UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION OF AN ORGANIC SOLVENT-TOLERANT PROTEASE FROM *PSEUDOMONAS AERUGINOSA* STRAIN K

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FSAS 2003 27

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, In Fulfilment of the Requirement for the degree of Doctor of Philosophy

March 2003

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctoral of Philosophy

CHARACTERIZATION OF AN ORGANIC SOLVENT-TOLERANT PROTEASE FROM *PSEUDOMONAS AERUGINOSA* STRAIN K

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March 2003

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Faculty : Science and Environmental Studies

This study involves the isolation and screening of an organic solvent-tolerant producer from eleven Benzene-Toluene-Xylene-Ethylbenzene (BTEX) tolerant bacteria and Polycyclic-Aromatic-Hydrocarbons (PAHs) degraders. The bacteria were initially isolated following qualitative screening on skim milk agar plates and quantitative screening for organic solvent stable protease in liquid media. An organic solvent-tolerant protease producer identified as *Pseudomonas aeruginosa* strain K was selected for further analysis based on the stability of its protease in 25% (v/v) benzene and toluene.

Maximum protease production by *Pseudomonas aeruginosa* strain K was achieved after 48 h incubation at pH 7.0 and 37°C. Static condition and 4.0% (v/v) bacterial inoculum gave the optimum enzyme yield. Culture media containing sorbitol as the carbon source; casamino acids as the organic nitrogen source and sodium nitrate the inorganic nitrogen source, gave the highest level of protease production. Corn steep liquor, beef extract and ammonium nitrate on the other hand inhibited protease

activity. However, the addition of metal ions such as K^+, Mg^{2+} and Ca^{2+} maximized enzyme synthesis.

The organic solvent-tolerant strain K protease was purified to homogeneity by ammonium sulphate precipitation and anion exchange chromatography with 124-fold increase in specific activity and about 40% recovery. The molecular weight of the purified enzyme as revealed by SDS-PAGE electrophoresis is about 51 kilodaltons (kDa). The strain K protease was an alkaline metalloprotease with an optimum pH and temperature of pH 10.0 and 70°C, respectively. The protease was activated by Zn^{2+} and Sr^{2+} while Fe^{3+} inhibited it. Activation effect was also observed when the purified enzyme was exposed to denaturing and reducing agents such as 6M urea, Triton-X-100 and Tween 20 for 1 h exposure to the purified enzyme. After 14 days of incubation, the purified organic solvent-tolerant enzyme was 1.11, 1.82, 1.50, 1.75 and 1.80 times more stable in 1-decanol, isooctane, decane, dodecane and hexadecane, respectively.

The gene coding for the organic solvent-tolerant protease was amplified from *Pseudomonas aeruginosa* strain K by polymerase chain reaction using consensus primers based on the multiple sequences alignment of alkaline and metalloprotease genes from *Pseudomonas* species. Nucleotide sequence analysis of the gene revealed an open reading frame containing 1440 bp, which codes for a polypeptide of 479 amino acid residues. The polypeptide composed of a N-terminal propeptide of 7 amino acid residues and a mature protein of 472 amino acid residues. Amino acid sequence comparison revealed that the organic solvent-tolerant protease gene shared high homology with alkaline and metalloprotease sequences from *Pseudomonas*

aeruginosa and *Pseudomonas fluorescens*. The recombinant strain K protease was successfully expressed in pGEX-4T-1 expression vector. In the presence of 1.0 mM IPTG, the recombinant strain K protease was released into the periplasm of the *Escherichia coli* BL21 (DE3) host.

