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Ph.D. Dissertation

Intracellular Targeting Mechanisms of Novel Multifunctional Peptides from *Bacillus* Genera Reveals Diverse Biological Insights

Graduate School of Chosun University

Department of Pharmacy

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Bacillus genera 균주 유래 기능성 펩타이드의 새포내 표적 메커니즘 및 생물학적 이해

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Advisor: Prof. Jin Cheol Yoo

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ABSTRACT

Intracellular Targeting Mechanisms of Novel Multifunctional Peptides from *Bacillus* Genera Reveals Diverse Biological Insights

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The present study reports that many fermented foods have both nutritional and non-nutritional elements, which can modulate complex molecular functions that are important to the consumer's well-being and health. However, in various regions of the world, about 90% of naturally fermented foods and alcoholic beverages are still dependent on traditionally home-made processing. Fermented foods and beverages contain microorganisms that are both functional and non-functional. During fermentation, active microorganisms change the chemical constitutions of animal/plant source materials, thereby improving nutrient bioavailability, sensory food quality, bio-preservative effects and improving food safety and the degradation of toxins anti-nutritional ingredients, creating antimicrobial and antioxidant compounds, stimulating food safety.

In this dissertation, we report the isolation, purification, and complete biochemical characterization of novel peptide MS07 and MS15 from the strain *Bacillus siamensis* CBSMS07 *and Bacillus velezensis* CBSMS15, *respectively*, isolated from Korean traditional fermented food kimchi. In addition, we studied antibiofilm and

antimicrobial activity mechanism study of MS07. Moreover, upon on confirmation of the novelty of peptide MS15, we studied the antimicrobial and antioxidant mechanism study through Nrf2 mediated HO-1 pathway of MS15.

Efforts to isolate a broad-spectrum antimicrobial peptide (AMP) from microbial sources have been on the rise recently. Here we report the identification, optimization of culture conditions, and characterization of an efficient AMP from *Bacillus* strain designated MS07 that exhibited antimicrobial and antibiofilm activity. The production of MS07 was maximized by evaluating the culture conditions by response surface methodology to get optimum media compositions. The biochemical integrity of MS07 was assessed by a bioassay indicating inhibition at ~6 kDa, like tricine SDS-PAGE. MALDI-TOF confirmed molecular weight, and purity stated the presence of a molecular mass of 6.099 kDa. Peptide MS07 exhibited its antimicrobial activity counter to both gram-positive and gram-negative bacteria. The MIC of MS07 for *Escherichia coli*, *Alcaligenes faecalis*, *MRSA*, *Pseudomonas aeruginosa* ranged from 16-32 µg/mL, demonstrating superior potency. The count of biomass was diminished by about 15% and 11%, with rising concentrations up to 8× MIC with *P. aeruginosa* and *E. coli* biofilm, separately. MS07 exhibited 8 µM and 6 µM minimum bactericidal concentration on biofilm counter to the gram-negative strain *P. aeruginosa, and E. coli* biofilm, respectively. Peptide MS07 was reduced and interrupted the biofilm development in a concentration- reliant by a Backlight live/dead stain using confocal microscopy. MS07 was shown to bind to membranes and to depolarize them. Against Gram-negative bacteria, peptide MS07 lipopeptides have superior antimicrobial activity. Overall, this

study lays the groundwork for the development of MS07 to treat life-threatening infections as a new generation of novel lipopeptide antibiotics.

The efficient culture and purification of antimicrobial peptides (AMPs), along with intense antioxidant activity, have drawn the interest to study antioxidant activity mechanism. We report the culture conditions optimization, efficient biosynthesis, and purification of an antioxidant peptide MS15 from *Bacillus velezensis* obtained from fermented food that would generate heme oxygenase-1 (HO-1) expression and lead to nuclear factor erythroid 2-related factor-2 (Nrf2) nuclear translocation. We explored the ability of kinetics and potency for the bacterial killing to work against various pathogenic bacteria. The MIC of MS15 ranged from 2.5–160 μg/mL for many pathogenic bacteria, showing greater potency. In macrophage RAW 264.7 cells, MS15 was exposed to assess its inhibitory effect against the generation of reactive oxygen species (ROS) in oxidative stress. In the sample treated group, the translation, and transcriptional levels of CAT (catalase), GPx (glutathione peroxidase), and SOD (superoxide dismutase) were significantly greater. In short, MS15 has significant antioxidant properties, reducing ROS production in RAW 264.7 cells, and raising the translation and transcriptional rates of antioxidant enzymes with stimulating HO-1 induction facilitated by Nrf2.

The current study aimed to isolate and purify a cytoprotective antioxidant peptide that acts against oxidative damage. To realize its activity counter to numerous pathophysiological disorders related to oxidative stress, we tried to verify by studying its antioxidant mechanism.

This study indicates that MS07 and MS15 could be candidates for natural antibiotics, antimicrobials, antioxidants, and probiotics, respectively. A promising antimicrobial candidate for therapeutic application may be regulated by peptide MS07. It also indicates that probiotics such as *Bacillus velezensis* CBS15 peptide MS15 could be used as natural foods and avoid oxidation reactions in food processing.

국문 초록

Bacillus genera 균주 유래 기능성 펩타이드의 새포내 표적 메커니즘 및 생물학적 이해

앰디마러프 칸

지도교수: 유진철

조선대학교 대학원 약학과

최근 다양한 연구를 통하여 발효식품들은 보통 영양학적 가치가 있는 다양한 성분을 포함하고 있어 소비자의 건강에 있어 중요하고 복잡한 생리적 기능을 조절하고 있음을 보고하고 있다. 한 편 여러 지역에서 생산되는 발효식품 및 알코올 음료의 약 90%는 집에서 만들 수 있는 전통적인 가공방식을 사용하고 있다. 이때 발효식품과 알코올 음료에는 기능성과 비기능성을 갖춘 다양한 미생물이 분리되는데, 발효 중에 역할을 하는 미생물은 동물/식물원료의 화학적 구성을 변화시켜 영양소 생체이용률, 생물보존효과를 향상시키고 식품안전성과

독소성분 분해효율을 향상시켜 항균물질 및 항산화물질을 생성하고 식품의 안전성에 기여한다.

본 논문에서는 한국의 전통 발효식품인 김치에서 분리한 *Bacillus siamensis* CBSMS07 과 *Bacillus velezensis* CBSMS15 균주를 통해 이들이 생산하는 기능성 펩타이드 MS07 과 MS15 를 분리하였으며 펩타이드의 정제 및 수준높은 생화학적 특성 등을 보고하고 있다. 또한 MS07 의 항균필름과 항균활동 메커니즘 연구를 진행하였으며, 펩타이드 MS15 의 생화학적 기능성 확인과 함께 펩타이드 MS15 의 Nrf2 매개 HO-1 경로를 통해 항균 및 항산화 메커니즘 연구를 연구하였다.

최근들어 미생물 공급원으로부터 광범위한 항균 펩타이드(AMP)를 분리하려는 노력이 증가하고 있다. 이러한 연구에 발맞춰 항균 및 항균필름 활동을 보인 MS07 로 명명된 바실러스 균주의 효율적인 AMP 분리, 배양 조건의 최적화 및 특성화에 대한 연구를 진행하였다. MS07 의 생산은, 최적배지 조성을 확인하기 위해 다양한 배양 조건을 평가하였으며, MS07 의 생화학적 분리는 tricine SDS-PAGE 를 통해 약 6 kDa 에서 단일 밴드를 확인하였다. 또한 MALDI-TOF 를 통해 6.099 kDa 의 분자량과 Purity 를 확인하였다. 미생물 유래 항균펩타이드인 MS07 은

그램 양성 박테리아와 그램 음성 박테리아 모두에 대응할 수 있는 항균활성을 보였으며 특히 *Escherichia coli, Alcaligenes faecalis, MRSA, Pseudomonas aeruginosa* 에 대한 MS07 의 MIC 는 16-32μg/mL 로 비교적 우수한 활성을 보였다. Biomass 수치는 *P. aeruginosa* 와 *E. coil* 에 대응하여 바이오필름의 경우 최대 8 배까지 농도가 상승하면서 약 15%, 11% 감소했다. MS07 은 그람 음성균주인 *P. aeruginosa* 와 *E. coil* 의 바이오필름에 대해 각각 8μM 과 6μM 의 최소 억제 농도를 바이오필름 카운터에서 확인할 수 있었다. MS07 은 confocal microscopy 를 활용한 concentration-reliant by a Backlight live/dead stain 에서 바이오필름의 생성의 억제를 확인하였다. 전반적으로, 본 연구는 생명을 위협하는 감염병으로부터 lippo-펩타이드를 활용한 새로운 세대의 항생제인 MS07 개발의 토대를 제공하고 있다.

최근들어 항균 펩타이드(AMP)의 효율적인 배양과 정제는 강력한 항산화 활성과 함께 항산화 활성 메커니즘을 연구함에 있어서 관심을 끌고 있는데, Heme oxygenase-1 (HO-1)을 생성하여 핵인자 에리트로이드 2 관련 인자-2(Nrf2) 핵번역을 유도하는 항산화 펩타이드 MS15 의 배양조건 최적화, 효율적인 생합성 및 정제에 관하여 보고하고 있다. 본 연구에서는 확인할 수 있는 광범위한 항균활성은 다양한 병원성 박테리아에 대항할 수 있는 동력학적 및 항균활성을 연구하였다. 특히

MS15 의 MIC 는 많은 병원성 박테리아에 대해 2.5~160μg/mL 로 넓은 범위에서 활성을 보임으로써 그 가능성을 입증하였다. 대식세포인 RAW264.7 Cell 에서 MS15 는 산화 스트레스에서 반응성 산소종(ROS) 생성에 대한 억제 효과를 확인하였는데, 샘플 처리 그룹인 CAT(카탈라제), GPx(글루타티온페록시디제), SOD(과산화디푸타제)의 활성 수준이 현저히 높았다. 요컨대 MS15 는 항산화 성질이 유의하여 RAW264.7 세포에서 ROS 생성을 감소시키고 Nrf2 가 촉진하는 자극적인 HO-1 유도를 통해 항산화효소의 활성을 상승시키는것으로 추측해볼 수 있다.

이번 연구는 산화기전 대해 대응할 수 있는 항산화 펩타이드의 분리와 정제를 목표로 하고있다. 산화 스트레스와 관련된 수많은 병태생리학적 장애에 대항하는 활동을 실현하기 위해 항산화 메커니즘을 연구하여 검증하려고 노력하였다.

본 연구는 MS07과 MS15가 각각 천연 항생제, 항균제, 항산화제 및 프로바이오틱스등의 우수한 후보소재로서의 가능성을 타진하고 있다. 치료제 적용을 위한 유망한 항균제 후보물질은 펩타이드 MS07로 제시될 수 있으며, *Bacillus*

velezensis CBS15펩타이드 MS15와 같은 프로바이오틱스를 자연식품으로 사용할 수 있고 식품가공에서 산화반응을 억제할 수 있음을 보여주고 있다.

Abbreviations

ABTS : 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic

ACE : Activity of The Angiotensin-Converting Enzyme

AMP : Antimicrobial peptide

APD : Antimicrobial Peptide Database

APP : Avian Pancreatic Polypeptide

AU : Arbitrary Unit

AUC : Area Under the Curve

BLAST : Basic Local Alignment Search Tool

BSH : Bile Salt Hydrolase

CaM : Calmodulin

CAT : Catalase

CFU : Colony Forming Unit

RSM : Response Surface Methodology

CPP : Calcium Binding Phosphopeptides

CUPRAC : Cupric Reducing Antioxidant Capacity

DC : Dendritic Cells

diSC3-5 : 3,30-Dipropylthiadicarbocyanine

DCFDA : Cellular Reactive Oxygen Species Detection Assay

DPPH : 2,2-Diphenyl-1-picrylhydrazyl

EDTA : Ethylenediaminetetraacetic acid

FICI : Ractional Inhibitory Concentration Index

FRAP : Ferric reducing antioxidant power

GMP : Calcium-Binding Phosphopeptides

GPx : Glutathione Peroxidase

HDP : Host Defense Peptide

IEC : Epithelial Cells

LAB : Lactic Acid Bacteria

LPS : Lipopolysaccharide

MALDI-TOF : Matrix-Assisted Laser Desorption - Ionisation-Time of Flight

MBEC : Minimum Biofilm Eradication Concentration

MDR : Multi Drug Resistance

MH : Mueller-Hinton

MHA : Mueller Hinton Agar

MHB : Murlller Hinton Broth

MIC : Minimum Inhibitory Concentration

MRS : Media-De Man-Rogosa-Sharpe

MRSA : *Methicillin-resistant Staphylococcus aureus*

MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NMDAR : N-Methyl-d Aspartate Receptors

NPN : 1-N-phenylnaphthylamine

NPY : NeuroPeptide Y

NQO1 : NAD(P)H-Quinone Dehydrogenase 1

Nrf-2 : NF-E2-related factor-2

NRP : Nonribosomal Peptide

NUC : Nucleic Acid

OM : Outer Membrane

ORAC : Oxygen radical absorbance capacity

PDB : Protein Data Bank

POMC : Proopiomelanocortin

POPC : Phosphatidylcholine

POPG : Phosphatidylglycerol

PPY : Pancreatic PolYpeptide

RNS : Reactive Nitrogen Species

RONS : Reactive Oxygen and Nitrogen Species

ROS : Reactive Oxygen Species

SDS-PAGE : Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SOD : Superoxide Dismutase

SUV : Small unilamellar vesicles

TDCA : Tauroglycocholic Acid

TEM : Transmission Electron Microscopy

VRE : *Vancomycin-Resistant Enterococci*

CHAPTER ONE

INTRODUCTION

1.1. Antibiotic discovery and the emergence of antibiotic resistance

A significant aspect of modern medicine, antibacterial treatment, has dramatically increased human health and life expectancy globally, providing benefits to individuals and society. Animal production agriculture has also been revolutionized using antibiotics in livestock and poultry, especially for growth promotion, but also to combat endemic diseases in increasingly larger classes of animals. This started with the discovery and development of sulfonamides and penicillin during the first half of the twentieth century, followed by the so-called Golden Era of drug discovery [1, 2], which accelerated the discovery of many novel classes of antibiotics (Figure 1.1).

However, the selection and dissemination of resistant bacteria have been favored by widespread (over- and mis-) use of antibacterial drugs over the past decades. With the ongoing lack of development and commercial acceptance of effective novel antibacterial medicines, susceptibility to widely applied antibiotics by clinically applicable bacteria remains a global emergency for health. With drug-resistant infections causing elevated morbidity, death, and overall healthcare costs in both human and veterinary medicine, the increasing incidence of antibiotic resistance in bacteria is a leading healthcare concern worldwide [3]. It is estimated that 25,000 Europeans and 23,000 Americans die due to infections with multidrug-resistant bacteria annually. While hard to quantify, bacterial antimicrobial resistance has been estimated to cost about EUR 1.5 billion per year to the European Union economy and about $21 billion and $34 billion per year to the US healthcare system [4]. Increased morbidity and mortality are predicted to emerge from antibiotic resistance, with related exorbitant rises in healthcare prices and indirect economic consequences worldwide.

Compounds discovered in the Golden Age of antibiotic development (the 1940s to 1960s) or versions of these compounds include virtually all antibiotics commonly used. However, in the last 20 years, only two new types of antimicrobials have been licensed, considering the tremendous high-tech development efforts that began in the 1990s (Figure 1.1). History has demonstrated that the development of resistance in the target microorganism has rapidly accompanied the launch of a new antibiotic. The needed steady flow of novel groups of antibiotics and derivatives has been outpaced by developing and spreading antibiotic resistance. While bacteria have hundreds of essential proteins, the number of targets for antibiotics commonly used is comparatively limited. The most successful antibiotics hit only three targets or pathways: ribosome (affecting protein synthesis), DNA gyrase or DNA topoisomerase (affecting DNA synthesis), or cell-wall synthesis [5]. Other antibiotics target RNA synthesis, the pathways of metabolism of folic acid, and the membrane of the bacterial cell (Figure 1.2).

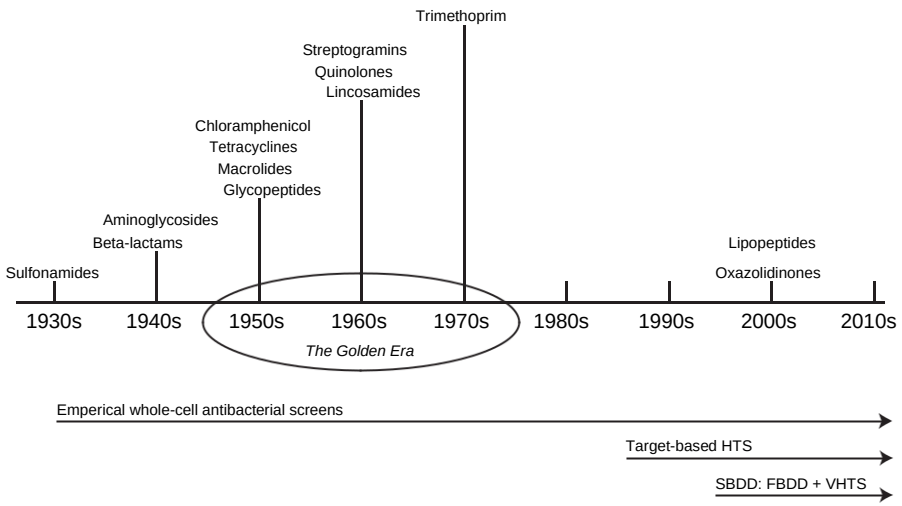


Figure 1.1. Antimicrobial drug development history, demonstrating the "Golden Age" of the development of antimicrobial drugs from ~1945-1976. Market clearance is past the timeframe for various types of antibiotics per decade. Under the timeline (HTS: High-

Throughput Screening, SBDD: Structure-Based Drug Discovery, FBDD: Fraction-Based Drug Discovery, VHTS: Virtual High-Throughput Screening), the essential techniques used in antimicrobial drug discovery are shown.

