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

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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

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

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March 2003

Chairman: Professor Abu Bakar Salleh, Ph.D.

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+, Mg2+ and Ca2+ 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 Zn2+ and Sr2+ while Fe3+ 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.
PENCIRIAN GEN STABIL PELARUT ORGANIK DARIPADA 
*PSEUDOMONAS AERUGINOSA* STRAIN K

Oleh

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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”,

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ekstrak daging and ammonium nitrat merencat aktiviti protease. Penambahan ion logam seperti K⁺, Mg²⁺ and Ca²⁺ 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 ditulenken 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 Zn²⁺ and Sr²⁺ mengaktifkan enzim ini manakala ion Fe³⁺ 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 ditulenken didapati lebih stabil sebanyak 1.11, 1.82, 1.50, 1.75 and 1.80 kali ganda untuk 1-decanol, isoctana, 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).
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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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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.

LEE POH GEOK

Date: 9 June 2003

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<td>A</td>
<td>Adenine base nucleotide</td>
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<tr>
<td>AP</td>
<td>Alkaline Buffer</td>
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<tr>
<td>Apr</td>
<td>Alkaline protease</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulphate</td>
</tr>
<tr>
<td>BCIP</td>
<td>Bromochloroindolyl phosphate</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain Heart Infusion</td>
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<tr>
<td>BTEX</td>
<td>Benzene-Toluene-Xylene-Ethylbenzene</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine base nucleotide</td>
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<tr>
<td>cm</td>
<td>Centimeter</td>
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<td>Da</td>
<td>Dalton</td>
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<tr>
<td>3,4-DCI</td>
<td>3,4-dichloroisocoumarin</td>
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<tr>
<td>DFP</td>
<td>Diisopropylflouro phosphate</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>L-3-carboxytrans-2, 3-epoxypropyl-leucylamido (4-guanidine) butane</td>
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<td>Ethylenediaminetetraacetic Acid</td>
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<td>G</td>
<td>Guanine base nucleotide</td>
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<td>g</td>
<td>Gram</td>
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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