OPTIMIZATION ON THE EXTRACTION OF BACTERIOPHAGE ENDOLYSIN AGAINST *Shigella dysenteriae* FROM CRUDE PHAGE LYSATE

By

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ABSTRACT

OPTIMIZATION ON THE EXTRACTION OF BACTERIOPHAGE ENDOLYSIN AGAINST *Shigella dysenteriae* FROM CRUDE PHAGE LYSATE

Pang Jing Wen

In order to release the viral progeny, bacteriophages produce endolysin which cleaves the cell wall peptidoglycan, resulting in the lysis of host cells. The ability of endolysins to cause cell lysis makes them a potential anti-microbial agent. Therefore, the aims of this study were to optimize the extraction of endolysin from crude phage lysate using different ammonium sulfate saturations and co-precipitated phage deactivation in protein samples via heat treatment at various temperatures and durations. In this study, the SDyM-18 phages were grown and amplified. Next, the crude phage lysate was prepared and the proteins were extracted with 0-20%; 20-40%; 40-60% and 60-80% ammonium sulfate precipitation. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and spot lytic assays were then conducted to determine the presence of endolysin.

Lastly, heat treatment was carried out on both SDyM-18 phage and precipitated protein samples to determine the phage deactivation temperature and to remove the co-precipitated bacteriophages, respectively. Based on the results obtained, it was found that the SDyM-18 phage was deactivated when heated at 70°C for 10 min. However, when the precipitated protein sample heated at 70°C for 8 min, it was shown that the co-precipitated bacteriophage has been completely deactivated. In this study, the lysis zone was observed only on EDTA-treated bacterial cells and this showed that the endolysin can only lyse the cells exogenously in the presence of outer membrane permeabilizer. Therefore, this confirmed that the protein band between 15 and 20 kDa in the 60-80% saturation precipitated protein was the SDyM-18 phage endolysin. This study also showed that the endolysin can withstand temperature up to 70°C for 8 min. In summary, the SDyM-18 phage endolysin was successfully precipitated at 60-80% ammonium sulfate saturation from crude phage lysate. It is a heat stable endolysin and only works in the presence of outer membrane permeabilizer.

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DECLARATION

I hereby declare that the project report 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 UTAR or other institutions.

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PANG JING WEN

APPROVAL SHEET

This project report entitled "**OPTIMIZATION ON THE EXTRACTION OF BACTERIOPHAGE ENDOLYSIN AGAINST *Shigella dysenteriae* FROM CRUDE PHAGE LYSATE**" was prepared by PANG JING WEN and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by:

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(Dr. Michelle Ng Yeen Tan) Date:……

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Date: _____

PERMISSION SHEET

It is hereby certified that **PANG JING WEN** (ID No: **14ADB06456**) has completed this final year project entitled "**OPTIMIZATION ON THE EXTRACTION OF BACTERIOPHAGE ENDOLYSIN AGAINST *Shigella dysenteriae* FROM CRUDE PHAGE LYSATE**" under the supervision of Dr. Michelle Ng Yeen Tan (Supervisor) from the Department of Biomedical Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

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(PANG JING WEN)

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LIST OF ABBREVIATIONS

×*g*	Times gravity (acceleration due to gravity)
ºC	Degrees Celcius
µg/mL	Microgram per millilitre
µL	Microlitre
µm	Micrometer
%	Percentage
APS	Ammonium persulfate
B. anthracis	*Bacillus anthracis*
B. cereus	*Bacillus cereus*
CBD	Cell wall binding domain
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EAD	Enzymatically active domain
EDTA	Ethylenediaminetetraacetic acid
g	Gram
h	Hour
HCl	Hydrochloric acid
ICTV	International Committee for Taxonomy of Viruses

kDa	Kilodaltons
L	Litre
LB	Luria Bertani
LPS	Lipopolysaccharide
M	Molar
M cells	Microfold cells
mA	Milliampere
MDR	Multidrug resistant
min	Minutes
mL	Millilitres
mM	Millimolar
MWCO	Molecular weight cut-off
NaCl	Sodium chloride
nm	Nanometer
OD_{600}	Optical density measure at wavelength 600 nm
OM	Outer membrane
P. aeruginosa	*Pseudomonas aeruginosa*
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pfu/mL	Plaque forming units per millilitre

pH	Power of hydrogen
PMN	Polymorphonuclear leukocytes
S. aureus	*Staphylococcus aureus*
S. dysenteriae	*Shigella dysenteriae*
S. pyogenes	*Streptococcus pyogenes*
SDS	Sodium dodecyl sulfate
sp.	Species
Stxs	Shiga toxins
TBS	Tris-buffred saline
TEMED	Tetramethylethylenediamine
UV	Ultraviolet
VAPH	Virion-associated peptidoglycan hydrolases
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

Shigellosis is a global human health issue. In the developing countries, it is one of the leading causes of mortality and morbidity among children who are below 5 years old (Mandal, et al., 2012). Shigellosis can be caused by enteroinvasive *Escherichia coli* or any one of the *Shigella* species, namely, *Shigella boydii*, *Shigella dysenteriae*, *Shigella flexneri* and *Shigella sonnei*. Among these four main species, *S. dysenteriae* especially serotype 1 is commonly associated with life-threatening complications as compared to others (Niyogi, 2005). This is because *S. dysenteriae* type 1 secretes Shiga toxins (Stxs) which cause vascular damage in the colon, kidneys and central nervous system, eventually results in hemorrhagic colitis or more severe conditions such as hemolytic uremic syndrome (Lamba, et al., 2016).

Nowadays, multidrug resistance issue of *Shigella* sp. has become a concern among scientific community. According to a recent study, *Shigella* serogroups have become resistant to the commonly prescribed inexpensive antibiotics such as ampicillin, tetracycline, co-trimoxazole and chloramphenicol (Kahsay and

Muthupandian, 2016). Antimicrobial agents are the mainstay of therapy for shigellosis. The emerging of multidrug resistance has therefore complicated the treatment of shigellosis as the options of antibiotics that can be used to treat shigellosis are limited. Up to now, only ceftriaxone remains as the drug of choice to treat shigellosis (Shakya, et al., 2016). Unfortunately, there is still no currently available protective vaccine against *Shigella* sp. (Rohmer, et al., 2014). Alternative ways to overcome this serious problem are still under way. One of the possible treatments is to use bacteriophages.

Bacteriophages, which are also known as phages, are typically the viruses that infect bacterial cells and disrupt their metabolism. Eventually, this causes the bacterial cells to burst at the end of the bacteriophage lytic cycle (Sulakvelidze, Alavidze and Glenn Morris Jr., 2001). They are the most abundant organisms with estimated population number about 10^{32} in the world (Shahrbabak, et al., 2013). They are also the most ubiquitous organisms as they are present anywhere including soil, water and within human or animal bodies (Mai, et al., 2015). Like all viruses, phages are obligate parasites, which means their replication occurs only after they infect a bacterium (Sillankorva, Oliveira and Azeredo, 2012). They multiply exponentially inside the bacteria by taking over the cellular machinery which includes protein-synthesizing system and energy-generating system (Sharma, et al., 2017). At the end of the infection cycle, phages release

their newly formed viral progeny through lysis of the host bacterial cells.

The lysis of the host bacterial cells needs two components from the lytic cassette of the phage (Schmelcher and Loessner, 2014). These two components are holins and endolysins. Holin is a small hydrophobic protein which induces the formation of pores in the cytoplasmic membrane by undergoing oligomerization. Whereas endolysin is an enzyme that cleaves the peptidoglycan from within, resulting in rapid lysis (Catalão, et al., 2013). Both of them work together in degrading the bacterial cell wall, in which holin creates holes on the inner membrane to allow access of endolysin to the peptidoglycan (Borysowski, Weber-Dabrowska and Górski, 2006).

The ability of endolysins to cleave the peptidoglycan makes them a potential antimicrobial agent. Purified endolysins can be applied externally to lyse the bacterial cells (Roach and Donovan, 2015). This has been demonstrated in one of the studies by Schmelcher, et al. (2012) whereby the exogenous application of purified endolysins to *Staphylococcus aureus* was able to kill the bacteria by causing cell lysis. The lack of outer membrane in Gram-positive bacteria renders them more susceptible to endolysins due to unrestricted access (Tiwari, et al., 2014). In contrast, for the Gram-negative bacteria, an exogenously applied

endolysin usually requires the help of chelating agents such as ethylenediaminetetraacetic acid (EDTA) to alter the outer membrane's permeability, thereby allowing the access of endolysin to the peptidoglycan (Dong, et al., 2015).

Currently, the treatments available for shigellosis are antibiotics and bacteriophage therapy. However, both treatments have their limitations. For instance, antibiotics have lose their effectiveness due to the emergence of multidrug resistant *S. dysenteriae* (Kahsay and Muthupandian, 2016). The lacking of internationally recognized human clinical trials also restricts the use of phage therapy to treat shigellosis (Roach and Donovan, 2015). These limitations have greatly limited their further applications in therapy and therefore urged the researchers to study the potential of using bacteriophage encoded bacteriolytic proteins as antimicrobial agents. Endolysin, which is one of the bacteriolytic proteins produced by bacteriophage, has the potential to be used as alternative antimicrobial agent to treat shigellosis due to its ability to cause cell lysis by cleaving the peptidoglycan cell wall which present exclusively on Gram-negative *S. dysenteriae*.

Therefore, in this study, the optimization of the endolysin extraction from crude phage lysate using ammonium sulfate precipitation method at various saturations was carried out. Secondly, the optimization of co-precipitated phage deactivation in protein samples via heat treatment at various temperatures and durations was also performed.

CHAPTER 2

LITERATURE REVIEW

2.1 Bacteriophages

2.1.1 Discovery of Bacteriophages

In 1896, a British bacteriologist named Ernest Hankin was the first scientist who discovered the bacteriophage in which he found an unidentified substance limiting the spread of *Vibrio cholera* (Sharma, et al., 2017). In 1898, which was two years later, a Russian bacteriologist named Nikolay Gamaleya also observed the same phenomenon on *Bacillus subtilis*. However, similar to Hankin, he was also unable to identify the causative agent of this phenomenon. The doubt remained until year 1915 where Frederick William Twort proposed that such phenomenon could be due to a virus. In 1917, bacteriophage was discovered "officially" by Felix d'Herelle (Sulakvelidze, Alavidze and Glenn Morris Jr., 2001).

2.1.2 Nature of Bacteriophages

Bacteriophages are the viruses that infect prokaryotes, particularly bacterial genera (Sharma, et al., 2017). The basic physical features of a typical bacteriophage are a "head" and a "tail". The "head" is composed of nucleic acids enclosed by a protein coat, called capsid. The nucleic acids, which are the viral genome can be either double-stranded DNA, single-stranded DNA or RNA. The capsid, which is made of capsomeres serves to protect the viral genome. It comes in many forms such as small hexagonal structures or filaments. The "tail" is a hollow tube for the nucleic acids to pass through during the injection stage of phage infection (Goodridge and Abedon, 2003).

Through the years, International Committee for Taxonomy of Viruses (ICTV) has developed a sophisticated bacteriophage classification system to illustrate the diversity of phages (Elbreki, et al., 2014). Many parameters have taken into consideration in phage classification, which include host range, physical characteristics, genetic content, genome size and resistance when classifying the phages (Sharma, et al., 2017). Based on this approach, phages have been organized into 14 different families: *Myoviridae*, *Podoviridae*, *Fuselloviridae*, *Lipothrixviridae*, *Leviviridae*, *Rudiviridae*, *Corticoviridae*, *Cystoviridae*, *Siphoviridae*, *Microviridae*, *Inoviridae*, *Guttavirus*, *Tectiviridae*, and lastly *Plasmaviridae* (Elbreki, et al., 2014). Figure 2.1 provides an overview on the

morphology of all the bacteriophage families.