A natural ecological occurrence that is prevalent in the world is antibiotic resistance. Bacteria have developed over billions of years to resist the action of antibacterial compounds that occur naturally. It has been shown that resistance to antibiotics in bacteria already existed before the advent of the current selective pressure caused by human use of antibiotics. This drastic rise in selective pressure caused by the widespread use of antibiotics over the past 70 years has intensified the production and spread of antibiotic-resistant bacteria [6]. Although antibiotic resistance is a dynamic phenomenon, it is possible to divide its pathways into three categories: innate, acquired, and adaptive resistance. Figure 1.2 shows some common antibiotic targets and pathways associated with antibiotic resistance.

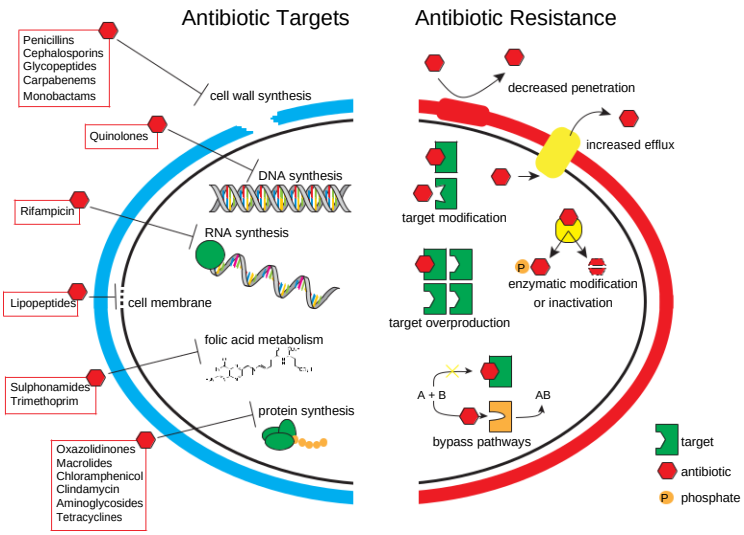


Figure 1.2. Antibiotic targets and tolerance pathways.

1.2. Alternatives to conventional antibiotics

The troubling issue of antibiotic resistance has contributed to increased research in alternative methods, such as the use of bacteriophages, synthetic antimicrobial peptides, and naturally occurring bacteriocins to fight pathogenic bacteria. Renewed excitement about identifying natural products and the vast amount of uncharacterized clusters of biosynthetic genes (BGCs) has also increased attempts to discover novel secondary antimicrobial metabolites from microbes. Another historically popular technique against bacteria causing infectious diseases is a vaccine, which is both relatively inexpensive and highly effective. While new instruments and vaccination techniques are continually being developed, there are still many obstacles, such as recurring, highly volatile, and novel pathogens, complicated (e.g., polymicrobial) diseases, and hard-to-reach target populations. As an alternative approach to reduce the risk of infection by pathogenic bacteria, the study in this research would concentrate on antimicrobial peptides [7, 8].

1.3. Bioactive peptide

Bioactive Peptides have been identified as unique fragments of protein that may affect health and have a definite influence on potential physical conditions or functions in the body. Peptidomics is the rigorous quantitative and qualitative research in a biological sample of all the peptides. A recent field arises from proteomics and is enabled by existing separation, analysis, and computing technology. Bioactive peptides are proteins in the cell that are synthesized in propeptides, then cleaved and converted into usable products[9]. Bioactive peptides perform essential roles as the signaling agents in pathogenesis and physiological activity.

1.4. Antimicrobial peptides

The use of antimicrobial peptides (AMPs) is, as described above, one of the alternatives to traditional antibiotics that are currently being studied and has drawn significant attention from the scientific community. Peptides with antibacterial and antifungal functions have an ancient genetic history and are commonly spread in all fields of life; the Antimicrobial Peptide Archive (http:/aps.unmc.edu/AP/) deposits sequences of 2692 antimicrobial peptides from six kingdoms. In many lifeforms, their effectiveness in antimicrobial action against pathogens has possibly led to their ubiquity. AMPs have been found in numerous multicellular organisms and are an essential component of innate immunity and form the first line of defense against pathogenic microbes [10].

AMPs typically function non-specifically (broad-spectrum activity) and quickly (within minutes) as an essential part of innate immunity. There is a wide range of recognized peptide sequences, and there is usually no sequence resemblance between peptides isolated from different species. This work focuses mainly on the antimicrobial function of AMPs. However, several AMPs have been shown to have other parts in addition to overt pathogen destruction, such as endotoxin neutralization, immune cell recruiting, cytokine production regulation, suppression of potentially dangerous inflammation, cell differentiation induction, and enhancement of adaptive immune responses, cell survival, wound healing, and angiogenesis, and are therefore also involved [11].

1.5. Antioxidant peptides

The significance of oxidation in our body and food is generally known. Oxidative metabolism is an important mechanism for cells to live. They may overtake protecting

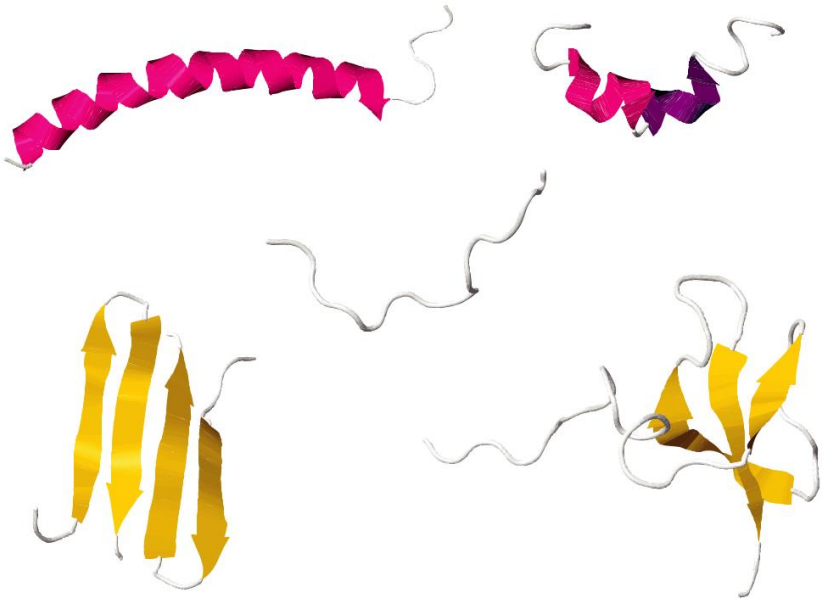
enzymes such as catalase, superoxide dismutase (SOD), and peroxidase that trigger damaging and lethal cellular effects (e.g., apoptosis) of cellular proteins, DNA, oxidizing membrane lipids, and enzymes, thus shutting down the cell phase. In tests, antioxidant peptides from caseins have been documented in digestive enzyme hydrolysis and milk fermentation with proteolytic LAB strains. Most of the peptides identified are derived from alpha-casein. Free radical scavenging activities have been shown to prevent enzymatic and non-enzymatic lipid peroxidation, most likely by being a favored target over radicals free of fatty acids [12]. In order to elucidate the function of peptides in oxidative stress conditions, more and more additional exploration is required.

1.6. Structures of AMPs

Characterize the vast range of AMPs based on characteristics such as origin, secondary structure, mode of action, or post-translational modification. Several peptide databases have been developed over the last two decades. The most popular three-dimensional secondary structures are historically known as AMPs: alpha-helices (e.g., LL-37, magainin, cecropin), β-strands with several stabilizing disulfide bridges (e.g., human alpha- and β-defensin, protegrin), loop structures with one internal disulfide bridge (e.g., bactenecin), and expanded structures abundant in glycine, proline, arginine tryptophan, or histidine [13].

Cationic alpha-helical AMPs are a commonly occurring and extensively studied class of HDPs. Such linear AMPs often behave as unorganized coils in an aqueous solution, but in the presence of phospholipid membranes, they may adopt an active secondary alpha-helix configuration. Clusters of cationic and hydrophobic amino acid residues in this form

are arranged three-dimensionally into distinct molecule clusters, resulting in an amphipathic structure. Often, multiple cationic alpha-helical AMPs can be found in amphibian skin secretions, such as the magainins being among the first to be identified HDPs. The antimicrobial and cytolytic function of these peptides is defined by the parameters of cationic, hydrophobicity, alpha-helicity, and amphipathicity, which can be altered to change antimicrobial potency or specificity by amino acid substitution. Naturally occurring amphibian AMPs and their analogs were tested for their action and selectivity against pathogenic bacteria in this thesis, and the human cathelicidin LL-37 was used as a cationic peptide model [14].



Protegrin-3 (2MZ6) Human beta-defensin (1KJ6)

Figure 1.3. Three-dimensional configurations of chosen AMPs that highlight the diversity of structures. In pink, β-helical motifs are shown, while β-sheets are seen in purple. The Protein Data Bank ID of the models shown is given in brackets.

1.7. Bacteriocins

Ribosomally synthesized prokaryote-origin AMPs are commonly referred to as bacteriocins and have been suggested as alternatives to traditional antibiotic treatment or synergistic complementary agents. Class I peptides (e.g., nisin, thuricin CD, bottromycin A2) are typically categorized on the basis of their post-translational modification, while class II peptides (e.g., pediocin PA-1, epidermicin NI01, enterocin AS-48) in their active form are mostly unmodified or cyclic. In common with traditional antibiotics, bacteriocins target specific mechanisms or processes, such as inhibition of cell wall biosynthesis and DNA or protein synthesis. Besides, certain bacteriocins have been found to disturb the integrity of the membrane [15]. In general, against a small range of phylogenetically closely related to the producer, bacteriocins have a narrow-spectrum function. They are thus thought to modulate the composition of niche-colonizing bacterial species without significantly disrupting the microbiota. The development of such AMPs is widespread among bacteria and, through multiple ecological functions, is considered to contribute to bacterial fitness, colonization ability, and probiotic functionality. Commensal development of precisely tailored AMPs against in situ pathogens is an attractive approach to combat infectious diseases has been considered. For example, the two-peptide bacteriocin thuricin CD that shows narrow-spectrum antimicrobial activity against *Clostridium difficile* was found to generate a *Bacillus thurigiensis* isolate from human feces without significantly affecting the resident microbiota that could contribute to *C. recurrence* [16]. The complicated diarrhoea is associated with it.

1.8. Non-ribosomal peptide biosynthesis

In addition to ribosomally synthesized peptides, utilizing massive multifunctional enzymes encoded by BGCs, many microbes can generate secondary metabolites of broad structural diversity, including peptide antibiotics. Non-ribosomal peptide synthetases (NRPSs), consisting of a linear assembly of units, each of which is further subdivided into distinct catalytic domains, constitute one class of BGC. Each module is responsible for the integration into the increasing peptide chain of one building block (e.g., amino or hydroxy acid), with the different domains catalyzing the unique activation of the substrate, covalent binding, and the forming of peptide bonds [17]. In this way, the NRPS contains both the assembly machinery and the product's basic biosynthesis template. As the potential building blocks are not limited to the 20 canonical amino acids, the structural diversity of NRPS related molecules is immense. More than 530 probable monomers and 1179 distinct peptides that are not ribosomally derived have been known to date. Many pharmaceutically useful antimicrobial substances used today in the pharmacy are products or derivatives of microbial NRPS. Vancomycin, a powerful medication against Gram-positive bacteria, belonged to the glycopeptide family of antibiotics and was already isolated and identified in the early 1950s. The cyclic lipopeptide daptomycin was found in *Streptomyces roseosporus* fermentations and is now used as a last-resort antibiotic against Gram-positive skin infections.

1.9. Production of peptides

A variety of methods of generating biologically active peptides from precursor proteins are used. The most frequent ones are—

a. With intestinal enzymes, enzymatic hydrolysis,

b. Via the microbial behavior of fermented food items,

c. Via the action of proteolytic microbe-derived enzymes.

It is possible to synthesize peptides upon confirmation of the composition of a bioactive peptide. Three methods are widely used at present.

i. Synthesizing compounds

ii. Recombinant technology with DNA and

iii. enzymatic synthesis

1.9.1. Enzymatic hydrolysis

Enzymatic hydrolysis is the most popular method of generating bioactive peptides by enzymatic hydrolysis of whole protein molecules. Numerous recognized bioactive peptides and enzyme combinations of various proteinases, such as pepsin, trypsin, pancreatin, chymotrypsin, and thermolysis, have been produced using digestive enzymes [18].

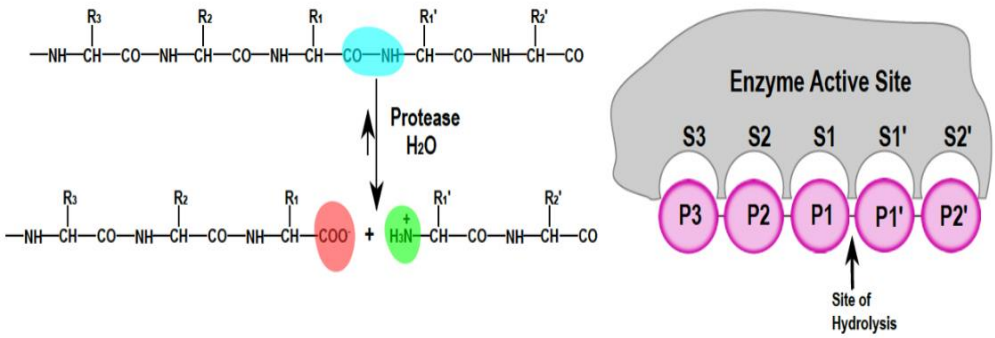


Figure 1.4: A protease regulated hydrolysis of a peptide bond. (A) A scheme of hydrolytic reaction. (B) The active of protease is composed of subsites (S). Every S has

an affinity for respective residues (P). By the mean of a mechanism called “Lock and Key” directions protease specificity.

Studies have shown that hydrolysis of proteins from milk using digestive enzymes can produce bioactive peptides. Several calcium-binding phosphopeptides (CPPs), antihypertensive peptides, immunomodulatory, antibacterial, and opioid peptides from a number of casein (alpha-, β- and j-casein) and whey proteins such as β-lactoglobulin (β-lg), alpha-lactalbumin (alpha-la), and glycomacropeptide (GMP) have been shown to release enzymes, pepsin, trypsin, and chymotrypsin.

1.9.2. Microbial fermentation

Since many dairy starter cultures have a highly proteolytic nature, it is possible to anticipate bioactive peptides to be generated when fermented dairy products are produced from microbial bioactive peptides release proteolysis of milk proteins. Many studies have mainly reported that the strains, *Lactobacillus helveticus*, produce antihypertensive peptides, the better known of which are the inhibitory tripeptides Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP). The antihypertensive ability of these peptides has been identified in many animal and human studies [19, 20]. Fermentation of milk with a commercial starter culture (a mixture of five *Lactobacillus* bacterial strains) accompanied by hydrolysis with a microbial protease enzyme to increase the ACE inhibitory action of hydrolysate has also been reported for different bioactive peptides produced in milk during fermentation by cheese starter bacteria, yogurt bacteria, and commercial probiotics.

Existing research data indicate that many fermented foods have nutritional and non-nutritious components that modulate the capacity for unique molecular activity essential to the consumer's well-being and well-being. Nearly 90 percent of naturally fermented foods, though, and alcoholic drinks are also typically home-made in various world regions. Naturally, fermented foods and liquids contain both functional and non-functional microorganisms. Active microorganisms transform the chemical constitutions of products from animal/plant origins during fermentation, thus improving the bio-availability of nutrients, sensory food quality, bio-preservative effects, enhancing food safety, and degrading toxicity components and anti-nutrition factors, producing antimicrobial and antioxidant substances, stimulating pro-food protection. Lactic acid bacteria (LAB) are also *Lactobacillus species*, *Enterococcus*, *Pediococcus*, *Lactococcus*, *Leuconostoc*, *Weissella*, etc. and alcoholic beverage-related bacteria. Many fermented foods and drinks are commonly present [21]. In food fermentation, some genera and plants' microorganisms are used as commercial starts (Table 1.2). Some of the items are internationally sold as health foods, nutritional foods, nutraceutical foods, and medicinal foods.

1.10. Fermented food of Korea

Many fermented foods and drinks are the essential component of the Korean cuisine that Koreans and many people around the world eat during the year. Fermented food items from Korea have also become popular and are well received by individuals around the world. The primary fermented food products are fundamentally classified into three broad categories as bellow (Korean fermented foods, Kimchi and Doenjang, in the Handbook

of Fermented Functional Foods), excluding the alcoholic drinks consumed today in Korea [22-24].

Category one: Fermented foods in this group consist of products derived from soy, including doenjang (soybean paste), chongkukjang (quickly fermented soybean paste), gochujang (hot soybean pepper paste), and ganjang (soy sauce) (Figure 1.5). Such fermented goods are conventionally prepared once a year; large clay pots are used during the year for storage and use.

Category two: Fermented foods of this type are typical and prepared from fish and shellfish (Figure 1.6A). These items are either eaten as such or blended with kimchi. Meat, shellfish, and their derivatives provide the consumer with a source of essential vitamins, high-quality proteins, minerals, and polyunsaturated fatty acids.

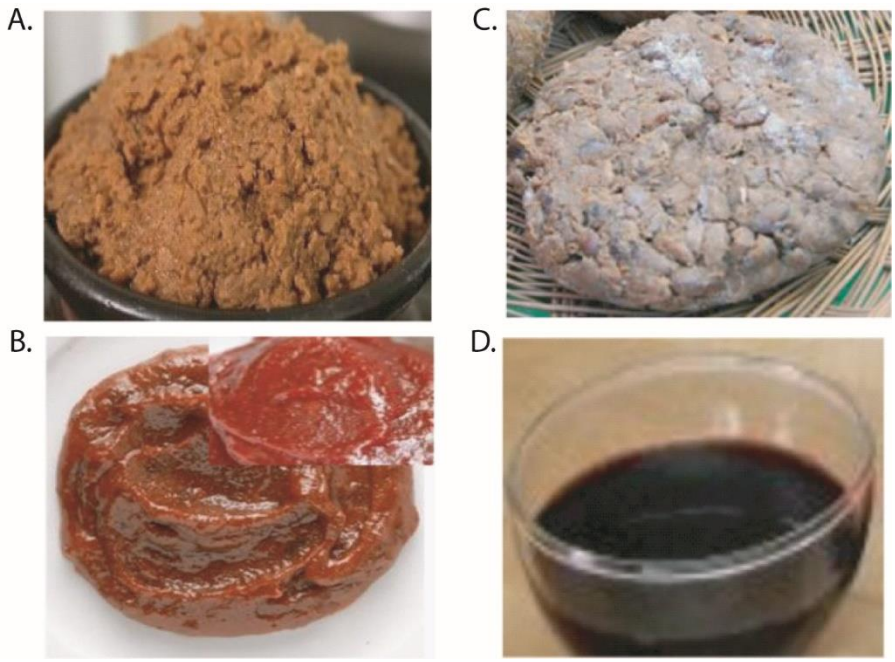


Figure 1.5: Different fermented food in Korea (Category one). In this category, fermented food consists of the soy-based products, which includes doenjang (soybean paste) (A), chongkukjang (quickly fermented soybean paste) (B), gochujang (hot pepper-soybean paste) (C), and ganjang (soy sauce) (D).

