RECOMBINANT PRODUCTION AND CHARACTERIZATION OF SUBTILISIN-LIKE SERINE PROTEASE FROM *Acinetobacter baumannii*

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A thesis submitted in fulfilment of the
requirements for the award of the degree of
Master of Philosophy

Faculty of Science
Universiti Teknologi Malaysia

JANUARY 2020

DEDICATION

"My dearest family and friends"

This is for all of you

ACKNOWLEDGEMENTS

My deepest thanks and praise for the blessing of almighty God to whom I indebted for my life, knowledge and thoughts, who has given me strength to stood up and face all the harshness in the pathway of life and research.

In preparing this thesis, I was in contact with many people, researchers, academicians, and practitioners. They have contributed towards my understanding and thoughts. In particular, I wish to express my sincere appreciation to my main thesis supervisor, Dr Haryati Jamaluddin, for invaluable advice, constructive discussion and untiring help throughout the duration of the research. Without her continued support and interest, this thesis would not have been the same as presented here.

I am indebted to my mother, who taught me that even the largest task can be accomplished if it is done one step at a time also to my father, who taught me that the best kind of knowledge to have is that which is learned for its own sake. During this period, they have been the source of courage and confidence for me.

Last but not least, a special thanks to all respected staffs of the Faculty of Science, UTM for their kind and warmness while assisting and providing all sorts of services during the research period. Thus, allowing me to perform my work in happy, healthy and homely environment

My fellow postgraduate student should also be recognised for their support. My sincere appreciation also extends to all my colleagues, Nurulfarhana Hussin, Monaliza Md. Din, Siti Mastura and others who have provided assistance at various occasions. Their views and tips are useful indeed. Unfortunately, it is not possible to list all of them in this limited space.

ABSTRACT

Acinetobacter genus specifically *Acinetobacter baumannii* is notoriously known in the past five decades as one of the most dangerous saprophytic organisms that cause broad arrays of diseases particularly nosocomial infections. One of the factors that may contribute to *A. baumannii* virulence are the secretory proteases that it synthesizes. In order to formulate the effective antibiotics targeting secretory proteases from *A. baumannii*, this protease need to be characterized for functional and structural studies. Therefore, in the present study, a subtilisin-like serine protease isolated from *A. baumannii* designated as "SPSFQ" was cloned, purified and characterized. The nucleotide sequences of *spsfq* revealed 1,104 bp open reading frame corresponding to 368 amino acid residues. Amino acid sequence comparison revealed that SPSFQ shared 38.6 - 60% sequences identity with those of other serine proteases reported to have broad substrate specificity; keratinolytic and collagenolytic properties. Homology model of mature SPSFQ revealed its structure composed of 10 β-strands, 8 α-helices with connecting loops resembling a typical architecture of subtilisin-like α/β motif. Expressed recombinant SPSFQ was purified using a combination of HiTrap HP and Q-Sepharose columns prior to characterization studies. The optimum temperature and pH for SPSFQ activity were achieved at 40°C and pH 9.0, respectively. SPSFQ activity was significantly reduced by phenylmethylsulfonyl fluoride (PMSF) while the presence of Ca^{2+} ion significantly enhanced the activity, suggesting that SPSFQ is a serine protease that require divalent metal ions, Ca^{2+} as a cofactor. Substrate specificity test concluded that purified SPSFQ has high catalytic activity for casein followed by gelatin (hydrolysed collagen) and keratin. As a conclusion, this study suggests that SPSFQ from *A. baumannii* is a potent hydrolytic protease. This data could serve as an impetus for further in-depth study on the function of extracellular proteases and their potential role in *A. baumannii* pathogenicity.

