MOLECULAR VERIFICATION AND BIOINFORMATIC ANALYSIS OF A SERINE PROTEASE GENE FROM *BACILLUS PUMILUS*

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DEDICATION

I dedicate my dissertation work to many people who influenced me differently. First and foremost, to my beloved Mother “Fateheia” who loved me, supported me, encouraged me for every single part of my life, and most importantly, she always prays for me.

I dedicate my work for my Father “Hassan” who have meant and continue to mean so much to me although he is no longer of this world, his memories continue to regulate my life and keep me going.

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ABSTRACT

Enzymatic wound debridement is a promising effective approach; however, there is a limited number of enzymes that can be used for debridement, and many existing enzymes in the market can cause side effects. Cardiovascular diseases are the main causes of death and disability. It is usually associated with an increased risk of blood clots. The current medications are limited in number and have serious bleeding risks. This study is focused on the verification of the previously cloned serine protease gene via PCR, sequencing the purified plasmid, and analyzing its amino acid sequence through different bioinformatics tools. Eventually, this protease is concluded to have the potential to be a new wound debridement and clot-dissolving agent that can be more affordable, effective, with a better side effects profile. The serine protease gene from *Bacillus pumilus* was previously cloned into pET-21b expression vector and transformed in *E. coli* HST08. The vector was successfully extracted from the host bacteria and quantified by Nanodrop spectrophotometer to record a concentration of 92.9 ng/µl, with acceptable 260/280 and 260/230 ratios; 1.85, 2.22 respectively. The purified plasmid was then used as a DNA template and subjected to amplification via PCR. The amplified gene resolved in the agarose gel and was found to constitute approximately 1077 bp. The sequencing result was showed 100% identity to serine protease from *Bacillus pumilus* (Accession No: kll00441.1). A bioinformatics study was conducted to generate fundamental data that will give some insights into the protein nature and structure. Cytoplasmic, transmembrane, and non-cytoplasmic regions were predicted by the Phobius online tools. ProtParam tool computed the molecular weight of the protease to be 37.8 kilodaltons, acidic in nature, moderately hydrophilic, and have a good stability index. SMART online tool predicted that the serine protease contains two highly conserved domains; trypsin-like peptidase, and PDZ domains. The 3D model of the serine protease was generated based on the HtrA protease Deg1 structure through the Swiss model server. The proposed model passes all three quality validation methods (Errat, Verify 3D, and Procheck). The three catalytic residues at the active site of the enzyme are found to be His^{91}, Asp^{121}, and Ser^{202}. Additionally, the generated model was superimposed to the human tissue plasminogen activator (Currently used thrombolytic agent) to propose a structure-function relationship. The superimposition between the two structures was good, and the catalytic triad has a good alignment. To conclude, the serine protease was found to be a good candidate as a potential fibrinolytic agent as its sequence was verified successfully with the reference gene, and the generated bioinformatic data have shown it can be expressed and purified successfully for further characterization.

