UNIVERSITI PUTRA MALAYSIA

EFFECTS OF STRUCTURAL MODIFICATION ON THE THERMOSTABILITY OF F1 PROTEASE FROM *Bacillus stearothermophilus* F1

NOOR AZLINA IBRAHIM

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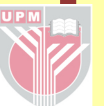

EFFECTS OF STRUCTURAL MODIFICATION ON THE THERMOSTABILITY OF F1 PROTEASE FROM *Bacillus stearothermophilus* F1

By

NOOR AZLINA IBRAHIM

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EFFECTS OF STRUCTURAL MODIFICATION ON THE THERMOSTABILITY OF F1 PROTEASE FROM *Bacillus stearothermophilus* F1

By

NOOR AZLINA BINTI IBRAHIM

August 2007

Chairman: Professor Abu Bakar Salleh, PhD

Faculty: Biotechnology and Biomolecular Sciences

A thermophilic *Bacillus stearothermophilus* F1 was found to produce an extremely thermostable serine protease. The F1 protease sequence was modeled onto the crystal structure of thermitase with 61% sequence identity. The F1 protease contains a catalytic triad comprising of Asp39, His72 and Ser226. The predicted structure of F1 protease comprised 10 α-helices and 10 β-sheets arranged in a single domain. Comparison of the predicted 3D structure of F1 protease with the crystal structure of serine proteases from mesophilic bacteria and archaea, led to the identification of features related to protein stabilization. Higher themostability was found to be correlated with an increased number of residues involved in ion pairs or networks of ion pair. In order to investigate F1 protease stability, two mutated (W200R and D58S) F1 protease were designed. The analysis of molecular dynamics simulations of W200R mutant revealed that an

additional three new ion pairs between Arg200 and Asp202, however there was no ion pair interaction at position Ser58. To confirm the role of ion pair, site-directed mutagenesis was carried out. Both mutated F1 proteases were designed, cloned into pGEX-4T1 and expressed in *E. coli* BL21 (DE3) pLysS. The optimum expression level for the wild type F1 protease, W200R and D58S mutants were 94 U/mL, 112 U/mL and 68 U/mL, respectively. The wild type F1 protease, W200R mutant and D58S mutant were purified by affinity chromatography and heat-treatment. A single band was visible at SDS-PAGE at approximately 33.5 kDa. The purified wild type, W200R mutant and D58S mutant showed 95%, 115% and 64% recovery with purification fold of 21.7, 33.8, and 17.2, respectively. In the presence of 2 mM $CaCl_2$, the wild type had half-lives of 60 min and 7 min at 85 ºC and 90 ºC. Meanwhile, the W200R mutant had half-lives of 75 min and 12 min at 85 °C and 90 ºC, which was more stable than wild type. The stability of W200R mutant was 1.25 times higher than that of the F1 protease. The enhanced thermostability can be correlated to the increase in the number of residues involving ion pairs and ion pairs networks. In contrast, the D58S mutant showed half-life of 45 min at 85 ºC but there was no enzymatic activity at 90 ºC. Thus, the D58S mutant was less thermostable than that wild type which could be due to the removal of ion pairs on this mutated F1 protease (computational work). Far-UV CD at 221 nm was used to detect the denatured proteins. As the temperature was increased from 50 °C to 90 °C, the change in ellipticity at 221 nm revealed a sigmoidal monophasic transition curve of mutant's proteins which indicated unfolding of a protein.

The melting point at pH 8.0 of the wild type F1 protease, W200R mutant and D58S mutant were 70 °C, 72 °C and 63 °C, respectively. Far-UV CD measurements studies indicated the overall features of the secondary structure of protein. The wild type contains 25.5% of α-helix, 17.7% of β-sheet, 22.5% of turn and 34.0% of random coil. There was some loss of β-sheet in W200R but a 3.8% and 1.7% increase in an α-helix and a random coil, respectively, indicated that Arg200 stabilized the α-helix content. The β-sheet was reduced to less than 4% and random coil rose up to more than 8%. A small decrease in the α-helix was observed in D58S mutant. This could be due to the substitution of Asp58 to Ser causing disruption of four ion pairs between Asp58 and Arg103, located at β-sheet. The results obtained confirmed the important role of intermolecular ion pairs in the stability of the whole structure of F1 protease. These results also showed that simulation procedures and molecular biology techniques can be used together to direct protein engineering and/ or site-directed mutagenesis.