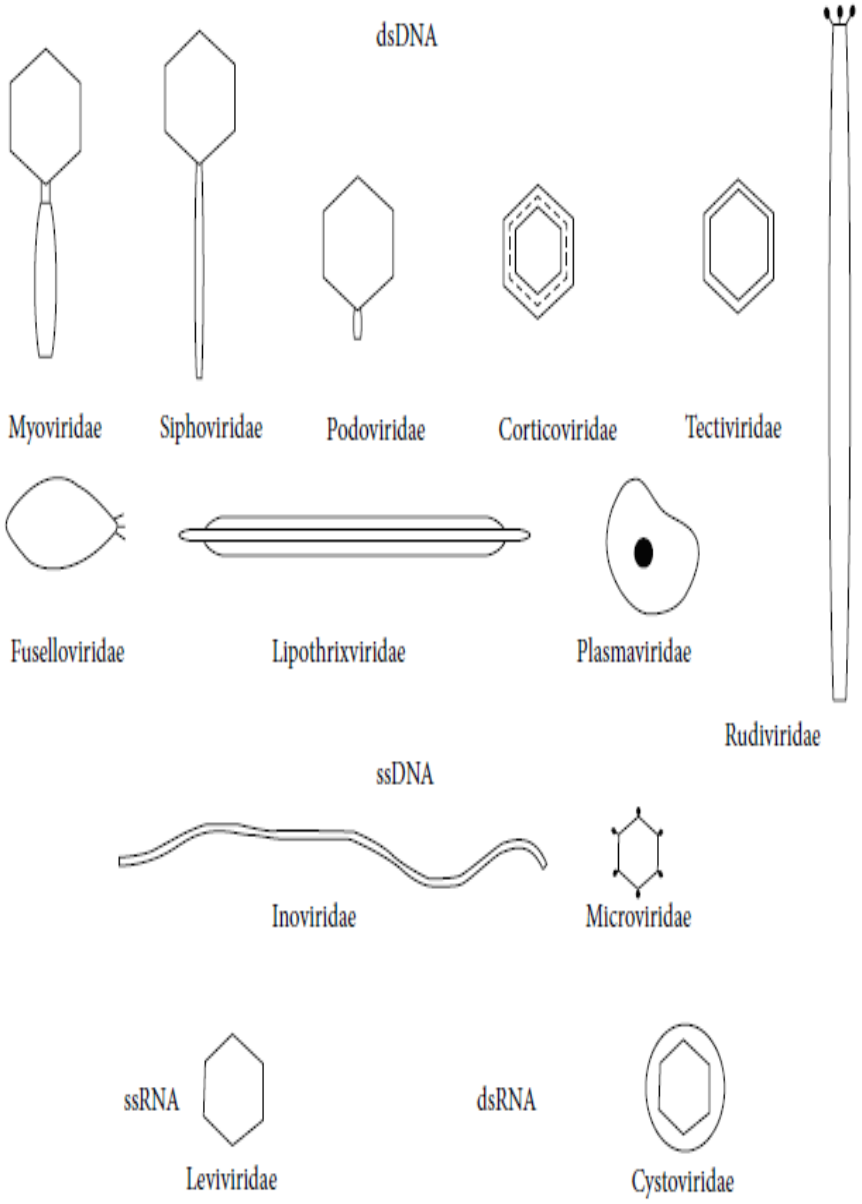

Figure 2.1: The morphology of 14 bacteriophage families. (Adapted from Elbreki, et al., 2014)

2.1.3 Life Cycle of Bacteriophages

Bacteriophages are obligate intracellular parasites. They highly depend on the host cells' biosynthetic machinery to replicate themselves (Roach and Donovan, 2015). Upon infection, phages can exhibit either one of two distinct life cycles depending on its type. Virulent phages will undergo a lytic cycle whereas temperate phages will undergo a lysogenic cycle (Elbreki, et al., 2014).

Regardless of which cycle, the life cycle of phages begins with attachment to their specific bacterial host cells through the interaction between specific receptors molecules on the host cell wall and specific phage proteins on the tip of phage tail (Wittebole, De Roock and Opal, 2014). Once the phages have been adsorbed stably to the cell surface, they begin to create pores in the cell wall to inject their nucleic acids into the host cells.

In the lytic cycle, the life cycle process is then continued with the expression of phage's early genes using the host cell machinery to produce nucleic acids and proteins. Assembly and genome packaging of phages take place after that. Meanwhile, phage's late enzymes like holin and endolysin will be synthesized to release the phage progeny from infected host cells. The lytic cycle is ended by the lysis of bacterial host cells and the liberation of phage progeny to

extracellular environment (Wittebole, De Roock and Opal, 2014).

On the other hand, in the lysogenic cycle, the life cycle process is followed by integration of phage genome into the bacterial chromosome to form prophage. In this state, the phages have established a stable long-term relationship with their host cells (Elbreki, et al., 2014). During cell division, the phage DNA is replicated together with the host's chromosome to ensure continue replication of the genetic material. The phages will not cause any deadly consequences to the infected host because the genes that are harmful to the host bacteria are not being expressed (Sharma, et al., 2017). This relationship will continue until the lytic cycle is initiated (Wittebole, De Roock and Opal, 2014). Figure 2.2 shows the life cycle of both virulent and temperate bacteriophages.

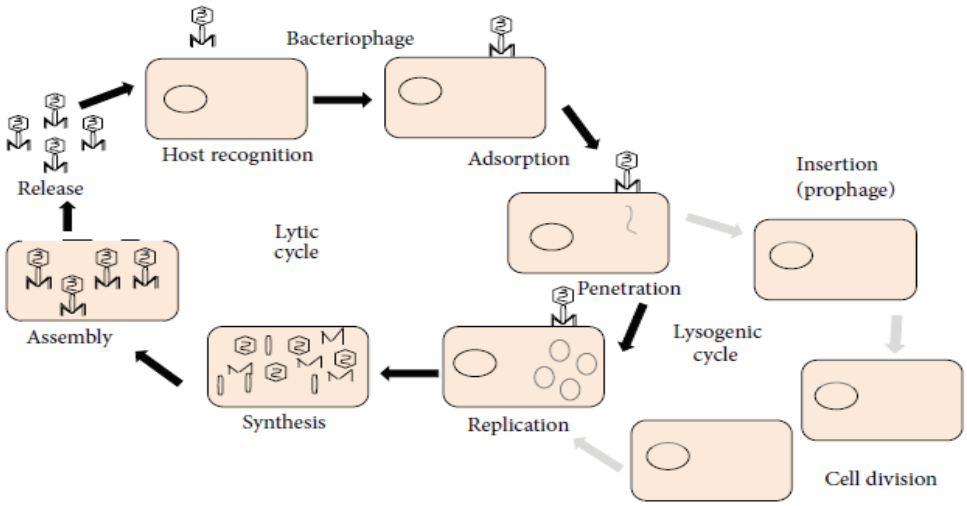

Figure 2.2: The lifecycle of virulent and temperate bacteriophage. (Adapted from Elbreki, et al., 2014)

2.1.4 Applications of Bacteriophages

In the medical field, the host specific characteristic of phages have been studied as the alternative treatment for bacterial infections, and this is known as phage therapy. Besides that, phages also contribute in other applications such as in bacterial detection and serve as drug delivery agent (O'Sullivan, et al., 2016). Phage virions and their encoded proteins are useful in the detection and identification of specific bacteria. In addition, phages are modified to deliver chemotherapeutic drug through attaching a drug to the phage surface. Furthermore, some of the modified phage carriers have been designed to cross the blood-brain barrier, which help in the treatment of Parkinson's and Alzheimer's diseases (O'Sullivan, et al., 2016).

In the food industry, phages have been used as biocontrol agents to reduce colonization of pathogenic bacteria on foods. During food processing, high titres of phages are applied directly onto food surfaces to achieve effective control against pathogens. Phages also serve as biosanitation agents to eliminate biofilms significantly, which are often found on the surfaces of equipment used in food processing. Apart from that, phages are an excellent food biopreservation agent since they are capable to cause cell lysis even at low temperature. As a result, the growth of pathogenic and spoilage bacteria can be limited, particularly to refrigerated foods (Sillankorva, Oliveira and Azeredo, 2012).

2.2 Bacteriophage Therapy

2.2.1 Importance of Bacteriophage Therapy

Over the last few decades, the emergence of multidrug resistant (MDR) bacteria is a great challenge in medical field. Many MDR bacteria cases have been reported worldwide. Furthermore, the remaining therapeutic options to treat those MDR bacteria are also limited and this has caused a global major healthcare crisis (Wittebole, De Roock and Opal, 2014). Therefore, this problem has urged many researchers to discover or develop novel agents to combat it. Phage therapy has therefore become a potential alternative treatment for these bacterial pathogens.

2.2.2 Advantages of Bacteriophage Therapy

Phage therapy has specific advantages compared to antibiotics. Firstly, phage therapy is able to minimize the bacterial load effectively. Phages, especially the lytic phages are bactericidal agents, whereby bacteria that have been infected by them would unable to retain their viability. Secondly, phage therapy is able to treat bacterial infection at a relatively low dosage. Phages are capable of increasing their number during the process of bacterial-killing, thus greatly increase the efficacy (Goodridge and Abedon, 2003).

Thirdly, phage therapy is harmless in terms of toxicity. As the phages are composed of nucleic acids and proteins only, they are considered inherently nontoxic, and would not cause any harm to patients (Loc-Carrillo and Abedon, 2011). However, the interaction of phages with body immune system may result in some harmful immune responses. It is recommended to use highly purified phage preparations for phage therapy in order to prevent anaphylactic responses towards bacterial components like endotoxins (Loc-Carrillo and Abedon, 2011).

Last but not least, phage therapy has minor disruption on microbiota. Due to the host specificity of phages, they are able to infect only certain strains of bacterial species. As a result, they only minimally affect the health-protecting normal flora bacteria. In contrast, antibiotics which often possess a broad spectrum activity tend to induce superinfections such as antibiotic-associated *Clostridium difficile* colitis (Loc-Carrillo and Abedon, 2011).

2.2.3 Limitations of Bacteriophage Therapy

Although phage therapy comes with many advantages, there are still some disadvantages of using phage therapy. For instance, phage therapy has the potential to elicit immune responses. As mentioned earlier, since they are viruses which are composed of proteins, phages may be seen as foreign invaders by the

body immune system (Wittebole, De Roock and Opal, 2014). As a result, they are subjected to neutralization and clearance by the body immune system, which in turn, decreases the effectiveness of phage therapy (Kaur, et al., 2012).

Besides that, phage therapy has the potential to modify bacterial hosts in ways that could cause them to become pathogenic or even resistant to antibiotics through a process known as transduction. Occasionally, the transduction process may involve the transfer of pathogenic genes or virulence factors, which may later transform a harmless host bacterium into a pathogen (Wittebole, De Roock and Opal, 2014). Similarly, if the transfer of genetic materials involve antibiotic-resistance genes, this may contribute to the development of more resistant bacteria.

Also, phage therapy has the potential to enhance the toxicity or virulence of phage-mediated bacterial lysis (Abedon, et al., 2011). Many bacteria produce toxins, for example, Shiga-like toxin or cholera toxin (Nilsson, 2014). During the course of treatment, lytic phages which cause the lysis of host bacteria in the end may result in the acceleration of bacterial-encoded toxin release. Eventually, this may exacerbate the infection. The rapid release of lipopolysaccharides which resulted from the breakdown of bacterial cell wall by phages can also

elicit an immune response (Nilsson, 2014).

Consequently, by taking into account the various disadvantages of phage therapy and also the lacking of internationally recognized human clinical trials, most of the countries still not approved the use of phage therapy in humans. As a result, researchers have shifted their focus in developing antibacterial agents using bacteriolytic and bacteriostatic proteins secreted by phages such endolysin (Roach and Donovan, 2015).

2.3 Endolysin

2.3.1 Nature of Endolysin

Endolysins, which are also called as lysins, are enzymes produced by bacteriophage (Young, 2013). The main function of endolysins is to degrade the peptidoglycan, thereby enabling the release of progeny virions. As mentioned earlier, in order for endolysins to access the peptidoglycan layer, another lysis factor called holins is required. During the late stage of lytic cycle, both of them are produced and accumulated in the cytoplasm of the host cell. At a genetically specific time, holins begin to create pores in the plasma membrane, thus, providing access for the endolysins to reach the peptidoglycan layer to cause rapid cell lysis (Elbreki, et al., 2014).

2.3.2 Structure and Classification of Endolysin

Generally, the structure of endolysins derived from phages against Gram-positive bacteria differs from those endolysins derived from phages against Gram-negative bacteria. Endolysins produced by phages against Gram-positive bacteria have a typical modular structure which consists of two distinct functional domains: enzymatically active domains (EADs) and cell wall binding domains (CBDs). EADs are typically situated at the N terminus whereas CBDs are located at the C terminus (Roach and Donovan, 2015). However, this multi-domain structure is only found among endolysins derived from phages against Gram-positive bacteria. On the other hand, endolysins derived from phages against Gram-negative bacteria have a globular structure which is composed of a single domain (EAD) and lack of CBDs (Schmelcher, Donovan and Loessner, 2012).

Endolysins are classified into five different classes based on their cleavage site within the peptidoglycan. They are (I) N-acetyl-β-D-muramidases (lysozymes); (II) lytic transglycosylases; (III) N-acetyl-β-D-glucosaminidases (glycosidases); (IV) N-acetylmuramoyl-L-alanine amidases and (V) L-alanoyl-D-glutamate endopeptidases and interpeptide bridge-specific endopeptidases (Elbreki, et al., 2014). The first three classes break the sugar moiety of peptidoglycan. Amidases hydrolyze the bond holding the sugar and peptide moieties of the peptidoglycan

whereas endopeptidases cleave the peptide moiety of the peptidoglycan (Borysowski, Weber-Dabrowska and Górski, 2006).

2.3.3 Characteristics of Endolysin

2.3.3.1 Endolysin Specificity

Commonly, endolysins have bactericidal activity against the host bacteria that the phages infected (Pastagia, et al., 2013). For instance, a *Staphylococcus aureus*-infecting phage endolysin can kill *S. aureus*. In addition, endolysins are genus- or species-specific. For example, a chimeric lysin, ClyS is a highly specific endolysin in which it targets only the *staphylococci sp.* but not others (Daniel, et al., 2010).