ABSTRAK

Debridemen luka menggunakan enzim adalah pendekatan merawat luka yang berkesan. Tetapi, bilangan enzim yang boleh digunakan untuk tujuan debridemen adalah terhad dan banyak enzim yang terdapat dalam pasaran boleh mengakibatkan kesan sampingan. Penyakit kardiovaskular adalah punca utama kematian dan kecacatan yang selalunya dikaitkan dengan risiko darah beku yang tinggi. Ubat-ubatan yang boleh digunakan untuk rawatan darah beku adalah terhad dan mempunyai risiko pendarahan yang serius. Kajian ini memfokuskan kepada pengesahan gen protease serin yang telah diklon sebelum ini dengan menggunakan kaedah PCR, penjujukan plasmid yang telah ditulenkan dan analisis jujukan asid amino menggunakan alat bioinformatik. Protease ini didapati mempunyai potensi untuk menjadi agen debridemen luka dan pencair darah beku yang baru dan selamat, di mana ianya adalah lebih berkesan, kosnya adalah lebih berpatutan dan profil kesan sampingannya adalah lebih baik. Gen protease serin daripada *Bacillus pumilus* telah diklonkan ke dalam vektor ekspresi pET-21b dan telah ditransformasikan ke dalam *E. coli* HST08. Vektor tersebut telah diekstrak daripada hos bakteria terbabit dan kepekatan plasmid itu telah dicatatkan sebagai 92.9 ng/µl dengan nisbah 260/280 dan 260/230 masing-masing di 1.85 dan 2.22, dengan menggunakan spektrofotometer Nanodrop. Kemudian, plasmid yang telah ditulenkan digunakan sebagai templat DNA di dalam amplifikasi gen menggunakan PCR. Gen yang diamplifikasi telah dipisahkan melalui elektroforesis gel agaros dan saiznya dianggarkan 1077 bp. Hasil jujukan gen pula menunjukkan 100 % identiti kepada protease serin daripada *Bacillus pumilus* (Nombor aksesi: kll00441.1). Kajian bioinformatik pula telah dijalankan untuk menghasilkan data asas yang dapat memberikan informasi tentang sifat dan struktur protein. Kawasan sitoplasma, transmembran dan bukan sitoplasma telah diramalkan menggunakan alat atas talian Phobius. Alat ProtParam pula menganggarkan berat molekul protease serin adalah 37.8 kilodaltons, bersifat asid dan hidrofilik sederhana, dan mempunyai indeks kestabilan yang baik. Alat atas talian SMART pula meramalkan protease serin ini mempunyai dua domain pemangkin yang terpelihara iaitu peptidase yang menyerupai tripsin dan domain PDZ. Model 3D protease serin ini telah dihasilkan berdasarkan struktur HtrA protease Deg1 menggunakan pelayan model Swiss. Model yang dicadangkan lulus ketiga-tiga kaedah pengesahan kualiti (Errat, Verify 3D dan Procheck). Tiga residu pemangkin di laman aktif enzim ini adalah His^{91}, Asp^{121}, dan Ser^{202}. Model yang dihasilkan telah ditindankan kepada pengaktif plasminogen tisu manusia (agen trombolitik semasa) untuk mencadangkan hubungan antara struktur dan fungsi. Penindihan antara dua struktur ini menghasilkan keputusan yang baik dan triad pemangkin yang dihasilkan juga menunjukkan penjajaran yang baik. Sebagai kesimpulan, protease serin ini didapati berpotensi sebagai agen fibrinolitik kerana jujukannya telah disahkan ke atas gen rujukan, dan data bioinformatik yang terhasil menunjukkan enzim ini boleh diekspresi dan ditulenkan dengan jayanya untuk pencirian selanjutnya.

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LIST OF ABBREVIATIONS

1D	-	One-dimension
3D	-	Three-dimensional
AA	-	Amino Acid Sequence
ABD	-	Acetylbutanediol
AMI	-	Acute Myocardial Infarction
API	-	Analytical Profile Index
BLAST	-	Basic Local Alignment Search Tool
bp	-	Base Pair
BSA	-	Body Surface Area
CCA	-	Collagenase Clostridiopeptidase A
CCO	-	Clostridial Collagenase Ointment
DC	-	Dupuytren's Contracture
DFP	-	Diisopropyl Fluorophosphate
DNA	-	Deoxyribonucleic Acid
DNAse	-	Desoxyribonuclease
DVT	-	Deep Vein Thrombosis
E. coli	-	Escherichia coli
EDTA	-	Ethylenediaminetetraacetic Acid
EMA	-	European Medicines Agency
EMBL	-	European Molecular Biology Laboratory
ExPASy	-	Expert Protein Analysis System
FDA	-	Food and Drug Administration
FIX	-	Factor IX
FVIIa	-	Factor VIIa
FVIII	-	Factor VIII
Fwd.	-	Forward
GMQE	-	Global Model Quality Estimation
GRAS	-	Generally Regarded As Safe
GRAVY II	-	Grand Average of Hydropathicity
H_2O_2	-	Hydrogen Peroxide

HSPs	-	Heat Shock Protein
HtrA	-	High Temperature Requirement
IgA	-	Immunoglobulin A
II	-	Instability Index
INR	-	International Normalized Ratio
LB	-	Luria-Bertani
MDT	-	Maggot Debridement Therapy
MEGA	-	Molecular Evolutionary Genetics Analysis
MI	-	Myocardial Infarction
MW	-	Molecular Weight
n.d.	-	No Date
NaOH	-	Sodium Hydroxide
NCBI	-	National Center for Biotechnology Information
NJ	-	Neighbor-Joining
ORF	-	Open Reading Frame
PCR	-	Polymerase Chain Reaction
PDB	-	Protein Data Bank
pI	-	Theoretical Isoelectric Point
PMSF	-	Phenylmethylsulfonyl Fluoride
QMEAN	-	Qualitative Model Energy Analysis
RCSB	-	Research Collaboratory for Structural Bioinformatics
Rev.	-	Reverse
RMSD	-	Root Mean Square Deviation
SMART	-	Simple Modular Architecture Research Tool
SOC	-	Standard of Care
spp.	-	Several Species
TAE	-	Tris-acetate-EDTA
TAME	-	p-tosyl-L-arginine Methyl Ester
TBSA	-	Total Body Surface Area
TLCK	-	N-alpha-tosyl-L-Lysine Chloromethyl Ketone
TNK	-	Tenecteplase
t-PA	-	Tissue Plasminogen Activator
u-PA	-	Urokinase