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

PENCIRIAN GEN STABIL PELARUT ORGANIK DARIPADA *PSEUDOMONAS AERUGINOSA* STRAIN K

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Pengajian mengenai penyaringan dan pemencilan bakteria penghasil protease stabil pelarut organik telah dijalankan ke atas sebelas pencilan yang stabil kepada benzena, toluena, xilena dan etilbenzena (BTEX). Bakteria yang berkeupayaan mengdegradasi hidrokarbon polisiklik aromatik telah dipencil berdasar kepada pemencilan kualitatif di atas agar susu (SMA) dan pemencilan kuantitatif kepada protease stabil pelarut organik di dalam media kaldu. Satu pencilan yang menghasilkan protease stabil pelarut organik telah dikenalpasti sebagai *Pseudomonas aeruginosa* strain K. Pencilan tersebut dipilih untuk kajian selanjutnya berdasarkan kepada keupayaan proteasenya yang stabil dalam 25% (I/I) benzena dan toluena.

Penghasilan maksimum protease *Pseudomonas aeruginosa* strain K tercapai pada pH 7.0 dan 37°C selepas 48 jam pengeraman. Keadaan statik dan 4.0% (I/I) inokulum bakteria menghasilkan protease pada tahap yang maksimum. Penghasilan protease tertinggi diperolehi apabila bakteria strain K dikulturkan dalam media yang menggunakan sorbitol sebagai sumber karbon, asid casamino dan sodium nitrat sebagai sumber nitrogen organik dan nitrogen tidak organik. “Corn steep liquor”,

ekstrak daging and ammonium nitrat merencat aktiviti protease. Penambahan ion logam seperti K^{+}, Mg^{2+} and Ca^{2+} meningkatkan sintesis enzim tersebut.

Protease stabil pelarut organik daripada strain K berjaya ditulenkan hingga homogen melalui pemendakan ammonium sulfate dan kromatografi penukaran anion dengan peningkatan aktiviti spesifik sehingga 124 kali ganda dan pulangan aktiviti sebanyak 40%. Berat molekul enzim yang ditulenkan ialah kira-kira 51 kDa ditentukan melalui kaedah SDS-PAGE. Protease strain K adalah protease jenis alkali dan metalo dengan pH dan suhu optimumnya pada pH 10.0 dan 70°C. Ion logam seperti Zn^{2+} and Sr^{2+} mengaktifkan enzim ini manakala ion Fe^{3+} merencatkannya. Tindakan keaktifan protease juga dapat dikesan dengan kehadiran agen denaturasi dan penurun, di mana pendedahan selama 1 jam kepada 6M urea, Triton-X-100 and Tween 20 meningkatkan aktiviti enzim. Selepas pengeraman selama 14 hari dengan pelarut organik, protease yang telah ditulenkan didapati lebih stabil sebanyak 1.11, 1.82, 1.50, 1.75 and 1.80 kali ganda untuk 1-decanol, isooctana, decana, dodecana and hexadecana berbanding dengan piawai.

Gen protease stabil pelarut organik daripada *Pseudomonas aeruginosa* strain K telah digandakan melalui tindakbalas berantai polimerasi dengan menggunakan primer-primer yang berdasarkan jujukan tindihan gen protease alkali dan metalo daripada spesies *Pseudomonas.* Analisis jujukan menunjukkan rangka bacaan terbuka bersaiz 1440 bp yang mengkodkan polipeptida yang mengandungi 479 residu asid amino. Polipeptida tersebut terdiri daripada 7 asid amino residu propeptida N-terminal dan 472 residu asid amino protein matang. Perbandingan asid amino menunjukkan homologi yang tinggi dengan protease alkali dan metalo daripada spesis

Pseudomonas aeruginosa dan *Pseudomonas fluorescens*. Protease daripada strain K rekombinan telah berjaya diekspreskan dengan vektor pengekspresan pGEX-4T-1. Kehadiran 1.0 mM IPTG menyebabkan protease strain K rekombinan dirembeskan ke dalam periplasma perumah *Escherichia coli* BL21 (DE3).

ACKNOWLEDGEMENTS

No written words to elaborate or express how privileged, grateful and honour, I am to be able to complete and write my thesis. This "mission impossible" will only be possible and come true by the power and assistance of the all mighty God. To my dearest mother and father, thank you for bringing me up to be who I am today. My success symbolizes and reflects on the undivided support and love from both of you.

