



UNIVERSITI PUTRA MALAYSIA

***EXPRESSION, CHARACTERIZATION AND ORGANIC SOLVENT
STABILITY OF PROTEASE FROM
Bacillus pumilus 115b***

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By

PEIMAN NAZARIRAD

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
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**EXPRESSION, CHARACTERIZATION AND ORGANIC SOLVENT
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Chairman: Prof. Raja Noor Zaliha Raja Abd. Rahman, PhD

Faculty: Biotechnology and Biomolecular Sciences

Organic solvent stable proteases have potential to be used in non-aqueous enzymatic reactions. Therefore, organic solvent stability study of these enzymes could contribute to a better understanding of their functions. *Bacillus pumilus* 115b produce organic solvent stable protease. The protease showed stability in 25% (v/v) benzene and toluene and it was activated by non-polar organic solvents. Protein engineering is useful to clarify the mechanisms by which the enzymes are stable in the presence of organic solvents. In the current study the organic solvent stable protease gene (1065 bp) from *Bacillus pumilus* 115b was cloned and expressed in *E.coli* BL21 (DE3). To achieve the maximum production

of recombinant protease, parameters such as temperature, inducer concentration (IPTG), induction time and OD_{600nm} were optimized. The optimum conditions assessment consisting of; cultivation temperature at 25°C, induction timing at late stage (OD_{600nm}, 0.75) of exponential growth, IPTG 0.8 mM and post induction time for 8 h were determined.

The recombinant organic solvent stable protease from *Bacillus pumilus* 115b (55 kDa) was purified by affinity chromatography using Nickle-Sepharose. Protein peak was formed from fraction 36-40. In the range of 375-400 mM imidazole, the target protein was eluted and purified. The recombinant purified protease was verified using SDS-PAGE and Western blot analyses. The purification of recombinant organic solvent protease (OSSP) was increased to 126.89 fold with 72.83% recovery.

The purified protease was shown to be active between 30 to 60°C with an optimal temperature of 55°C. Thermostability profile indicated that the protease was stable at 37, 45 and 50 °C for 30 min. Further increase in temperature above 60 °C resulted in a reduction of the activity.

The pH activity of the purified protease was 7 to 10 with an optimum pH of 9.

Low protease activity was detected at pH below 6.0. Moreover less than 50 % of maximal protease activity was detected at pH above 10.0. The result showed that, the activity retained 68.3, 38.3 and 10 % of maximal activity at pH 10, 11 and 12, respectively. No activity was detected at pH 4 and very low protease activity was observed at pH 5. pH stability study showed that recombinant protease was fairly stable at alkaline pH condition. The enzyme was stable between pH 7.0 to 11. Further increment in pH value (12) caused 58.07 % loss of the maximal activity. Meanwhile 45.16% of maximal activity was retained in pH 6; treatment of the enzyme at pH below 5 almost destabilized the protease activity.

Metal ion study revealed that Ca^{++} ion increased the activity of purified recombinant protease to 118.18% compared to control while Sr^{++} and Na^{++} gave negligible enhancement effects on the activity of protease. Whereas, variable inhibitory effects were observed in the presence of Zn^{++} (77.28%), Mn^{++} (71.82%), Cu^{++} (53.64%), K^{+} (45.64%), Fe^{+++} (45.46) and Co^{++} (32.78%).

In regard to inhibitors, phenylmethanesulfonyl fluoride (PMSF) caused 100% inactivation of the protease. The purified protease was inhibited 89.2% by Diisopropylfluorophosphate (DFP). Inhibitory effects were also observed in the presence of ethylenediaminetetraacetic acid (EDTA) and bestatin with 43.4 and

31.67%, respectively. As PMSF completely deactivated the recombinant protease activity, this protease was grouped as serine protease.

The casein, a major protein component in milk was the most susceptible to hydrolysis compared to other substrates (albumin, haemoglobin, azocasein and azocoll). It was found that hemoglobin was less suitable substrate compared to casein. Recombinant protease 115b also revealed substrate specificity toward Albumin and azocasein. This protease also showed the ability of hydrolysing large molecules such as azocoll.

Organic solvent stability study showed that the recombinant protease was stable in the presence of various organic solvents. It was found that the residual activity reduced to 50, 66 and 79% of the initial when enzyme was exposed to acetonitrile, diethylamine and butanol, correspondingly ($\log P_{o/w} < 2$). The residual activity of recombinant purified protease was enhanced against nonpolar organic solvents including n-dodecane, n-tetradecane and n-hexadecane ($\log P_{o/w} > 4$) 175, 197 and 219%, respectively. On the other hand, application of solvents having a $\log P$ (2 to 4) showed fluctuations of 56 -115% in comparison to control.