UV	-	Ultraviolet
VTE	-	Venous Thromboembolism

LIST OF SYMBOLS

~	-	About
α	-	Alpha
Å	-	Angstrom
*	-	Asterisk
β	-	Beta
:	-	Colon
°C	-	Degree Celsius
$	-	Dollar
∞	-	Endless
g	-	Gram
Kb	-	Kilobase
µg	-	Microgram
µl	-	Microliter
µm	-	Micrometer
mg	-	Milligram
ml	-	Milliliter
min	-	Minute
M	-	Molarity / Mole
nm	-	Nano meter
ng	-	Nanogram
.	-	Period
®	-	Registered trademark
rpm	-	Rotation per minutes
s	-	Second
xg	-	Times gravity
™	-	Trademark

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of the Problem

Proteases or peptidases are a ubiquitous class of biological enzymes. Protease has a wide spectrum of hydrolytic actions, and they are selective and specific in their action on their protein substrates. Due to its diversity, proteases have been utilized extensively in three main sectors; research, medical therapeutic, and industries. Proteases are used in the pharmaceutical field, industrial biotechnology, food, and detergent industries. Therapeutically, proteases are used for wound debridement, as a fibrinolytic agent to dissolve blood clots, management of hemophilia, lyse of bacterial biofilm, and for digestion improvement (Jisha *et al.*, 2013; Li *et al.*, 2013).

Wounds frequently occur due to different reasons, including diabetic foot, pressure ulcer, and burns. Wounds are of two major types; acute and chronic wounds and they have different modes of healing. In the UK, there are around 250,000 burn victims per year (Hettiaratchy and Dziewulski, 2004). Moreover, wound burn care is considered expensive care (Sanchez *et al.*, 2008). In the United States, 6 million people have chronic wounds. Diabetic foot wounds are common diabetes mellites complications throughout the world, greatly affecting patients and society's economics (Boulton, 2005). Chronic wound treatment and management are estimated to cost around $9,7 billion in the US in 1 year (Bickers, 2004). Dead tissues that are present within the wound such as necrotic tissue, eschar, and slough play a vital role in preventing or delaying the natural healing process as they act as a medium for bacteria and form a physical barrier inhibiting new tissue formation (Weir *et al.*, 2007; Shi *et al.*, 2009).

Thrombosis (blood clot) is responsible for three major cardiovascular disorders; ischemic heart disease (Acute coronary syndrome), stroke, and venous thromboembolism (VTE) (SCfWT, 2014). Venous thromboembolism is one of the major global diseases, with about 10 million cases per year. The yearly economic burden of venous thromboembolism in the USA has been estimated to be 7–10 billion US dollars (Grosse *et al.,* 2016). Stroke is one of the serious diseases that lead to death, the most common form of stroke is an ischemic stroke that can lead to irreversible brain damage and death due to the formation of fibrin clot within blood vessels. Thrombosis is generally treated by two types of medications; anticoagulant and thrombolytic agents. Anticoagulants reduce the fibrin clot formation, hence preventing stroke reoccurrence, while thrombolytic agents can dissolve the already formed fibrin clot within the occluded vessel (Kim *et al.,* 2018).

1.2 Problem Statement

Proteases from bacteria have many potential applications in the medical field, especially in wound debridement and cardiovascular disease treatment. Therapies that currently exist for both medical conditions are limited and have several disadvantages hence there is a need to find alternative improved therapies from sustainable sources such as bacteria to treat those conditions.

Wound debridement, especially in the chronic one, is the most important parameter to aid healing. Current conventional practices for wound debridement include; mechanical debridement, autolytic debridement, biosurgical debridement, surgical /sharp debridement, and the enzymatic debridement (Stephen-Haynes and Thompson, 2007). These debridement methods are suffering from some limitations, such as pain, bleeding, and damage to the healthy tissues associated with surgical debridement (Ayello and Cuddigan, 2004). Autolytic debridement is slow and costly as it needs multiple dressing applications. No selectivity for both tissue types (dead and healthy) in mechanical debridement, and also it is a time-consuming process (Gwynne and Newton, 2006). More recently, maggots therapy has been developed as a more efficient treatment for unresponsive leg and foot ulcers (Sherman, 2003).