Category three: Kimchi is the third form of kimchi, which is eaten most and most commonly not only in Korean but around the world (Figure 1.6B). It is the primary traditional fermented food in Korea. It is made with Chinese cabbage (*Brassica rapa L. spp. pekinensis* [Lour.] Han) and/or radish as its main ingredient, with numerous types of vegetables on the side. The method of Kimchi fermentation takes a short period to complete.

Figure 1.6: Fermented Food prepared from fish and shellfish (A). Korean traditional fermented kimchi (B).

1.11. Kimchi fermentation and its microorganisms

Kimchi fermentation is typically broken into four acidity-based stages; early-stage (acidity<0.2), immature stage (acidity 0.2–0.4), optimal stage of ripening (acidity 0.4–0.9), and eventually, over-ripening or rancid stage (acidity>0.9). The profile of the LAB varies with pH and acidity during the kimchi fermentation process. During initial fermentation (pH 5.6-4.2 and acidity 0.48-0.89 percent), *Leuconostoc mesenteroides* is detected, and *Lactobacillus sakei* dominates in fermentation later on (pH 4.15 and acidity 0.98 percent). A different LAB subset related to the fermentation of kimchi is greatly affected by temperature. Lab. Uh. Lab. In kimjang kimchi, *sakei predominates* (as a long-term preserved kimchi for the winter season), and the strain tends to be sufficient for low fermentation temperatures at temperatures of 5-9 ° C and storage at temperatures of -2 ° C. *Lactobacillus plantarum* and Leu have been shown by the normal recognition of bacterial isolates from kimchi. The prevalent genus was *Mesenteroides*. New research from Park and his colleagues reveals that the approach is used by microbial communities of 13 forms of Korean commercial kimchi's at pH 4.2-4.4 [22].

Pyrosequencing, and Wei bacteria. Koreensis (27.2%) accounted for the highest proportion in kimchis of overall LAB; 14.7% of Lab followed. *Sakei*, 8.7% *Weissella Cibaria*, Lab. 8.7% *Graminis* (13.8%), *Lactobacillus gelidum* (6.3%), followed by other species (Figure 1.7).

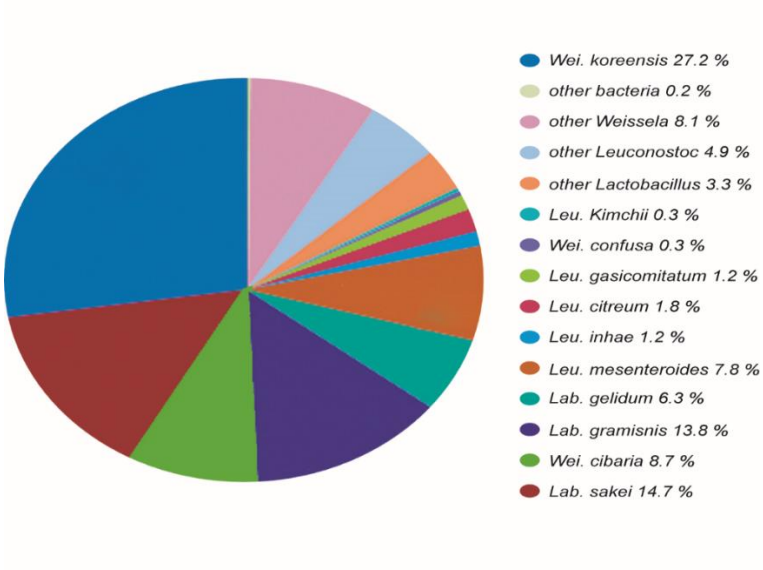


Figure 1.7: Microbial communities of 13 kinds of Korean commercial kimchi.

Yellow-green vegetables are primarily used for the preparation of fermented kimchi, which has been shown to prevent cancer, boost immune function, enhance antimicrobial activity, slow ageing, and prevent constipation. Fermented kimchi has improved its taste and functionality and has become a good food for probiotics [25].

1.12. AMP modes of action

While AMPs lack primary sequence evolutionary conservation and therefore have a wide variety of three-dimensional structures, they typically share common characteristics that are important for their mode of operation, such as cationic, amphipathicity, and short duration. In contrast to higher eukaryotes' membranes, AMPs show a greater precision for binding and inserting into the plasma membrane of bacteria and fungi. This is primarily due to the higher content of anionic phospholipids exposed on the outer leaflet of bacterial and fungal plasma membranes compared to plants and animals' plasma

membranes [26], where the negatively charged headgroups of the phospholipids are mainly located on the inner leaflet. Other elements that are negatively charged, such as lipopolysaccharide (LPS) or wall and lipoteichoic acid (WTA, LTA), are also found only on cell surfaces that are bacterial but not eukaryotic. The electrostatic activity between these elements of the negatively charged cell envelope and positively charged amino acids is commonly assumed to be a key driver of AMP binding and membrane surface accumulation. In addition, a variety of other factors, such as variations in transmembrane potential and the involvement of cholesterol in the target membrane, which change membrane fluidity and stability, can be involved in the selectivity and behavior of AMPs [27].

Different models have been proposed to explain the molecular mode of lethal action of AMPs, but the precise mechanisms are still not well known. However, both models agree on the initial main case of stoichiometric aggregation of AMPs by their binding to phospholipids on the outer leaflet of the plasma membrane, and plasma membrane permeabilization is triggered until a threshold is hit. Traditionally accepted models explain permeabilization by micellisation ('carpet' model), by the formation of pores consisting exclusively of peptides arranged perpendicular to the membrane ('barrel-stave' pore model), by the aggregation of peptides parallel to the surface of the membrane triggering the folding of phospholipids, and by the bending of peptide-phospholipid pores ('toroidal' pore model) (Figure 1.8). Regardless of the process, significant internal homeostasis destruction results, with the targeted microbe having a lethal result [10, 28].

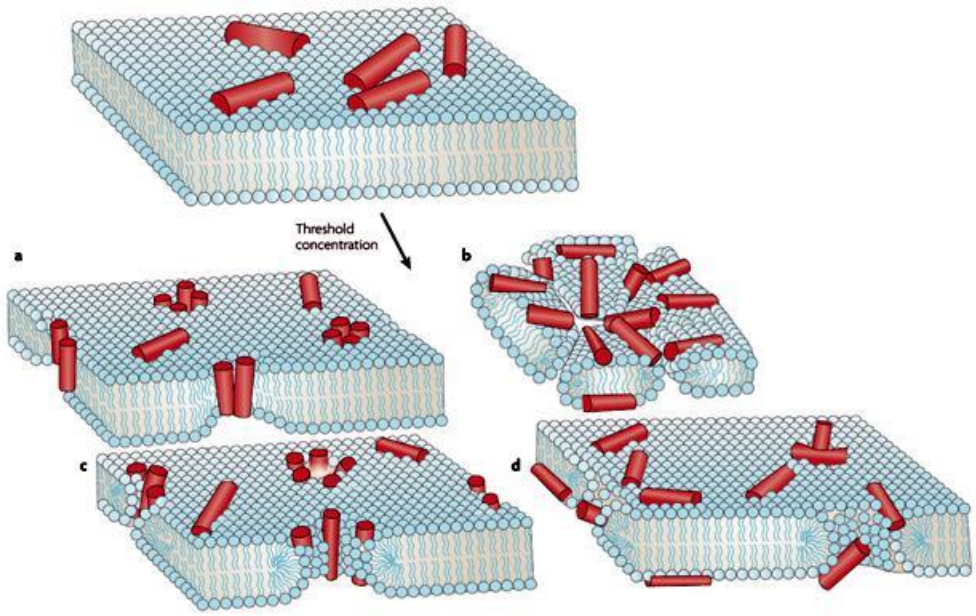


Figure 1.8. Suggested templates for disrupting the phospholipid membrane by AMPs. A threshold concentration of AMPs on the interface of the membrane is initially necessary. (a) Barrel-stave pore, (b) Style carpet, (c) Toroid pore, (d) Toroid pore disorder. Source: Melo et al (2009).

It has lately become apparent that the value of the non-specific contact of AMPs with the phospholipid membrane and eventual disruption of the membrane might be overestimated and that the connection with other specific targets may be responsible for the bactericidal behavior of several AMPs. Nisin, a lantibiotic developed by *Lactococcus lactis* strains that have been commonly used for more than 40 years as a food preservative, is one example. Nisin interacts with the precursor lipid II of the membrane-bound cell wall, thereby inhibiting peptidoglycan synthesis. Also, nisin forms membrane pores composed of lipid II and nisin molecules, allowing membrane potential to dissipate and small metabolites to efflux quickly [29]. Several defenses from different sources have been

shown to directly attack the lipid II block building block of the bacterial cell wall, precisely inhibiting the cell wall's biosynthesis. Fungal defensin plectasin, for example, exhibited very potent antimicrobial activity against Gram-positive pathogens and was able to cure mice from *S. pneumonia*. Peritonitis have been caused by pneumonia without evidence of host toxicity. Without violating the bacterial membrane's integrity, this antibacterial action is entirely due to lipid II binding and blocking of the peptidoglycan synthesis pathway [30, 31].

1.13. Bacterial resistance mechanisms to AMPs

Because of their killing mechanism, the rapid production of microbial resistance to AMPs has always been believed to be impossible because bacteria will have to modify the composition and/or arrangement of retained structures such as the membrane and cell-wall, causing adverse effects. Until recently, this idea was reinforced by the lack of evidence of bacterial resistance to AMPs and was one reason why they attracted a significant amount of research interest. The appealing concept of designing AMPs as novel antibiotics in the 21st century that would circumvent the global issue of antibiotic resistance now needs to be reconciled with recent proof of many pathways of resistance to AMPs [32, 33]. Because of the selective pressure arising from exposure to ambient bacteriocins and host AMPs, the different resistance mechanisms to AMP-mediated killing most likely originated in bacteria and involve surface charge change, aggressive efflux, and membrane fluidity alteration, proteolytic digestion, and surface protein and polysaccharide trapping. In recent reviews, the latest information about methods used by bacteria to combat the lethal action of AMPs is thoroughly defined. Some primary resistance mechanisms are discussed briefly here (Figure 1.9) [34].

Provided that many AMPs are cationic, changing anionic cell surface constituents to decrease the electrostatic attraction of such peptides is a well-described resistance mechanism in both Gram-positive and Gram-negative bacteria. The cell-wall components LTA and WTA normally have an overall negative charge in Gram-positives. Still, the gene products of the dlt operon impart a positive charge by adding d-alanyl esters. This decreases the bacterium's sensitivity to cationic AMPs by electrostatic repulsion and by increasing the density of the cell wall, which, due to altered LTA conformation, sterically hinders AMP entry to the membrane [27]. The alteration of the anionic membrane phospholipid phosphatidylglycerol (PG) by the multi peptide resistance factor (MprF) adds a trace of l-lysine l-alanine, resulting in a net-positively charged PG, is another electrostatic repulsion mechanism observed in both Gram-positives and Gram-negatives. Secreted or membrane-bound proteases can, by effectively cleaving and inactivating them, provide another simple but successful resistance technique against AMPs. For example, in an ex vivo wound fluid model, proteases from multiple clinically relevant human pathogens, such as *Pseudomonas aeruginosa* elastase, were able to break down the human cathelicidin LL-37 and improve bacterial survival [35].

Lipid II is an important and well-preserved part of the peptidoglycan biosynthesis pathway needed in eubacteria for cell wall formation. For several structurally unrelated antibiotics and AMPs, Lipid II provides many binding sites. Their association sometimes leads to defunct cell wall biosynthesis with serious adverse effects on the target bacterium. Change the overall cell surface charge and decrease the binding affinity of AMPs; bacteria may alter lipid II [36, 37]. Structural modifications to the cell wall

components usually are constitutive compared to the controlled resistance induced by surface charge alteration of proteolytic AMP degradation.

Figure 1.9 illustrates these and other typical resistance mechanisms to AMPs.

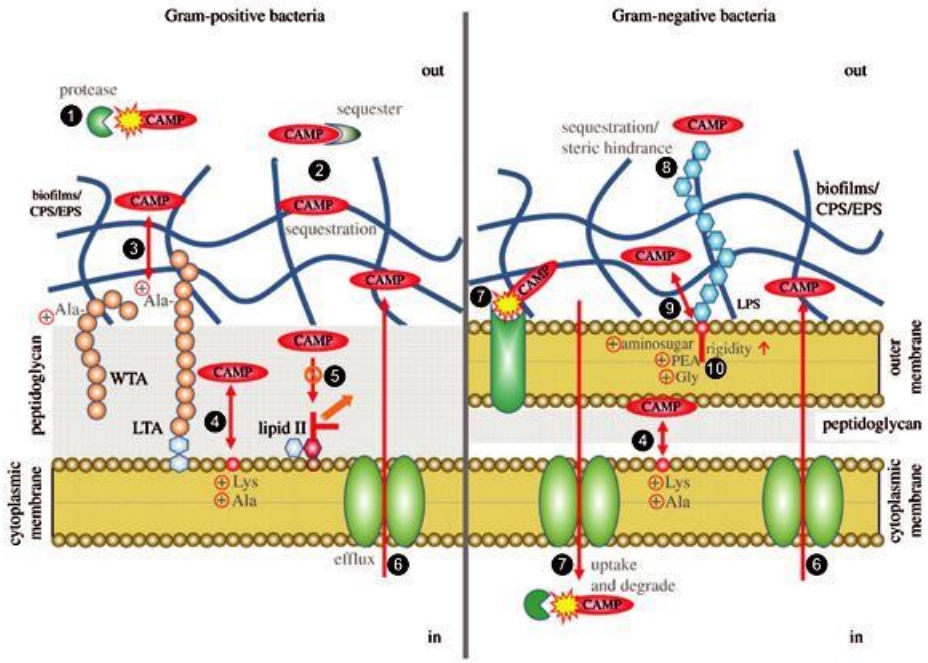


Figure 1.9. Gram-positive and Gram-negative pathways of tolerance to cationic AMPs (CAMP) summary. 1) proteolytic degradation; 2) sequestration; 3) electrostatic repulsion by d-alanylation of teichoic acid; 4) electrostatic repulsion by amino-acylation of membrane phospholipids; 5) lipid II alteration to avoid binding; 6) elimination of AMP by efflux transporters; 7) proteolytic degradation by membrane-bound or intracellular proteases; 8) sequestration or binding prevention;

Interestingly, AMPs have been suggested to function as environmental markers to evoke gene expression of virulence in the host, resulting in a more "invasive phenotype" for

bacteria. Furthermore, changes in gene expression caused by these AMPs may lead to improved adaptive tolerance to traditional antibiotics such as macrolides, fluoroquinolones, or aminoglycosides. Surprisingly, when exposure to subinhibitory concentrations of LL-37 was detected, the acquisition of adaptive DNA mutations leading to improved bacterial mucoid and rifampin tolerance was detected. Natural innate immunity can also be impaired by the medicinal use of AMPs, such as exposure to *Staphylococcus aureus* to pexiganan, a synthetic derivative to magainin, contributing to cross-resistance to human defensin-1 [37].

1.14. Other limitations of AMPs as therapeutic agents

Despite the scientific excitement for AMPs as effective new candidates for antibiotic drugs and the growing number of patents citing such therapeutic promise, many AMPs do not make it to the market due to failure in clinical trials. This may be attributed to a variety of causes, such as absorption, poor stability of the in-situ peptide and proteolytic degradation, patient hemolytic and cytotoxic events, or processes of bacterial resistance. This highlights that their multitude of biological roles, the mode of antibacterial action, and the resistance mechanisms formed by bacteria are not fully known [38]. Thus, before the medicinal use of AMPs can be made, several significant problems need to be addressed.

Extract antimicrobial peptides, and two approaches were initially available: separation from the host organism or chemical synthesis. It took several fractionation and purification steps to separate these defensive molecules from host tissues, such as frog skins and insect lymph fluid, followed by microbiological experiments to determine their

bactericidal properties. Many cationic peptides from different species have been discovered using this tool. However, this AMP isolation procedure is cumbersome, involves vast amounts of the source material, typically has low yields, and is thus insufficient for clinical trials [39]. On the other hand, using the existing solid-phase methods, chemical peptide synthesis is quicker, easier to automate, and involves simplified purification steps. This approach facilitates experiments on structure-activity interactions due to toxicity without biological constraints. It also can contain artificial amino acids, while ribosome-generated peptides are confined to a subset of alpha-amino acids and alpha-hydroxy acids. However, the restriction of chemical AMP synthesis is the high cost and complexity of integrating complicated post-translational improvements. The production of peptides containing several disulfide bonds and thus complex secondary structures, for instance, would significantly increase the cost of production [40].

Alternatively, a cost-effective method for practical and therapeutic studies may be the heterologous expression of eukaryotic AMPs by microorganisms, such as lactic acid bacteria. Due to the low costs, rapid growth on inexpensive substrates, controllable laboratory environments, and the relatively well-characterized genetic histories and availability of a wide variety of vectors and host strains, the development of AMPs by genetically modified microorganisms is impressive. In the development of small, labile AMPs, fusion proteins are popular to provide stability, shield the AMP from intracellular proteolytic degradation, increase expression and promote downstream purification processes, but more significantly, to mask the host expression with the intrinsic toxicity of these peptides [41]. However, technological issues, such as their inherent antibacterial

behavior, lack of post-translational modification machinery in heterologous producers, infection with bacterial LPS, and the inefficient expenditure of energy and protein synthesis machinery induced by the fusion protein strategy, still exist in optimizing bacteria such as AMP generating cell-factories. Despite the possibility of isolating or developing AMPs by chemical synthesis or microbial cell factories, the expense of generating adequate amounts of AMPs for medicinal application exceeds the cost of antibiotics currently in use [42].