To find the amino acid residue(s) responsible for the organic solvent stability of the protease, random mutation was carried out using error-prone PCR (EP-PCR) method. A mutated transformant which retained its protease activity but different in stability (a change in residual activity in acetonitrile) was selected. The mutant (M2-17) showed decreased stability in the presence of acetonitrile. The mutant protease was also less stable in the presence of various organic solvents compared to recombinant protease.

The residual activity of mutant protease decreased in polar solvents. It was found that the residual activity reduced to 18, 27.27 and 34% of initial while enzyme was exposed to acetonitrile, diethylamine and butanol, correspondingly ($\log P_{o/w} < 2$). The residual activity of mutant protease was enhanced against nonpolar organic solvents including n-dodecane, n-tetradecane and n-hexadecane ($\log P_{o/w} > 4$) 113.63, 136.36 and 156.8%, respectively. Furthermore, solvents having a log P (2 to 4) showed result in fluctuations between 31.81 - 70.45% compare to control. Study of organic solvent on the stability of mutant protease revealed that polar solvents could destabilize the mutant protease more than the recombinant.

Optimization and characterization (except organic solvent stability) of mutant M2-17 showed almost similar result with recombinant protease. This finding revealed the major role of a polar amino acid (Lysine 244) residue merely affecting in the organic solvent stability of protease from *Bacillus pumilus* 115b.

By comparison of the sequences of mutant and recombinant proteases, it was revealed that a point mutation on the polar amino acid (Lysine 244 to Isoleucine) had occurred which could significantly change the organic solvent stability. This finding revealed the polar amino acid (Lysine 244) residue is responsible for organic solvent stability of protease from *Bacillus pumilus* 115b.

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

**EKSPRESI, PENCIRIAN DAN KESTABILAN PELARUT ORGANIK
PROTEASE DARI *Bacillus pumilus* 115b**

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Protease stabil pelarut organik berpotensi untuk digunakan dalam tindak balas enzim tanpa air. Oleh itu, kajian kestabilan enzim di dalam pelarut organik boleh menyumbang kepada pemahaman yang lebih baik tentang fungsi enzim-enzim tersebut. *Bacillus pumilus* 115b menghasilkan protease stabil pelarut organik.

Protease terhasil menunjukkan kestabilan dalam 25% (v/v) benzena dan toluena, dan ia telah diaktifkan oleh pelarut organik tidak berkutub. Kejuruteraan protein berguna untuk menjelaskan mekanisme bagaimana enzim itu stabil dalam kehadiran pelarut organik. Dalam kajian semasa, gen protease stabil pelarut

organik (1065 bp) daripada *Bacillus pumilus* 115b telah diklon dan diekspres dalam *E.coli* BL21 (DE3). Untuk mencapai penghasilan protease rekombinan yang maksimum, parameter seperti suhu, kepekatan pencetus (IPTG), masa induksi dan OD_{600nm} telah dioptimumkan. Penilaian keadaan optimum terdiri daripada: suhu pembiakan pada 25°C, induksi masa pada peringkat akhir pertumbuhan eksponen (OD_{600nm}, 0.75), kepekatan IPTG 0.8 mM dan masa pos-induksi selama 8 j telah ditentukan.

Protease rekombinan stabil pelarut organik daripada *Bacillus pumilus* 115b (55 kDa) telah dituliskan dengan teknik kromatografi afiniti menggunakan nikel-Sepharose. Puncak protein adalah terdiri daripada pecahan-pecahan 36-40. Dalam julat 375-400 mM imidazole, protein sasaran berjaya dituliskan. Penulenan protease rekombinan telah dipastikan menggunakan SDS-PAGE dan analisa Western blot. Penulenan protease rekombinan pelarut organik (OSSP) telah meningkat 126.89 kali ganda dengan penghasilan sebanyak 72.83%.