However, it has been reported that endolysins have activity against several Gram-positive bacteria. In a previous study conducted by Yoong, et al. (2004), the enterococcal phage lysin PlyV12 which is a broad-acting lysin had shown bactericidal activity not only against *enterococci sp.* but also other Gram-positive bacteria like *Streptococcus pyogenes* and *Streptococcus agalactiae*. In addition, there are also endolysins which act on both Gram-positive bacteria and Gram-negative bacteria. A recent study by Dong, et al. (2015) demonstrated that P28 endolysin exhibited a broad antibacterial activity against Gram-positive

bacteria and Gram-negative bacteria.

2.3.3.2 Endolysin Immunogenicity

Endolysins are proteins, therefore, they can induce the production of antibodies against them. For example, Loeffler, Djurkovic and Fischetti (2003) detected the presence of IgG against phage lysin after several doses of pneumococcal phage Cpl-1 lysin injection into the mice. In addition, there were also several other studies reported that the antibodies towards different lysins which against *S. pyogenes*, *Bacillus anthracis* and *S. aureus* had raised (Schmelcher, Donovan and Loessner, 2012).

However, Jado, et al. (2003) reported that antibodies that were raised in mice after administration of two pneumococcal lysins, Cpl-1 and Pal, did not compromise its recoverability after repeated infection and repeated administration of lysin treatment. Apart from that, there were also no other adverse effects or signs of anaphylaxis observed. Therefore, they suggested that the antibodies generated could be non-neutralizing antibodies which will not affect the lytic activity of lysins or prevent their use as antimicrobial agents in treating infections (Fenton, et al., 2010). In short, the antibodies that are produced against the corresponding lysins did not inhibit the antibacterial

activity of lysins.

2.3.3.3 Endolysin Resistance

Two previous studies have attempted to find out whether any bacterial strains which resistant to endolysins exist. The first study that carried out by Schuch, Nelson and Fischetti (2002) has exposed *Bacillus* sp. to several chemical mutagens which increased the frequency of antibiotic-resistance. Surprisingly, all the organisms remained fully sensitive to the endolysins. In another study, *Streptococcus pneumoniae*, *S. pyogenes* and *B. anthracis* were tested with repeated exposures to sub-lethal concentrations of endolysins specific to each species. The surviving bacteria remained unchanged in their susceptibility when they were challenged again with the respective endolysins (Fischetti, 2005). In the present, there are still no reports of bacterial strains that are resistant to endolysins.

The lack of resistant bacteria towards endolysins could be probably due to the coevolution of bacteriophages together with their hosts. The phages might evolved their endolysins for binding and hydrolyzing highly conserved and immutable bonds in the bacterial cell wall (Schmelcher, Donovan and Loessner, 2012), thereby making the development of resistance to phage endolysins a rare

event.

2.3.4 Applications of Endolysin

In the medical field, endolysins can be used to treat the bacterial infections in human or animals, especially those life-threatening infections caused by multidrug resistant bacteria. Besides that, these enzymes have also been clinically applied as rescue therapy for patients who have experienced recurrent infections. Endolysin has also been used in disinfection of implanted medical devices such as cardiac valves or catheters. As they could target clinically relevant bacteria, they are able to remove pathogens in the implanted medical devices, thus reducing the risk of bacterial colonization on these surfaces (Pastagia, et al., 2013).

Recently, the application of endolysins has also been extended to the food industry, especially with regard to food safety. For instance, endolysins are being used for the detection and control of food-borne pathogens. The high specificity and binding affinity of endolysins make them an ideal candidate for the development of new detection tool. Apart from that, endolysins have also been used in food processing to prevent food contamination caused by pathogens or spoilage bacteria (Schmelcher and Loessner, 2016).

2.3.5 Researches on Endolysin

Nowadays, many studies have been carried out on endolysin due to its usefulness in various aspects. The major objectives of these studies were to isolate the endolysin from bacteriophages and identify the characteristics of the extracted endolysin such as its specificity, thermal stability and pH stability.

Previously, endolysin was extracted and purified directly from crude phage lysate. For example, Gupta and Prasad (2011) obtained P-27/HP endolysin (derived from phage P-27/HP) from crude phage lysate using ammonium sulfate precipitation method and then purified it using gel filtration chromatography. The purified endolysin was reported to be effective against *Staphylococcus aureus* and it exhibited maximum lytic activity at pH 7.0 and temperature between 35-40°C.

Currently, many researchers studied the endolysin using recombinant technology at which they first isolated the endolysin gene, followed by cloning the gene and expressed it in selected host cells, after that they purified the recombinant endolysin and characterize it. For instance, Yuan, Peng and Gao (2012) expressed the entire plyBt33 endolysin gene (isolated from phage BtCS33 of *Bacillus thuringiensis*) in *Escherichia coli* and showed that the recombinant

PlyBt33 endolysin only exhibited activity against Gram-positive *Bacillus* strains. The optimal conditions for PlyBt33 endolysin activity were pH 9.0 and 50°C. Furthermore, a similar study conducted by Guo, et al. (2017) also cloned and expressed the lysPA26 endolysin gene (isolated from bacteriophage JD010 of *Pseudomonas aeruginosa*) in *E. coli* BL21 and found that the recombinant LysPA26 endolysin was able to lyse several Gram-negative bacteria which has been treated with outer membrane permeabilizer and unable to lyse Gram-positive bacteria. It was active between pH of 6.0 to 8.0 and can withstand temperature up to 100°C for 10 min.

There were also several studies have reported that endolysin could lyse the intact cells even without the outer membrane permeabilizer. According to Lim, et al. (2014), the SPN9CC endolysin (from SPN9CC bacteriophage) was able to lyse intact Gram-negative *E. coli* without EDTA pretreatment. Moreover, Morita, et al. (2001) also discovered that *Bacillus amyloliquefaciens* phage endolysin exerted antibacterial activity towards *P. aeruginosa* PAO1 without using the outer membrane permeabilizer.

2.4 *Shigella dysenteriae*

Shigella dysenteriae is one of the species under the genus *Shigella*. It is a rod-shaped Gram-negative bacteria which are small, uncapsulated, non-motile, non-sporulating and facultative anaerobic. *S. dysenteriae* is a highly infectious organism which can cause acute bacillary dysentery, which is popularly known as shigellosis. It is transmitted predominantly through fecal-oral route (Goodridge, 2013). It can also be spread by direct contact with an infected person (Zaidi and Estrada-García, 2014).

S. dysenteriae can be further classified into different serotypes based on the biochemical differences and variations in their O-antigen. Currently, there are 17 serotypes that have been discovered so far (Taneja and Mewara, 2016). Among all serotypes, *S. dysenteriae* type 1 has attracted the most attention among the researchers due to its epidemic-causing potential and its association with most serious dysentery cases (Dutta, et al., 2003). The pathogenicity of *S. dysenteriae* type 1 is mainly due to their ability to invade the colonic epithelial cells and to synthesize Shiga toxins (Stxs) during infection.

S. dysenteriae type 1 possess a virulence plasmid that carries the genes which are necessary for the cell invasion (Zaidi and Estrada-García, 2014). In addition

to these virulence factors, *S. dysenteriae* type 1 is also capable in producing Stxs, which are responsible for the most severe manifestation of shigellosis. Stxs work by binding to the glycolipid receptor on the surface of the targeted cell, thus inhibiting the protein synthesis (Sandvig, et al., 2010). Stxs are also associated with the development of hemolytic uremic syndrome which could lead to microangiopathic hemolytic anemia, thrombocytopenia and acute kidney injury in patients (Keir, Marks and Kim, 2012).

2.4.1 Shigellosis

As mentioned earlier, shigellosis is endemic around the world. According to World Health Organization (2017), approximately one million people are estimated to die from shigellosis every year. In addition, there are about 580,000 cases of shigellosis reported among military personnels and travelers. Among all these episodes, the incidence of shigellosis is the highest among children of 1 to 4 years of age followed by elderly people of 70 years old and above (Zaidi and Estrada-García, 2014).

2.4.1.1 Pathogenesis

Shigellosis is characterized by the inflammation induced upon invasion of *Shigella* which then results in the damage of the colonic epithelial cells (Ud-Din

and Wahid, 2014). The invasion of colonic epithelial cells by bacteria and the production of Shiga toxins (Stxs) by *Shigella* are the critical events responsible for the clinical manifestation of shigellosis.

The pathogenesis of shigellosis begins with the invasion of *Shigella* in the lumen of large intestine by transcytosis across the microfold cells (M cells) to reach the underlying submucosa. *Shigella* can escape from phagocytosis by resident macrophages and proliferate within the cells that they invaded, which eventually lead to cell death. After the cells died, the bacteria released in the submucosa layer will start to invade the epithelial cells through endocytosis. Bacterial invasion of colonic epithelial cells then stimulates the release of pro-inflammatory cytokines which attracts the polymorphonuclear leukocytes (PMN) to the infection site. Meanwhile, the production of Stxs also increases the expression of cytokines, which further enhances the inflammatory response (Zaidi and Estrada-García, 2014). Lastly, transmigration of PMN across the epithelium leads to major tissue destruction and inflammation (Ud-Din and Wahid, 2014). Consequently, the resulting colitis and ulceration of the mucosa contribute to symptoms such as bloody, mucoid stools and febrile diarrhea (Niyogi, 2005).

2.4.1.2 Treatment of Shigellosis

Previously, antimicrobial agents are effective in treating shigellosis. Since the early 1940s, many antibiotics have been used to treat shigellosis such as sulphonamides, tetracycline, chloramphenicol, ampicillin, co-trimoxazole, nalidixic acid and fluoroquinolones (Taneja and Mewara, 2016). Unfortunately, the efficacy of these drugs have been affected due to the emergence of increasing resistance to antibiotics. At the present, the emergence of multidrug resistant *Shigella* has been reported from all over the world (Taneja and Mewara, 2016). Therefore, there is an urgent need to seek for alternative solution to overcome this problem.

Bacteriophage therapy has been shown to have high potential in replacing antibiotics therapy. For example, Dubos, Straus and Pierce (1943) had shown that the usage of phages to treat *S. dysenteriae* infection in mice was effective. In another study, Miliutina and Vorotyntseva (1993) who combined phage therapy and antibiotics to treat shigellosis found that this combination was effective in treating cases, in which antibiotic treatment alone was ineffective. In summary, these studies have demonstrated that phage therapy can be a feasible option to use in treating shigellosis.

Recently, the potential use of endolysin to treat shigellosis has been reported. A previous study conducted by Yang, et al. (2012) has shown that LysEC1 endolysin (derived from Stx Phage) exerted efficient lytic activity on several *E. coli* O157:H7 strains. Several other studies also reported that endolysin was able to cause lysis on *Shigella* genus. For example, Son, et al. (2012) found that LysB4 endolysin (derived from the *Bacillus cereus*-infecting bacteriophage B4) lysed *Shigella flexneri* and *Shigella boydii* effectively in the presence of EDTA. Similarly, Park, et al. (2012) also discovered that LysBPS13 endolysin which produced by a bacteriophage that infects *B. cereus* was active against *Shigella* strains when these bacteria were treated with EDTA. Therefore, all of these studies have proved that endolysin could be an alternative treatment for shigellosis.

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial Strain and Bacteriophage

The bacterial strain *Shigella dysenteriae* and SDyM-18 bacteriophage sample used in this study were obtained from Dr. Tan Gim Cheong, Head of Department of Biomedical Science, Faculty of Science, University Tunku Abdul Rahman. The SDyM-18 bacteriophage sample against *S. dysenteriae* was previously isolated from sewage water in Sungai Petani, Kedah.

3.2 Reagents, Chemicals, Equipments and Laboratory Wares

The reagents and chemicals that used in this study with their respective manufacturers are listed in Table 3.1. Besides that, the equipments and laboratory wares that were used in this study with their respective brands and models are tabulated in Table 3.2.

Table 3.1: List of reagents and chemicals used in this study.

Chemicals/ Reagents	**Manufacturers/ Country**
Acrylamide	Amersham bioscience, UK
Ammonium persulfate (APS)	Sigma Aldrich, USA
Ammonium sulfate [$(NH_4)_2SO_4$]	Bio Basic Canada Inc, Canada
Bis-acrylamide	AMRESCO, USA
Coomassie blue R-250	Bio Basic Canada Inc, Canada
Ethylenediaminetetraacetic acid (EDTA)	Acros Organic, USA
Glycine	Bio Basic Inc, Canada
Hydrochloric acid (HCl)	*AnalaR*®. *Normapur*, UK
Luria Bertani (LB) agar	Laboratorios Conda, Spain
Luria Bertani (LB) broth	Laboratorios Conda, Spain
Lysozyme	Bio Basic Inc, Canada
Methanol	*EMPARTA*® ACS, Korea
Polyethylene glycol 6000 (PEG 6000)	Bio Basic Canada Inc, Canada
Protein marker	Invitrogen, USA
Sodium dodecyl sulfate (SDS)	Bio Basic Canada Inc, Canada
Sodium chloride (NaCl)	Merck Millipore, Germany
Tetramethylethylenediamine (TEMED)	Thermo fisher scientific, USA
Tris-base	MediGene, Germany

Table 3.2: List of equipment and laboratory wares used in this study.