AMPs do not follow the Lipinski "Rule of Five" for drug-likeness from a pharmacological perspective that predicts strong absorption and permeation. Besides, certain natural AMPs are susceptible to host or bacterial proteases and peptidases enzymatic degradation in vivo. The systemic toxicity and hemolytic activity of certain AMPs are another drawback, restricting their use to treat systemic invasive diseases. As topical therapies for skin and wound diseases, AMPs still hold the most significant promise for these reasons.

1.15. The importance of the microbiota

While there has been a significant scientific emphasis on the production of antibiotic resistance in particular pathogenic bacteria for many decades, there is now concern about the collateral effects on commensal bacteria of the widespread use of broad-spectrum antibiotics. Also, the aggregation in commensal microbes of antibiotic resistance genes (i.e., the resistome) and their subsequent horizontal transition to opportunistic or pathogenic bacteria is of interest. The microbiota is generally defined as the abundant and dynamic populations of commensal microbes that occupy external mammalian surfaces, such as the gastrointestinal tract, the oral cavity, or the skin [43]. The composition of

microbiota can vary significantly between people and between different niches within an organism and is the result of mutual co-evolution, co-adaptation, and host codependency.

As early as 1907, Élie Metchnikoff theorized that through modifying the intestinal microbiota, the beneficial effects of the commensal lactic acid bacteria may be used to promote well-being. Strategies for researching and characterizing microbiota are increasingly developing. In particular, the development of cultivation-independent strategies, such as those targeting the 16S rRNA gene sequence of bacteria and random metagenomic DNA approaches, has intensified scientific progress in this area over the past decades. For microbiota profiling, next-generation sequencing (NGS) technologies that allow high-throughput data collection, such as 454 pyrosequencing and Illumina sequencing [44], have been widely adopted as (outsourced) sequencing costs continue to decrease.

Moreover, our understanding of the essential role of microbiota in intestinal growth and homeostasis and the immune system's work has been dramatically advanced by studies in gnotobiotic mice. We now recognize that microbiota is involved in multiple host-beneficial processes, such as fermentation and conversion of food or feed products, development of vital nutrients and metabolites, immune system maturation and regulation, and tolerance pathogens to colonization [45]. On the other hand, dysbiosis of the gastrointestinal microbiota has been found to contribute to the pathophysiology of both intestinal and extraintestinal diseases, such as inflammatory bowel disease, obesity, and type II diabetes, as well as the unexpected rise in the abundance of particular intestinal pathobionts.

It has since been clear that environmental factors may affect the structure and role of the microbiota and change the host's health status. It has been shown that nutrition, especially dietary fibers, and probiotics, is an essential factor in the regulation of the intestinal microbiota. Several experiments have demonstrated that the microbiota's structure and diversity can be negatively impacted by antibiotic chemotherapy. Increased vulnerability to pathogenic infections has already been shown almost 60 years ago due to the lack of colonization tolerance following exposure to antibiotics, and this phenomenon is now well-accepted.

1.16. Rationale and approach undertook in this thesis

A broad spectrum of antimicrobial activity against pathogenic microbes has been demonstrated by peptides developed by members of the genus *Bacillus*. Both ribosomal and nonribosomal, *bacillus*-derived AMPs can be synthesized and categorized according to peptide biosynthesis, structure, and molecular weight. By forming channels inside and/or destroying the bacterial cell wall, AMPs kill bacteria. Although several hypotheses to explain the molecular processes caused by AMPs have been suggested, it is currently uncertain which of the hypothesized mechanisms, if any, is responsible for their biological activity. Several models have been proposed, with the exact process likely to depend on the peptide, concentration, and bacterium. It has also been demonstrated that bacteria react to AMPs and even gain tolerance to their toxic effects. Natural products from microbial sources tend to be the most favorable alternative to current antibiotics to address the growing threat of infectious bacteria to humans. In this respect, because of their broad specificity and specific and rapid killing behavior against different pathogens, AMPs from the *Bacillus* species are ideal therapeutic resources. However, to determine

the safe and efficient use conclusively in medical settings of *Bacillus*-derived AMPs, such as bacteriocins, lantibiotics, lipopeptides, and biosurfactants, further research on their mode of action, toxicity, and immunogenicity need to be performed in humans.

AIMS AND OUTLINE OF THIS THESIS

As a potential option for treating multidrug-resistant (MDR) pathogenic bacteria, the therapeutic use of AMPs has been suggested. This study's first purpose was to examine the degree to which AMPs that occur naturally could successfully treat infections caused by various pathogenic bacteria. About 200 bacterial strains have previously been isolated from Korean fermented foods obtained in our laboratory from different Korean provinces. About 90 percent of naturally fermented foods and alcoholic drinks still rely on traditional domestic processing in various regions of the world. Naturally, foods that are fermented contain both functional and non-functional microorganisms. After fermentation, useful microorganisms transform the chemical constitutions of materials from animal/plant origins, thus improving the food's sensory consistency and the bioavailability of nutrients. Antimicrobial and antioxidant compounds are also made, probiotic functions are activated, and defenses are improved, with some combinations encouraging bioactive health. Thus, the aims of this research are,

- To scan and classify the strain of microbial strain developing comparatively higher bacteriocin of interest,
- To purify the enzymes by using the least possible purification steps with optimum purity and yield,
- To classify the purified peptide biochemically

- To explore the antimicrobial mechanism, upon confirmation of the novelty of the peptide MS07 and MS15,
- To study the mechanism of antioxidants and antibiofilm activity,
- A literature analysis on future perspectives,

CHAPTER TWO

THE STRAIN CBSMS07 AND PURIFICATION OF MS07

2. 1. INTRODUCTION

There is a recent tradition of fermented vegetables. About 2000 years ago, salted and fermented vegetables were used in Korea. From the 4th century A.D. It is fair to pursue In Korea, by following the various ancient accounts of kimchi preparation, kimchi was cooked and consumed. Kimchi's taste depends on the fermentation products used, the fermentation condition, and the lactic acid bacteria (LAB) used in the fermentation. The fundamental ingredients of kimchi preparation are Baechu cabbage and radish, but other vegetables such as green onion, leek, cucumber, and so on are also used to manufacture different kinds of kimchi. High concentrations of minerals, minerals, dietary fibers, and other essential elements occur in kimchi [22]. In several previous studies, Kimchi has been reported to have anticancer, antioxidant, antiatherosclerotic, antidiabetic, anti-obesity, and so on impact. It contains very high levels of lactic acid (108 to 109 CFU / g) formed by bacteria such as *Lactobacillus brevis*, *Leuconostoc mesenteroides*, *Enterococcus faecalis*, *Lactobacillus plantarum*, *Streptococcus faecalis*, *Pediococcus cerevisiae*, *Lactobacillus software*, and *Weissella koreanis*, the most significant characteristic of kimchi for human health. Therefore, eating kimchi is a good way to add more vegetables and probiotics into the diet to promote well-being.

No proof exists suggesting that *Bacillus siamensis* has been discovered as a bacterium with antibiofilm activity. In our report, we paid interest in Kimchi, a traditional Korean vegetable food that was fermented by various microorganisms such as *Bacillus* and *Lactobacillus* under multiple conditions. Fermented foods are often a powerful source of microorganisms, and different microbes that can produce antimicrobials and other useful substances have been tried by many researchers to isolate them. The latest research

reports on probiotic identification have optimized the media for fermented Kimchi isolated *Bacillus* CBSMS07, the probiotic producing bacteriocin MS07.

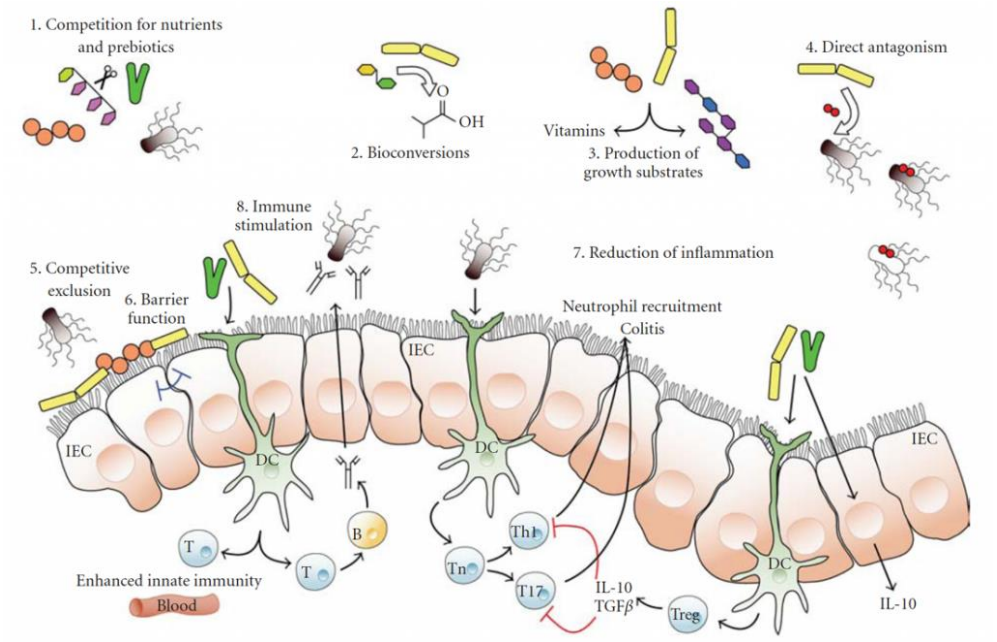


Figure 2.1: The graphical diagram shows the microbiota's mechanisms of action by probiotic bacteria that are conceivable or established. (1) Competition for dietary components as substrates for development, (2) Bioconversion of fermentation products with inhibitory sugar properties, for instance,(3) For other microorganisms, the production of growth substrates, such as vitamins, (4) control of bacteriocin-like compound antagonism, (5) competitive prohibition of binding sites, (6) enhanced barrier role, (7) reduction of inflation DC: dendritic cells, IEC: cells of the epithelium, T: T-cells.

2.2. MATERIALS AND METHODS

2.2.1. Materials

Chemicals and solvents used for our study were in extra pure grades. Bacterial media Muller-Hinton (MH) and Man-Rogosa-Sharpe (MRS) were procured from Dickinson & co., Becton, USA. Bacterial strain CBSMS07 was obtained from Korean fermented food (Kimchi). All reagents that used for the experimental purpose were of analytical quality.

2.2.2. Bacterial isolates and nutrition media

The bacterial strain CBSMS07, proficient in making AMP, was obtained from Korean fermented food kimchi. The method of isolation for the bacterial strain was acted following our earlier statement [46]. About 1 g of kimchi was incubated at 37°C for 24 h by mixing with 0.85% NaCl. Serial dilutions were executed, and steaking was performed to check the suitable CFU/mL (colony-forming unit). The stock was mixed with glycerol (20%) and kept at -75°C. The identification of CBSMS07 was performed based on consistent morphology with the Bergey's Manual of Systematic Bacteriology. For further identification, Sequencing study of the 16S rRNA gene was executed [47]. Optimization of culture media with different percentage of carbon and nitrogen sources (0.25%, 0.5%, 0.75%, 1%, 1.25%, 1.5%, 1.75%, and 2%) and different percentages of metal ion sources (0.005%, 0.0075%, 0.01%, 0.0125%, and 0.015%) for the bacterial strain was performed with various carbon sources (maltose, glucose, sucrose, lactose, fructose, starch, mannitol, sorbitol), nitrogen sources (peptone, tryptone, beef extract, yeast extract, malt, soymeal, oatmeal), and metal ion sources ($MgCl_2$, KCl, NaCl, $CaCl_2$, KH_2PO_4, Na_2HPO_4, $ZnSO_4$, $FeSO_4$) in different proportion. In contrast, MRS and MH were used as control.

2.2.3. Bioprocess design and optimization by response surface methodology

The peptide MS07 obtained from bacterial strain CBSMS07 showed more significant activity containing mannitol as carbon sources, peptone as nitrogen sources, and NaCl as metal ion sources. The box-Behnken design was utilized to verify the highly efficient media arrangement amount employing Design-Expert software (Version 12.0, Minneapolis, Stat-Ease Inc., USA). By using this model, the most important independent variables, named A, B, and C, are incorporated, and each factor can be assessed at three different levels, lower (-), central (0) and higher (+) whereas Bacteriocin activity (AU/mL) was the dependent variable (Table 2.1) [48, 49].

Table 2.1. Different levels (-1, 0, +1) of the independent variables were used for investigational ranges

Code no.	Independent Variables	Lower level (-1)	Center point (0)	Higher-level (+1)
A	Mannitol (g/L)	10	15	20
B	Peptone (g/L)	5	10	15
C	NaCl (g/L)	0.05	0.1	0.15
D	pH	5	7	9
E	The incubation period (hr.)	24	30	36

Totally seventeen investigational runs were presented in a partial factorial model (Table 2.2). The software provided predicted bacteriocin activity (AU/mL). The impact of each significant variable on bacteriocin activity was discovered by the prospect levels higher than 95% ($p< 0.05$).

Table 2.2. Experimental runs and the result of the Box-Behnken experiment

Run	Mannitol (g/L) A	Peptone (g/L) B	NaCl (g/L) C	Bacteriocin activity (AU/mL)	
				Actual*	Predicted
1	20	10	0.15	10110.00	9920.00
2	15	10	0.1	10740.00	10818.00
3	10	5	0.1	8400.00	8402.50
4	15	10	0.1	10800.00	10818.00
5	20	15	0.1	9600.00	9597.50
6	15	15	0.15	9260.00	9452.50
7	15	5	0.15	8370.00	8717.50
8	20	10	0.05	9940.00	10290.00
9	15	5	0.05	9460.00	9267.50
10	10	15	0.1	9140.00	9297.50
11	10	10	0.05	9620.00	9810.00
12	15	10	0.1	10950.00	10818.00
13	20	5	0.1	9380.00	9222.50
14	15	10	0.1	10610.00	10818.00
15	15	10	0.1	10990.00	10818.00
16	15	15	0.05	10150.00	9802.50
17	10	10	0.15	9630.00	9280.00

*Actual bacteriocin activity was obtained from the mean of the triplicate experiment. The two-level of each variable were used to get the final optimum media. P-value 0.02, f-value 9.41.

The bacterial strain CBSMS07 was cultured in a fully optimized media at 37°C containing 1.773% mannitol, 1.249% peptone, and 0.012% NaCl (MPN media). Production was regulated in 2L Erlenmeyer flasks, including 400 mL culture media with constant rocking at 155 rpm. The antimicrobial compound was designated as MS07 that produced by the strain CBSMS07.

2.2.4. Purification of peptide MS07

Mass culture was carried out in MPN media with continuous shaking for 30 h at 37°C. The supernatant was taken out by centrifugation (Supra 22k, Korea; Hanil Science industrial) (10,000×g) at 4°C for 30 min. Salting out process was conducted by mixing diammonium sulfate (20-60%) with harvested culture supernatant for overnight stirring at 4°C. The active precipitate was collected employing centrifugation at 10,000×g for 50 min at 4°C, resuspended with buffer 10mM Tris-HCl (pH 7.4). Hereafter, Ultrafiltration (Millipore corp.) through the molecular cut-off (MWCO) membrane (10 and 1 kDa) was carried out for further column chromatography. The active fraction was loaded on a column Sephadex G-50 (2.5×85 cm) (Pharmacia, Sweden) next to the column DEAE-Sephadex A-50 (1.5×37 cm) using the equal buffer. Further, active fractions were collected and concentrated, employing chloroform precipitation. Also, precipitate applied to the Pierce peptide desalting spin column (Thermo Scientific, Rockford, USA) and were analyzed for purity.

Table 2.3. Purification stages of peptide MS07

Purification steps	Vol (mL)	Total protein (mg)	Total activity (AU)	Specific activity (AU/mg)	Purification fold	Recovery (%)
Cell free supernatant	910	410.45	350000	852.72	1	100
Ammonium sulphate pallet	45	75.75	171000	2257.43	2.64	48.86
Sephadex G-50	9	8.16	92000	11274.51	13.22	26.29
DEAE- Sephadex A-50	2.5	1.14	27000	23684.21	27.77	7.71

2.2.5. Tricine-SDS polyacrylamide gel electrophoresis and bioassay

Estimation of peptide MS07 concentration was employed by the Bradford method [50]applying bovine serum albumin (BSA) as standard. Hereafter, Tricine SDS-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) [51] was performed to evaluate the purity and molecular weight of peptide MS07. The gel was stained along with Coomassie Brilliant Blue R-250 and destained with the mixture of distilled water, methanol, and glacial acetic acid (8:1:1) destaining solution. According to our previous report [52], the in-situ inhibitory exposure of activity was presented against the indicator organisms (1.5×10^7 CFU/mL). The gel was rinsed with Tris-HCl buffer (50 mM/L; pH 7.4) several times containing Triton X-100 (2.5%). Later, the gel was composed of agar (0.6%) on MH broth (Difco, USA) and incubated for 10 h at 37 °C.

2.2.6. Intact molecular weight determination by MALDI-TOF

Molecular weight determination of MS07 was assessed through the MALDI-TOF MS method using Ultraflex III (Burker, Germany). MALDI-TOF/MS assessments were presented in the range of m/z 500-6000 and 2000-40000 to both the mass spectrum.

2.2.7. Stability and solubility of peptide MS07

The thermostability of MS07 was subjected to analyze various treatments of temperature 0, 10, 20, 40, 50, 60, 80, and 100°C for 60 min and 121°C/105kPa for 15 min prior to evaluating the remaining activity. The samples were tested against the indicator microbial strain Micrococcus luteus ATCC 9341. Likewise, pH stability was checked at a wide array of buffers, for instance, citric acid-sodium phosphate (pH 4.0-6.0), Tris-HCl (pH 7.0-9.5), and KCl-NaOH (pH 11.0-12.5). The solubility of MS07 was checked with different organic and inorganic compounds in which bacitracin was the control.