ABSTRAK

Genus *Acinetobacter* khususnya *Acinetobacter baumannii* telah terkenal sejak lima dekad sebagai salah satu organisma saprofit merbahaya yang mengakibatkan pelbagai jenis penyakit terutamanya jangkitan nosokomial. Salah satu faktor yang boleh menyumbang kepada kevirulenan *A. baumannii* adalah penghasilan protease yang dirembeskan. Untuk memformulasikan antibiotik yang berkesan terhadap protease sasaran, protease ini perlu dicirikan untuk kajian fungsi and struktur. Oleh itu, dalam kajian ini, protease serin menyerupai-subtilisin yang dipencilkan dari *A. baumannii* yang dinamakan sebagai SPSFQ telah diklon, ditulen dan dicirikan. Jujukan nukleotida memaparkan satu bingkai bacaan terbuka dengan 1,104 bp dan ini bersamaan dengan 368 asid amino. Perbandingan jujukan asid amino menunjukkan bahawa SPSFQ mempunyai 38.6 – 60% identiti jujukan dengan beberapa protease serin yang dilaporkan mempunyai pengkhususan substrat yang luas; juga memiliki sifat keratinolitik and kolagenolitik. Model struktur homologi SPSFQ matang didapati mempunyai 10 bebenang β, 8 heliks α dan sambungan gelung yang menyerupai seni bina tipikal bagi motif α/β menyerupai-subtilisin. SPSFQ rekombinan yang diekspreskan telah ditulenkan dengan menggunakan gabungan dua turus pemisah iaitu HiTrap HP dan turus Q-Sepharose sebelum kajian pencirian dijalankan. Suhu dan pH optimum bagi SPSFQ yang telah ditulenkan telah direkodkan pada 40°C dan pH 9.0. Aktiviti SPSFQ direncatkan secara ketara oleh fenilmetilsulfonil florida (PMSF) sementara dipertingkatkan dengan kehadiran ion Ca^{2+}, ini jelas menunjukan bahawa SPSFQ merupakan protease serin yang memerlukan ion logan dwivalen, Ca^{2+} sebagai ko-faktor. Ujian kekhususan substrat menyimpulkan bahawa SPSFQ mempunyai aktiviti pemangkin yang tinggi ke atas kasein diikuti oleh gelatin (hidrolisis kolagen) dan keratin. Sebagai kesimpulan, penyelidikan ini mencadangakan bahawa SPSFQ daripada *A. baumannii* merupakan protease hidrolitik yang kuat. Data ini boleh dijadikan sebagai pemangkin untuk mengkaji lebih lanjut mengenai peranan dan fungsi protease dan penglibatan protein ini dalam kepatogenan *A. baumannii*.

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LIST OF ABBREVIATIONS

A.	-	*Acinetobacter*
BLAST	-	Basic Local Alignment Search Tool
BSA	-	bovine serum albumin
CV	-	Column volume
DNA	-	deoxyribonucleic acid
EDTA	-	Ethylenediaminetetraacetic acid
et al.	-	and friends
His	-	Histidine
i.e	-	in that
IPTG	-	Isopropyl β-D-1-thiogalactopyranoside
LB	-	Luria-Bertani
MWCO	-	molecular weight cut-off
NaCl	-	sodium chloride
NaOH	-	sodium hydroxide
OD	-	optical density
ORF	-	open reading frame
PAGE	-	Polyacrylamide gel electrophoresis
PBS	-	Phosphate Buffer Saline
PMSF	-	phenylmethanesulfonylfluoride
SDS	-	sodium dodecyl sulfate
SDS-PAGE	-	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sp.	-	species
TEMED	-	N,N,N',N'-tetramentylene-ethylenediamine
Tris	-	tris(hydroxymethyl)methylamine
WHO	-	World Health Organization

LIST OF SYMBOLS

bp	-	Base pair
cm	-	centimetre
Da	-	dalton
g	-	gramme
H	-	hour
kDa	-	kilodalton
L	-	litre
M	-	molar
mg	-	milligramme
min	-	minute(s)
mL	-	millilitre
mM	-	Millimolar
Mw	-	Molecular weight
R^2	-	coefficient of determination
rpm	-	revolutions per minute
U	-	unit of enzyme activity
V	-	volt
v/v	-	volume per volume
w/v	-	weight per volume
µg	-	microgramme
µL	-	microlitre
µmol	-	micromole
%	-	percentage
xg	-	times gravity
℃	-	degree Celsius
≈	-	Approximate

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CHAPTER 1

INTRODUCTION

1.1 Problem Background

Proteases are natural occurring biocatalysts which ubiquitous in occurrence in all living organisms (Hinneman and Norskov, 2006). Apart from maintaining cellular function and differentiation, proteolytic enzymes are crucial in providing accessible peptide nutrient and defense mechanism for microorganism (Sabotic and Kos, 2012). Investigation of novel proteases with unique catalytic properties has been continued to be the central issue not only in scientific field of protein chemistry but also in applied field such as pharmaceutical, agrochemical, bulk chemical and nutraceutical (Sawant and Negendran, 2014). With unique attributes such as low substrate specificity, highly stable in alkaline and organic solvent and high thermostability, serine protease specifically produced by bacteria has draws the attention of researchers worldwide (Gupta, Beg and Lorenz, 2002). Thus, becoming the most common class of peptidase that has been studied to date (Rawling et al., 2017).