Equipments/ Laboratory wares	**Brand/ Model**
High speed centrifuge	Beckman Coulter, USA
0.45μm syringe filter with Supor® Membrane	Acrodisc®, USA
5 mL syringe	Terumo, Japan
Cotton swab	CITOSWAB, China
Dialysis tubing	Thermo Scientific, USA
Filter paper	Whatman, China
Shaking heater block	BioShake, Germany
Hot plate stirrer	Harmony, Japan
Incubator (37°C)	Memmert, Germany
Laminar flow cabinet	ESCO, Singapore
Media bottle	SCHOTT DURAN®, Germany
Microcentrifuge	Thermo Scientific, USA
Microcentrifuge tubes	Greiner Bio-One, UK
Micropipette (10 μL)	Gilson, Canada
Micropipette (200 μL)	Labmate, India
Micropipette (1000 μL)	Proline® Plus, Germany
Microwave oven	Sharp, Japan
Parafilm	Bemis, USA
PCR tubes	Axygen, Inc, USA.

Table 3.2; Continued:

Equipments/ Laboratory wares	Brand/ Model
Petri dishes	NEST Biotechnology, USA
pH meter	Sartorius, Germany
SDS-PAGE electrophoresis set	Bio-rad, USA
Shaking incubator (37°C)	N-Biotek, Korea
Spectrophotometer	Biochrom, UK
Vortex mixer	Gemmy Industrial Corp., Taiwan

3.3 Methodology

3.3.1 Growing and Amplification of Bacteriophage

3.3.1.1 Preparation of LB Broth

Luria Bertani (LB) broth was prepared by adding 10 g of LB broth powder into 500 mL of deionized water. The medium was mixed well and autoclaved for 15 min at 121°C, 975 kPa. The LB broth was then stored at room temperature until use.

3.3.1.2 Preparation of LB Agar

Luria Bertani (LB) agar was prepared by adding 17.5 g of LB agar powder into 500 mL of deionized water. The medium was mixed well and autoclaved. The

LB agar was then poured into sterile petri dishes and allowed to solidify in laminar flow cabinet. After the agar has solidified, the agar plates were stored at 4°C until use.

3.3.1.3 Preparation of LB Top Agar

Luria Bertani (LB) top agar was prepared by adding 1 g of LB broth powder and 1.75 g of LB agar powder into 100 mL of deionized water. The mixture was autoclaved and kept at room temperature until use. The top agar was melted using a microwave oven prior to use.

3.3.1.4 Preparation of Tris-buffered saline (TBS) (pH 7.6)

Tris-buffered saline solution (50 mM Tris-HCl, 150 mM NaCl) was prepared by adding 6.05 g of Tris and 8.76 g of NaCl into 800 mL of deionized water. The pH of the solution was then adjusted to 7.6 by using 1 M HCl. After the desired pH was achieved, the solution was topped up to 1 L with deionized water. Lastly, the solution was autoclaved and stored at room temperature until use.

3.3.1.5 Preparation of 20% PEG 6000 with 10% NaCl

In order to prepare 20% PEG 6000 solution, 100 g of NaCl was added into 1 L of deionized water and mixed well. Then, 200 g of PEG 6000 powder was added into the NaCl solution. The mixture was mixed well and then filtered through 0.45μm syringe filter.

3.3.1.6 Preparation of Overnight Culture and Log Phase Bacterial Culture

The glycerol stock of *S. dysenteriae* was streaked over LB agar and incubated at 37°C for 16-18 h to obtain single colonies of bacteria. The overnight bacterial culture was then prepared by inoculating a single colony of *S. dysenteriae* into fresh LB broth and incubated in a shaking incubator at 37°C with agitation speed at 200 rpm for 16-18 h. The log phase bacterial culture was prepared by adding the overnight culture into fresh LB broth in a ratio of 1:20 and incubated at 37°C with agitation speed at 200 rpm until the OD_{600} of the bacterial culture reached 0.4-0.6.

3.3.1.7 Amplification of Bacteriophage

In order to amplify the bacteriophage sample, 500 mL of log phase *S. dysenteriae* culture was grown as described in Section 3.3.1.6. Next, 50 μL of phage sample was added into the log phase bacterial culture and incubated in a shaking

incubator with agitation speed at 200 rpm at 37°C until total lysis was observed. After that, the culture was centrifuged at 8,603 ×*g* for 10 min to remove cell debris. The supernatant was obtained for precipitation of bacteriophage whereas the pellet was discarded.

3.3.1.8 Precipitation of Bacteriophage

The bacteriophage precipitation was done by adding PEG 6000 (20% w/v) with 10% NaCl into the supernatant at a ratio of 1:4. The mixture was then stirred for 2 h at 4°C to precipitate the phage particles. After that, centrifugation of the mixture was carried out at 14,000 ×*g* for 30 min at 4°C. The supernatant was discarded whereas the pellet obtained was suspended in TBS buffer solution.

3.3.1.9 Enumeration of Bacteriophage

The titre of bacteriophage was determined by performing plaque assay. Firstly, 10 mL of log phase *S. dysenteriae* culture was prepared as described in Section 3.3.1.6. Then, serial dilutions of phage sample was conducted up to a dilution factor of 10^{-10}. Three consecutive samples with dilution factors of 10^{-8} to 10^{-10} were chosen to do plaque assay. Two hundred microlitres of diluted phage sample was added into 2 mL of log phase bacterial culture and incubated at 37°C for 20 min to allow adsorption of phage onto the bacteria. During the incubation,

LB top agar was melted in a microwave oven. After the incubation was done, 2 mL of melted top agar was added into the culture and immediately mixed by using a vortex mixer. Next, the mixture was quickly poured onto the agar plate and swirled gently to cover the entire base agar evenly. Once the top agar has solidified, the plates were incubated at 37°C for 16-18 h. The number of plaques was determined on the next day and the phage titer was calculated based on the equation below:

$$\frac{pfu}{mL} = \frac{Number\ of\ plaques\ x\ Dilution\ factor}{Volume\ plated}$$

3.3.2 Extraction of Endolysin

3.3.2.1 Preparation of Crude Phage Lysate

Five hundred millilitres of log phase *S. dysenteriae* culture was prepared as described in Section 3.3.1.6. After that, 50 µL of amplified phage sample was added into the log phase bacterial culture. The culture was incubated in a shaking incubator at 37°C with agitation speed at 200 rpm until total lysis occured. Then, centrifugation was carried out at 8,603 ×*g* for 10 min to remove cell debris. The supernatant which was the crude phage lysate was kept for ammonium sulfate precipitation while the pellet was discarded.

3.3.2.2 Ammonium Sulfate Precipitation

The proteins from crude phage lysate was precipitated using 0-20%; 20-40%; 40-60% and 60-80%.ammonium sulfate saturation. The amount of solid ammonium sulfate needed for each saturation percentage was calculated using an online ammonium sulfate calculator (accessed at http://www.encorbio.com/protocols/AM-SO4.htm). The solid ammonium sulfate was first grinded into fine powder form with a mortar and pestle. Then, the ammonium sulfate powder was added slowly with stirring into the lysate on ice until the ammonium sulfate was fully dissolved. The mixture was then stirred for another 1 h on ice. Next, the mixture was centrifuged at 12,000 ×*g* for 20 min at 4°C. After the centrifugation, the supernatant was kept for 20-40% ammonium sulfate precipitation whereas the pellet obtained was suspended with appropriate volume of TBS buffer solution and stored at 4°C. Same procedures were repeated for the remaining ammonium sulfate saturation. After the ammonium sulfate precipitation was completed, all the protein samples were subjected to dialysis as described in Section 3.3.2.3 to remove the excess ammonium sulfate in the samples.

3.3.2.3 Dialysis

Dialysis tubing (10,000 MWCO) was cut into appropriate sizes with a pair of sterile scissors. One end of the dialysis tubing was first moistened with TBS

buffer solution and clamped tightly. The precipitated protein samples were then loaded into the dialysis tubing. After that, the other end of the dialysis tubing was clamped tightly. Next, the dialysis tubing was placed into 500 mL of TBS buffer solution and dialysed at 4°C with gently stirring. After 6 h of dialysis, the TBS buffer solution was replaced with new TBS buffer solution. Then, the dialysis was continued at 4°C overnight. The next day, the dialyzed protein was removed from the tubing and SDS-PAGE was carried out to analyze the precipitated proteins.

3.3.2.4 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.3.2.4.1 Preparation of 4X Resolving Gel Buffer Solution (pH 8.8)

Resolving gel buffer solution (1.5 M Tris-base, 0.4% SDS) was prepared by adding 18.171 g of Tris base and 4 mL of 10% SDS solution into approximately 60 mL of sterile deionized water and mixed thoroughly. The pH of the solution was then adjusted to 8.8 by using 1 M HCl. After the desired pH was achieved, the solution was topped up to 100 mL with sterile deionized water. Lastly, the solution was filtered through 0.45 μm syringe filter and kept at 4°C until use.

3.3.2.4.2 Preparation of 4X Stacking Gel Buffer Solution (pH 6.8)

Stacking gel buffer solution (0.5 M Tris-base, 0.4% SDS) was prepared by adding 6.057 g of Tris base and 4 mL of 10% SDS solution into approximately 60 mL of sterile deionized water and mixed thoroughly. The pH of the solution was then adjusted to 6.8 by using 1 M HCl. Next, the solution was topped up to 100 mL with sterile deionized water. Lastly, the solution was filtered through 0.45 μm syringe filter and kept at 4°C until use.

3.3.2.4.3 Preparation of 10X and 1X Running Buffer Solution

In order to prepare 10X running buffer (0.025 M Tris-base, 0.1% SDS), 15 g of Tris base and 72 g of glycine was added into 500 mL of sterile deionized water and mixed thoroughly. In order to run SDS-PAGE, 10X running buffer was diluted to 1X running buffer by mixing 100 mL of 10X running buffer with 900 mL of sterile deionized water. Lastly, 10 mL of 10% SDS solution was added into the 1X running buffer solution.

3.3.2.4.4 Preparation of Staining and Destaining Solution

Staining solution (0.1% Coomassie Brilliant Blue R-250, 1% acetic acid, 40% methanol) was prepared by adding 100 mg of Coomassie Brilliant Blue R-250 into 1 mL of acetic acid. The solution was then mixed with 40 mL of methanol

and 59 mL of deionized water. Destaining solution (10% acetic acid, 40% methanol) was prepared by mixing 10 mL of acetic acid with 40 mL of methanol and 50 mL of deionized water.

3.3.2.4.5 Casting of SDS-PAGE Gel

The casting frame was first set up on the casting stand. Resolving gel solution and stacking gel solution were prepared according to Table 3.3 and Table 3.4 respectively. Then, the resolving gel solution was loaded into the gap between the glass plates. After that, 70% ethanol was added immediately to overlay the resolving gel in order to remove the air bubbles and smoothen the gel surface. The gel was then allowed to solidify at room temperature. Once the resolving gel has solidified, the ethanol was removed completely by using a filter paper. The stacking gel solution was later loaded into the gaps between the glass plates and the comb was inserted. The gel was again allowed to solidify at room temperature. After the stacking gel has solidified, the whole gel was transferred into gel tank and 1X running buffer was added into the tank.

Table 3.3: List of components required to prepare the resolving gel.

Components	**Volume (µL)**
30% Bis-acrylamide (30% T, 2.67% C)	1875
Sterile deionized water	940
4X resolving gel buffer	940
10% Ammonium Persulfate (APS)	23.5
Tetramethylethylenediamine (TEMED)	3.8
Total volume	3782.3

Table 3.4: List of components required to prepare the stacking gel.

Components	**Volume (µL)**
30% Bis-acrylamide (30% T, 2.67% C)	415
Sterile deionized water	1460
4X stacking gel buffer	625
10% Ammonium Persulfate (APS)	16.7
Tetramethylethylenediamine (TEMED)	3.5
Total volume	2520.2

3.3.2.4.6 SDS-PAGE Analysis

First of all, 7 µL of 4X sample loading dye was mixed with 21 µL of protein sample. The sample was then boiled at 100°C for 10 min. After that, 3 µL of

protein marker and 16 μL of each protein sample were loaded into their respective wells. Electrophoresis was later carried out at 16 mA/gel. The electrophoresis was stopped once the loading dye was ran off. The gel was stained with staining solution for 5 min followed by destained with destaining solution until no blue background was observed.