2.3. RESULTS AND DISCUSSION

2.3.1. Strain identification and culture media

Bacterial strains that proficient in producing AMPs were collected and insulated from Korean fermented food (kimchi). The strain CBSMS07 presented a high degree of identity on morphological resemblance to *Bacillus* strain. The 16S rRNA sequence of the isolate showed the closest character to *Bacillus siamensis* KCTC 13613 (Accession no. AJVF01000043) with a pairwise similarity of 99.9%. A phylogenetic tree constructed from the 16S rRNA sequence analysis has been shown in Figure 2.2. Optimization of nutritional media was carried out with numerous carbon sources (0.25%-2%), nitrogen

sources (0.25%-2%), and metal ion sources (0.005%-0.015%) for antimicrobial peptide (AMP) MS07, which was produced by bacterial strain B. siamensis CBSMS07. Among all the nutritional sources, 1.773% mannitol, 1.249% peptone, and 0.012% NaCl were checked to be the most significant carbon sources, nitrogen sources, and metal ion sources, respectively. Antimicrobial peptide MS07 was grown on optimized media at 37°C for 30h and 160 rpm. Culture conditions showed a significant effect for producing bacteriocin.

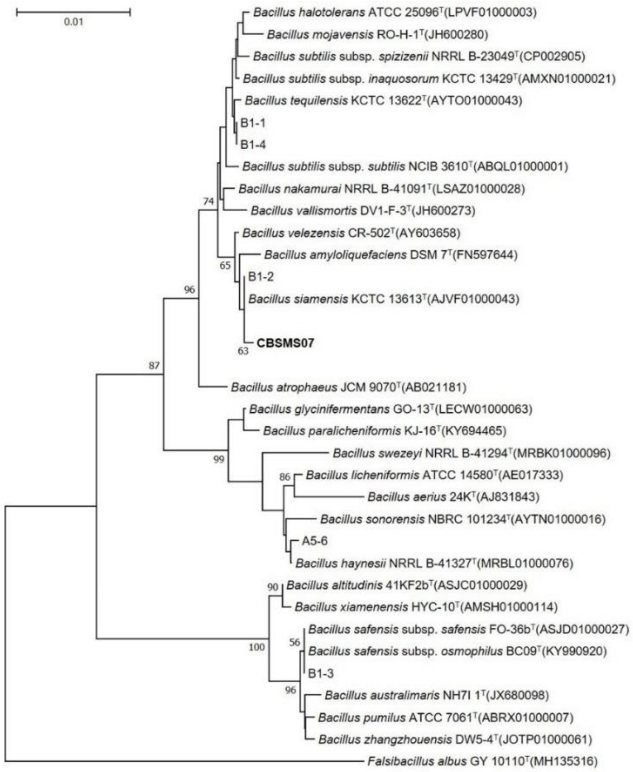


Figure 2.2. Phylogenetic tree created on almost full 16S rRNA gene sequences displaying interactions among CBSMS07 and a few intently associated taxa of the genus *Bacillus.* Here, the percentages amount at the nodes are the quantities of bootstrap assistance value depend on 1000 resampled data sets retrieved from GenBank. Bar symbolizes 0.01substitution per nucleotide place.

2.3.2. Box-Behnken design and response surface analysis

Corresponding to the theory of RSM, the higher amount obtained from the levels of the three independent variables Mannitol (A), peptone (B), and NaCl (C) were further examined each of three distinct levels (Table 2.2). The bacteriocin activity differed significantly from 8370 to 10990 AU/mL at various concentrations of several elements in the culture medium. The fitness of bacteriocin activity was calculated by ANOVA test. From the ANOVA test, the straight terms A, B, C, and interface term AC, AB, and BC were observed to be statistically significant ($p< 0.05$) by analysis in bacteriocin action. After evaluation of data, we found that the interaction of AB, AC, and BC matched the maximum peak for bacteriocin activity appeared at NaCl concentration of 0.0123 g/L, Mannitol concentration of 1.773 g/L and peptone concentration of 1.249 g/L, where the gradual increase in their level the highest bacteriocin activity was achieved (Figure 2.3). By employing multiple regressing evaluations to the investigational data, the ultimate equation to describe bacteriocin activity:

Bacteriocin activity= -1857.50+795.80A+1002.70B+23620.00C-

$5.20AB+160.00AC+200.00BC-23.46A^2-44.06B^2-1.626E+05C^2$

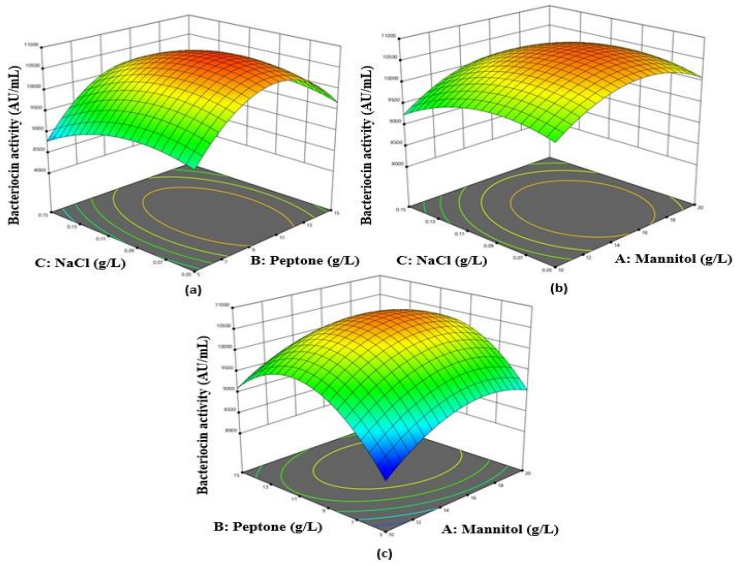


Figure 2.3. Response surface method plots (3D) displaying the distinct and collaborative impacts of variables on the bacteriocin activity (AU/mL). (a) the interaction effect between NaCl and peptone concentrations (gL^{-1}) on actual bacteriocin activity (AU/mL) (b) the interaction effect between NaCl and mannitol concentrations (gL^{-1}) on actual bacteriocin activity (AU/mL) (c) the interaction effect between peptone and mannitol concentrations (gL^{-1}) on actual bacteriocin activity (AU/mL).

Accordingly, the match of the model is conveyed by coefficient determination (R^2) as exponential, which was determined to be 0.92, which was closer to 1.00 to predict better response.

2.3.3. Purification and molecular weight determination of MS07

Production of MS07 was cultured in improved media at the temperature of 37°C with constant shaking. The purification of MS07 from cultured supernatant of CBSMS07 (ammonium sulfate; 20-60% saturation) has been reviewed in Table 2.3. The peptide

MS07 was purified to uniformity by two phases of chromatographic partition on Sephadex G-50 (Figure 2.4a) and the DEAE-Sephadex A-50 column (Figure 2.4b). The active fractions were collected based on protein concentrations and antimicrobial activities. The peptide MS07 was purified in the total activity of 27000 AU with 27.77-fold and 7.71% activity revival. Tricine SDS-PAGE gel electrophoresis assessment appeared a single band protein of MS07 relating to a molecular weight of 6.099 kDa (Figure 2.5a). The protein band was proved to be an AMP by bioassay activity of the SDS-PAGE gel showing a clear lysis zone in the MH agar plate by the same region against *Mycobacterium smegmatis* (Figure 2.5b).

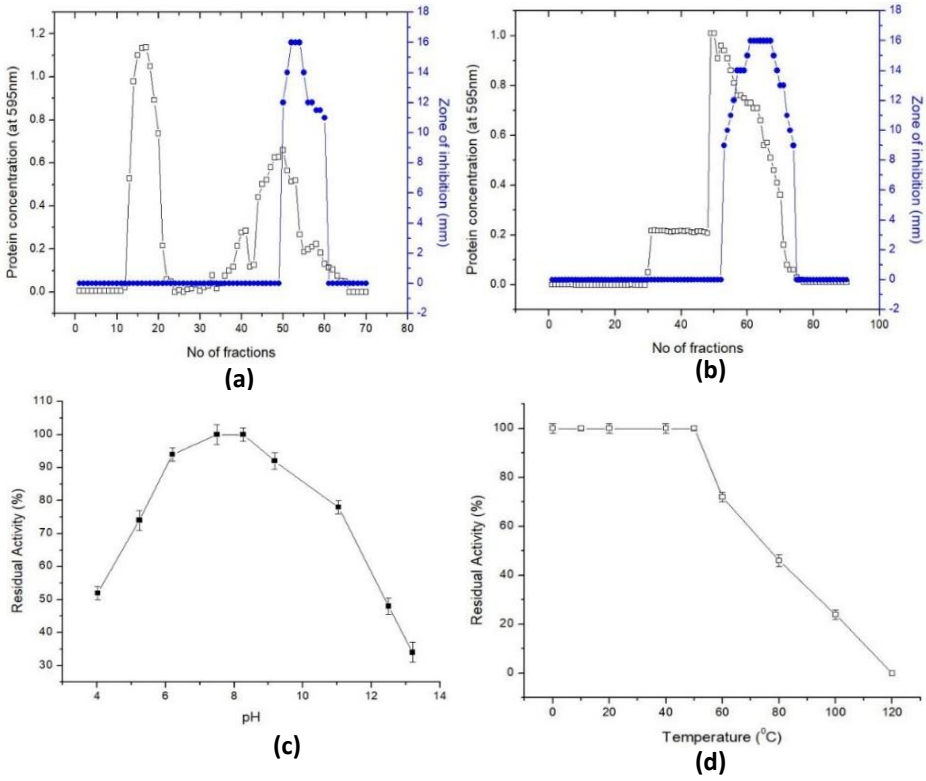


Figure 2.4: Gel filtration elution summary of peptide MS07 from (a) a Sephadex G-50 (2.5×85 cm) and (b) a DEAE-Sephadex A-50 column (1.5×37 cm). Stability of (c) pH and (d) temperature on the activity of peptide MS07.

2.3.4. Molecular weight determination by MALDI-TOF

MALDI-TOF assessed the molecular weight of MS07 by acquisition mass range of 500Da~40kDa. The molecular weight of peptide MS07 was at the peak of 6099.689Da (Figure 2.5d), which showed a single major peak (n+1). Rather than a significant peak, it showed some minor detected peaks also which had similar masses to lipopeptide (fatty acid) compounds. It had low-intensity peaks at m/z= 1067.675, 1102.762, and 1118.721 corresponded to C13, C14, and C15 molecules (Figure 2.5c), which were observed in the spectra of the sample by the alignment with standard units. The peak at m/z=1067.675 that relates to potassium adducts Iturin A C13 was highly plentiful as a fatty acid chain in the spectrum. Otherwise, the peak at m/z=1102.762 corresponds to the sodium adducts Fengycin A C14 also was resembled a fatty acid chain. We found the peak at m/z=1118.721 corresponds to Puwainaphycin F analogs.

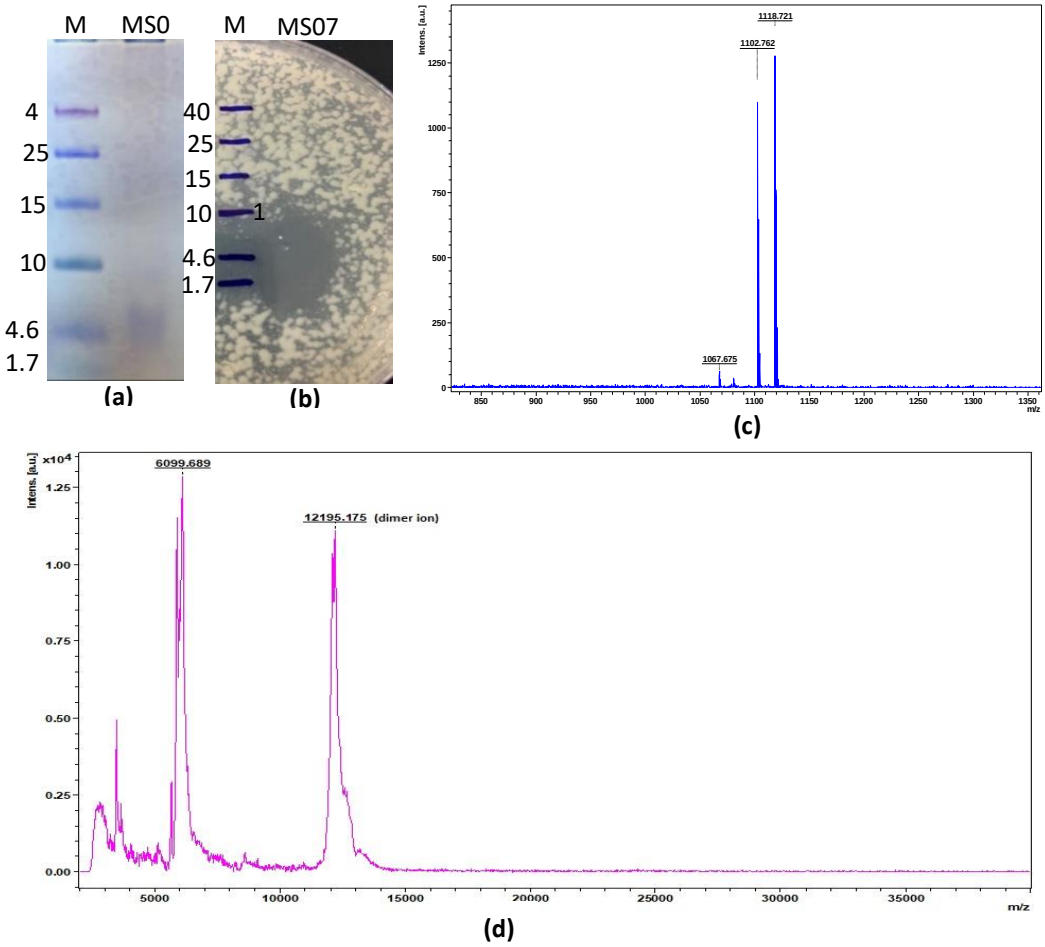


Figure 2.5: (a) Tricine SDS-PAGE; Lane 1: standard protein indicator, Lane 2: Purified MS07 after desalting and (b) In-situ analysis (bioassay) against *Mycobacterium smegmatis* ATCC 9341; Lane 1: protein standard marker point, Lane 2: activity of purified MS07. (c) MALDI-TOF/MS showing m/z= 1067.675, 1102.762, and 1118.721 correspondence to lipopeptide, (d) Intact molecular weight determination by MALDI-TOF; purified MS07: 6099.689 Da, dimer ion: 12195.175 Da.

2.3.5. Stability and solubility study of MS07

Verifying the thermal stability of MS07, remaining activity checked according to the retained antimicrobial activity. MS07 remained stable up to 50°C (Figure 2.4d). Although its activity was gradually decreased after incubation for 60 min, it employed about 72% of the activity at 60°C. Complete loss of activity occurred in contact with to autoclave environment at 121°C and 105kPa for 15 min. The ability to retain the activity of MS07 across a broad array of pH values could be the consequence of the mutable protein denaturation. To analyze the stability of pH, peptide MS07 was incubated at different pH buffers (Figure 2.4c). It was observed that MS07 endures a broad array of pH values (4.02 to 11.04). MS07 showed a broad range of pH and temperature.

Peptide MS07 was discovered to be soluble in distilled water, methanol, ethanol, acetone, chloroform, and butanol, whereas not soluble in ethyl acetate, acetonitrile, and n-hexene.

2.4. CONCLUSION

Cyclic lipopeptide MS07 has potent antimicrobial and antibiofilm activities, which was purified from a *Bacillus* bacterial strain that was freshly obtained from Korean fermented food Kimchi. It mostly acted against gram-negative bacteria, but it has a strong value against gram-positive bacteria also. In summary, the result presented here demonstrated the activity of a cyclic lipopeptide MS07, which was effective against broad spectrum pathogenic bacteria as well as maintained greater antibiofilm activity.

CHAPTER THREE

THE STRAIN CBSMS15 AND PURIFICATION OF MS15

3.1. INTRODUCTION

Antimicrobial peptides kill microorganisms and disrupt cells' cohesion in a relatively brief amount of time, leaving very little space for immunity to develop in the target microbes. On the other hand, in conventional antibiotics, the mechanism of action is even more complicated. The growth of bacteria is impeded either by inhibiting protein synthesis, DNA synthesis, synthesizing the bacterial cell wall, or acting as metabolites or enzyme inhibitors. Consequently, the risks of developing bacterial resistance are even higher by using traditional antibiotics. Accumulating evidence shows that through many pathways, such as the carpet mechanism, barrel-Stave, and toroidal mechanism, antimicrobial peptides exert their cell lytic action (figure 3.1). Other models have also been proposed, such as the self-promoted cationic peptide absorption or the aggregate model. Some antibacterial peptides also inhibit bacterial cell formation, like conventional antibiotics.

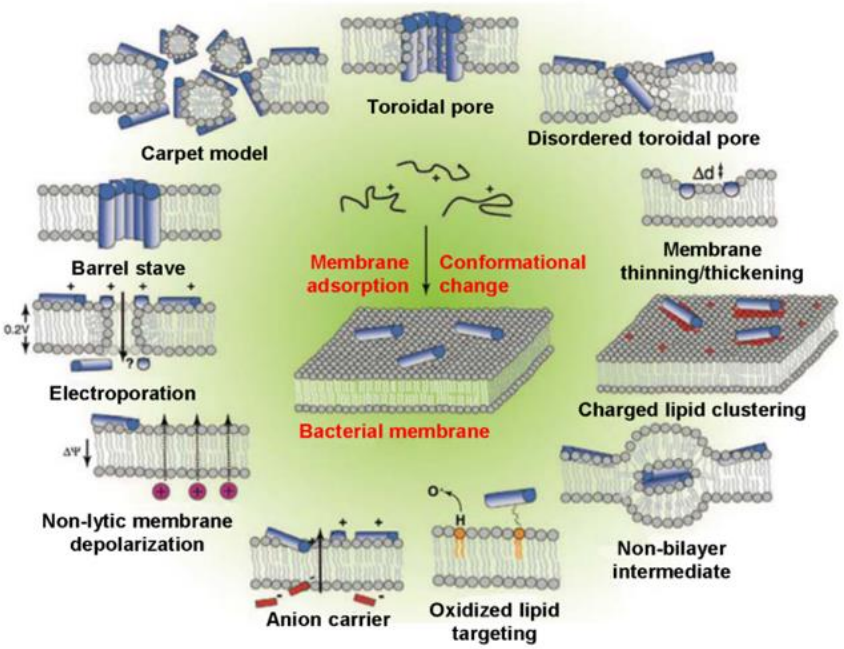


Figure 3.1: For the antimicrobial peptide-induced killing of bacteria, the carpet model, the barrel-stave model, and the toroidal model. Blue and red, respectively, demonstrate the hydrophilic and hydrophobic regions of the peptide.