Protease tertulen telah dikenalpasti aktif pada suhu antara 30 dan 60°C dengan suhu optimum 55°C. Profil kestabilan suhu menunjukkan bahawa protease adalah stabil pada 37, 45 dan 50°C selama 30 minit. Peningkatan suhu selanjutnya melebihi 60°C menyebabkan pengurangan aktiviti enzim tersebut

Protease tertulen mempunyai aktiviti pada pH 7-10 dengan pH optimum pada 9. Aktiviti protease yang rendah telah dikesan pada pH di bawah 6.0. Selain itu, kurang daripada 50% daripada aktiviti protease maksimum dikesan pada pH 10 dan ke atas. Hasil eksperimen menunjukkan bahawa, aktiviti dapat dikekalkan sebanyak 68.3, 38.3 dan 10.0% daripada aktiviti maksimum pada pH 10, 11 dan 12, masing-masing. Tiada aktiviti dikesan pada pH 4 dan aktiviti protease yang sangat rendah dikenalpasti pada pH 5. Kajian kestabilan pH menunjukkan bahawa protease rekombinan adalah agak stabil pada keadaan pH beralkali. Enzim adalah stabil antara pH 7.0 hingga 11. Kenaikan selanjutnya dalam nilai pH (12) menyebabkan kehilangan aktiviti sebanyak 58.07% daripada aktiviti maksimum. Sementara itu, 45.16% daripada aktiviti maksimum dikekalkan pada pH 6; rawatan enzim pada pH di bawah 5 hampir menyahstabilkan aktiviti protease.

Kajian ion logam menunjukkan bahawa ion Ca^{++} meningkatkan aktiviti protease rekombinan tertulen kepada 118.18% berbanding pemalar, manakala Sr^{++} dan Na^{++} memberikan kesan peningkatan terabai pada aktiviti protease. Manakala, kesan-kesan pembolehkan penyahaktif telah dikenalpasti dalam kehadiran Zn^{++} (77.28%), Mn^{++} (71.82%), Cu^{++} (53.64%), K^{+} (45.64%), Fe^{+++} (45.46) dan Co^{++} (32.78%).

Dalam kes penyahaktif, phenilmethilsulfonil florida (PMSF) telah menyebabkan penyahaktifan protease sebanyak 100%. Protease tertulen telah dinyahaktif sebanyak 89.2% oleh Diisopropilfluorofosfat (DFP). Kesan penyahaktifan juga dikesan dalam kehadiran asid etilinediamintetrasetik (EDTA) dan bestatin sebanyak 43.4 dan 31.67%, masing-masing. Disebabkan PMSF telah menyahaktifkan aktiviti protease rekombinan sepenuhnya, protease ini telah diklasifikasikan sebagai protease serin.

Kasein, komponen protein utama dalam susu adalah yang paling mudah terdedah kepada hidrolisis berbanding substrat-substrat lain (albumin, hemoglobin, azokasien dan azokol). Telah didapati bahawa hemoglobin adalah substrat yang kurang sesuai berbanding kasein. Protease rekombinan 115b juga menunjukkan spesifikasi substrat cenderung kepada albumin dan azokasien. Protease ini juga menunjukkan keupayaan menghidrolisis molekul besar seperti azokol.

Kajian kestabilan pelarut organik menunjukkan bahawa protease rekombinan adalah stabil dalam kehadiran pelbagai pelarut organik. Telah didapati bahawa aktiviti berbaki berkurang kepada 50, 66 dan 79% berbanding aktiviti asal apabila enzim terdedah kepada acetonitril, diethylamin dan butanol, masing-masing (log

$P_o / w < 2$). Aktiviti berbaki protease rekombinan tertulen telah dipertingkatkan terhadap pelarut organik tidak berkutub termasuklah n-dodekan, n-tetradekan dan n-hexadekan ($\log P_o / w > 4$) 175, 197 dan 219%, masing-masing. Sebaliknya, penggunaan pelarut yang mempunyai $P \log (2-4)$ menunjukkan turun naik 56-115% setelah dibandingkan dengan pemalar.

Untuk mencari asid-asid amino yang bertanggungjawab bagi kestabilan protease terlarut organik, mutasi rawak telah dijalankan menggunakan kaedah cenderung-ralat PCR (EP-PCR). Satu mutan transforman dengan aktiviti protease kekal tetapi berbeza kestabilannya (perubahan dalam aktiviti berbaki dalam asetonitril) telah dipilih. Mutan (M2-17) menunjukkan penurunan kestabilan dalam kehadiran asetonitril. Protease mutan juga kurang stabil dalam kehadiran pelbagai pelarut organik berbanding protease rekombinan.