3.3.3 Spot Lytic Assay

3.3.3.1 Spot Lytic Assay for EDTA-treated Bacterial Cells

Spot lytic assay was conducted to determine which ammonium sulfate saturation precipitated protein sample contained the endolysin. Firstly, log phase *S. dysenteriae* culture was prepared as described in Section 3.3.1.6. Then, 1 mL of log phase bacterial culture was centrifuged at 10,000 ×*g* for 1 min. After that, the supernatant was discarded while the pellet was washed with 200 μL of Tris buffer solution containing 50 mM EDTA. The pellet was then incubated at room temperature for 20 min. Centrifugation was again carried out at 10,000 ×*g* for 1 min to remove the EDTA and the remaining pellet was resuspended in TBS buffer solution. The EDTA-treated bacteria were spread onto LB agar evenly with a cotton swab. Subsequently, 5 μL of each precipitated protein sample was spotted onto the agar. At the same time, 5 μL of lysozyme (25μg/mL) and TBS buffer were also spotted to act as positive and negative controls respectively. The plates were then allowed to air dry and then incubated at 37°C for 16-18 h.

3.3.3.2 Spot Lytic Assay for non EDTA-treated Bacterial Cells

Similar procedures were repeated for bacterial culture without EDTA treatment. Log phase *S. dysenteriae* culture was first grown as described in Section 3.3.1.6. Then, centrifugation was carried out at 10,000 ×*g* for 1 min to harvest the bacterial cells. The supernatant was discarded while the pellet was suspended in TBS buffer solution. The non EDTA-treated bacteria was later spread onto the LB agar evenly by using a cotton swab. After that, 5 μL of each precipitated protein sample, together with amplified phage sample (positive control) and TBS buffer (negative control) were spotted onto the agar. The plates were allowed to air dry and then incubated at 37°C for 16-18 h.

3.3.3.3 Heat Treatment of Bacteriophage Sample

Heat treatment was carried out on PEG-precipitated SDyM-18 phage to determine its deactivation temperature. Firstly, 5 μL of PEG precipitated SDyM-18 phage was aliquoted separately into five PCR tubes. Then, all the PCR tubes were heated at 40°C, 50°C, 60°C, 70°C and 80°C for 10 min using a heater block. The non EDTA-treated bacterial cells were spread onto the LB agar using a cotton swab. Lastly, 5 μL of each heated phage sample was spotted onto the agar. The plates were allowed to air dry and then incubated at 37°C for 16-18 h.

3.3.3.4 Heat Treatment of Precipitated Protein Samples

Heat treatment was conducted on the precipitated protein samples to deactivate the co-precipitated phages that were present in the samples. All the precipitated protein samples were heated at 60°C and 70°C for different durations (2, 4, 6, 8 and 10 min). Firstly, 10 µL of 0-20% saturation precipitated protein sample was aliquoted separately into 10 different PCR tubes. Then, five of the PCR tubes were heated at 60°C for different durations (2, 4, 6, 8 and 10 min) whereas the remaining five tubes were heated at 70°C for different durations (2, 4, 6, 8 and 10 min) using heater block. Both EDTA-treated and non EDTA-treated bacteria were spread onto the LB agar by using cotton swabs. Lastly, 5 µL of each heated 0-20% saturation precipitated protein samples was spotted onto the LB agar and allowed to air dry. Similar procedures were repeated for the remaining precipitated protein samples. All the plates were then incubated at 37°C for 16-18 h.

CHAPTER 4

RESULTS

4.1 Enumeration of Amplified Bacteriophage Sample

Plaque assay was conducted over several dilution factors to identify the titer of amplified SDyM-18 bacteriophage sample. The titer obtained was 2.1 x 10^{12} pfu/mL, which was calculated based on the equation stated in Section 3.3.1.9.

4.2 Extraction of Endolysin by Ammonium Sulfate Precipitation

The presence of endolysin in the precipitated protein samples was determined based on SDS-PAGE gel. Figure 4.1 shows the results of SDS-PAGE gel of the precipitated protein samples. There were bands observed for all the protein samples. However, the bands for lane 1 (0-20% saturation precipitated protein) and lane 2 (20-40% saturation precipitated protein) were very faint compared to the remaining samples. Numerous bands were observed in lane 3 (40-60% saturation precipitated protein) and lane 4 (60-80% saturation precipitated protein). However, it can be seen that 40-60% saturation precipitated protein sample contained more and thicker protein bands than 60-80% saturation

precipitated protein. Hence, it can be concluded that 40-60% ammonium sulfate saturation has precipitated out the most proteins.

The molecular weight of SDyM-18 phage endolysin was approximately 17.4 kDa. In lane 4 (60-80% saturation precipitated protein), there was one band located near to 20 kDa, which could be due to the presence of endolysin of SDyM-18 phage. Therefore, this study demonstrated that the SDyM-18 phage endolysin could be precipitated at 60-80% ammonium sulfate saturation.

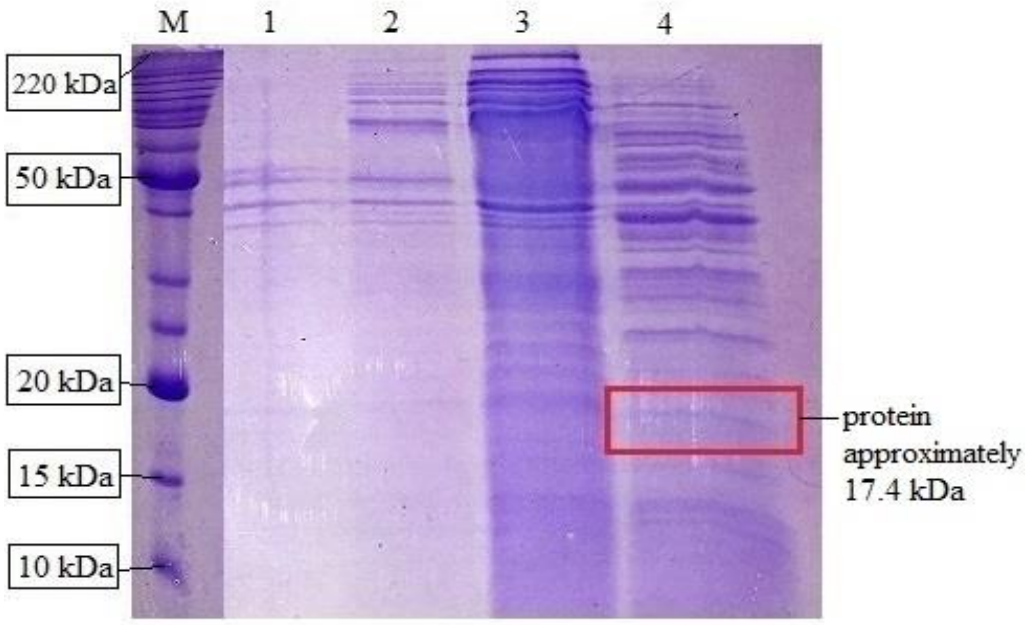

Figure 4.1: SDS-PAGE analysis of protein samples. Lane M: Protein marker; Lane 1: 0-20% ammonium sulfate saturation precipitated protein sample; Lane 2: 20-40% ammonium sulfate saturation precipitated protein sample; Lane 3: 40-60% ammonium sulfate saturation precipitated protein sample; Lane 4: 60-80% ammonium sulfate saturation precipitated protein sample.

4.3 Spot Lytic Assay

4.3.1 Spot Lytic Assay for Bacterial Culture with and without EDTA Treatment

Spot lytic assay was carried out to determine which ammonium sulfate saturation has successfully precipitated the endolysin. In this study, EDTA-treated and non EDTA-treated bacterial cells were prepared to conduct the spot lytic assay.

Figure 4.2 shows the results of spot lytic assay for both EDTA-treated and non EDTA-treated bacterial cells. Generally, it can be seen that there were clear zones observed for all precipitated protein samples on EDTA-treated bacterial cells (Figure 4.2A). This indicated that all the precipitated protein samples were able to cause lysis. However, the lysis zone caused by these precipitated protein samples were similar to the lysis pattern produced by SDyM-18 phage instead of lysozyme. Besides that, the precipitated protein samples also formed clear zones on non EDTA-treated bacterial cells (Figure 4.2B). Therefore, this further confirmed that the lytic zones produced were due to the co-precipitated bacteriophages in the protein samples.

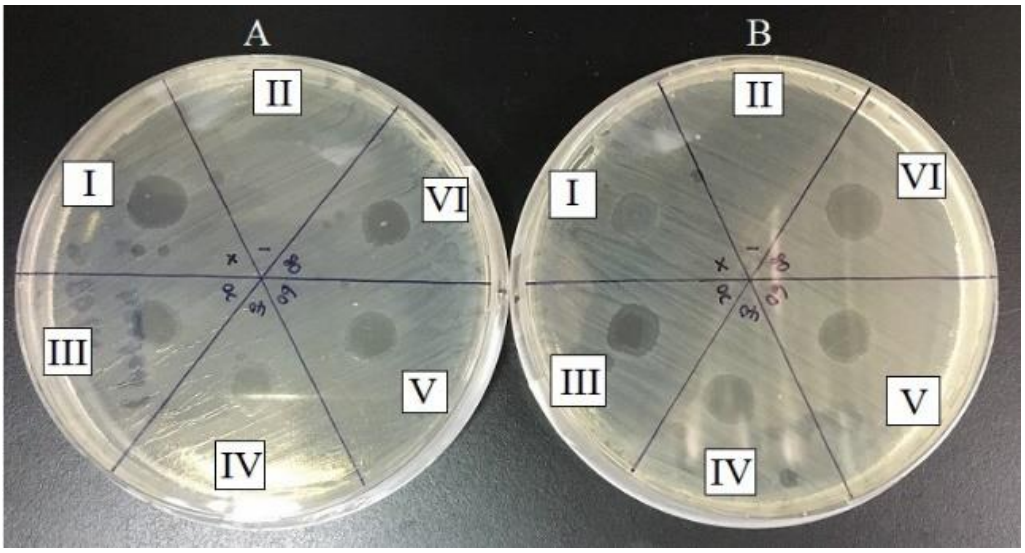

Figure 4.2: Spot lytic assay for non EDTA-treated and EDTA-treated bacterial cells. The plates were incubated at 37°C for 16-18 h. (A) Non EDTA-treated bacterial lawn spotted with 5 µL of (I) PEG precipitated SDyM-18 phage as positive control; (II) TBS buffer as negative control; (III) 0-20% ammonium sulfate saturation precipitated protein; (IV) 20-40% ammonium sulfate saturation precipitated protein; (V) 40-60% ammonium sulfate saturation precipitated protein; (VI) 60-80% ammonium sulfate saturation precipitated protein. (B) EDTA-treated bacterial lawn spotted with 5 µL of similar samples as (A) except (I) 0.125 µg/mL lysozyme as positive control.

4.3.2 Determination of Deactivation Temperature for SDyM-18 Phage

Heat treatment was performed on PEG precipitated SDyM-18 phage sample to determine the deactivation temperature of the phage. Figure 4.3 shows the results of heat treatment of PEG precipitated SDyM-18 phage for 10 min at different temperatures. There were clear zones observed for phage samples heated separately at 40°C, 50°C and 60°C. These lysis zones showed that the phage was still active at these particular temperatures as they were able to infect the host cells and lyse them. However, phage sample heated at 70°C and 80°C did not produce any clear zones on the bacterial lawn. This indicated that no lysis

occurred which demonstrated that the phage was successfully deactivated at these particular temperatures. Based on the results obtained, it was shown that SDyM-18 phage was deactivated at 70°C.

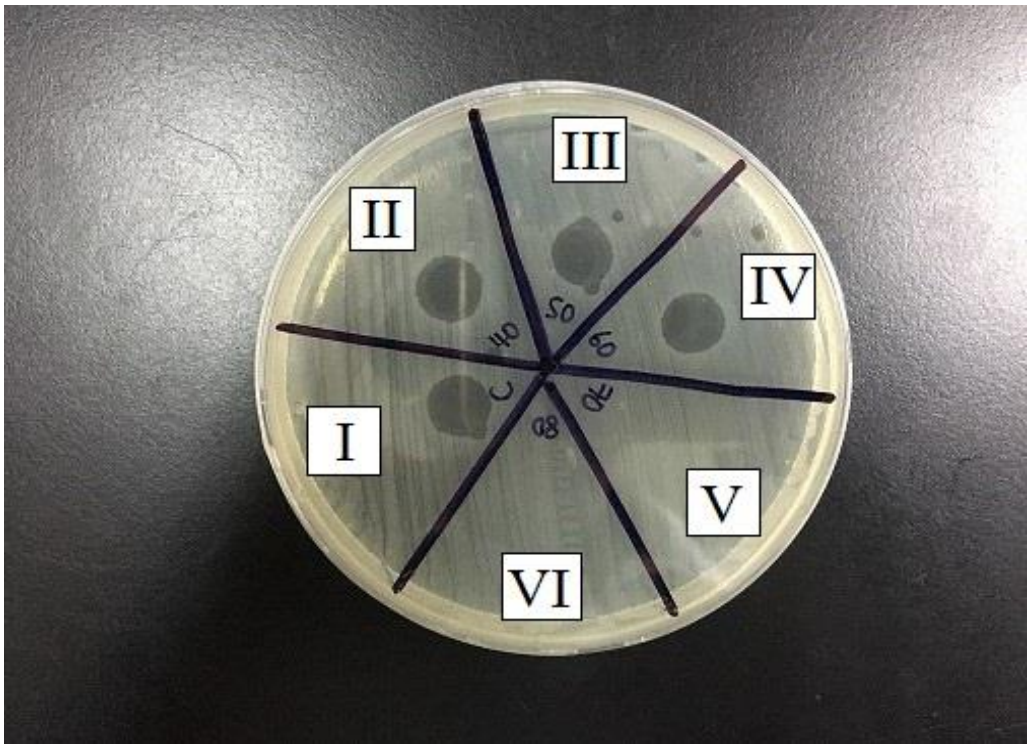

Figure 4.3: Heat treatment of PEG precipitated SDyM-18 phage. The plate was non EDTA-treated bacterial lawn spotted with 5 µL of (I) PEG precipitated SDyM-18 phage as positive control and SDyM-18 phage sample heated at (II) 40°C; (III) 50°C; (IV) 60°C; (V) 70°C; (VI) 80°C for 10 min. The plate was incubated at 37°C for 16-18 h.