3.2. MATERIALS AND METHODS

3.2.1. Materials

All chemicals employed for the investigational objective were of the highest analytical grade. Bacterial strain CBSMS15 was obtained by Kimchi's isolation process (traditional Korean food, local market, Gwangju, South Korea).

3.2.2. In Vitro Process of Isolation, Screening, and Culture Media Optimization

The bacterial strain CBSMS15, which had a first nucleotide sequence similarity with *Bacillus* sp., was isolated from Kimchi. It could produce AMP (antimicrobial peptide), which was highly active against a wide range of pathogenic microorganisms. Kimchi is a distinctive typical fermented Korean food characterized by different microorganisms' species, such as *Lactobacillus* and *Bacillus subtilis*. We used cabbage kimchi for our study to isolate *Bacillus* strain. The isolation process was described briefly in our previous report [46], whereas 0.85% NaCl was mixed with 1 g of fermented kimchi sample and incubated overnight at 37 °C. Subsequently, the samples were serially diluted up to 10^{-8} in Mueller–Hinton (MH) broth. The proper CFU/mL (colony forming units) were assessed by streaking from those diluted aliquots. Hereafter, for the further stock, glycerol was used with appropriately diluted aliquots by 20% and 80%, respectively. The strain was discovered as *Bacillus* bacterial strain, based on morphologically characterized

bacteria. It was analyzed following Bergey's manual of systematic bacteriology [47]. Moreover, the identification was established with the phylogenetic tree by 16S ribosomal RNA sequence analysis (figure 3.2).

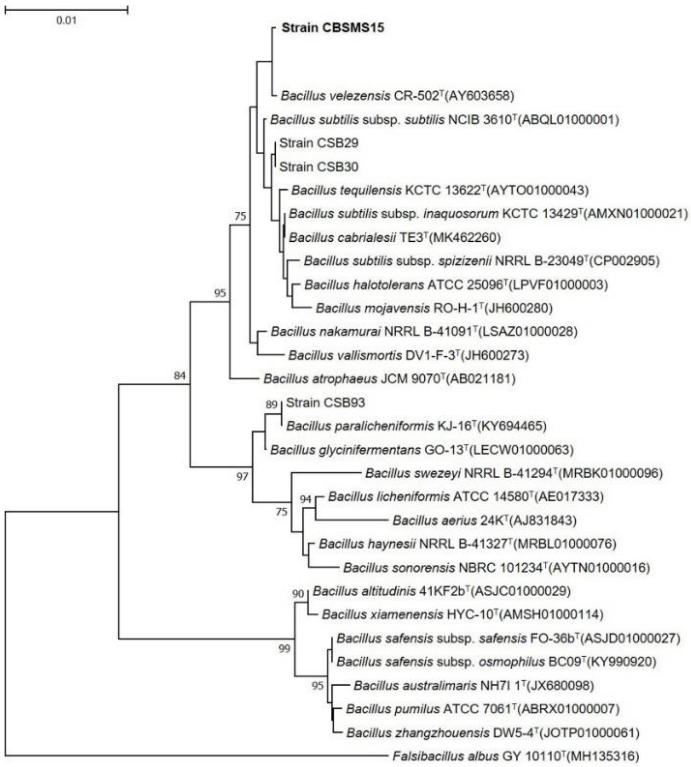


Figure 3.2. Phylogenetic tree, established on a full sequence of 16S rRNA genes, indicating the correlation among the CBSMS15 strain and closely linked *Bacillus*-type taxa. Node numbers are percentage bootstrap values based on 1000 replicates above values. The bar reflects 0.01 replacements by nucleotide type.

Antimicrobial activity was established by the method of disc diffusion. Briefly, paper disc filter (8 mm, Roshi Kaisha, Tokyo, Japan) soaked on the face of the petri dish with AMP (40 µL) was positioned, including the MH agar plate, overlay with *Mycobacterium* smegmatis [53]. Optimization of production culture media has enormous implications in

the enhanced culture of AMP. Using de Man–Rogosa–Sharpe (MRS) media as control, culture medium optimization was brought out with different carbon, nitrogen, and metal ion sources [54]. In brief, numerous carbon sources (sucrose, maltose, sorbitol, mannitol, starch, fructose, glucose, lactose), nitrogen sources (beef extract, peptone, malt, oatmeal, yeast extract, tryptone, soybean meal), and metal ion sources (NaH_2PO_4, Na_2HPO_4, $ZnSO_4$, $MgSO_4$, KH_2PO_4, $MgCl_2$, NaCl, $FeSO_4$, $CaCl_2$) were utilized in various amounts. Culture optimization was accomplished with diverse nitrogen sources (1%), and carbon sources (1%), with numerous metal ion sources (0.01%) till the last optimization. Finally, a metal ion, nitrogen, and carbon source were utilized to verify the final percentage of culture media by response surface methodology.

3.2.3. Optimizations of Culture Media Design by Response Surface Methodology and Statistical Analysis

A response surface optimizer was applied to verify the optimum environments for soluble starch as carbon sources, yeast extract as nitrogen sources, and NaCl as metal ion sources compositions [54]. Design-Expert software (Stat-Ease Inc, Minneapolis, MN, USA) version 12.0 was used to verify the percentages of different sources by Box-Behnken method design. Each variable, denoted A, B, and C were assimilated. Every single factor can be evaluated, down level (−1), midpoint (0), and up level (+1), at three different levels, while the dependent variable was for bacteriocin activity (AU/mL) (Table 3.1). Totally 17 individual experimental runs were presented in a fractional factorial design. The program offered a prediction of bacteriocin activity (AU/mL). The probability levels above 95% (* $p < 0.05$) discovered the effect of every single considerable variable on the activity of bacteriocin.

Table 3.1. Experimental design and independent variables of choice for optimizing culture media.

Code	Variables of Choice (Independent)	Up Level (+1)	Midpoint (0)	Down Level (−1)	Generated Optimum Factor
A	Soluble starch (g/L)	25	15	5	13.42
B	Yeast extract (g/L)	15	10	5	7.64
C	NaCl (g/L)	0.15	0.1	0.05	0.089

3.2.4. Culture and Purification of Peptide MS15

Seed culture produced on De Man, Rogosa, and Sharpe (MRS) media was continuously shaken at 160 rpm for 12 h at 37 °C. The purification process was staged for the culture of peptide MS15 for 32 h at 37 °C, and shaking speed was 170 rpm, including 1.5% soluble starch, 1% yeast extract, 0.01% NaCl obtained by response surface methodology. Then, cell-excluded supernatant was accumulated after centrifugation (10,000× *g*) (supra 22K, Hanil Sci. Ind., Korea) for 30 min at 4 °C. Active precipitation fraction was recovered by mixing diammonium sulfate at the saturation of 20–80% using centrifugation at 10,000× *g* at 6 °C for 50 min. Aliquots dialyzed with 10 mM Tris-HCl (pH 7.2) buffer and subjected to ultrafiltration (Millipore co., Billerica, MA, USA) with 1 kDa and 10 kDa membrane. Following dialysis and filtration, the biologically active fraction was applied to a Sephadex G-50 (3 × 90 cm) column. The active fractions were combined and employed to ion-exchange separation using the DEAE Sephadex A-50 column (1.2 × 35 cm) and eluted, including the equal buffer. Further, active aliquots were utilized to a spin column named the Pierce spin desalting column and were investigated for purity check.

3.2.5. Electrophoresis with Tricine SDS-PAGE and Bioassay (in Situ) Analysis

The protein content was assessed using BSA (bovine serum albumin) as a reference protein by the Bradford method [50]. Tricine SDS-PAGE determined the molecular mass of the peptide [51]. The peptide was calculated with molar mass and purity employing Tricine SDS-PAGE. The bioassay was achieved alongside the designator organism (5.5×10^7 CFU/mL) by covering the treated gel [55]. The gel was washed with 50 mM Tris-HCl buffer (pH 7.5) from tricine SDS–PAGE, consisted of 2.5% Triton X-100 for numerous times on 0.6% agar on Mueller–Hinton (DIFCO, CA, USA) covering the reference organism's media and kept at 37 °C.

3.2.6. MALDI-TOF/MS for Molecular Weight Determination

The MALDI-TOF method was utilized to check the molecular weight of peptide MS15 as well as the purity check of the final peptide sample [56]. To assess the process, we used the Ultraflex III (Burker, Germany). The evaluations were performed with a mass spectra range of m/z 500–40,000.

3.2.7. pH and Thermal Stability of MS15

By investigating the temperature stability of MS15, aliquots were subjected to different treatments of temperature 0–100 °C and 121 °C for 60 and 15 min, respectively, before assessing the residual activity. The specimens were evaluated aligned with the *Escherichia coli* KCTC 1923 predictor microbial strain. Likewise, a large variety of buffers such as sodium phosphate-citric acid (pH 4.2–6.5), Tris-HCl (pH 7.2–9.4), and KCl-NaOH (pH 10.8–12.4) were tested for pH stability.

3.3. RESULTS AND DISCUSSION

3.3.1. Strain Isolation and Identification

Microbial strains that were obtained and isolated from Kimchi (traditional Korean food) that have the capability of making AMP was preserved. Strain CBSMS15 was initially discovered based on its morphological and biochemical nature, and according to the results, the strain exhibited a close identity with some *Bacillus* strains with 16S rRNA gene sequences analysis. The 16S rRNA analysis showed the most intimate nature to *Bacillus velezensis* CR-502^T (Accession no. AY603658) with a homologous resemblance of 99.9%. A phylogenetic tree was shown in Figure 3.2, built from the 16S rRNA sequence evaluation.

3.3.2. Experimental Design and Box-Behnken Analysis by Response Surface Methodology

Following the RSM theory, the higher value derived from the degree of the independent amount of choice, soluble starch (A), yeast extract (B), and NaCl (C) were additionally inspected at three distinctive levels. The bacteriocin production was substantially different in the medium from 7622 to 12367 AU/mL at varying levels of multiple elements (Table 3.2).

Table 3.2. Assessment of the generated data by the Box-Behnken experiment.

Run	Soluble Starch (g/L)	Yeast Extract (g/L)	NaCl (g/L)	Bacteriocin Activity of MS15 (AU/mL)	
	A	B	C	Actual Value[x]	Predicted Value
1	15	10	0.1	12138.00	12205.80
2	5	15	0.1	9460.00	9166.00
3	15	10	0.1	11940.00	12205.80
4	5	10	0.05	8650.00	8782.25
5	15	5	0.05	10450.00	10072.25
6	5	10	0.15	10113.00	10029.25
7	25	10	0.05	11146.00	11229.75
8	15	10	0.1	12268.00	12205.80
9	15	5	0.15	11329.00	11167.25
10	25	5	0.1	9336.00	9630.00
11	5	5	0.1	7622.00	7867.50
12	15	10	0.1	12316.00	12205.80
13	25	15	0.1	11560.00	11314.50
14	15	15	0.05	11742.00	11903.75
15	15	15	0.15	11941.00	12318.75
16	25	10	0.15	11625.00	11492.75
17	15	10	0.1	12367.00	12205.80

[x] The experimental data were presented by three replications per inspection. Different levels of three variables were used to get optimum culture conditions, whereas ***$p < 0.001$; F-value 31.18.

The predicted median was noted as 11359.9 AU/mL ANOVA study had measured the fitness of bacteriocin activity. Analyzing data with ANOVA, the linear research of independent variables A, B, C, and cross term BC (Figure 3.3a), AB (Figure 3.3b), AC (Figure 3.3c) in bacteriocin activity was found to be statistically significant (*** $p <$ 0.001; F-value 31.18). 2D dimensions were produced with cross term BC (Figure 3.3d), AB (Figure 3.3e), and AC (Figure 3.3f). After data assessment, we initiate that the interface of AB, BC, and AC interactions corresponding to the highest bacteriocin activity peak at 13.424 g/L soluble starch, 7.636 g/L yeast extract, and 0.089 g/L NaCl (Figure 3.3). The final equation in periods of essential components for bacteriocin activity was established. The comparison in terms of real considerations can be employed to create estimates regarding the reply for provided levels of every element. For each factor, the levels should be defined at this moment in the initial components.

$$\text{Bacteriocin activity} = -151.987 + 681.670\,A + 879.920\,B + 19782.000\,C + 1.930\,AB - 492.000\,AC - 680.000\,BC - 18.466\,A^2 - 34.586\,B^2 + 9740.000\,C^2$$

Therefore, focus on the model maximizing the adjusted coefficient determination, $\mathbf{R^2}$ showed 0.9857, and the predicted R^2 showed 0.9444, which is near to 1.00, which improved the model.

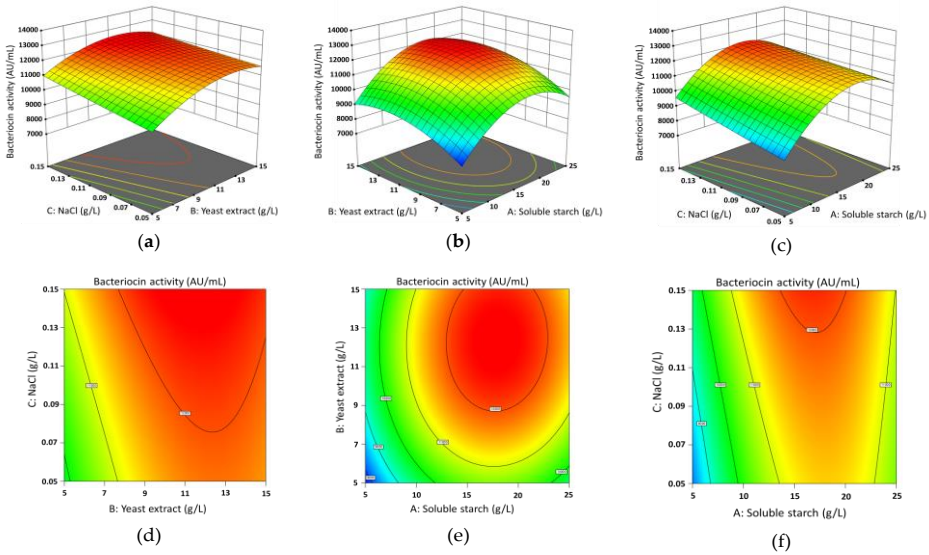


Figure 3.3. The system (3D) plot for response surface methodology showing the mutual and distinct effects of the variables on Bacteriocin activity (AU/mL). (**a**,**d**) optimized bacteriocin activity with the interaction between of yeast extract (g/L) and NaCl (g/L), (**b**,**e**) optimized bacteriocin activity with the interaction between of soluble starch (g/L) and yeast extract (g/L), (**c**,**f**) optimized bacteriocin activity with the interaction between of soluble starch (g/L) and NaCl (g/L).

3.3.3. Culture Media, Purification, and Molecular Weight Resolve of MS15

The maximum bacteriocin activity was achieved by culture media containing 1.342% soluble starch, 0.763% yeast extract, and 0.0089% NaCl and cultured for 32 h at 37 °C with 150 rpm. Peptide MS15 was purified from the culture supernatant and fractioned with the salting-out process. Two-step chromatography with size exclusion chromatography (Figure 3.4a) and anion exchange chromatography (Figure 3.4b) leads to a recovery of 5.92% activity with 24.49-fold and 16,000 AU total activity (Table 3.3). Clear, single, and purified peptide band observed by Tricine SDS-PAGE to a molecular

weight of 6091Da and lysis zone (bioassay) was showed within the same area where it showed the single pure peptide band against a reference bacteria *Mycobacterium smegmatis* (Figure 3.4c).

Table 3.3. Purification steps of peptide MS15.

Purification steps	Vol (mL)	Total Protein (mg)	Total Activity (AU)	Specific Activity (AU/mg)	Purification Fold	Recovery (%)
Cell free crude sample	935	388.5	270000	694.98	1	100
Ammonium sulphate pallet aliquots	52	98.45	156000	1584.56	2.28	57.78
Sephadex G-50 gel	8	7.23	38000	5255.88	7.56	14.07
DEAE Sephadex A-50 gel	2	0.94	16000	17021.27	24.49	5.92

All data represent the status of the sample after the designated procedure has been carried out. The purification fold was estimated based on starting crude sample activity.

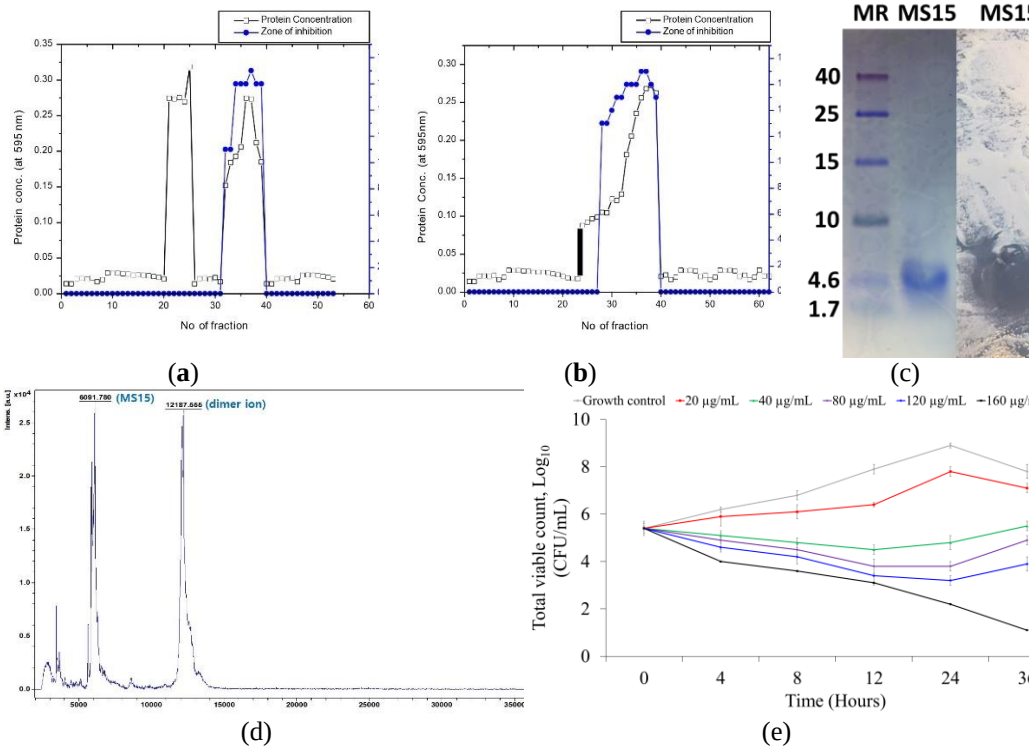


Figure 3.4. The elution profile of peptide MS15 from (**a**) the Sephadex G-50 column (3 × 90 cm) and (**b**) the DEAE Sephadex A-50 column (1.2 × 35 cm). (**c**) Tricine SDS-PAGE gel electrophoresis and bioassay (in situ):; Lane 1: protein marker (low range protein ladder), Lane 2: purified peptide MS15, Lane 3: in situ analysis showed clear inhibition zone in the same area in the right side picture. (**d**) MALDI-TOF/MS method is used to obtain the molecular weight of purified MS15. (**e**) Bacterial kill kinetics with various concentrations of MS15 against *E. coli* for 36 h.

