



UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION AND CRYSTALLIZATION OF W200R PROTEASE

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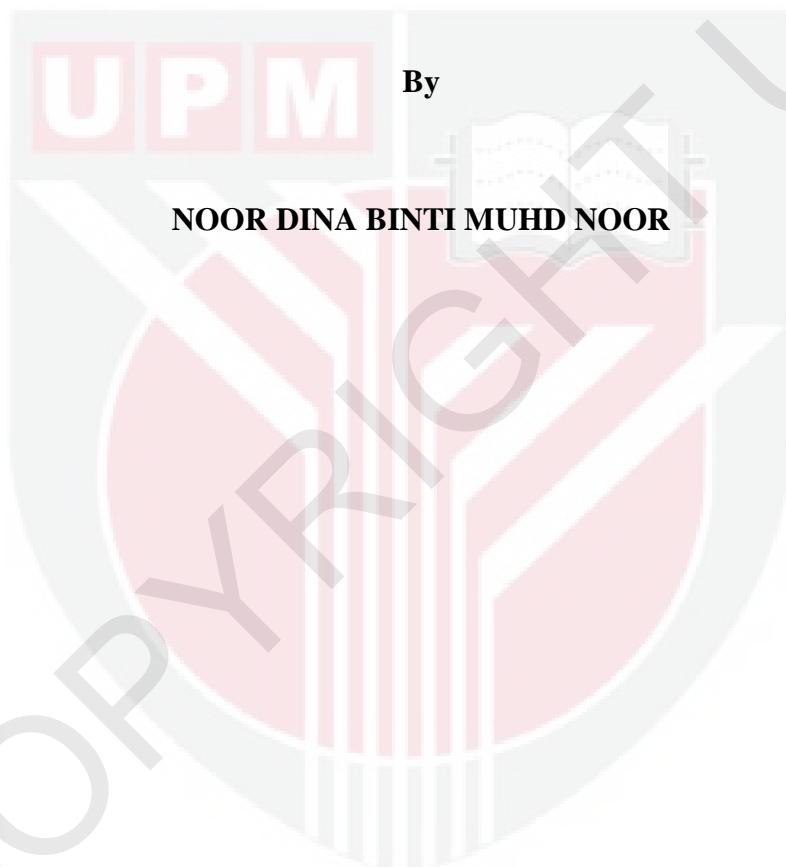
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**MASTER OF SCIENCE
UNIVERSITI PUTRA MALAYSIA**

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**CHARACTERIZATION AND CRYSTALLIZATION
OF W200R PROTEASE**



**Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfillment of the Requirements for the Degree of Master of Science**

February 2012

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment
of the requirement for the degree of Master of Science

