MOLECULAR VERIFICATION AND BIOINFORMATICS ANALYSIS OF A METALLOPROTEASE GENE FROM *ACINETOBACTER BAUMANNII*

DIANA JALAL MOHAMMED

A dissertation submitted in partial fulfilment of the
requirements for the award of the degree of
Master of Science

Faculty of Science
Universiti Teknologi Malaysia

NOVEMBER 2020

DEDICATION

“To those who truly love me, to my father, my mother and my sisters”

This is for all of you

ACKNOWLEDGEMENT

First and foremost, praises and thanks to the God, the Almighty, for His showers of blessings throughout my research work to complete the research successfully.

I would like to express my deep and sincere gratitude to my research supervisor, Dr. Haryati Jamaluddin for her patience, motivation, enthusiasm, and immense knowledge. It was a great privilege and honour to work under her guidance. Besides, I would like to thank Siti intan rosdianah Binti damis and Nur syafiqa Binti Muhammad (postgraduate students) for their kind assist during the laboratory session.

I am extremely grateful to my mother for her love, prayers, patience, support, caring and sacrifices for educating and preparing me for my future. I am very much thankful to my sisters for their love, prayers and encouragement to continue my study.

Last not the least, especial thank to my friends who were beside me in my whole master journey since first day, I am deeply grateful for their support, love, patience, and encouragement to continue my study and my research.

ABSTRACT

The accumulation of eschar, dead tissue, microbes on the surface of wound lead to delay the wound healing particularly in diabetic wounds and foot ulcers. The process of removing these dead tissues to enhance the healing process is called debridement. A variety of approaches can be used for debridement including surgical, mechanical, enzymatic and maggot therapy. Recently enzymatic debridement is gaining more attention especially in situations where surgical debridement may not be suitable. The current available enzymatic debridement agents are limited, and they can also cause side effects to patients. Hence, there is a need to search for a new debridement agent that have high efficiency and specificity that can cause lesser side effects, one of the alternative methods is via protease enzymatic debridement. This research focused on verification of a cloned metalloprotease gene via PCR and sequencing as well as characterisation of the metalloprotease gene product via bioinformatic tools. The plasmid containing the cloned metalloprotease gene from *Acinetobacter baumannii* was successfully extracted from *E.coli HSTO8* and amplified via PCR. The metalloprotease gene amplicon showed the correct size of approximately 717 bp on agarose gel. The concentration of the amplified gene was measured by nanodrop spectrophotometer showing that it has a concentration of 96.8 ng/µl, as well as 260/280 and 260/230 ratios of 1.93 and 2.18 respectively. The sequencing analysis result illustrated that the cloned gene is 100% identical to metalloprotease from *Acinetobacter baumannii* (accession number WP_000722324.1). The result of the in silico study showed that, the metalloprotease from *Acinetobacter baumannii* is a membrane protein, consisting of 238 amino acids with estimated molecular weight of 27.2 kDa. The nonpolar amino acids content is higher than the polar amino acids which illustrated that our metalloprotease is hydrophobic in nature, and it is stable with instability index of 39.58. The metalloprotease from *Acinetobacter baumannii* has Zinc-dependent metalloprotease domain Phe^{162} to Asn^{235} which characterized by the presence of zinc binding motive (H^{180}, E^{181}, H^{184}, G^{187}, H^{190}). The generated model consists of five beta sheets and four alpha helixes, alpha helix number three (α 3), alpha helix number four (α 4) and beta sheet number five (β 5) are located in the Zinc-dependent metalloprotease domain. The active site of metalloproteinases group of protein contains a catalytic divalent metal ion which is usually zinc atom, the zinc atom in the generated model attached to the three histidine residues of the active site (H^{180},H^{184} and H^{190}) with distance 2Å, 2.3Å and 2Å respectively. This metalloprotease belonged in the same M12 family as well as having the same catalytic motif as a fibrinolytic enzyme isolated from snake venom which may indicate that this metalloprotease has the potential ability to have fibrinolytic activity.