3.3.4. Mass Spectroscopy Analysis by MALDI-TOF-MS

Purified MS15 were investigated primarily on a MALDI-TOF-MS that linear/reflector mode was applied. The analysis was done with linear mode (2–200kDa) and reflector mode (500–6000Da) to detect the purity and molecular weight of MS15. The molecular weight was at the peak point of 6.091kDa for peptide MS15 (Figure 3.4d) that demonstrated a major peak of the peptide (n + 1).

3.3.5. Stability Analysis of MS15

Residual activity was examined corresponding to the preserved bacteriocin activity to verify the temperature stability of MS15, and it persisted in being stable up to 60 °C. However, its activity was declining slowly and retained about 84% of the bacteriocin activity after 30 min of incubation at 70 °C. Total loss of activity appeared for 15 min at 121 °C when it got exposure to autoclave conditions. The capability to maintain the MS15 activity in a wide selection of pH values can result in the denaturation of the variable protein. The peptide MS15 was incubated at various pH buffers to evaluate the stability

of the pH. MS15 has been found to tolerate an extensive range of pH values (4.2–12.4). MS15 exhibited a wide pH and temperature array.

3.4. CONCLUSION

In conclusion, it can be mentioned that the *Bacillus* CBSMS15 strain has been identified as a possible candidate for probiotics, which is largely similar to *Bacillus velezensis* sp. The *Bacillus* probiotic short list may be joined by Plantarum. Via bacteriocin development, *Bacillus* CBSMS15 may be designed as a probiotic candidate. In order to be confident of AMP, more experiments on purification and hydrophilic existence of the peptide are pursued. Further studies are being carried out based on these observations.

CHAPTER FOUR

ANTIBIOFILM & ANTIMICROBIAL MECHANISM STUDY OF MS07

4.1. INTRODUCTION

Either through the creation of pores in the membrane or through the destabilization of the whole membrane, antimicrobial peptides induce bacterial cell lysis. In addition to their direct antimicrobial function, antimicrobial peptides, including anti-inflammation, immunomodulation, wound healing, cytokine release, chemoattraction, angiogenesis and histamine release from mast cells, play a multifunctional role in animals (Figure 4.1).

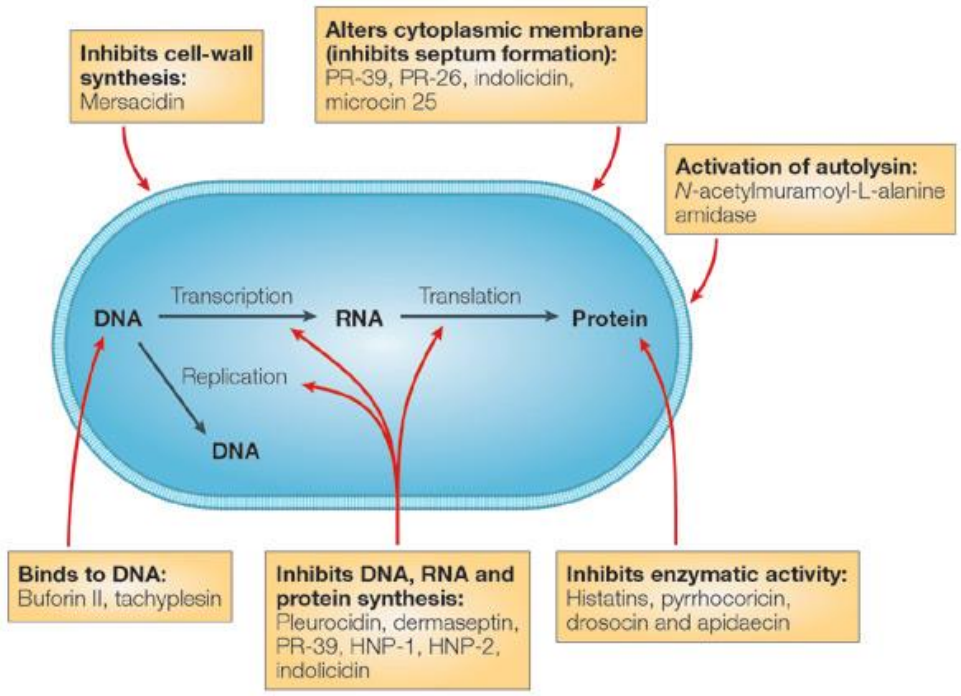


Figure 4.1: Alternative mechanisms of action of antimicrobial peptides.

It is essential to determine the structures and conformations of these peptides in solution, in lipid vesicles, or in the presence of membrane mimetic environments to understand the molecular basis of action and functioning of antimicrobial peptides. For the analysis of their conformations or secondary configurations, circular dichroism has been used. Data

obtained from these studies show that some antimicrobial peptides such as BMAP-27, BMAP-28, cecropins, magainins, and others have unordered/random in an aqueous medium coil structures and adopt helical structures in a structure-promoting environment or lipid vesicle presence. Many other methods have also been successfully used to analyze the composition of several antimicrobial peptides, such as Fourier transformed infrared spectroscopy, X-ray crystallography, Raman spectroscopy, and directed circular dichroism spectroscopy [57, 58]. The structure obtained in each context from these techniques is well associated with their biological activities.

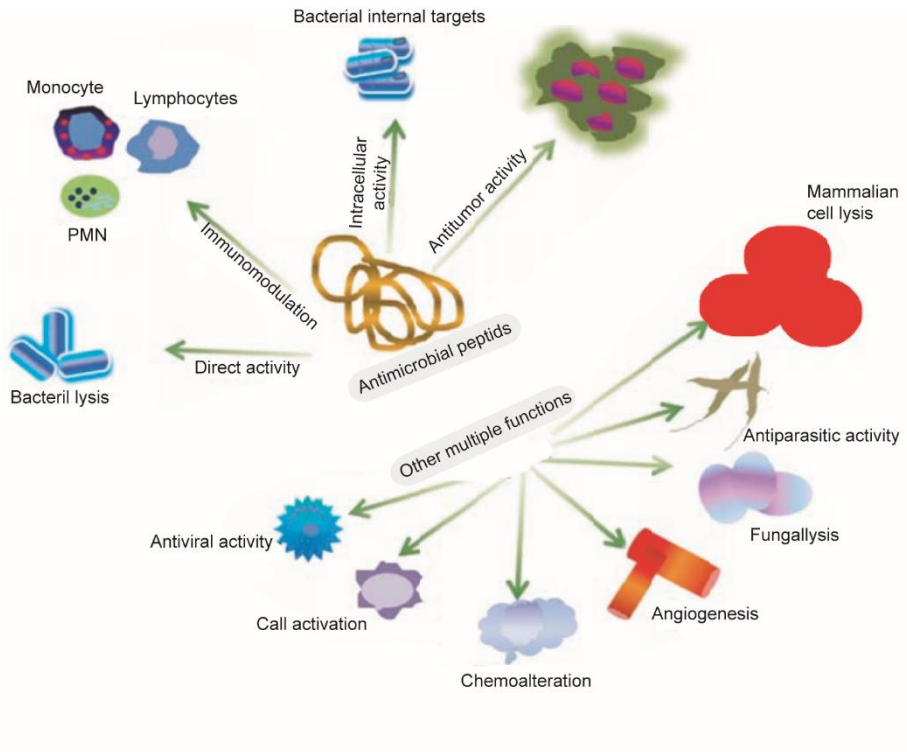


Figure 4.2: Demonstration of multifunctional activity of antimicrobial peptides.

In our culture, natural goods are used widely. About 60% of licensed medicines and drug candidates are either natural products or derived from them. Approximately 100,000 secondary metabolites (organic compounds not specifically involved in the growth, development, or reproduction of an organism) were characterized as having a molecular weight of less than 2500 Da, half of which were microbes and half of which were plants. In managing pathogenic microorganisms and contagious diseases, AMPs and' bacteriocins' have raised attention to their potential use. A wide variety of bacteria create AMPs ubiquitously. A species with considerable attention to human well-being and medicinal applications, *Bacillus* gives various AMPs of numerous simple chemical structures. *Bacillus* has identified multiple peptides with biological activities and is under active study for their antimicrobial activity [59, 60]. Some other significant pathogens, such as Listeria monocytogenes and Streptococcus pyrogens, have also been identified as bacteriocin or bacteriocin-like substances.

There is an extensive tradition of using fermented vegetables as a fundamental source of beneficial bacteria. Koreans have been eating salted and fermented vegetables, such as kimchi, for over 20 decades. Baechu cabbage and radish are the main ingredients of kimchi, but other vegetables such as green onion, leek, and cucumber are also used to prepare different kimchi forms. High amounts of dietary fibers, vitamins, minerals, and other functional ingredients comprise Kimchi. Previous research on kimchi has shown that kimchi has had significant benefits, including antioxidant, antiatherosclerotic, anticancer, antimicrobial, antidiabetic, and anti-obesity benefits [25].

The rapid growth and celebration of bacterial resistance and the advent of increasing multi-drug resistant pathogens have produced a critical need to recognize new groups of antimicrobial agents active against microbial targets of new resistance while avoiding existing resistance mechanisms action. Elucidating the mechanism(s) of antimicrobial drugs will help characterize both the chemical and the host interface of the pathogen, devise improved antimicrobials, assess appropriate drug formulations, and consider the production of microbial resistance as well. The analysis of action mechanisms of emerging drugs, not extracted from target-based discovery, should be begun early in their development stage to promote the modification of the drug scaffold to enhance the selectivity of action and pharmaceutical profile [61]. The production and mechanisms of action of AMPs from microbes isolated from fermented foods have not been widely investigated.

We organized a two-step analysis in this segment of work. Initially, the strains from kimchi were isolated, the potential AMP was sampled, identified, characterized, and distilled, and the distilled AMP designated as MS07 was further described. Finally, we studied the antimicrobial mode of action upon the assurance of the novelty of purified MS07.

4.2. MATERIALS AND METHODS

4.2.1. Antimicrobial inhibitory spectrum

To determine the antimicrobial inhibitory spectrum, the agar dilution method [62] applied for checking the MIC (minimum inhibiting concentration) of peptide MS07. Bacitracin

and vancomycin were taken as reference antibiotics to compare the MIC value of MS07. Twofold serial dilution was performed using 5% DMSO to check MIC and MBC of lipopeptide MS07. MIC was distinct as the reciprocal of the dilution that could reduce the noticeable growth of selected microorganisms (1.5×10^8 CFU/mL). MIC was carried out against both gram-negative and gram-positive microorganisms. Tested microorganisms were applied to the MH-agar medium containing different concentrations of peptide MS07 and reference antibiotics, further incubated at 37°C. Gram-negative bacteria: *A. faecalis* ATCC 1004, *E. coli* KCTC 1923, *S. typhimurium* KCTC 1925, *Extended-spectrum beta-lactamase* V4 *(Escherichia coli), Extended-spectrum beta-lactamase* 31, *Extended-spectrum beta-lactamase* W_1, *Pseudomonas aeruginosa* KCTC 1637; Gram-positive bacteria: *B. subtilis* ATCC 6633, *E. faecalis* ATCC 29212, MRSA B15, *M. luteus* ATCC 9341, *M. smegmatis* ATCC 9341, *S. aureus* KCTC 1928, VRE 4. MBC (Minimum bactericidal concentration) was obtained by using the broth dilution method. MBC was assessed as the minimum concentration inhibiting the bacterial growth entirely ($\geq$ 99.9%) [63].

4.2.2. Time kill kinetics assay

The activities of peptide MS07 were investigated in time-kill studies tested with *Escherichia coli* cells in the log (Log_{10}) growing phase [64]. Concentrations half the MIC, equivalent to MIC, two times the MIC, four times the MIC, and eight times the MIC of the peptide were made. Furthermore, an inoculum volume of 5.0×10^5 CFU/mL was selected and incubated at 37°C. 1.0mL aliquots of the medium were prepared with different time intervals of initial, two, four, six, eight, and twenty-four hours for bacteria and inoculated into 10mL nutrient medium. Incubation was performed at 37°C for 24h.

The colony-forming-unit (CFU) of the bacteria was determined. The growth control (GC) test was acted for the organisms without reference antibiotic or peptide. The graph of the $\log_{10}$ (CFU/mL) was plotted against the growth time.

4.2.3. Synergism of peptide MS07 with Melittin

The synergistic effect between the peptide MS07 and melittin was evaluated by the combination assay [65]. It is noteworthy that for the synergism assay, twofold dilutions of MIC concentration samples were prepared to treat against pathogenic microorganism *Escherichia coli.* The synergistic antibacterial activity against *E. coli* was tested at half of the MIC and the MIC concentration of the peptide MS07 with half of the MIC and the MIC concentration of melittin. The study was also conducted in the absence of melittin as control.

4.2.4. Antibiofilm properties of peptide MS07

Antibiofilm activity of gram-positive bacteria *Staphylococcus aureus* KCTC 1928 and gram-negative bacteria *Escherichia coli* KCTC 1923, *Pseudomonas aeruginosa* KCTC 1637 was assessed with ampicillin and oxacillin in combination with MS07. Bacteria cells (100µL) were positioned in 96 well TCPs in MH broth accompanied with glucose (0.2%). The plate was incubated at 37°C for 24h. The solution, not including cells, were treated as control. The culture was removed, cleaned with PBS, resuspended by 100 µL methanol (100%). Further, the culture media was incubated for 15 min to calculate the biofilm formed. After the elimination of methanol, biofilm development was measured by crystal violet (0.4%) staining for 30 min. It was washed with Distilled water (DW), and the well

plate was thoroughly air-dried. Absorbance was measured at 620nm by adding 100% ethanol to each well. The experiment was carried out triplicate to validate the process.

4.2.5. Viability assesses of biofilm cells interpreted with MS07

Viability test of the preformed biofilm cells interpreted with peptide MS07 was investigated on *P. aeruginosa* KCTC 1637 and *E. coli* KCTC 1923 at sub-inhibitory concentrations [66]. In Brief, a 96-well plate was poured with 600µL of 1.0×10^6 CFU/mL in Tryptic Soy Broth (TSB), and incubation was performed at 37°C for 24h. The medium was separated, and well plates were soaked two times with PBS (pH 7.5). Peptide MS07 (200µL of various MIC) dissolved TSB was mixed. After inoculation at 37°C for 12h, the supernatant was removed, and 200µL of PBS was mixed with 50µg of methyl-thiazol-tetrazolium (MTT). Afterward, viability assay was established on the development of insoluble purple formazan from a decrease of MTT by respiratory reduction of the bacterial cells. The crystal form of formazan was suspended in DMSO. The absorptions were taken at 570nm with a microplate reader. Three separate tests were conducted for each concentration.

To determine minimum biofilm eradication concentration (MBEC), gram-positive bacteria *M. luteus* ATCC 9341, and gram-negative bacteria *P. aeruginosa* KCTC 1637 and *E. coli* KCTC 1923 were used. A 96-well revival microtiter plate was used, like every well, including 200µL TSB medium [67]. The microplate was closed and incubated for 4 hours at 37°C. The progression of bacteria in a properly showed regrowth of planktonic cells from persisting biofilm. Afterward, Minimum biofilm eradication concentration (MBEC) was described as the lowest peptide intensity stopping regrowth of bacteria from

the interpreted biofilm. Minimum bactericidal concentration on biofilm (MBCb) was described as the minimum strength of peptide that essential to reduce viable biofilm cell numbers by 99.9% killing (≥3 $\log_{10}$).

Table 4.1. Antibiofilm activity of MS07 with various bacterial species

Bacterial species	**Minimum biofilm eradication conc. (MBEC, µM) ***	**Minimum Bactericidal conc. on biofilm (MBCb, µM) ****
Gram-negatives		
Escherichia coli KCTC 1923	4	8
Pseudomonas aeruginosa KCTC 1637	1.5	6
Gram-positive		
Micrococcus luteus ATCC 9341	1.5	2

*The lowest AMP concentration inhibiting regeneration of bacteria from the preserved biofilm contained by 4 hours.

**The lowest AMP concentration needed to decrease the count of viable cells by ≥3loh10 after 2hr.

4.2.6. Inhibition of bacterial biofilm imaging by confocal microscopy

Confocal laser scanning microscopy was used for imagining the formation of bacterial biofilm (*Pseudomonas aeruginosa*) in the absence and presence of peptide MS07. *Pseudomonas aeruginosa* (1.5× 10^8 CFU/mL) was incubated in 96-well microplate or three well chamber slides per well under microaerobic conditions for three days to grow biofilm. Then the supernatants were aspirated and treated with different MIC concentrations of MS07 for 48h. After the incubation period, the resident biofilm was measured under flood cells and stained employing Live/Dead BacLight viability stain (Life Technologies Corporation) visualized using a confocal microscope process [68].

4.2.7. Time-kill assay

The morphologically related *Escherichia coli* KCTC 1923 colonies were suspended with MHB at a concentration of 1×10^8 CFU/mL (compared to the freshly prepared McFarland 0.5 standard) diluted with MHB at 1:100. The following suspension was combined with either MS07, polymyxin B, ampicillin, and chloramphenicol (approximately 10×MIC) with a ratio of 1:1 and incubated at normal temperature [69]. 25 µL of each crop was streaked onto a fresh agar plate after 8, 15, 38, 46, and 54 minutes of incubation and permitted air dry. As a negative control, an untreated cell sample was used at 1×10^8 CFU/mL. At 37°C, plates were incubated overnight, and each time-point counted the number of individual colonies.