However, it still has many major drawbacks such as increased pain and/or exudate, not applicable to wounds with exposed blood vessels, and the use of life maggots can be deemed uncomfortable and distasteful to some patients and health care practitioners (Brown, 2013). The enzyme debridement method is safer, effective, easy to apply, and it is a good alternative when surgery or sharp debridement is not a debridement option. Nevertheless, only a limited number of approved debriding enzymes are available commercially, such as collagenase and bromalin products, and most of them suffer from some drawbacks like being nonselective, pain, have an allergic reaction, or the ability to digest only one type of protein substrate like collagen (Wright and Shi, 2003; Smith, 2008).

In the cardiovascular disease front, presently the only FDA approved stroke treatment is tissue-type plasminogen activator (t-PA), Alteplase. Tissue-type plasminogen activator is a serine protease. It has a vital role in the blood coagulation process (Lin and Hu, 2014; Kim *et al.,* 2018). Despite the effectiveness of the t-PA in stroke treatment, however, it can cause serious adverse reactions. According to FDA, Alteplase (Activase®) can cause; (i) Internal bleeding including; retroperitoneal, intracranial, genitourinary, respiratory and gastrointestinal bleeding, or external bleeding at the puncture site in the veins and arteries. (ii) Orolingual angioedema has been reported in acute myocardial infarction and ischemic stroke cases ("Label for ACTIVASE (alteplase) for injection - FDA", 2015). Up to date, there are no successful t-PA alternatives for stroke management that overcome t-PA side effects (Wu *et al.,* 2009; Kotb, 2015).

Anticoagulants as well can cause serious side effects. The most common and serious adverse reaction of anticoagulants is bleeding, as in the case of warfarin (The most used drug) (Wysowski *et al.,* 2007). Warfarin has a narrow therapeutic index meaning that small changes in systemic concentration can lead to significant changes in pharmacodynamic response, i.e., inability to maintain international normalized ratio (INR) between 2 and 3 can predispose to either thrombosis (INR<2) or hemorrhage (INR>3) (Schachter and Pirmohamed, 2012). The injectable anticoagulant drug, heparin suffer as well from side effects due to its broad biological actions such as bleeding (major adverse reaction), skin lesions, thrombocytopenia, osteoporosis,

allergic and anaphylactic reactions, the elevation of liver enzymes, and alopecia (Alban, 2012).

Despite the efficacy of controlling these life-threatening diseases by current medications, however, there are still serious side effects, mainly bleeding, so they have to be used carefully, and the dose must be monitored carefully and precisely. Therefore, there is a great need to investigate new and safe thrombolytic agents, primarily proteases, since there are broad sources for proteases, especially the microbial fibrinolytic enzymes have attracted a great deal of therapeutic enthusiasm during the last decade (Dhamodharan and Naine, 2019). Many studies showed that a different type of proteases could be a good potential fibrinolytic agent (Khan *et al.*, 2019; Chandramohan *et al.*, 2019; Thu *et al.*, 2020). Accordingly, this study is focused on the verification of the previously cloned serine protease gene from *Bacillus pumilus* by PCR amplification and gene sequencing as well as the study of the protein gene product using bioinformatic tools. Results from this research will provide information through bioinformatic analysis on the potential of this protease as a wound debridement agent or fibrinolytic agent for cardiovascular therapies.

1.3 Objectives of the Study

a) Verification of the cloned serine protease gene via PCR and sequencing.
b) Protein bioinformatics analysis of cloned serine protease gene product.

1.4 Significance of the Study

This work is proposed to verify the serine protease gene previously cloned by Nur Syafiqah Muhammed, 2019 (unpublished data) through PCR and sequencing. The serine protease gene was cloned from *Bacillus pumilus* that isolated from local Malaysian traditional food, Belacan. Additionally, this study will provide some fundamental biological data by using different bioinformatic tools and software to find out more about gene sequence, different physical and chemical properties, identify domains, and most importantly to predict the protein 3D structure by using homology modeling to propose the structure-function relationship as a fibrinolytic agent, and to the best of our knowledge, this gene has not been cloned and studied before. This recombinant serine protease can be then expressed, and purified in large quantities for further structural and functional characterization research in the hope of developing a new wound debridement and safe clot-dissolving agent that can be more affordable, acceptable, effective, and with better side effects profile. Moreover, this study is part of a project that eventually aims to increase the therapeutic options for the limited number of the current wound debridement enzymes and fibrinolytic drugs, as the current medications for clot-dissolving are suffering from many side effects, mainly bleeding, while the wound debridement approaches, have many limitations like pain, bleeding, slow and costly, no selectivity for both tissue types (dead and healthy), time-consuming or unacceptability by the patients.