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

KESAN-KESAN PENGUBAHSUAIAN STRUKTUR KE ATAS KESTABILAN PROTEASE F1 DARI *Bacillus stearothermophilus* F1

Oleh

NOOR AZLINA BINTI IBRAHIM

Ogos 2007

Pengerusi: Profesor Abu Bakar Salleh, PhD

Fakulti: Bioteknologi dan Sains Biomolekul

Bacillus stearothermophilus F1 yang termofilik dilaporkan menghasilkan serin protease yang amat thermostabil. Jujukan protease F1 dimodelkan berdasarkan struktur kristal thermitase yang mempunyai 61% identiti jujukan. Protease F1 mengandungi triad katalitik, iaitu Asp39, His72, Ser226. Struktur jangkaan bagi protease F1 mengandungi 10 bebenang-β and 10 heliks-α yang disusun dalam satu domain. . Perbandingan struktur jangkaan bagi protease F1 dengan struktur serin protease dari bakteria mesofilik dan arkea, membolehkan pengenalpastian ciri-ciri berkaitan dengan kestabilan protein. Kestabilan yang tinggi dijumpai berkait rapat dengan pertambahan nombor-nombor residu yang terlibat dalam pasangan ion atau jaringan pasangan ion. Bagi mengkaji kestabilan protease F1, dua mutasi (W200R and D58S) protease F1 direka. Analisis dari simulasi dinamik molekul bagi mutasi W200R didapati terdapat tiga penambahan

pasangan ion baru di antara Arg 200 dan Asp 202, manakala tiada pasangan ion berinteraksi pada kedudukan 58. Untuk memastikan peranan pasangan ion, mutasi tapak-terarah dilaksanakan dan dipelajari ke atas mutasi-mutasi ini. Kedua-dua mutasi protease F1 ini dicipta, diklon ke dalam pGEX-4T1 dan diekspres dalam *E. coli* BL21 (DE3) pLysS. Tahap pengekspresan yang optimum bagi protease F1, mutasi W200R dan mutasi D58S masing-masing ialah 94, 112 and 68 U/mL. Ketiga-tiga protein ini ditulenkan dengan kromatografi afiniti dan rawatan haba. Satu jalur protein wujud pada SDS-PAGE, pada kedudukan 33.5 kDa. Kesemua protein tulen ini menunjukkan perolehan semula protein masing-masing sebanyak 95, 115 dan 64% dengan tahap ketulenan sebanyak 21.7, 33.8 dan 17.2. Dengan kehadiran 2 mM $CaCl_2$, protein asli menunjukkan jangka hayat selama 60 min dan 7 min pada 85 ºC and 90 ºC. Manakala, mutasi W200R mempunyai jangka hayat selama 75 min dan 12 min pada 85 ºC and 90 ºC, di mana lebih stabil daripada protein asli. Dalam kes ini, mutasi W200R lebih stabil sebanyak 1.25 kali berbanding protein asli. Kestabilan ini didapati berkait rapat dengan penambahan nombor residu yang melibatkan pasangan ion dan jaringannya. Berlainan dari mutasi D58S yang menunjukkan jangka hayat selama 45 min pada 85 ºC dan tiada aktiviti protease dikesan pada 90 ºC. Maka, mutasi D58S kurang termostabil dari protease asli. Keputusan ini mungkin disebabkan kemusnahan pasangan ion pada mutasi D58S. CD UV-jauh pada 221 nm digunakan untuk mengesan kemusnahan protein protease asli, mutasi W200R dan mutasi D58S. Peningkatan suhu dari 50 hingga 90 °C,