**CHARACTERIZATION AND CRYSTALLIZATION
OF W200R PROTEASE**

By
NOOR DINA BINTI MUHD NOOR

February 2012

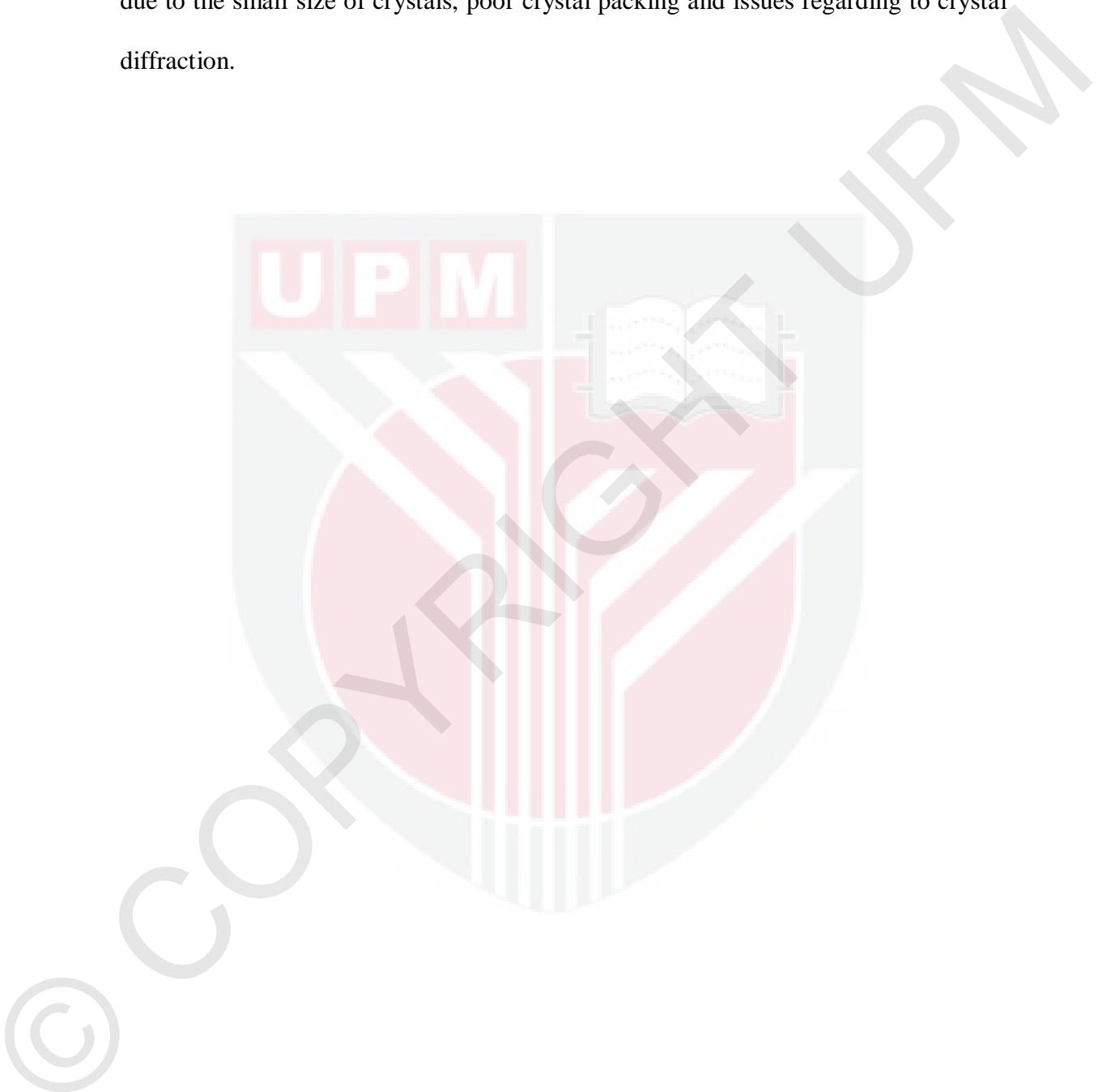
Chair: Professor Raja Noor Zaliha Raja Abd Rahman, D. Eng

Faculty: Biotechnology and Biomolecular Sciences

Structural comparison of wild-type F1 protease and thermophilic homologues leads to hypothesis about the role of ion pairs for the thermostability of the enzymes. Higher thermostability was found to correlate with an increased number of the residues involved in ion pairs or ion pair's networks. Therefore, W200R protease is a mutant designed to have three new additional ion pairs compared to the F1 protease. This designed mutant underwent substitution of Trp (Tryptophan) at position 200 with Arg (Arginine). Both of protease and W200R were cloned into pGEX-4T1 vector and transformed into *E. coli* BL21(DE3)pLysS. For the purpose of characterization, F1 protease was purified by using Glutathione Sepharose resin and heat treatment whereby W200R was purified in three steps; Glutathione Sepharose resin, ion exchange by using Q-Sepharose Fast Flow resin and Phenyl Sepharose High Performance. The last product of purification for F1 protease and W200R was confirmed to be the mature form which is approximately 27 kDa. Characterization of W200R's thermostability was done in comparison with F1 protease. The optimum

