI12 was a monomer with the size of ~30kDa as judge native and SDS PAGE respectively.

LipPI12 holds huge prospect of greater finding therefore characterization of LipPI12 lipase and protease were done. Temperature profile of the bifunctional LipPI12 showed that the lipase functions optimally at 20°C and reached halflife after 30 min whereas the protease was more active at 40°C but reaches halflife even faster after 15 mins of incubation. pH profile showed that both LipPI12 lipase and protease were active at near neutral condition. Medium chain length fatty acid (C12) seemed to be the best substrate for LipPI12 lipase. The presence

of organic solvents did not affect both the lipase and protease activities. The lipase was more stable at solvents with higher log P value whereas the protease was slightly activated at low log P value particularly with dimethylsulfonyl. Activity of LipPI12 lipase and protease were also activated in the presence of $CaCl_2$ but its protease counterpart seemed to be more active in the presence of other metal ions such as $ZnCl_2$ and $MgCl_2$. Effect of surfactants showed LipPI12 lipase was activated by Tween 80 and SLS and in contrast, LipPI12 protease was almost deactivated in all surfactants tested. Inhibitor studies revealed that LipPI12 lipase was partially inhibited with EDTA and PMSF whereby the LipPI12 protease was inhibited by pepstatin and was also partially inhibited by EDTA and PMSF. Amino acid comparison showed patterns of cold adaptation with increasing number of glycine and lesser proline. Circular dichroism and fluorescence spectroscopy analysis, strengthens the findings which entails protein psychrophilicity.

The findings of unique LipPI12 has led to better understanding of the enzyme as shown from its bifunctional properties. The contrasting figure of LipPI12 lipase and protease reveals greater elucidation on protein structure and function. Thus it is concluded that LipPI12 lipase and protease is a remarkable enzyme which has highlighted way of surviving the cold and also promises potential application in the future.

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

ENZIM LIPASE TAHAN SEJUK DWIFUNGSI BERAKTIVITI SEBAGAI PROTEASE DARI MIKROORGANISMA ANTARTIKA PI 12

Oleh

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Pengerusi: Profesor Raja Noor Zaliha Raja Abd Rahman, PhD

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Mikroorganisma tahan sejuk yang dinamakan PI 12 telah diperolehi dan dipencilkan dari ais lautan Antartika berdekatan Stesen Casey Antartika. Mikroorganisma ini yang tahan pada suhu sejuk ini merembeskan enzim lipase ekstrasellular. Aktiviti ditentukan melalui kaedah kualitatif dan kuantitatif. Kawasan yang berbentuk halo terbentuk pada agar tributirin menandakan kehadiran enzim lipase yang tahan dan aktif pada suhu rendah dengan aktiviti 0.051 U/ml apabila di asai pada suhu 4°C. Gen yang mengkodkan 16S rRNA yang diamplifikasikan melalui PCR menunjukkan ia adalah dari genus *Janthinobacteria* dengan persamaan sebanyak 99% dengan data yang sedia ada. Walaubagaimanapun, mikroorganisms ini telah di kenalpasti secara mendalam dan ia menunjukkan sejenis yis, *Leucosporodium antarcticum*. Gen yang

mengkodkan enzim lipase telah diklon. Hasil penjujukan nukleotida, satu bingkai bacaan terbuka dijumpai dan mempunyai saiz sebesar 783 pasangan nukleotida yang mengkodkan enzim lipase. Sebanyak 0.1 U/ml aktiviti lipase dicatat apabila diasai pada suhu 4^{o}C. LipPI12 telah berjaya dirembes keluar hasil daripada transformasi bersama plasmid pJL3 yang mengekspres protein pengeluar Bacteriocin (BRP)

Gen lipase tidak menunjukkan persamaan identiti yang tinggi sebagai gen lipase sepertimana yang diharapkan. Namun, ia menjadi lebih menarik apabila gen lipase ini menunjukkan homologi yang tinggi kepada protease. Maka banyak kajian perlu dijalankan untuk memahami dengan lebih mendalam bagi penemuan baru ini. Untuk melihat enzim ini secara 3 dimensi, model LipPI12 telah dibina berdasarkan acuan protease tahan sejuk dari *Pseudomonas* sp TACII 18. Berdasarkan model 3D yang dibentuk, ia telah menunjukkan sebahagian adaptasi enzim pada sejuk iaitu pertambahan struktur fleksibel dan juga struktur yang tidak padat yang mana ia boleh mambantu menambah kelenturan dan untuk aktif pada suhu rendah. Penulenan enzim LipPI12 ini telah dijalankan menggunakan teknik kromatografi affiniti Nikel Sepharose dan berjaya ditulenkan ke tahap homogen. Anggaran saiz LipPI12 sebesar ~30kDa diperoleh hasil dari pemerhatian pada gel natif dan SDS.