Aktiviti berbaki protease mutan berkurangan dalam pelarut kutub. Telah didapati bahawa aktiviti berbaki berkurangan kepada 18, 27, 27 dan 34% berbanding aktiviti asal, apabila enzim terdedah kepada asetonitril, diethylamin, dan butanol, masing-masing ($\log P_o / w < 2$). Aktiviti berbaki protease mutan telah dipertingkatkan terhadap pelarut organik tidak berkutub termasuklah n-dodekan, n-tetradekan dan n-hexadekan ($\log P_o / w > 4$) 113.63, 136.36 dan

156.8%, masing-masing. Tambahan pula, pelarut yang mempunyai P log (2-4) mengakibatkan turun naik antara 31.81%-70.45 jika dibandingkan dengan pemalar. Kajian pelarut organik ke atas kestabilan protease mutan menunjukkan bahawa pelarut berkutub boleh menjejaskan kestabilan protease mutan lebih daripada rekombinan. Pengoptimuman dan pencirian (kecuali kestabilan pelarut organik) mutan M2-17 menunjukkan hasil yang hampir sama dengan protease rekombinan. Penemuan ini menunjukkan peranan utama asid amino berkutub (lisin 244) hanya mempengaruhi kestabilan protease pelarut organik daripada *Bacillus pumilus* 115b.

Perbandingan jujukan mutan-mutan dan protease rekombinan telah menunjukkan bahawa mutasi titik pada asid amino berkutub (lisin 244 kepada isoleucine) telah berlaku dan boleh mengubah kestabilan pelarut organik secara signifikan. Penemuan ini mendedahkan asid amino berkutub (lisin 244) bertanggungjawab untuk kestabilan protease pelarut organik daripada *Bacillus pumilus* 115b.

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I certify that a Thesis Examination Committee has met on 7 August 2012 to conduct the final examination of Peiman Nazarirad on his thesis entitled “Expression, Characterization, and Organic Solvent Stability of Protease from *Bacillus pumilus* 115b” in accordance with the Universities and University College 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 Doctor of Philosophy.

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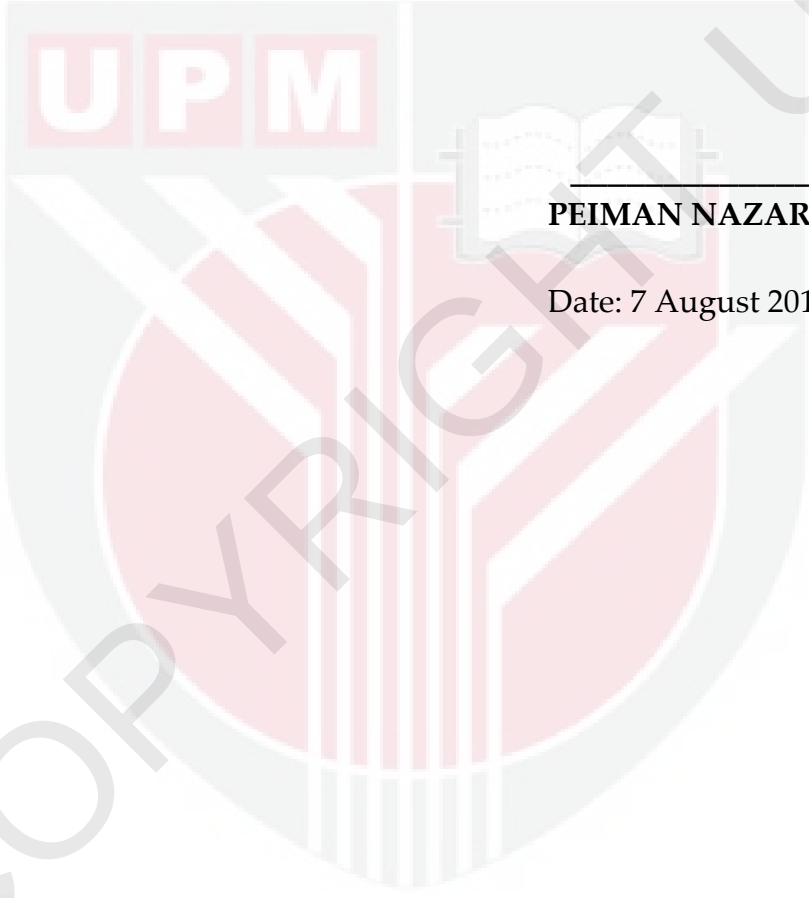
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DECLARATION

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 Universiti Putra Malaysia or other institutions.



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Date: 7 August 2012

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