4.3.3 Spot Lytic Assay for Bacterial Culture with and without EDTA Treatment using Heat-treated Protein Samples

Spot lytic assay was conducted again with precipitated protein samples that have been heat-treated to remove the co-precipitated bacteriophage, to unmask the lytic activity of endolysin, so that the ammonium sulfate saturation which

precipitated out the endolysin can be determined. All the precipitated protein samples were heated at two different temperatures (60°C and 70°C) with a range of duration (2, 4, 6, 8 and 10 min). The heat-treated protein samples were tested against both EDTA-treated and non EDTA-treated bacterial cells.

Figure 4.4 and 4.5 demonstrate the results of spot lytic assay for EDTA-treated and non EDTA-treated bacterial cells using precipitated protein samples that have been heat-treated at 60°C at different durations (2, 4, 6, 8 and 10 min). It can be observed that there were clear zones observed for all areas spotted with precipitated protein samples at both EDTA-treated and non EDTA-treated plates. Besides that, the lysis pattern produced by the precipitated protein samples was similar to that caused by SDyM-18 phage but different from the lysis caused by lysozyme. Therefore, this indicated that the lysis was caused by SDyM-18 phage but not endolysin.

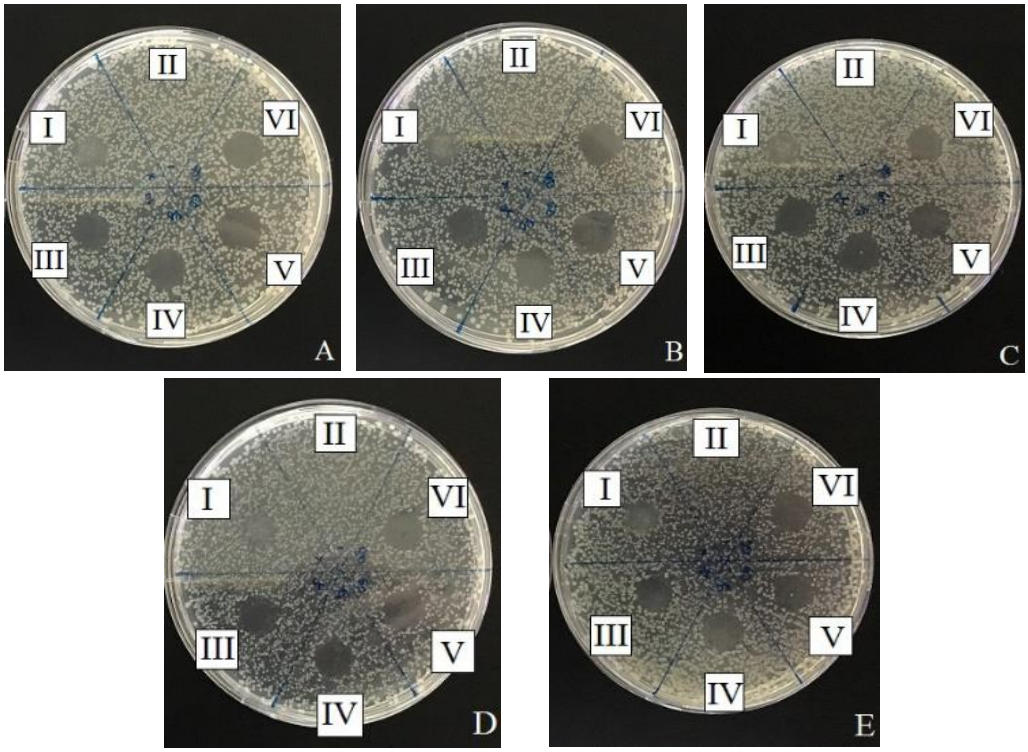

Figure 4.4: Spot lytic assay for EDTA-treated bacterial cells using precipitated protein samples that underwent heat treatment at 60°C for different durations: (A) 2 min; (B) 4 min; (C) 6 min; (D) 8 min; (E) 10 min. EDTA-treated bacterial lawn was spotted with 5 µL of (I) 0.125 µg/mL lysozyme as positive control; (II) TBS buffer as negative control; (III) 0-20% ammonium sulfate saturation precipitated protein; (IV) 20-40% ammonium sulfate saturation precipitated protein; (V) 40-60% ammonium sulfate saturation precipitated protein; (VI) 60-80% ammonium sulfate saturation precipitated protein. The plates were incubated at 37°C for 16-18 h.

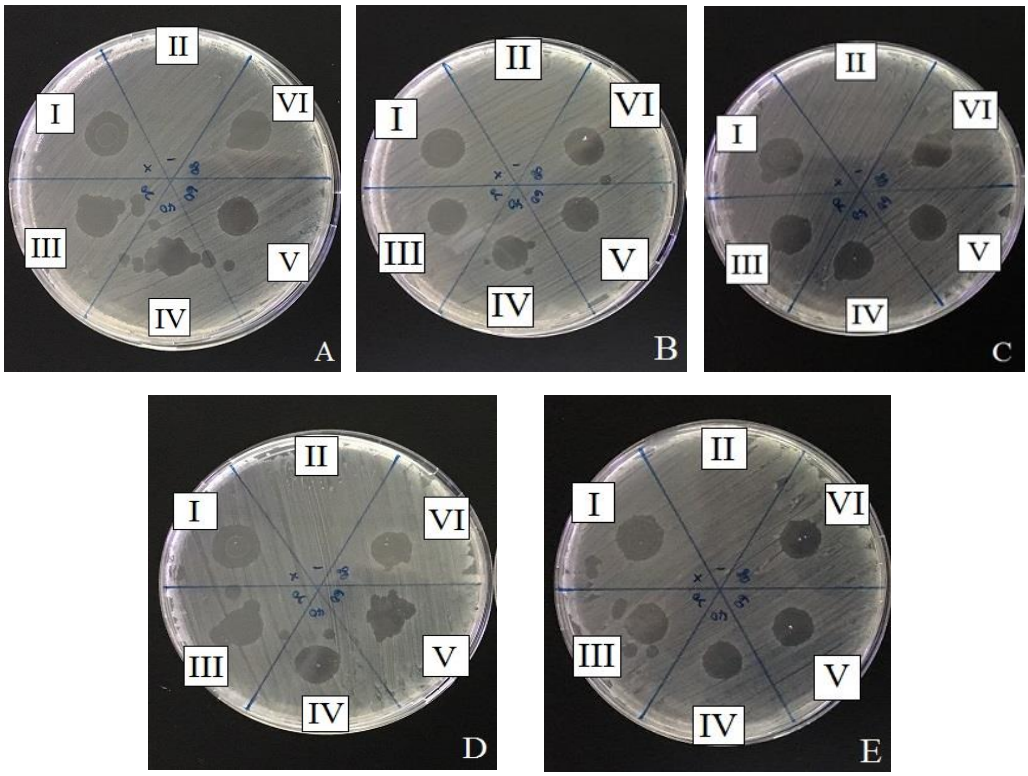

Figure 4.5: Spot lytic assay for non EDTA-treated bacterial cells using precipitated protein samples that underwent heat treatment at 60°C for different durations: (A) 2 min; (B) 4 min; (C) 6 min; (D) 8 min; (E) 10 min. Non EDTA-treated bacterial lawn was spotted with 5 µL of (I) PEG precipitated SDyM-18 phage as positive control; (II) TBS buffer as negative control; (III) 0-20% ammonium sulfate saturation precipitated protein; (IV) 20-40% ammonium sulfate saturation precipitated protein; (V) 40-60% ammonium sulfate saturation precipitated protein; (VI) 60-80% ammonium sulfate saturation precipitated protein. The plates were incubated at 37°C for 16-18 h.

Figure 4.6 and 4.7 demonstrate the results of spot lytic assay for both EDTA-treated and non EDTA-treated bacterial cells using precipitated protein samples heated at 70°C with different durations (2, 4, 6, 8 and 10 min). Based on Figure 4.6A (2 min heat-treated protein sample on EDTA-treated plate) and 4.7A (2 min heat-treated protein sample on non EDTA-treated plate), it can be seen that there

were clear zones observed for all precipitated protein samples on both EDTA-treated and non EDTA-treated plates. The lysis pattern was also similar to the lysis pattern produced by SDyM-18 phage. Therefore, the lysis occurred was due to the presence of phage and this showed that heating duration for 2 min was insufficient to remove the phages.

Based on Figure 4.7B (4 min heat treated protein sample on non EDTA-treated plate) and 4.7C (6 min heat treated protein sample non EDTA-treated plate), there were clear zones produced on spotted areas. This showed that the phage particles present in the precipitated protein samples were still not completely deactivated. Therefore, it can be concluded that any lysis observed on EDTA-treated bacterial lawn (as shown in Figure 4.6B and 4.6C) was not due to endolysin. In short, precipitated protein samples heated for 4 min and 6 min at 70°C were also unable to completely remove the phages.

Based on Figure 4.6D (8 min heat treated protein sample on EDTA-treated plate) and 4.7D (8 min heat treated protein sample on non EDTA-treated plate), it can be seen that the precipitated protein samples spotted on non EDTA-treated bacterial lawn did not produce any clear zones on their respective areas. This indicated that the phages present in the precipitated protein samples have been

successfully deactivated. In contrast, there was a clear zone observed on EDTA-treated plate spotted with 60-80% saturation precipitated protein. This lysis may be possibly caused by endolysin since the co-precipitated phages have been deactivated. Therefore, this study showed that a heating duration of 8 min at 70°C was sufficient to deactivate all the phage particles present in the precipitated protein samples.

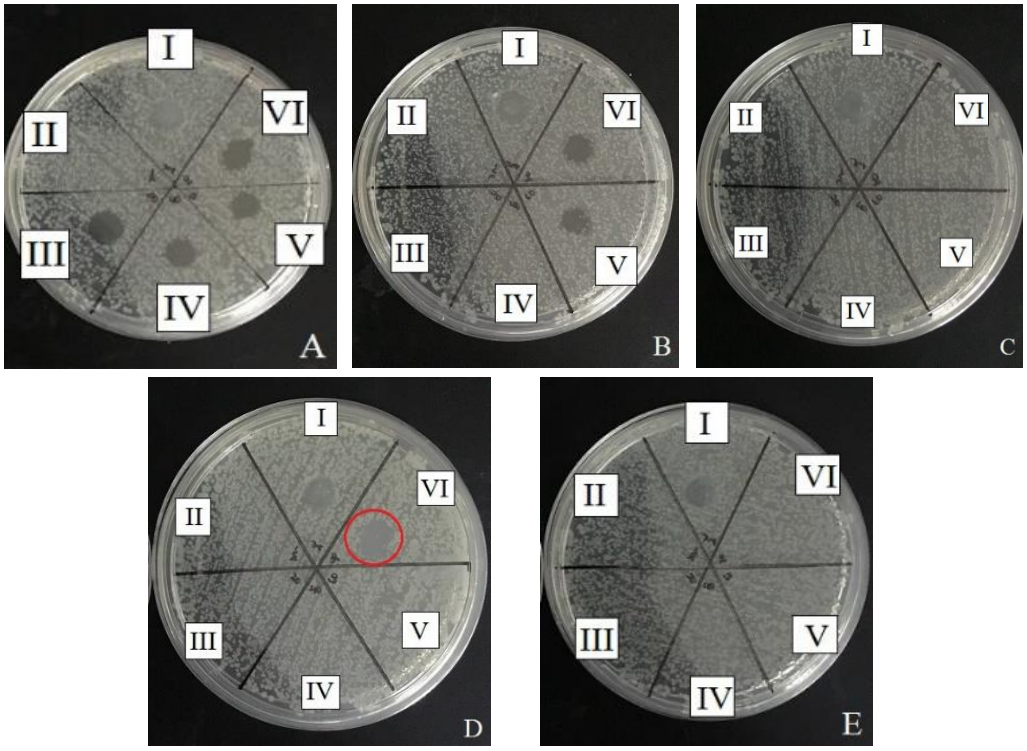

Figure 4.6: Spot lytic assay for EDTA-treated bacterial cells using precipitated protein samples that underwent heat treatment at 70°C for different durations: (A) 2 min; (B) 4 min; (C) 6 min; (D) 8 min; (E) 10 min. EDTA-treated bacterial lawn was spotted with 5 µL of (I) 0.125 µg/mL lysozyme as positive control; (II) TBS buffer as negative control; (III) 0-20% ammonium sulfate saturation precipitated protein; (IV) 20-40% ammonium sulfate saturation precipitated protein; (V) 40-60% ammonium sulfate saturation precipitated protein; (VI) 60-80% ammonium sulfate saturation precipitated protein. The plates were incubated at 37°C for 16-18 h.