ABSTRAK

Pengumpulan eskar, tisu mati dan mikrob di atas permukaan luka boleh melambatkan proses penyembuhan luka, terutamanya luka diabetik dan ulser kaki. Proses untuk membuang tisu-tisu mati bagi mempercepatkan penyembuhan luka dikenali sebagai debridemen. Pelbagai kaedah boleh digunakan untuk debridemen termasuklah kaedah pembedahan, kaedah mekanikal, kaedah enzim dan terapi berenga. Sejak kebelakangan ini, debridemen menggunakan enzim telah mendapat perhatian terutamanya di dalam situasi di mana kaedah debridemen melalui pembedahan didapati tidak sesuai. Agen debridemen enzim yang digunapakai sekarang adalah terhad dan boleh mengakibatkan kesan sampingan kepada pesakit. Oleh itu, pencarian agen debridemen baru yang mempunyai kecekapan dan kekhususan yang tinggi, dan yang mampu mengurangkan kesan sampingan menjadi satu keperluan pada masa kini. Salah satu alternatif adalah melalui kaedah debridemen menggunakan enzim protease. Kajian ini tertumpu kepada pengesahan gen metaloprotease yang telah diklon menggunakan kaedah PCR dan penjujukan, dan juga pencirian produk gen metaloprotease tersebut menggunakan alat bioinformatik. Plasmid yang mengandungi gen metaloprotease daripada *Acinetobacter baumanii* yang diklon telah diekstrak daripada *E. coli* HST08 dan gen tersebut telah diamplifikasi menggunakan PCR. Amplikon gen metaloprotease tersebut menunjukkan saiz yang tepat di atas gel agaros, iaitu kira-kira 717 bp. Kepekatan gen yang telah diamplifikasi itu ditentukan menggunakan spektrofotometer nanodrop. Kepekatannya adalah 96.8 ng/µl dan nisbah 260/280 dan 260/230 yang dicatatkan adalah pada 1.93 dan 2.18. Hasil analisis penjujukan pula menunjukkan gen yang diklon itu adalah 100 % sama dengan metaloprotease daripada *Acinetobacter baumanii* (Nombor aksesi WP_000722324.1). Dapatan daripada kajian “insilico” pula menunjukkan metaloprotease daripada *Acinetobacter baumanii* ini adalah protein membran yang mengandungi 238 asid amino dengan anggaran berat molekul 27.2 kDa. Kandungan asid amino tidak berkutub adalah lebih tinggi berbanding asid amino berkutub. Ini menunjukkan metaloprotease ini bersifat hidrofobik dan stabil, dengan indeks ketidakstabilan pada 39.58. Metaloprotease daripada *Acinetobacter baumanii* ini mempunyai domain metaloprotease Phe^{162} sehingga Asn^{235} yang dicirikan oleh kehadiran motif pengikatan zink (H^{180}, E^{181}, H^{184}, G^{187}, H^{190}). Model yang terhasil mengandungi lima helaian beta dan empat heliks alfa. Heliks alfa nombor tiga (α 3), heliks alfa nombor empat (α 4) dan helaian beta nombor lima (β 5) terletak di domain metaloproteinase yang bergantung kepada zink. Laman aktif kumpulan protein metaloproteinase mengandungi ion logam divalent yang selalunya adalah atom zink. Atom zink di dalam model yang terhasil tercantum dengan tiga residu histidin di dalam laman aktif (H^{180},H^{184} dan H^{190}), dengan jarak masing-masing adalah 2 Å, 2.3 Å dan 2 Å. Metaloprotease ini dikelaskan dalam keluarga M12 yang sama dan enzim ini mempunyai motif pemangkin yang sama dengan enzim fibrinolitik yang diasingkan daripada bisa ular. Ini menunjukkan metaloprotease ini berpotensi mempunyai aktiviti fibrinolitik.