temperature of W200R protease in the presence and absence of 2 mM CaCl₂ was 75°C. Meanwhile, F1 protease exhibited a lower temperature optimum which is 70°C with the presence and absence of Ca²⁺. In term of thermostability in the presence of 2 mM Ca²⁺, the activity of W200R and F1 protease retained 100% of its activity between 50°C to 80°C and 50°C to 65°C, respectively. Ca²⁺ affected both enzymes by increasing and stabilizing the protease activity. The optimum pH of W200R was at pH 8 and the activity was stable in pH ranging from pH 7.0-10. The activity of W200R retained more than 100% of its activity compared to the control in the presence of miscible organic solvents log *P* < 2 such as pyridine, ethanol, methanol and DMSO. In comparison with the wild type, stability in the presence of immiscible organic solvents log *P* > 2 such as n- heptanes, hexane and benzene was exhibited. W200R protease was strongly inhibited by Phenylmethylsulfonyl fluoride (PMSF) showing that it belongs to the serine protease superfamily. W200R which was purified by using a series of purification of affinity chromatography, IEX and HIC was subjected to crystallization trial. Crystallization was set up with and without inhibitor. Crystallization screening without inhibitor on microbatch showed best crystal growth in Formulation 42 (Crystal Screen 2 kit) consists of 1.5 M Ammonium sulfate, 0.1 M Tris pH 8.5, 12% (v/v) glycerol. Optimization on crystallization temperature showed that crystallization was possible from 20°C to 40°C whereby the crystal grew as a single and bigger crystal at 20°C and fewer crystals were produced at temperature of 30°C. The number of crystals was observed to increase at 40°C. It took 30, 18 and 6 days for crystallization at 20°C, 30°C and 40°C, respectively. The crystals of W200R protease with inhibitor were obtained after 25 days of incubation at 20°C in Formulation 38 from Crystal screen 2 kit consisting of 0.1 M HEPES pH 7.5, 20% (w/v) PEG 10000. AEBSF, 5 mM was

found to be the best inhibitor in contributing the crystal growth of W200R protease. However, from X-ray diffraction studies on crystals for both without and with inhibitor, it showed that the diffraction scattering intensities was low which might be due to the small size of crystals, poor crystal packing and issues regarding to crystal diffraction.



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai
memenuhi keperluan untuk ijazah Master Sains

**PENCIRIAN DAN PENGHABLURAN
W200R PROTEASE**

Oleh

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Februari 2011

Pengerusi: Profesor Raja Noor Zaliha Raja Abd Rahman, D.Eng

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Perbandingan struktur protease F1 dengan struktur serin protease dari bakteria mesofilik seumpamanya, telah menghasilkan satu hipotesis tentang peranan pasangan ion untuk kestabilan enzim. Kestabilan protein terhadap suhu tinggi didapati berkaitan dengan peningkatan jumlah residu yang mempunyai pasangan ion atau jaringan pasangan ion. Oleh itu, penghasilan mutan W200R protease mempunyai penambahan tiga pasangan ion berbanding dengan F1 protease. Mutan ini melibatkan penggantian Trp (Tryptophan) pada kedudukan ke-200 digantikan dengan Arg (Arginin). Kedua-dua F1 protease dan W200R diklonkan ke dalam vektor pGEX-4T1 dan ditransformasikan ke dalam *E. coli* BL21(DE)pLysS. Untuk tujuan pencirian protein, F1 protease telah ditulenkan dengan menggunakan resin Glutathione Sepharose dan kaedah rawatan haba manakala W200R ditulenkan dalam tiga langkah; kaedah resin Glutathione Sepharose, pertukaran ion menggunakan resin Q-sepharose Fast Flow dan Phenyl Sepharose High Performance. Hasil akhir dari penulenan bagi F1 protease dan W200R disahkan sebagai bentuk ‘mature’ yang

mana saiz dianggarkan adalah 27 kDa. Pencirian untuk thermostabil W200R dilakukan dan dibandingkan dengan F1 protease. Suhu optimum bagi W200R dalam kehadiran dan ketidakhadiran 2 mM CaCl₂ adalah 75°C. Sementara itu, F1 protease menunjukkan suhu optimum yang lebih rendah iaitu 70°C dalam kehadiran dan ketidakhadiran Ca²⁺. Dalam pencirian thermostabil dengan kehadiran 2 mM CaCl₂, aktiviti W200R dan F1 protease masing-masing bertahan pada tahap 100% dalam lingkungan suhu 50°C hingga 80°C dan 50°C hingga 65°C. Ca²⁺ memberikan kesan terhadap enzim dengan meningkatkan dan menstabilkan aktiviti protease. pH optimum W200R adalah pH 8 dan aktivitinya stabil antara pH 7.0 – 10. Aktiviti W200R meningkat lebih daripada 100% apabila dalam kehadiran pelarut organik Log *P* < 2 seperti pyridine, etanol, metanol dan DMSO. Berbanding dengan gen yang asli, ianya menunjukkan kestabilan dalam kehadiran pelarut organic Log *P* > 2 seperti n-heptana, heksana dan benzana. Aktiviti W200R protease direncat dengan hebat oleh phenylmethylsulfonyl fluoride (PMSF) menunjukkan bahawa ia tergolong dalam superfamili serin protease. W200R yang telah ditulenkann dengan satu siri penulenan yang terdiri daripada affiniti kromatografi, IEX dan HIC digunakan untuk percubaan penghabluran. Eksperimen penghabluran dijalankan dengan kehadiran dan tanpa perencat. Pemeriksaan penghabluran tanpa perencat dilakukan dengan menggunakan “ microbatch” dan menunjukkan pertumbuhan hablur dalam Formula 42 yang mengandungi 1.5 M Ammonium sulfat, 0.1 M Tris pH 8.5, 12% (v/v) gliserol. Pengoptimuman suhu untuk penghabluran protein menujukkan bahawa penghabluran boleh dilakukan daripada suhu 20°C sehingga 40°C yang mana pertumbuhan kristal wujud secara tunggal dan lebih besar saiznya pada suhu 20°C dan jumlah kristal semakin banyak dihasilkan pada suhu 30°C. Jumlah kristal yang dipantau menunjukkan peningkatan pada suhu 40°C. Ianya mengambil masa selama