Pencirian enzim LipPI12 yang mempunyai dwi fungsi biokatalitik ini telah dijalankan. Suhu optimum untuk LipPI12 lipase dicatatkan pada 20°C manakala suhu optimum untuk aktiviti protease adalah pada 40°C. LipPI12 lipase menunjukkan tahap ketidakstabilan pada suhu optimum mancapai separuh hayat selepas 30 minit. LipPI12 protease juga berada pada jangka hayat 15 minit pada suhu optimal. Pencirian pH menunjukan kedua-dua enzim LipPI12 berfungsi pada julat pH neutral Ujian terhadap substrat menunjukkan LipPI12 lipase berupaya mengkatalisis substrat dengan panjang rantai C12. Namun demikian, ujian terhadap pNP esters menunjukkan lipase LipPI12 boleh mengkatalisis rantaian yang lebih panjang iaitu C16. LipPI12 lipase masih berfungsi dengan kehadiran pelarut organik berlog P tinggi manakala protease hanya berfungsi pada log P rendah. Activiti lipase LipPI12 turut aktif dengan kehadiran logam $CaCl_2$ manakala protease lebih kepada $ZnCl_2$ dan $MgCl_2$. Kesan terhadap surfaktan turut mempengaruhi peningkatan aktiviti protease apabila didedahkan kepada Tween 80 dan SLS berbanding dengan aktiviti lipase yang menunjukkan penurunan bagi hampir semua jenis surfaktan yang diuji kecuali Tween 20. Lipase turut mengalami perencatan sebahagian aktiviti apabila melibatkan perencat PMSF dan EDTA berbanding protease direncat sepenuhnya oleh pepstatin Perbandingan jumlah asid amino LipPI12 dengan enzim suhu sederhana dan suhu tinggi menunjukkan adaptasi LipPI12 pada suhu sejuk adalah dibantu dengan pengurangan prolina serta pertambahan glisina. Analisis spectropolarimetri *"circular dichroism"* dan spektrofotometri

fluoresen mendedahkan sebahagian struktur LipPI12 yang fleksibel yang mana ia membolehkan ia aktif dan tahan pada suhu rendah.

Penemuan enzim LipPI12 telah meningkatkan pemahaman mengenai enzim sebagaimana ditunjukkan oleh dwifungsinya. Sifat yang berbeza pada lipase dan protease mendedahkan banyak pengetahuan mengenai struktur protein dan fungsinya. Kesimpulannya LipPI12 adalah satu enzim yang unik dan menarik yang telah menunjukkan cara lain untuk protein berfungsi pada suhu rendah dan sememangnya penemuan ini menjanjikan potensi tinggi untuk diaplikasikan.

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I certify that a Thesis Examination Committee has met on 19th February 2009 to conduct the final examination of Mohd Shukuri bin Mohamad Ali on his (or her) thesis entitled "**A Cold Active Bifunctional Lipase with Protease Activity from Antarctic Microorganism PI 12**" 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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Date: 14 May 2009

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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MOHD SHUKURI BIN MOHAMAD ALI

Date:

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3D	three-dimensional
a	adenine
Ala (A)	alanine
ANS	8-anilino-1-naphthalenesulfonic acid
Amp	ampicillin
Arg (R)	arginine
Asn (N)	asparagine
Asp (D)	aspartic acid
bp	base pair
c	cytosine
Cα	carbon alpha
Cys (C)	cysteine
°C	degree centrigrade
CD	circular dichroism
dH_2O	distilled water
DNA	deoxyribonucleic
EDTA	ethylene diamine tetraacetic acid
g	gram
Gln (Q)	glutamine