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LIST OF ABBREVIATIONS

US	-	United States
UK	-	United Kingdom
PCR	-	Polymerase Chain Reaction
NHS	-	National Health Service
FDA	-	Food and Drug Administration
WHO	-	World Health Organization
u-PA	-	Urokinase Plasminogen Activator
t-PA	-	Tissue Plasminogen Activator
ICH	-	Intracerebral Haemorrhage
NK	-	Nattokinase
StK	-	Streptokinase
rt-PA	-	Recombinant Tissue Plasminogen Activator
TNK-tPA	-	Tenecteplase
MPs	-	Metallopeptidases
NCBI	-	National Center for Biotechnology Information
CCA	-	Collagenase clostridipeptidase A
NICU	-	Neonatal Intensive Care Unit
DGD	-	Debriding Gel Dressing
EPS	-	Extracellular polymeric substances
MDR	-	Multidrug resistant
OMPA	-	An outer-membrane protein
LB	-	Luria-Bertani
EXPAY	-	Expert Protein Analysis System
EMBL	-	European Molecular Biology Laboratory
MEGAX	-	Molecular Evolutionary Genetics Analysis
NJ	-	Neighbour-Joining
Sp.	-	Species
BLAST	-	Basic Local Alignment Search Tool
A.	-	Acinetobacter
PDB	-	Protein Data Bank

His	-	Histidine
kDa	-	Kilodalton
B.	-	Bacillus
RMSD	-	Root Mean Square deviation
et al.,	-	And others
EDTA	-	Ethylenediaminetetraacetic acid
UV	-	Ultraviolet
TAE	-	Tris-acetate-EDTA

LIST OF SYMBOLS

Kb	-	Kilobase
µg	-	Microgram
µl	-	Microliter
µm	-	Micrometre
mg	-	Milligram
ml	-	Milliliter
min	-	Minute
M	-	Mole
nm	-	Nano meter
ng	-	Nanogram
.	-	period
®	-	Registered trademark
rpm	-	Rotation per minutes
Xg	-	Times gravity
MW	-	Molecular Wight
mM	-	millimolar

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Skin is the largest organ in the body that acts as a physical barrier between the external environment and the human body. Skin is protecting the human body from harmful microbes, thermal, mechanical, and chemical damages. Skin damages may occur for several reasons such as burn, and chronic wounds (Nasalapure *et al.,* 2017). Centres for Medicare and Medicaid Services defined chronic wounds as wounds that have not healed within 30 days. In the US, almost 6.5 million patients had a chronic wound and evidently, 25 billion dollars were spent annually on the treatment of the chronic wound (Fauzi *et al.,* 2015). It has been estimated that nearly 2.5 million Americans are inclined to venous ulcers, 1.3 to 3 million are suffering from pressure ulcers, and one million diabetics are at a risk for developing neuropathic ulcers over a 3years period. The cost of chronic wound management is expensive and, in the US the wound management market is estimated to reach up to 4.4 billion dollars in 2019 (Dabiri *et al.,* 2016). Worldwide, around USD 2.8 billion were spent on wound management in 2014 and it is estimated to increase to reach up to USD 3.5 billion by 2021. Globally, the wound management market is expected to further increase to USD 22 billion by 2024 (Sen, 2019).

Wounds can heal naturally, however, failure of these wounds to heal properly can lead to complications like sepsis and osteomyelitis that can be dangerous to patients and challenging to manage as well as the cure for health care providers. (Nusbaum *et al.,* 2012). Dead and necrotic tissue are terms used to describe the tissue without blood supply. Infection, ischaemia, hypoxia, and dehydration of the wound may lead to the accumulation of such tissues (Atkin, 2014). Wound debridement is the procedure of removing the necrotic tissue (devitalized tissue), foreign body and microbes from the chronic wound. Wound debridement is essential first step in the

proper chronic wound treatment (healing), it aims to expose the underlying viable tissue (Doerler *et al.*, 2012; David and Chiu, 2018). Wound debridement minimizes the bacterial burden within the wound, controls the on-going inflammation and malodour, and enhances the formation of granulation tissue. (Madhok *et al*., 2013).

Metalloproteases are one of the most important hydrolytic enzymes. Metalloproteases are used in different industrial applications such as detergents, leathers, food processing, bioremediation, and cosmetics. Moreover, they play a role in the degradation of proteins and involved in the modulation of cell growth, inflammation, immunity, and hormone processing. Also, several metalloproteases are targets for drug development (Vélez-Gómez *et al*., 2019). Metalloprotease are found widely in nature including plants, animals, fungi, and microbial sources that are the most significant source of metalloproteases. Collagenases are microbial protease that originated from *Clostridium histolyticum*. Collagenases have been studied widely as a wound debridement enzyme. It is used effectively in the treatment of third-degree burns, diabetic ulcers, pressure ulcers, and ischemic arterial ulcers (Shi *et al.*, 2010).