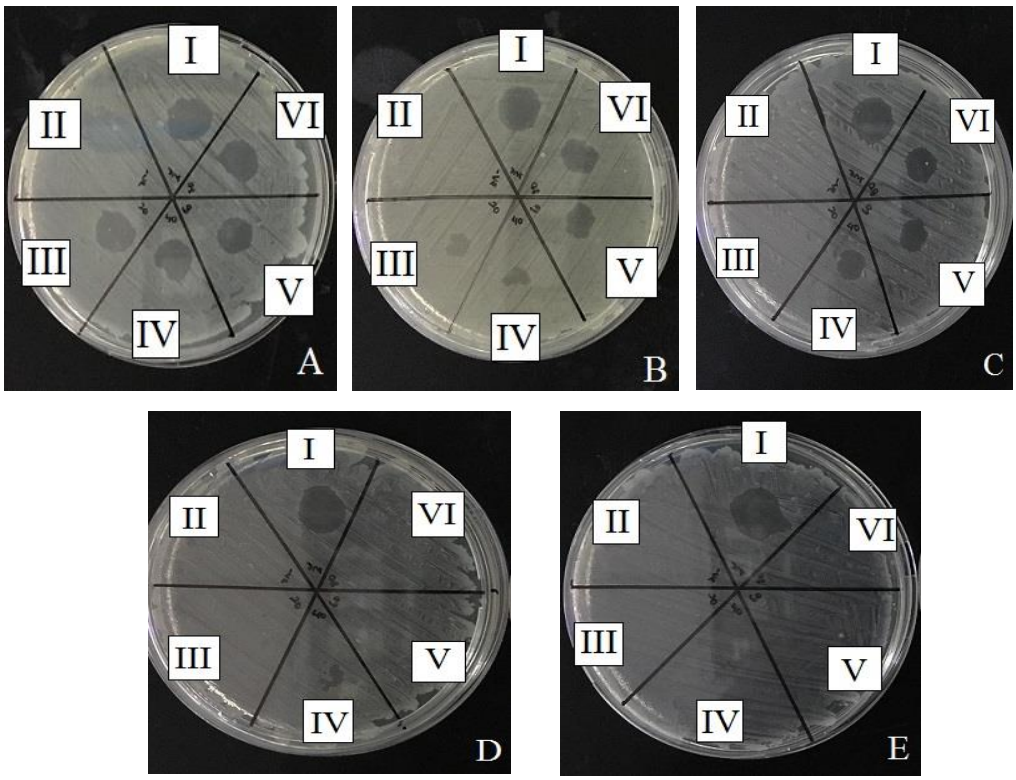

Figure 4.7: Spot lytic assay for non EDTA-treated bacterial cells using precipitated protein samples that underwent heat treatment at 70°C for different durations: (A) 2 min; (B) 4 min; (C) 6 min; (D) 8 min; (E) 10 min. EDTA-treated bacterial lawn was spotted with 5 µL of (I) PEG precipitated SDyM-18 phage as positive control; (II) TBS buffer as negative control; (III) 0-20% ammonium sulfate saturation precipitated protein; (IV) 20-40% ammonium sulfate saturation precipitated protein; (V) 40-60% ammonium sulfate saturation precipitated protein; (VI) 60-80% ammonium sulfate saturation precipitated protein. The plates were incubated at 37°C for 16-18 h.

Based on Figure 4.6E (10 min heat treated protein sample on EDTA-treated plate) and 4.7E (10 min heat treated protein sample on non EDTA-treated plate), it can be seen that all the precipitated protein samples which heated for 10 min did not produce any clear zones on non EDTA-treated bacterial lawn. This indicated that the phages have been deactivated. However, there was also no lysis observed for

EDTA-treated bacterial cells. This could be due to the denatured endolysin when the protein sample was heated for more than 8 min at 70°C.

CHAPTER 5

DISCUSSION

5.1 Enumeration of Amplified Bacteriophage Sample

In this study, the SDyM-18 phage sample obtained was first amplified to increase its titer before extraction of endolysin was carried out. This is because high titer phage is required for several downstream applications such as preparation of genomic DNA for sequencing and infection of host bacteria (Janež and Loc-Carrillo, 2013). The amplified phage titer obtained was 2.1 x 10^{12} pfu/mL. According to Bonilla, et al. (2016), a high titer phage ranges between 10^{10} and 10^{11} pfu/mL. Besides that, total lysis was observed to occur in cultures inoculated with phage titer ranging between 10^{6} and 10^{9} pfu/mL (Elliman, 2006). Thus, this yield of bacteriophage was sufficient for the infection of host *Shigella dysenteriae* culture to produce an optimum concentration of endolysin.

5.2 Extraction of Endolysin by Ammonium Sulfate Precipitation

A precipitating agent is usually added in order to precipitate a particular protein from a mixture. One of the most common precipitating agents used is ammonium

sulfate. It works by altering the ionic strength of aqueous salt solutions (El-Gayar, 2015). Ions affect the protein solubility (Duong-Ly and Gabelli, 2014). Upon the addition of salt, the solubility of protein increases along with ionic strength, and this is termed as salting-in (Wingfield, 2001; Duong-Ly and Gabelli, 2014). At high salt concentrations, the solubility of protein decreases due to high ionic strength, which withdraw the water molecules surrounding the protein and leads to protein precipitation. This is termed as salting-out (Wingfield, 2001; El-Gayar, 2015). Therefore, the salting-out method can be used to separate different proteins based on their solubility in high concentration of salt (Duong-Ly and Gabelli, 2014).

Many studies have reported the usage of ammonium sulfate to precipitate the endolysin from lysate. For instance, in a study conducted by Mishra, et al. (2014), precipitation of endolysin secreted by phages infecting *Staphylococcus aureus* was carried out using double ammonium sulfate precipitation. Moreover, Lood, et al. (2014) also employed ammonium sulfate precipitation method to precipitate PlyPy lysin (a lysin from phage infecting *Streptococcus pyogenes*). Ammonium sulfate is commonly used as the precipitating agent as it would not cause denaturation of protein and precipitated protein can be easily recovered by dissolving the pellet in suitable buffer (El-Gayar, 2015). Furthermore, ammonium sulfate can also stabilize the native conformation of the protein,

thereby retaining the biological activity of the protein (Wingfield, 2001).

In this study, endolysin was extracted using four different ammonium sulfate saturations, which were 0-20%; 20-40%; 40-60% and 60-80%. This was carried out with the purpose to optimize the extraction of endolysin. After precipitation, dialysis was performed prior to SDS-PAGE analysis to remove excess ammonium sulfate present in the protein samples (Berg, Tymoczko and Stryer, 2002). This is because any residual salt can interfere with protein activity (Dako, et al., 2012). It is also reported that dialysis can aid in concentrating proteins by surrounding the dialysis membrane with a hygroscopic reagent to remove the water across the membrane, thereby enhancing the detection of particular protein in SDS-PAGE (Andrew, Titus and Zumstein, 2002; Nielsen, 2003).

Based on Figure 4.1, it can be observed that the amount of protein increased as the ammonium sulfate saturation percentage increased. The proteins precipitated by 0-20% and 20-40% ammonium sulfate saturation were the least compared to the remaining samples as indicated by the intensity and the number of bands on the gel. Proteins of high molecular weight usually will be precipitated by 25% ammonium sulfate saturation (Bradshaw and Stahl, 2015). Therefore, it can be concluded that majority of the proteins present in the lysate were low molecular

weight proteins. Besides that, it can be observed that there were numerous thick bands in 40-60% saturation precipitated protein sample. This showed that high concentration of protein was present in the sample. This was similar to a study done by Suryani, Ambarsari and Lindawati (2017) in which they obtained the highest total protein in the fraction with 60% ammonium sulfate saturation. Another study also discovered that the yield of total soluble proteins was the highest with 50% ammonium sulfate saturation (Park, et al., 2015). Hence, it can be concluded that 40-60% ammonium sulfate saturation precipitated the most protein.

Based on Figure 4.1, it can be observed that there was a band in lane 4 (60-80% saturation precipitated protein) located near to 20 kDa. As mentioned before, the molecular weight of SDyM-18 phage endolysin was approximately 17.4 kDa, hence, it could be the endolysin of SDyM-18 phage. This result was further supported by the outcome obtained in the spot lytic assay using 60-80% saturation precipitated protein sample heat-treated at 70°C for 8 min, in which the protein sample only showed lysis on EDTA-treated bacteria but not on non EDTA-treated bacteria.

Any protein with molecular weight below 30 kDa was considered as low molecular weight protein (Chen, et al., 2015; Cheon, et al., 2016). Commonly, low molecular weight protein will be precipitated out at higher salt concentration compared to larger molecular weight proteins (Wingfield, 2001). Therefore, this in accordance with the results of this study, whereby the endolysin with estimated size of 17.4 kDa was present in 60-80% ammonium sulfate saturation.

5.3 Spot Lytic Assay

5.3.1 Spot Lytic Assay for Bacterial Culture with and without EDTA Treatment

In this study, both EDTA-treated and non EDTA-treated bacteria were used in spot lytic assay. This was done with the purpose to identify whether the precipitated endolysin was able to lyse the bacteria with or without the presence of the outer membrane permeabilizer.

Lysozyme possesses antibacterial properties due to its N-acetylmuramoylhydrolase activity (Masschalck and Michiels, 2003). It hydrolyzes the bond between N-acetylmuramic acid and N-acetylglucosamine residues, causing the breakdown of peptidoglycan, thereby lysing the bacteria (Benkerroum, 2008). Similar to lysozyme, endolysin is also a peptidoglycan

degrading enzyme (Tiwari, et al., 2014). In general, both lysozyme and endolysin are essentially effective against Gram-positive bacteria as the peptidoglycan can be easily accessed. In contrast, they lose their effectiveness against Gram-negative bacteria due to the presence of an outer membrane (OM) layer (Benkerroum, 2008; Nelson, et al., 2012). Thus, lysozyme was used as the positive control when conducting the spot lytic assay for EDTA-treated bacterial culture in this study.

The presence of OM in Gram-negative bacteria normally contributes to its insensitivity to the exogenous action of lysozyme as well as endolysin (Alakomi, et al., 2000). This is because OM functions as an effective permeability barrier which prevents the entry of macromolecules like enzymes from accessing the peptidoglycan layer (Alakomi, et al., 2000; Briers, Walmagh and Lavigne, 2011). According to Alakomi, Saarela and Helander (2003), the barrier effect of OM is largely due to the presence and features of lipopolysaccharide (LPS) molecules on the surface.

The permeability of the OM can be altered by some external agents. Agents which change the permeability and result in the loss of protective function of OM are known as permeabilizers. They disrupt the integrity of OM by either

releasing LPS from OM or intercalating the membrane (Alakomi, et al., 2000). There are many potent permeabilizers such as ethylenediaminetetraacetic acid (EDTA), sodium citrate, sodium hexametaphosphate (HMP), citric acid, poly-1-lysine and polymyxin B nonapeptide (PMBN) (Guha, et al., 2002). However, EDTA was found to be the most effective permeabilizer among them. This has been demonstrated by several studies which compared the effectiveness of EDTA with other permeabilizers such as polyethylenimine (PEI), succimer (meso-2,3-dimercaptosuccinic), poly-l-lysine and found that EDTA was the most suitable chemical to use compared to the rest (Alakomi, et al., 2006; Briers, Walmagh and Lavigne, 2011). Therefore, EDTA was chosen as the membrane permeabilizer in this study. EDTA works by chelating divalent cations from their binding site in the OM, causing the release of LPS and eventually leads to OM disruption (Alakomi, et al., 2000; Alakomi, Saarela and Helander, 2003; Briers, Walmagh and Lavigne, 2011).

As shown in Figure 4.2, all the precipitated protein samples were able to lyse both EDTA-treated and non EDTA-treated bacterial cells. Since the OM normally inhibits the action of endolysin, the precipitated protein sample should not be able to cause lysis on non EDTA-treated bacterial cells. Several studies had demonstrated that endolysin-mediated cell lysis on Gram-negative bacteria was only enhanced with the presence of membrane permeabilizer like EDTA.

