UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION AND CRYSTALLIZATION OF W200R PROTEASE

NOOR DINA BINTI MUHD NOOR

FBSB 2011 51
CHARACTERIZATION AND CRYSTALLIZATION OF W200R PROTEASE

NOOR DINA BINTI MUHD NOOR

MASTER OF SCIENCE
UNIVERSITI PUTRA MALAYSIA

2012
CHARACTERIZATION AND CRYSTALLIZATION
OF W200R PROTEASE

By

NOOR DINA BINTI MUHD NOOR

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 \textit{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 CaCl$_2$ was 75°C. Meanwhile, F1 protease exhibited a lower temperature optimum which is 70°C with the presence and absence of Ca$^{2+}$. In term of thermostability in the presence of 2 mM Ca$^{2+}$, 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. Ca$^{2+}$ 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

NOOR DINA BINTI MUHD NOOR

Februari 2011

Pengerusi: Profesor Raja Noor Zaliha Raja Abd Rahman, D.Eng
Fakulti: Fakulti Bioteknologi dan Sains Biomolekul

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 ditembakkan 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 CaCl$_2$ adalah 75°C. Sementara itu, F1 protease menunjukkan suhu optimum yang lebih rendah iaitu 70°C dalam kehadiran dan ketidakhadiran Ca$^{2+}$. Dalam pencirian thermostabil dengan kehadiran 2 mM CaCl$_2$, 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. Ca$^{2+}$ 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 ditulenkan 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 menunjukkan 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.
ACKNOWLEDGEMENTS

First of all, I would like to express my gratitude to Him for the blessings throughout my studies and this project. Allah had guided me, in his own ways, in good and bad times.

At this opportunity, I would like to express my biggest appreciation to my main supervisor, Professor Dr Raja Noor Zaliha Raja Abd Rahman, for her constant supervision, helpful suggestions and constructive criticisms on my work. She like a mother to me who always keep supporting and the patience she has given me had kept me moving. She is the person I would always turn to when I faced any major lab work problems. My sincere appreciation also goes to Professor Dato’ Abu Bakar Salleh and Professor Dr. Mahiran Basri who always support me from behind. I also would like to express my sincere gratitude to Dr. Mohd Shukuri Mohamad Ali and Dr. Adam Leow Thean Chor for their helpful explanation on the theoretical studies and also for their helpful comments for each of the weekly progress report presented.

I would like to extend my heartfelt gratitude to Prof. Dr. Higuchi and Prof Dr Inaka, with the help of their valuable suggestions, guidance and encouragement I have able to perform this project work.

My biggest gratitude is to my parents, sisters and brothers who made me the person I am today. I would have gained for nothing if not because of them. Thank you so much. Thanks too, to all my friends and also my lab mates in Lab 140 and IBS, who made my life so colourful. Thank you, all.
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.

Members of the Thesis Examination Committee were as follows:

Prof. Dr Raha bt Hj. Abdul Rahim
Department of Cell and Molecular Biology,
Faculty of Biotechnology and Biomolecular Sciences, UPM
(Chairman)

Assoc. Prof. Dr Suhaimi bin Mustafa
Department of Microbiology,
Faculty of Biotechnology and Biomolecular Sciences, UPM
(Internal Examiner 1)

Assoc. Prof. Dr Rosfarizan bt Mohamad
Department of Bioprocess Technology,
Faculty of Biotechnology and Biomolecular Sciences, UPM
(Internal Examiner 2)

Prof. Dr Sheila Nathan
School of Bioscience and Biotechnology,
Faculty of Sciences and Technology, UKM
(External Examiner)

SEOW HENG FONG, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 21 May 2012
This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

**Raja Noor Zaliha Raja Abd. Rahman, D.Eng**  
Professor  
Faculty of Biotechnology and Biomolecular Sciences  
Universiti Putra Malaysia  
Serdang Selangor  
(Chairman)

**Dato’ Abu Bakar Salleh, PhD**  
Professor  
Faculty of Biotechnology and Biomolecular Sciences  
Universiti Putra Malaysia  
Serdang Selangor  
(Member)

**Mahiran Basri, PhD**  
Professor  
Faculty of Sciences  
Universiti Putra Malaysia  
Serdang Selangor  
(Member)

---

**BUJANG BIN KIM HUAT, PhD**  
Professor and Dean  
School of Graduate Studies  
Universiti Putra Malaysia  

Date:
DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that is has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or other institutions.