My heartiest gratitude goes to my supervisors: Assoc. Prof. Dr. Che Nyonya Abdul Razak and Prof. Dr. Abu Bakar Salleh. I am truly thankful to Dr. Che Nyonya for giving me the opportunity to study on this interesting and advent project. My appreciation also goes to Prof. Dr. Abu Bakar Salleh for his willingness to help, listen and assist in every way, in the midst of his heavy responsibilities and duties. Above all, thank you for the advice, guidance, ideas, criticism and encouragement throughout the project. Not forgetting my supervisory committee members: Prof. Dr. Mahiran Basri and Assoc. Prof. Dr. Raja Noor Zaliha Abdul Rahman and Dr. Basyaruddin for their constructive comments, constant support and invaluable guidance. In taking this text from the raw manuscript stage to the final ready thesis stage, I have received tremendous assistance from the meticulous checking of the text to numerous suggestions for modifications and special learning aids from all of you.

I would like to dedicate my appreciation to Assoc. Prof. Dr. Tan Wen Siang, Prof. Dr Khatijah Yusoff and my dearest friends. I am grateful to Dr. Tan Wen Siang for providing me with wealth of comprehensive information, invaluable and

insightful contributions to the last chapter. Especially to five of the best and brightest friends: Ong Swee Tin, Kho Chiew Ling, Dr. Lau Wei Hong, Dr. Majid, Dr. Mao, Brother Laith and Leow. Thanks for your unending patience, good humour and support during the seemingly unending process of getting this research finished! Special recognition is addressed to all my Bacteriology lab mates and friends in Virology Lab, thank you for your friendship. I am also indebted to all the staff in Department of Biochemistry and Microbiology.

I certify that an Examination Committee met on 20th March 2003 to conduct the final examination of Lee Poh Geok on her Doctor of Philosophy thesis entitled "Characterization of an Organic Solvent-tolerant Protease from *Pseudomonas aeruginosa* strain K" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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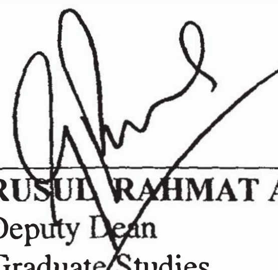

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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Date: 9 June 2003

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LIST OF ABBREVIATIONS

A	Adenine base nucleotide
AP	Alkaline Buffer
Apr	Alkaline protease
APS	Ammonium Persulphate
BCIP	Bromochloroindolyl phosphate
BHI	Brain Heart Infusion
BTEX	Benzene-Toluene-Xylene-Ethylbenzene
bp	Base pair
C	Cytosine base nucleotide
cm	Centimeter
Da	Dalton
3,4-DCI	3,4-dichloroisocoumarin
DFP	Diisopropylflouro phosphate
dH_2O	Distilled water
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothreitol
E.64	L-3-carboxytrans-2, 3-epoxypropyl-leucylamido (4-guanidine) butane
EDTA	Ethylenediaminetetraacetic Acid
FPLC	Fast Protein Liquid Chromatography
G	Guanine base nucleotide
g	Gram

g/L	Gram per litre
GST	Glutathione-S-Transferase
GTE	Glucose-Tris-HCl-EDTA
h	Hour
HPLC	High Performance Liquid Chromatography
IAA	Iodoacetic acid
IPTG	Isopropyl β-D Thiogalactoside
kDa	Kilodaltons
kbp	Kilobase pairs
L	Litre
M	Molar
mA	Milliampere
mg	Milligram
mL	Millilitre
mM	Millimolar
min	Minute
NBT	Nitroblue tetrazolium salts
nm	Nanometer
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAHs	Polycyclic-Aromatic-Hydrocarbons
PCMB	*p*-chloromercuribenzoate
PCR	Polymerase Chain Reaction
PMSF	Phenylmethylsulfonyl fluoride
SDS	Sodium dodecyl sulphate