30, 18 dan 6 hari masing-masing untuk penghabluran pada suhu 20°C, 30°C dan 40°C. Penghabluran kristal W200R dengan perencat pula diperoleh selepas 25 hari hasil daripada pengeraman pada suhu 20°C dalam Formula 38 yang mengandungi 0.1 M HEPES pH 7.5, 20% (w/v) PEG 10000. AEBSF, 5 mM didapati sebagai perencat terbaik dalam menyumbang kepada pertumbuhan kristal W200R protease. Walaubagaimanapun, kajian daripada hasil difraksi melalui sinar-X ke atas kristal tanpa perencat dan dengan perencat, menunjukkan pengamatan difraksi yang rendah yang mana mungkin berpunca daripada saiz kristal yang kecil, penyusunan molekul dalam kristal yang tidak bagus dan beberapa isu berkaitan dengan difraksi kristal.

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I certify that a Thesis Examination Committee has met on 29th February 2012 to conduct the final examination of Noor Dina binti Muhd Noor on her thesis entitled “Characterization and Crystallization of W200R protease” 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 student be awarded the Master of Science.

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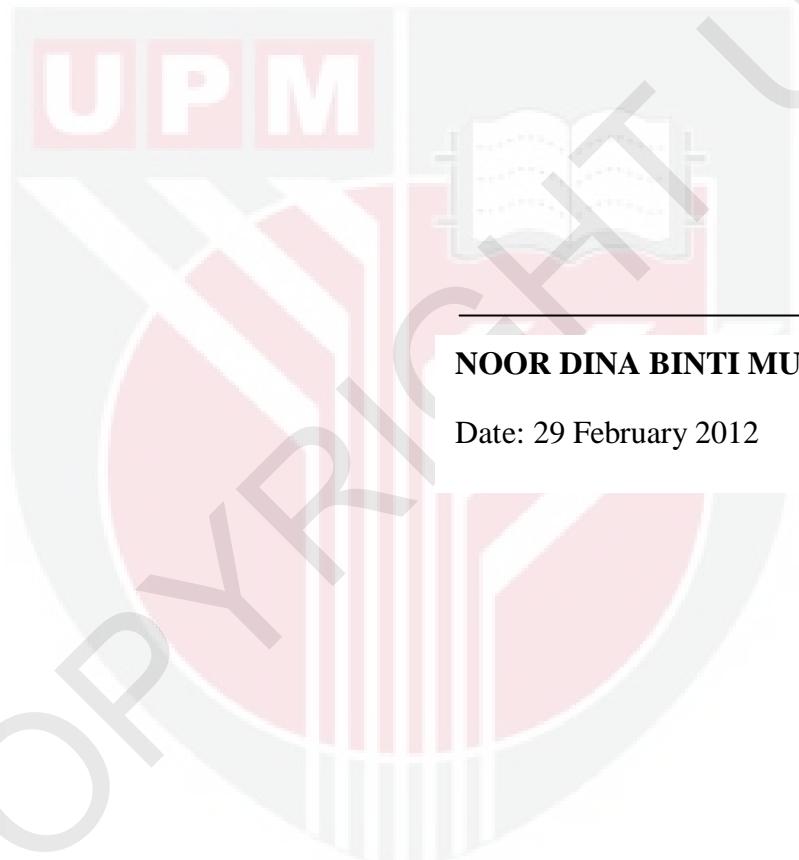
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DECLARATION

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 other institutions.



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Date: 29 February 2012

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