NOOR DINA BINTI MUHD NOOR

Date: 29 February 2012
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1 INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td>1.1 Objectives</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2 LITERATURE REVIEW</strong></td>
<td>6</td>
</tr>
<tr>
<td>2.1 Proteases</td>
<td>6</td>
</tr>
<tr>
<td>2.2 Classification of proteases</td>
<td>7</td>
</tr>
<tr>
<td>2.2.1 Serine Proteases (E.C 3.4.21)</td>
<td>7</td>
</tr>
<tr>
<td>2.2.2 Cysteine Proteases (E.C 3.4.22)</td>
<td>9</td>
</tr>
<tr>
<td>2.2.3 Aspartic Proteases (E.C 3.4.23)</td>
<td>9</td>
</tr>
<tr>
<td>2.2.4 Metallo Proteinases (E.C 3.4.24)</td>
<td>10</td>
</tr>
<tr>
<td>2.3 Thermostable proteases</td>
<td>10</td>
</tr>
<tr>
<td>2.4 Site-directed mutagenesis (SDM)</td>
<td>13</td>
</tr>
<tr>
<td>2.5 Ion pairs in protein thermostabilization</td>
<td>15</td>
</tr>
<tr>
<td>2.6 Organic solvent proteases</td>
<td>16</td>
</tr>
<tr>
<td>2.7 Effect of temperature on enzyme activity</td>
<td>23</td>
</tr>
<tr>
<td>2.8 Purification of proteases</td>
<td>24</td>
</tr>
<tr>
<td>2.9 Application of proteases</td>
<td>27</td>
</tr>
<tr>
<td>2.10 Protein crystallization</td>
<td>28</td>
</tr>
<tr>
<td>2.10.1 History and application in protein crystallography</td>
<td>28</td>
</tr>
<tr>
<td>2.10.2 Principle of protein crystallization</td>
<td>30</td>
</tr>
<tr>
<td>2.10.3 Parameters in crystallization</td>
<td>32</td>
</tr>
<tr>
<td>2.10.4 Bottleneck in protein crystallization</td>
<td>36</td>
</tr>
<tr>
<td>2.10.5 Temperature screening for protein crystal</td>
<td>38</td>
</tr>
<tr>
<td>2.10.6 Crystallization of serine protease and complex with inhibitor</td>
<td>40</td>
</tr>
<tr>
<td>2.11 W200R protease</td>
<td>41</td>
</tr>
</tbody>
</table>
3 MATERIALS AND METHODS

3.1 Materials

3.1.1 List of media and chemicals
3.1.2 Equipments
3.1.3 Kits

3.2 Preparation of media, buffers and solutions

3.3 Screening of the recombinant W200R protease

3.3.1 Microorganisms and culture condition
3.3.2 Plasmid extraction
3.3.3 Single colony PCR
3.3.4 Purification of the PCR product
3.3.5 Restriction enzyme analysis
3.3.6 Observation on skim milk agar

3.4 Culture preparation for recombinant F1 protease and W200R protease

3.5 Purification of recombinant F1 protease and W200R protease

3.5.1 Purification of recombinant F1 protease
3.5.2 Purification of W200R protease

3.6 Protease Activity

3.7 Determination of protein concentration

3.7.1 Preparation of protein reagent
3.7.2 Protein assay

3.8 Polyacrilamide gel electrophoresis

3.9 Activity staining gel

3.10 Characterization of W200R protease

3.10.1 Comparison studies of recombinant F1 and W200R protease

3.10.1.1 Optimal temperature for enzyme activity
3.10.1.2 Thermostability of the enzyme
3.10.1.3 Organic solvent stability of the enzyme

3.10.2 Optimal pH for W200R
3.10.3 pH stability for W200R protease
3.10.4 Enzyme inhibitor

3.11 Crystallization of W200R protease

3.11.1 Screening for crystallization condition
3.11.2 Temperature effect on crystal growth
3.11.3 Co-crystallization of W200R protease with inhibitor
3.11.4 X-ray diffraction

4 RESULTS AND DISCUSSION

4.1 Screening of W200R protease

4.2 Purification of recombinant F1 protease and W200R protease

4.2.1 Purification of recombinant F1 protease
4.2.2 Purification of W200R protease 70
4.3 N-terminal sequencing of W200R protease 82
4.4 Characterization of W200R protease 84
  4.4.1 Comparison studies of recombinant 84
    F1 and W200R protease
    4.4.1.1 Optimal temperature for protease activity 84
    4.4.1.2 Thermostability of enzyme 85
    4.4.1.3 Stability of W200R protease in 89
      various organic solvents
  4.4.2 Effect of pH on enzyme activity 92
  4.4.3 pH stability of enzyme 94
  4.4.4 Inhibitors of W200R protease 96
4.5 Homogeneity of protein for crystallization 98
4.6 Crystallization of W200R protease 102
  4.6.1 Crystallization screening of W200R protease 102
  4.6.2 Temperature effect on crystal growth of 106
    W200R protease
  4.6.3 Co-crystallization of W200R protease 112
    with inhibitor

5 CONCLUSIONS 117

REFERENCES 120
APPENDIX 1 139
APPENDIX 2 140
APPENDIX 3 141
PUBLICATIONS 146
BIODATA OF STUDENT 158