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Appendix B Media and Buffer Solutions

APPENDIX B1 MEDIA

i Luria-Bertani (LB) Broth

25 gram of LB broth was dissolved in 1000 ml of distilled water, autoclaved and store at 4°C.

ii Luria-Bertani (LB) Agar

37 gram of LB agar was dissolved in 1000 ml of distilled water, autoclaved and store at 4°C.

APPENDIX B2 Working Buffer

i 0.5 M EDTA (250 ml, pH 8.0)

46.5 g EDTA
200 mL Distilled water
The mixture was stirred vigorously using magnetic stirring. pH was adjusted to 8.0 using NaOH. The volume was brought up to 250 mL with distilled water, stored at room temperature.

ii 10X TAE Buffer (1000 mL)

20 ml 0.5M EDTA (pH 8.0)
48.4 g Tris base (hydroxymethyl) aminomethane
11.42 ml Glacial acetic acid
All components were added together then the volume was made up 1000 ml with distilled water. To prepare 1 liter of 1X, 100 ml of 10X TAE buffer stock was added to 900 ml of distilled water, and store at room temperature.

Appendix C General Procedure

APPENDIX C1 Plasmid Purification Protocol using NucleoSpin® Plasmid (Macherey-Nagel, Germany)

i Cultivate and Harvest Bacterial Cells

Use 5–10 mL of a saturated *E. coli* LB culture. Pellet cells in a standard benchtop microcentrifuge for 30 s at 11000 xg. Discard the supernatant and remove as much of the liquid as possible.

ii Cell Lysis

Add 500 µL Buffer A1. Resuspend the cell pellet completely by vortexing or pipetting up and down. Make sure no cell clumps remain before addition of Buffer A2. Add 500 µL Buffer A2. Mix gently by inverting the tube 6–8 times. Do not vortex to avoid shearing of genomic DNA. Incubate at room temperature for up to 5 min or until lysate appears clear. Add 600 µL Buffer A3. Mix thoroughly by inverting the tube 6–8 times. Do not vortex to avoid shearing of genomic DNA.

iii Clarification of Lysate

Centrifuge for 10 min at 11000 xg at room temperature.

iv Bind DNA

Place a NucleoSpin® Plasmid / Plasmid (NoLid) Column in a collection tube (2 mL) and decant the supernatant from step 3 or pipette a maximum of 750 µL of the supernatant onto the column. Centrifuge for 1 min at 11000 xg. Discard flow-through and place the NucleoSpin® Plasmid Column back into the collection tube. Repeat this step to load the remaining lysate.

v Wash Silica Membrane

Add 500 µL Buffer AW preheated to 50°C and centrifuge for 1 min at 11000 xg. Discard flowthrough and place the NucleoSpin® Plasmid / Plasmid (NoLid) Column back into the collection tube. Add 600 µL Buffer A4. Centrifuge for 1 min at 11000 xg. Discard flow-through and place the NucleoSpin® Plasmid / Plasmid (NoLid) Column back into the empty collection tube.

vi Dry Silica Membrane

Centrifuge for 2 min at 11000 xg and discard the collection tube. Note; residual ethanolic wash buffer might inhibit enzymatic reactions.

vii Elute DNA

Place the NucleoSpin® Plasmid / Plasmid (NoLid) Column in a 1.5 mL microcentrifuge tube and add 50 µL Buffer AE preheated to 70°C. Incubate for 2 min at 70°C. Centrifuge for 1 min at 11000 xg.

APPENDIX C2 Analytical Agarose Gel Electrophoresis

i Weigh 5 gm of the agarose powder and dissolve it in 50 ml of TAE buffer. Wait till cool down and then add 2 µl of Midori Green Advance (Nippon Genetics Europe). Insert the comb in the sealed casting tray and pour the mixture.

ii Once the gel solidified, remove the comb. Put the solid gel in the electrophoresis tank containing 1x TAE buffer.

iii Mix 1-2 µl of the sample and 1 µl of loading dye, then load the sample into the respective wells, and finally add 3 µl DNA ladder.

iv Run the agarose gel at 80 Volte for approximately 50 minutes.

v Visualize the DNA bands under a UV lamp.