menunjukkan lengkok berbentuk ‘sigmoidal monophasic’ yang menunjukkan proses pencairan bagi protein. Titik penyahaslian bagi protease asli, mutasi W200R dan mutasi D58S pada pH 8.0 masing-masing ialah 70, 72 dan 63 °C. CD UV-jauh juga membenarkan penilaian bagi keseluruhan struktur sekunder protein. Protease asli mengandungi 25.5% heliks-α, 17.7% bebenang-β, 22.5% lingkaran dan 39.7% struktur rawak. Terdapat sedikit kehilangan bebenang-β bagi mutasi W200R tetapi terdapat peningkatan sebanyak 3.8% dan 1.7% dalam α-heliks dan struktur rawak, menunjukkan Arg200 menstabilkan kandungan α-heliks. β-bebenang menunjukkan penurunan kurang dari 4% dan struktur rawak meningkat lebih daripada 8% tetapi terdapat sedikit penurunan dalam α-heliks dalam mutasi D58S. Ini disebabkan penggantian Asp58 kepada Ser menyebabkan kemusnahan empat pasangan ion antara Asp58 dan Arg103 yang terletak di bebenang-β. Keputusan-keputusan ini jelas menunjukkan pentingnya peranan pasangan ion dalam kestabilan keseluruhan struktur protease F1. Keputusan ini juga menunjukkan kaedah simulasi dan teknik-teknik biologi molekul boleh digunakan bersama dalam kejuruteraan protein dan mutasi tapak-terarah.

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I certify that an Examination Committee has met on 30th August 2007 to conduct the final examination of Noor Azlina Binti Ibrahim on her Doctor of Philosophy thesis entitled "Effects of Structural Modification on the Thermostability of F1 Protease from *Bacillus stearothermophilus* F1" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the student be awarded the degree of Doctor of Philosophy.

Members of the Examination Committee were as follows:

Norhani Abdullah, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Zulkarnain Zainal, PhD
Professor
Faculty of Science
Universiti Putra Malaysia
(Internal Examiner)

Rahmita Wirza O.K Rahmat, PhD
Faculty of Computer Science and Information Technology
Universiti Putra Malaysia
(Internal Examiner)

Noorsaadah Abd. Rahman, PhD
Professor
Faculty of Science
Universiti Malaya
(External Examiner)

____
HASANAH MOHD. GHAZALI, PhD.
Professor and Deputy Dean
School of Graduate Studies
University Putra Malaysia

Date: 22 November 2007

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy.

The members of the Supervisory Committee were as follows:

Abu Bakar Salleh, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Raja Noor Zaliha Raja Abdul Rahman, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Mahiran Basri, PhD
Professor
Faculty of Science
Universiti Putra Malaysia
(Member)

Mohd Basyaruddin Abdul Rahman, PhD
Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Member)

AINI IDERIS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 13 Disember 2007

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.

____

NOOR AZLINA BINTI IBRAHIM

Date:

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LIST OF ABBREVIATIONS

3D	three-dimensional
a	adenine
Å	Angstrom
Ala (A)	alanine
Amp	ampicillin
Arg (R)	arginine
Asn (N)	aspragine
Asp (D)	aspartic acid
bp	base pair
c	cytosine
Cα	carbon alpha
Cys (C)	cysteine
°C	degree centrigrade
CD	circular dichroism
dH_2O	distilled water
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
g	gram
g	guanine
Gln (Q)	glutamine
Glu (E)	glutamic acid
Gly (G)	glycine

h	hour
His (H)	histidine
Ile (I)	ileleucine
IPTG	isopropyl-*β*-D-thiogalactopyranoside
kb	kilo base pair
kDa	kilodalton
Leu (L)	leucine
Lys (K)	lysine
mg	milligram
min	minute
ml	mililiter
mM	miliMolar
M	Molar
Met (M)	methionine
MD	molecular dynamics
MW	molecular weight
ng	nanogram
NMR	nuclear magnetic resonance
pI	isoelectric point
Phe(F)	phenylalanine
Pro (P)	proline
PCR	polymerase chain reaction
rpm	revolutions per minute
RE	restriction enzyme

RMSD	root means square deviation
s	second
Ser (S)	serine
SDM	site-directed mutagenesis
SDS	sodium dodecyl sulphate
t	thymine
Thr (T)	threonine
Tm	melting temperature
Trp (W)	tryptophan
Tyr (Y)	tyrosine
TAE	tris acetate-EDTA
TCA	trichloroacetic acid
TEMED	N, N, N, N' tetramethyl-ethylene diamine
µg	microgram
µl	microliter
uv	ultraviolet
U	unit of activity
v/v	volume per volume
V	volts
Val (V)	valine
w/v	weight per volume