For example, Son, et al. (2012) found that LysB4 endolysin-mediated lysis was detected for all tested Gram-negative bacteria such as *Shigella flexneri*, *Shigella boydii*, *Pseudomonas aeruginosa* and *Escherichia coli* when these cells were treated with 0.1 M EDTA. Furthermore, treatment with 0.5 mM EDTA had significantly increased the antibacterial activity of three endolysins (PVP-SE1gp146, 201φ2-1gp229 and OBPgp279) against *Salmonella* Typhimurium, *E. coli* and *P. aeruginosa* (Walmagh, et al., 2012). Last but not least, Lim, et al. (2012) also discovered that the use of EDTA has improved the lytic activity of SPN1S endolysin against *Salmonella*. Therefore, based on the results obtained, it was demonstrated that ammonium sulfate could precipitate not only the endolysin, but also the bacteriophages.

Similar findings were also observed in two previous studies carried out by Gan (2015) and Muhilarasi (2015) whereby bacteriophage could be co-precipitated together with endolysin during ammonium sulfate precipitation. This is because bacteriophages consist of mostly proteins (Wittebole, De Roock and Opal, 2014; Czajkowski, Ozymko and Lojkowska, 2016), hence, they can be precipitated out from solutions with high salt concentration due to the electrolyte-nonelectrolyte interaction where the nonelectrolyte (phage particles) will become less soluble at high salt concentration (Czajkowski, Ozymko and Lojkowska, 2016).

As mentioned before, ammonium sulfate precipitation method has been used in many studies to precipitate endolysin from crude lysate. This is because it conserves the native conformation and biological activity of the enzyme (Wingfield, 2001). Therefore, the co-precipitation of bacteriophage from crude lysate can be avoided by performing an extra purification step. For instance, Ogata, Umeda and Hongo (1974) obtained the purified endolysin by performing centrifugation at 60,000 ×*g* for 60 min on the precipitated protein solution to completely remove the phage particles. Similarly, Ogata, Tahara and Hongo (1975) also removed the phage particles present in the precipitated protein samples by centrifugation at 55,000 ×*g* for 60 min. Moreover, there are studies also reported that the removal of co-precipitated bacteriophage can be done by chromatography method. For example, Nelson, Loomis and Fischetti (2001) and Arima, Yanai and Beppu (1976) obtained the purified endolysin by subjecting the crude lysate to ion-exchange chromatography followed by gel filtration chromatography.

5.3.2 Determination of Deactivation Temperature for SDyM-18 Phage

Since the lytic activity exerted by endolysin cannot be determined due to the presence of bacteriophage, heat treatment was carried out to deactivate the phage. In this study, heat treatment was carried out first on PEG precipitated SDyM-18 phage sample to determine the deactivation temperature of the phage.

Figure 4.3 demonstrates the results of heat treatment on PEG precipitated SDyM-18 phage. The phage in samples which were heated at 40°C, 50°C and 60°C was not deactivated at these particular temperatures and was still able to cause lysis. A typical characteristic for environmental bacteriophages is their high thermal resistance (Jończyk, et al., 2011). Many of them can survive at temperatures between 40 and 90°C (Ly-Chatain, 2014). The SDyM-18 phage used in this study was an environmental bacteriophage which was isolated from sewage water. Therefore, this may explain why heat treatment of phage at these temperatures did not lead to their deactivation.

The phage samples heated at 70°C and 80°C did not produce lysis on the host cells, which showed that the phage was deactivated at these particular temperatures. This findings were in accordance with other studies that showed deactivation of bacteriophage can occur at temperature above 60°C. For instance, a previous study done by Taj, et al. (2014) found that T4 bacteriophage which can infect *E. coli* BL21 was completely deactivated at 70°C. Furthermore, Jeon, et al. (2016) detected little or no lytic activity was detected in two *Myoviral* bacteriophages which infect *Acinetobacter baumannii* at 70°C. Rode, et al. (2011) also discovered that Shiga toxin phage could not tolerate temperature at 80°C for 10 min. An increase in temperature generally reduces the viral activity (Basdew and Laing, 2014). This is because phages are mainly composed of

protein. Exposure to relatively high temperature can cause denaturation of phage proteins, which are responsible for infection of host (Basdew and Laing, 2014).

5.3.3 Spot Lytic Assay for Bacterial Culture with and without EDTA Treatment using Heat-treated Protein Samples

Since the deactivation temperature for SDyM-18 phage was at 70°C, thus all the precipitated protein samples were subjected to heat treatment at 70°C. At the same time, the precipitated protein samples were also subjected to heat treatment at 60°C. Besides that, the precipitated protein samples were also heated over several durations to determine whether the phages can be deactivated with a shorter heat treatment duration.

There was possibility that the SDyM-18 phage maybe deactivated at a lower temperature instead of 70°C. This is because the phage titer in precipitated protein samples maybe lesser than the phage titer of PEG precipitated phage sample. In this study, the crude lysate obtained after total lysis was centrifuged at 8,603 ×*g* for 10 min to remove cell debris. This centrifugation speed may also sediment some of the phage particles present in the lysate together with other cell debris, which eventually reduce the phage titer precipitated with the protein using ammonium sulfate. This is further supported by a previous study

conducted by Heggen, et al. (2014) whereby they harvested their phages by centrifuging the lysates at 8,000 ×*g* for 10 min.

In addition, a study carried out by Bourdin, et al. (2014) reported that high-speed pelleting of phages can result in titer loss. The centrifugation speed of 8,000 ×*g* can be considered as high speed centrifugation (Malmendier, Alaupovic and Brewer, 2013). In a previous study by Gan (2015), the lysate obtained through centrifugation at 8,603 ×*g* for 30 min showed that the phage titer present in precipitated protein samples was generally lesser than the phage titer of PEG-precipitated phage sample. Therefore, it is presumed that the phage titer present in the precipitated protein samples in this study was lesser than the PEG precipitated phage titer.

Each Figure 4.4 and 4.5 demonstrates the results of spot lytic assay for EDTA-treated and non EDTA-treated bacterial cells using precipitated protein samples that were heat-treated at 60°C for different durations. It was hypothesized that a lower titer phage can be deactivated at lower temperature. A previous study done by Rode, et al. (2011) found that phages in ground beef (approximately 10^7 pfu/mL) were not detected after heated at 60°C for 10 min, while phages in broth (approximately 10^9 pfu/mL) which experienced the same heating conditions

were still active. Hence, the co-precipitated phages present in all the precipitated protein samples should able to be removed through heat treatment of 60°C with a maximum heating duration of 10 min.

On the contrary, all the precipitated protein samples heated at 60°C still produced lysis on both EDTA-treated and non EDTA-treated bacterial cells. As described in Section 5.3.2, SDyM-18 phage was completely deactivated when heated at 70°C for 10 min. This clearly explained the reason why heat treatment at 60°C on the precipitated protein samples was not successful in deactivating the phage particles, regardless of the heating duration. Hence, it can be concluded that the SDyM-18 phage activity and survival were independent of its titer. The ability of bacteriophages to survive can be influenced by many factors including acidity, sunlight irradiation, ions and temperature but not its titer (Iriarte, et al., 2007; Jończyk, et al., 2011).

Figure 4.6 and 4.7 demonstrate the results of spot lytic assay for both EDTA-treated and non EDTA-treated bacterial cells using precipitated protein samples heated at 70°C for different durations. Generally, a heating duration of 2, 4 and 6 min were unable to deactivate the co-precipitated phages as indicated by the lysis formed on non EDTA-treated bacterial cells. However, the precipitated

protein samples heat-treated for 8 min did not produce lysis on non EDTA-treated plate. This indicated that the phage was successfully deactivated. Prolonged exposure to high temperature generally decreases the lytic activity of phages (Wan Ibrahim, et al., 2017). As the heating duration was increased from 2 min to 8 min, the phages were unable to cause any lysis. Therefore, this study had showed that a heating duration of 8 min was sufficient to deactivate all the phage particles present in the precipitated protein samples.

As a result of successful complete phage deactivation, the lytic activity of endolysin on EDTA-treated bacterial cells was observed. Lysis was observed at the area spotted with precipitated protein at 60-80% ammonium sulfate saturation. Since the phage was shown to be deactivated at this particular temperature and heating duration, the lysis could be caused by endolysin. Although the crude phage lysate may contain other bacterial proteins like bacteriocins and phage proteins such as holins, polysaccharide depolymerases and virion-associated peptidoglycan hydrolases (VAPH) which also can result in bacterial lysis (Yang, et al., 2014; Drulis-Kawa, Majkowska-Skrobek and Maciejewska, 2015), however, the presence of these proteins in the lysate was not investigated in this study. This is because there is still no reports regarding the *S. dysenteriae* can produce bacteriocins. Besides that, the genome of SDyM-18 phage is also undergoing investigation in the present. Therefore, information

such as the molecular weight of these proteins which can indicate their presence in the precipitated protein samples determined through SDS-PAGE is still limited.

In addition, the SDS-PAGE gel of precipitated protein samples which was described in Section 4.2 showed that there was a protein band with approximate molecular weight lesser than 20 kDa present only in precipitated protein sample at 60-80% ammonium sulfate saturation which could be the SDyM-18 phage endolysin (17.4 kDa). Thus, the finding from SDS-PAGE gel correlates with the result in spot lytic assay, which further confirmed that the lysis could be due to endolysin. Therefore, it was suggested that the endolysin of SDyM-18 phage was successfully precipitated out at 60-80% ammonium sulfate saturation.

Based on Figure 4.6E and 4.7E, it can be seen that a heating duration of 10 min at 70°C has led to the degradation of endolysin. Endolysin is an enzyme, a protein which is generally sensitive to high temperature. The sensitivity of endolysin to high temperature could be influenced by its structure. Endolysins which derived from phages that infect Gram-positive bacteria and Gram-negative bacteria have different structures. The typical feature of endolysin from phages infecting Gram-positive bacteria is the modular structure which is

composed of two distinct functional domains (Borysowski, Weber-Dabrowska and Górski, 2006). As a result, this contribute to its higher molecular weight and therefore higher thermal stability. Findings from several studies also showed similar phenomenon. For example, BacL49 endolysin from Gram-positive *Streptococcus iniae* phage were found to be 54 kDa in size and is heat stable at 100°C for 60 min (Wright, 2010). Moreover, LysBPS13 endolysin from a Gram-positive *Bacillus cereus* bacteriophage demonstrated remarkable thermostability whereby it still exhibited lytic activity even after incubation at 100 °C for 30 min (Park, et al. 2012).

In contrast, for endolysins which derived from phages that infect Gram-negative bacteria, they are mostly small globular proteins with single-domain (Schmelcher, Donovan and Loessner, 2012). As a result, this contributes to its lower molecular weight and therefore lower thermal stability. A typical example of endolysin against Gram-negative bacteria is T7-endolysin derived from bacteriophage T7 which infects *E. coli* B cells. It is a single-domain, globular protein of 17 kDa in size which denatured when heated at 70°C for 5 min (Kleppe, Jensen and Pryme, 1977). Furthermore, LysO1 endolysin which against *Salmonella* is 21.3 kDa in size and found to be least stable at 40°C (Settle, 2012).

In this study, since SDyM-18 phage was found out to be an environmental bacteriophage which is heat-resistant, therefore the endolysin derived from SDyM-18 phage which showed thermal stability up to 70°C for 8 min can be considered as heat-stable endolysin.

5.4 Limitations of Study

A more effective method such as gel filtration chromatography can be adopted in order to remove the co-precipitated bacteriophage. However, the size of SDyM-18 phage was unable to be determined through this study. Therefore, this limitation has restricted the use of gel filtration chromatography.

5.5 Future studies

Alternative ways can be investigated to determine their effectiveness in bacteriophage removal. For example, the precipitated protein samples can be subjected to UV (wavelength ranges from 200-280 nm) exposure. Moreover, chromatography technique such as gel filtration or ion-exchange can also be considered. In addition, affinity chromatography can be conducted to purify the endolysin due to its target specificity. Last but not least, other properties of the extracted endolysin such as host specificity and pH stability can be investigated in the future studies. The extracted endolysin may also be sequenced to

determine its amino acid sequence.

CHAPTER 6

CONCLUSION

Ammonium sulfate precipitation method can be used to extract the endolysin from crude phage lysate. However, the co-precipitation of bacteriophage together with endolysin could mask the lytic activity of endolysin. In this study, the SDyM-18 phage was found to be deactivated when heated at 70°C for 10 min. Therefore, the heat treatment of the precipitated protein samples at 70°C for 8 min can successfully deactivated the bacteriophage and revealed the lytic activity of endolysin. The findings from SDS-PAGE and spot lytic assay revealed that the SDyM-18 phage endolysin was successfully precipitated at 60-80% ammonium sulfate saturation.

Besides that, the lysis zone was observed only on EDTA-treated bacterial cells but not on non EDTA-treated bacterial cells. Therefore, it can be concluded that the SDyM-18 phage endolysin can only works with outer membrane permeabilizer. Moreover, it was also found that the SDyM-18 phage endolysin is heat-stable at which it could withstand temperature up to 70°C for 8 min.

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