1.2 Problem Statement

Debridement is the first step in the process of wound healing by promoting new tissue growth in the wound and preventing infection. Different methods are currently available for removing dead and necrotic tissues such as surgical, mechanical, autolytic, maggot debridement therapy, and enzymatic debridement (Munir *et al.*, 2016; David and Chiu, 2018). Autolytic debridement aims to maintain wound moisture and support the gradual softening of eschar using the natural enzyme present in wound fluid nevertheless, autolytic debridement is slow in action and requires close monitoring as the risk of infection may increase, while surgical debridement can cause serious unwanted effects like bleeding, scarring, and healthy tissue damage. Mechanical debridement is another approach for debridement, it is carried out by applying wet to dry dressings or pressure irrigation (Singh and Singh, 2012; Langer *et al*., 2013; Schulz *et al*., 2017). Labor intensive, painful, time-consuming, and moisture might overstrain the tissues that surround the wound are

considered as the main limitations of mechanical debridement (David and Chiu, 2018). Another option is maggot therapy which has been used widely and has been reported to be gentler and more efficient for wound debridement, however, it has some limitation as it can be painful, not widely available, and patients can feel uncomfortable from the sensation of crawling maggot on their wound during treatment (Paul *et al*., 2009). The utilization of enzymes in wound debridement is a successful alternative method. However, there is a limited option in the market, many side effects like allergy and pain, and selectivity toward one component of the wound (collagenase enzyme is selective against collagen only and not to keratin, fibrin, or fat debris found in necrotic tissue) (Falabella, 2006; Huett *et al*., 2017).

1.3 Objectives of Study

(i) Verification of cloned metalloprotease gene via PCR and sequencing.

(ii) Protein bioinformatics analysis of cloned metalloprotease gene products.

1.4 Scope of Project

The laboratory work of this study started with culturing *E. Coli HSTO8* bacteria containing the recombinant plasmid. The plasmid was extracted by using Wizard® plasmid purification kit (Promega, USA), after that the primers were designed to amplify metalloprotease gene by using polymerase chain reaction (PCR), then the purified plasmid was sent for sequencing to confirm the correct sequence and orientation.

Following laboratory work, bioinformatic analysis was done to study the properties and characteristics of metalloprotease protein. Several tools (software and database) were employed such us Expasy translate tool to obtain the correct reading frame. Protein blast (BLASTP) of NCBI was used to compare and identify the sequence similarity against NCBI protein databases. SignalP 5.0 server to determine

the presence of signal peptides and its cleavage sites and Cello2Go for protein locolization prediction. A phylogenetic tree was generated by using MEGA X, Expasy ProtParam tool to analyse amino acids composition, Phyre^2 online tool and Gor4 were used to predict the secondary structure. NCBI conserved domain for domain prediction, Clustal Omega tool was used to carry out multiple sequence alignment in order to determine the regions of similarity between different amino acid sequences. I-TASSER server was employed to determine or predict the 3D structure of metalloprotease to postulate function of the protein and guide future experimental work. ERRAT and PROCHECK were employed to evaluate the stereochemical quality of the 3D structure.

1.5 Significance of Study

Debridement is generally considered as the essential procedure in the wound healing process (Hsu *et al*., 2015). Several methods of debridement such as autolytic, mechanical, surgical, enzymatic, and maggot's debridement therapy had been introduced (Shi and Carson, 2009; Munir *et al*., 2016; David and Chiu, 2018). Those current conventional methods that are available for wound debridement still have many limitations and disadvantages. Therefore, there is a great need to search for a gentler and more effective debridement method to overcome all of the previous setbacks that can occur with the known conventional methods. An alternative method that has the potential to be developed for wound debridement is debridement using enzymatic proteases. Hence, in this study, the previously cloned gene of a metalloprotease from *Acinetobacter baumannii* TUO4 isolated from *Tapai Ubi* (Malaysian traditional cassava-fermented food) was verified via PCR and sequencing while the properties of the protein gene product were studied by using bioinformatic tools to characterise whether the protein has the potential to be a wound debridement agent.

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