All 57 species fall under *Acinetobacter* genus are generally known as an obligate aerobic, glucose-nonfermenting cocco-bacilli Gram-negative bacterium (Doughari et al., 2011). Owing to its potential to survive in adverse environmental condition, bacteria of the genus *Acinetobacter*, specifically *A. baumannii, A. pittii and A. nosocomialis* have emerged as leading cause of nosocomial (hospital-acquired) infections (Almasaudi, 2018). Though, the natural habitat of clinically relevance multi-resistant *Acinetobacter* spp. are yet to be defined, this genus has been well recognized as ubiquitous microorganism (Hrenovic et al., 2014). According to *Bergey's Manual of Systematic Bacteriology*, members of *Acinetobacter* genus are considered as free-living saprophytes, which make up part of the normal flora of various habitats in the environment such as soil, water, food and sewage (Juni, 2015).

Due to the clinical importance manifest by *Acinetobacter* genus especially *A. baumannii*, researchers are extensively focused on studying the epidemiology and antibiotic resistance of the species. The discovery of an extracellular coagulation targeting metallo-endopeptidase (CpaA) expressed by clinically isolated *A. baumannii* showed that the protease was able to cleave purified factor F(V) and fibrinogen to hinder the coagulation cascade in human plasma (Tilley et al., 2014). This unbalance production of coagulation-related peptidase disrupt well-regulated hemostasis mechanism that acts as 'first-line' defence system in human against bacterial infections (Tilley et al., 2014). While, secreted serine protease termed as 'PKF' was found to suppress the formation of biofilm in the early stage of attachment and dispersing it prior complex matrix formation (King, Pangburn and McDaniel, 2013). Protease with potential to manipulate biofilm formation provides a check and balance mechanism in order to ensure that biofilm composition occur under optimum condition that are favourable to the survival of the bacteria (King et al., 2013).

While limited information available in open literature concerning production of degradative enzyme by this bacterium that could facilitate cellular growth by liberating complex nutrient available from the external environment. In light of this back drop, presented data here assume significance. In this context an attempt was made particularly aimed at an extracellular serine protease, termed as SPSFQ from *A. baumannii* by analyzing its primary sequence and 3-dimensional structure using bioinformatics tools. Functional characterization of the protease was also analysed using standard biochemical assay under different process parameters.

1.2 Aims of Study

The objectives of the research are:

(a) Amplification and cloning of selected gene encoding exoenzyme designated as *spsfq* into *Escherichia coli* expression plasmid.

(b) Analyze *spsfq* gene and gene product using bioinformatics tools

(c) Perform expression of recombinant SPSFQ and purification of the enzyme via affinity and ion-exchange chromatography

(d) Characterization of active recombinant SPSFQ protease

1.3 Scope of Study

In this work, a set of degenerated primer were designed in order to amplify putative extracellular subtilisin-like serine protease encoding gene from *A. baumannii* genome. Respective enzyme encoding gene designated as *spsfq* was cloned into the bacterial expression plasmid, pET22b (+) in-frame with the internal polyhexahistidine tag (His**.**tag). The recombinant plasmid was then transformed into competent *E.coli* BL21(DE3) strain and confirmation for correct orientation of the cloned gene was carried out by PCR technique and sequencing.

This research continued with preliminary structural studies of SPSFQ using *in silico* approach. Several bioinformatics softwares were employed to analyse the gene sequence as well as predicted protein structure. By doing this *in-silico* studies, more biological data can be combined to form a comprehensive characterisation of the protein of interest. The data presented in this thesis consisted of nucleotide and amino acid sequences which involve DNA and protein sequence alignment, protein domain identification and a predicted 3-D homology model of the SPSFQ enzyme.

Escherichia coli BL21(DE3) strain containing the recombinant construct was used to express recombinant SPSFQ. The recombinant SPSFQ possessing a 6X Histidine-tag was subjected for purification by Hi-Trap column chromatography and ion-exchange chromatography. The molecular weight of the protein of study was estimated by protein prediction software (ProtParam) and was supported by *in vitro* studies via SDS-PAGE technique. Purified recombinant SPSFQ was subjected to protease assays in order to evaluate its characteristics.

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