4.2.8. Effect of Lipopolysaccharide (LPS) binding peptide MS07

Antibacterial activity of the peptide MS07 against *Escherichia coli* KCTC 1923 in the presence of LPS was determined. *E. coli* cell was cultured and diluted with tryptic soy broth (Becton, Dickinson, Sparks, MD) to include 6.0×10^7 CFU/mL. The lipopolysaccharides from *E. coli* O111: B4 (Sigma, St. Louis, MO) was mixed with cell suspension at a final concentration of 5, 10, 25, or 100 µg/mL. Peptide MS07 was added to a final concentration of 64 µg/mL, which was two times the MIC of peptide MS07 against *Escherichia coli*. Ampicillin at 10 µg/mL and Polymyxin E at 2 µg/mL were applied as negative and positive controls, separately. The combinations were incubated with agitation at 200 rpm for 60 min at 37 °C. Stayers cells were measured by spread plating on tryptic soy agar [70].

4.2.9. Binding of peptide MS07 with LPS and Gram-negative bacteria

A FITC-labeled LPS (0.5 µg/mL) was employed in this analysis. Various concentration of peptide MS07 (70 µL) or polymyxin B was added with FITC-labeled LPS in to a 96-well plate [71]. This polymyxin B was applied to determine the binding resemblance of peptide MS07 to purified LPS, which was obtained from *E. coli* O111:B4. Aliquots of polymyxin B -LPS combination was checked from the well of a microplate reader. The final concentrations of LPS and polymyxin B were 5 µg/mL and 0.1 µg/mL, respectively. Addition of LPS to polymyxin B diminished its fluorescence due to polymyxin B-LPS binding. A variation in fluorescence because of the shift of polymyxin B from the polymyxin B -LPS complex was recorded using a luminescence spectrometer (LS55; Perkin-Elmer, Wellesley, MA), at 490nm excitation wavelength and 515nm emission wavelength. Likewise, the binding affinity of peptide MS07 to live *Escherichia coli* KCTC 1923 cells were assessed utilizing the similar process in which the LPS component was replaced with 10^6 *Escherichia coli* cells.

4.2.10. Preparation of Liposomes and binding to phospholipid bilayer

Small unilamellar vesicles (SUVs) were prepared by dissolving dry lipids in chloroform/MeOH (2:1, v/v), evaporating the solvent under a stream of nitrogen, lyophilizing overnight, resuspending in buffer (10 mg/ml), vortex mixing, and sonicating. Lipid films were prepared from POPC, POPC / POPG (4:1, wt: wt), and POPC / lipid A (4:1, wt: wt), which mimic the outer leaflets of the plasma membranes of *Escherichia coli*. Large unilamellar vesicles (LUVs) were prepared a by dissolving lipid film in the buffer by incorporating Tris-HCl buffer (10 mM; pH 7.5), which includes NaCl (20 mM), DPX quencher (45 mM), and ANTS fluorophore (12.5 mM), evaporating the solvent

under a stream of nitrogen, lyophilizing overnight, resuspending in a buffer, vortex mixing to produce multilamellar vesicles (MLVs), and sonicating. The vesicles were extruded 20 times through a mini extruder with a 0.2 mm polycarbonate membrane for making LUV after 5 freeze-thaw cycles in CO Large unilamellar vesicles (LUVs) were prepared a by dissolving lipid film in the buffer by incorporating Tris-HCl buffer (10 mM; pH 7.5), which includes NaCl (20 mM), DPX quencher (45 mM), and ANTS fluorophore (12.5 mM), evaporating the solvent under a stream of nitrogen, lyophilizing overnight, resuspending in a buffer, vortex mixing to produce multilamellar vesicles (MLVs), and sonicating. The vesicles were extruded 20 times through a mini extruder with a 0.2 mm polycarbonate membrane for making LUV after 5 freeze-thaw cycles in CO Large unilamellar vesicles (LUVs) were prepared a by dissolving lipid film in the buffer by incorporating Tris-HCl buffer (10 mM; pH 7.5), which includes NaCl (20 mM), DPX quencher (45 mM), and ANTS fluorophore (12.5 mM), evaporating the solvent under a stream of nitrogen, lyophilizing overnight, resuspending in a buffer, vortex mixing to produce multilamellar vesicles (MLVs), and sonicating. The vesicles were extruded 20 times through a mini extruder with a 0.2 mm polycarbonate membrane for making LUV after 5 freeze-thaw cycles in CO_2.

4.2.11. Membrane leakage by ANTS/DPX by lipopeptide MS07

The ability of polymyxin B, octapeptin C4 and peptide MS07 to translocate through lipid bilayers was evaluated by transfer of fluorescence, as earlier explained (X1). SUVs were prepared by freeze-thaw cycles and homogenized by extrusion. Instantly after the extrusion, utilizing the gel filtration chromatography on Sephadex G-25, vesicles containing ANTS/DPX were isolated from DPX and free ANTS. At an emission

wavelength of 520 nm and an excitation wavelength of 353 nm on a Shimadzu RF-5300PC spectrofluorometer, the release of the ANTS and DPX from the LUVs was fluorometrically monitored. The dose-dependent binding of lipopeptides to POPC/lipid A (4:1), POPC/POPG (4:1), and POPC was contrasted with two-fold lipopeptide concentrations varying between 100 μg/mL to 0.05 μg/mL. By adding 10% Triton X-100 (w/v), the maximum fluorescence intensity corresponding to a 100 percent leakage was determined. The percentage of apparent leakage was calculated using F, F1 and F2 whereas F denoted intact vescicle flurescence, F1 denoted initial flurescence and F2 denoted final flurescence by addition of detergent. The percentage of hemolysis was calculated as 100× [(F1- F)/ (F2- F)][72].

4.2.12. Membrane assays

NPN was applied to 2×10^6 Escherichia coli KCTC 1923 cells / mL (final NPN concentration of 20 mM) for external membrane permeability assays and incubated for 15 min with differing concentrations (64 to 0.015 mg / mL) of fluorescence emission intensity recorded lipopeptides (excitation wavelength of 340 nm and an emission wavelength of 405 nm) using a Shimadzu fluorescence spectrophotometer (RF-5300PC, Shimadzu Sci. Ins., Kyoto, Japan). Mid-log step (OD~0.6) Escherichia coli KCTC 1923 cells were obtained by centrifugation and suspended in HEPES (5 mM), glucose (20 mM), pH 7.4 for the cytoplasmic membrane depolarization assay. For 30 minutes, the absorption of diSC3-5 dye into bacterial cells was checked. A Shimadzu fluorescence spectrophotometer (RF-5300PC) was utilized to determine the fluorescence intensity (excitation wavelength of 620 nm and an emission wavelength of 670 nm). Suspensions of 1×10^7 Escherichia coli KCTC 1923 cells/mL were incubated for 1 h at 37 ° C with

antibiotics and lipopeptides at differing concentrations (10 to 0.0001 mg / mL) (n = 3) for the internal membrane permeability assay [73]. At room temperature, SYTOX green, a DNA binding dye that is fluorescent when bound to nucleic acids and only reaches cells with damaged plasma membranes, was applied at 2 mM to the Escherichia coli compound-treated cells for 5 minutes. For their fluorescence emission profiles, cells were compared with excitation wavelengths of 488 nm and emission wavelengths of 530 nm containing 30 nm bandpass by flow cytometry [74]. They were executed employing a Cyto FLEX flow cytometer (Beckman Coulter, Brea, CA, USA).

4.2.13. Hemolytic assay against red sheep blood cells

The hemolytic activity of the peptide MS07 was measured by the lysis of RBC as the amount of hemoglobulin released [75]. Fresh red sheep blood cells were washed with PBS (35 mM phosphate buffer, 0.15 M NaCl, pH 7.3) for three times, centrifuged at 116×g for 5 min, dispensed into 96-well plates as 100 µl of 4% (w/v) of RBC suspension in PBS and 100 µl of peptide solution was added to each well. A two-fold serial dilution of the peptides (the concentration test range was 0.5 µg/mL to 512 µg/mL) in PBS. The plate was incubated at 37 °C for 1 h, and then the cells were pelleted by centrifugation at 1000×g for 5 min. The supernatants (100 µl) were transferred to another clear 96-well plates. Hemoglobin was determined by measuring absorbance at 414 nm using a microplate reader (Bio-Tek Instruments, USA). Zero hemolysis was calculated using PBS(APBS), and 100% hemolysis was established using 0.1% (v/v) Triton X-100 (Atriton). The percentage of hemolysis was calculated as 100× [(Asample- APBS)/ (Atriton- APBS)].

4.2.14. In vitro Cytotoxicity Assay

The cytotoxicity of the peptide against RAW 264.7 cells and Mammalian cells (HeLa, SH-SY5Y) cells was tested by using the standard assay [76]. Cells were plated into 96-well microplates (Nunclon Delta Surface, Thermo Fisher Scientific, China) at 5×10^3 cells/well and incubated overnight. An increasing concentration of peptides (10-100µM) was added to those cells and incubated for 3h. After the media was substituted with new media, the cells were incubated for 24h. A total of MTT (20µL, 5mg/mL) solution was mixed and incubated for 4h at 37 °C. The medium was centrifuged, separated, and then 150 µL of DMSO was mixed to each well to dissolve the formazan crystals formed. The absorbance was measured on a microplate reader named KisanBio plate reader (KisanBio, Seoul, South Korea) at 570 nm. The results are the mean ± standard deviation of three independent experiments. Cell viability was determined by using the following equation:

$$\text{Viability (\%)} = A_{treated}\text{-}A_{blank}/A_{untreated}\text{-}A_{blank}*100$$

4.2.15. Flow cytometry

Peptide generated cell membrane damage was observed by the detection of PI (propidium iodide) influx [77]. Intermediate log phase of *Escherichia coli* KCTC 1923 cells was grown and washed three times with PBS buffer and dilute in the same buffer to OD= 0.3 (10^5 CFU/mL). Bacteria were incubated with LPS, 1 ×MIC, 2 ×MIC, 3 ×MIC and 4 ×MIC or without peptide for 1 h at 37 °C. Cells were centrifuged and incubated with fixed PI concentration (10 µg/mL) for 30 min at 4 °C. Later, bacteria cells were centrifuged at 10000×g and diluted in 1 mL of PBS. The analysis was performed using a FACS flow cytometer (Becton-Dickinson, USA) to

acquire data at a laser excitation wavelength of 488nm.

4.2.16. Confocal microscopy

Escherichia coli KCTC 1923 and *Pseudomonas aeruginosa* KCTC 1637 cells suspension was cultured in LB broth to mid logarithmic phase and harvested by centrifugation. Cells were collected and washed three times with 10mM PBS buffer (pH 7.5). MS07 was labeled with fluorescein isothiocyanate (FITC). Afterward, Bacteria cells (1×10^7 CFU/mL) were incubated with FITC labeled peptide MS07 (2 ×MIC) at 37 °C for 30 min. After being incubated, the cells were pelleted down by centrifugation and then washed three times using PBS buffer (pH 7.5) and static on a glass slide. Cell images with FITC-labeled peptide were observed using a laser confocal microscope (LSM 510, Carl Zeiss, Germany). Fluorescent images were found with a bandpass filter at 488nm for excitation of FITC [78].

4.2.17. Evaluation of Ultrastructure by Transmission electron microscopy (TEM)

Concentration-dependent bactericidal effects of lipopeptide MS07 were studied through conducting transmission electron microscopy (TEM) [79]. *E. coli* cells were cultured in a sterilized LB (Luria-Bertani) broth medium by incubating for 12 h at 37 °C to obtain a concentration of 5×10^7 CFU/mL. *E. coli* cells were washed three times with 10 mM PBS (phosphate-buffered saline) buffer (pH 7.3). Aim to verify the concentration-dependent effects, Lyophilized peptide MS07 were dissolved in this same buffer and added to the bacterial pellets, which were resuspended to a final peptide concentration of 32–128 μg/mL. *E. coli* were incubated with numerous concentrations of the peptide (0, 32, 64, and 128 μg/mL) for 60 min at 37 °C. cells were washed for 3×5 min in 10 mM PBS

buffer and washed with distilled water twice. Furthermore, *E. coli* cells were fixed with 2.5% glutaraldehyde by interacting with bacterial pellets overnight at 4 °C in 0.01 M PBS buffer (pH 7.3) and postfix with 1% osmium tetroxide for 1 hour. After fixing and washing, the cells were incubated overnight with 2% uranyl acetate and dehydrated in graded series of ethanol and permitted in white resin. Embedding was maintained by heating for 48 hours at the temperature at 60°C. Aim to analyze the section thickness of 100 nm, and the samples were transferred on a copper grid and stained with 2% uranyl acetate and 1% lead citrate.

4.3. RESULTS

4.3.1. Antimicrobial spectrum, time-kill kinetics assay, and synergistic effect of MS07

The antimicrobial spectrum of peptide MS07, established in terms of MIC, was a strongly effective counter to gram-negative bacteria (MIC of 16-32 µg/mL). It relatively effective counter to gram-positive bacteria (MIC of 16-128 µg/mL). We compared the MIC of our recently purified peptide MS07 to that of bacitracin and vancomycin (Table 4.2). Peptide MS07 has a stronger effect than commercial bacitracin and vancomycin on various gram-negative pathogens such as *Alcaligenes faecalis*, *Escherichia coli*, *Salmonella typhimurium*, *Extended-spectrum beta-lactamase* (V4, 31, W1) (*E. coli*), *Pseudomonas aeruginosa*, on the other hand, some gram-positive pathogens such as MRSA B15, *Micrococcus luteus*, *Bacillus subtilis*, *Mycobacterium smegmatis*. The minimum bactericidal activity (MBC) of MS07 against various pathogens was 320-1280 µg/mL that shows high MBC activity.

Table 4.2. Antimicrobial spectrum of peptide MS07

Microorganisms	MIC (μg/mL)			MBC (μg/mL)
	MS07	Bacitracin	Vancomycin	MS07
Gram-negative bacteria				
Alcaligenes faecalis ATCC 1004	32	> 128	64	640
Escherichia coli KCTC 1923	32	> 128	64	320
Salmonella typhimurium KCTC 1925	32	64	32	640
Extended-spectrum beta-lactamase V4 *(Escherichia coli)*	16	64	32	640
Extended-spectrum beta-lactamase 31	16	32	32	640
Extended-spectrum beta-lactamase W1	32	64	32	320
Pseudomonas aeruginosa KCTC 1637	32	> 128	> 128	640
Gram-positive bacteria				
Bacillus subtilis ATCC 6633	16	16	0.5	640
Enterococcus faecalis ATCC 29212	32	4	2	160
Methicillin-resistant Staphylococcus aureus B15	16	64	64	640
Micrococcus luteus ATCC 9341	16	32	2	320
Mycobacterium smegmatis ATCC 9341	32	64	2	> 1280
Staphylococcus aureus KCTC 1928	> 128	> 128	> 128	> 1280
Vancomycin-resistant Enterococci 4	> 128	64	> 128	> 1280

MIC- minimum inhibitory concentration; MBC- minimum bactericidal concentration

The time-kill kinetics report of peptide MS07 counter to the test organism *E. coli* at test concentrations demonstrated a decline in the count of viable cells around the initial 2, 4, 6, 8, and 24 hours correspondingly afterward an up to the 24 h for *E. coli* (Figure 4.3a).

MS07 was tested for their antimicrobial activity using colony count assays. At MIC level (32 µg/mL), MS07 killed bacteria up to 6h. At low peptide concentrations, an initial decline in the count of surviving *E. coli* cells was noticed, whether after 6-8 h surviving *E. coli* cells retrieved from the peptide action and started growing again. Regrowth was observed up to 2×MIC, whereas 4×MIC and 8×MIC showed a complete reduction of the bacterial count.

Visualizing the synergistic effect of MS07 and melittin, *E. coli* cell suspensions were spread on the MH agar plate. The result showed that the combination of peptide MS07 and melittin had intense synergistic antibacterial activity against *E. coli* (Figure 4.3b). It indicates that it could be demonstrated potentially valuable synergistic effect rather than the peptide MS07 applied separately.

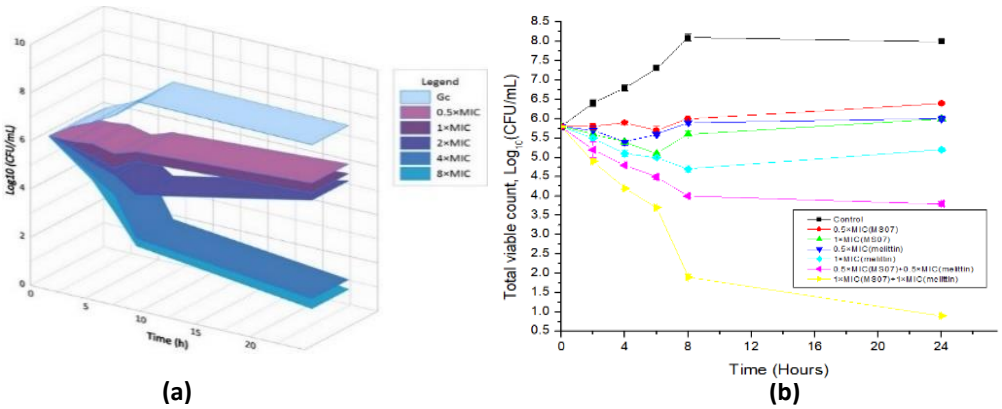


Figure 4.3: (a) Killing kinetics of MS07 in multiply with different MIC concentration against *Escherichia coli* compared with growth control (GC) for 24hr. (b) The synergistic effect of MS07 with melittin that synergy improves killing kinetics rather than acting alone against *Escherichia coli* for 24 hr.

4.3.2. Antibiofilm activity of peptide MS07

For biofilms, bacteria develop as multicellular accumulates inside an extracellular substance which shields the host defense cell. MS07 was assessed for its antibiofilm activity counter to gram-positive and gram-negative bacteria. Our result demonstrated that biofilm development was significantly diminished when treated in a combination of referenced antibiotics. Therefore, the ability of MS07 to inhibit biofilm development might be efficient more with a combination of ampicillin or oxacillin. SO, a combination of peptide MS07 and other antibiotics can signify the therapeutic application. ANOVA test was conducted to compare the mean, $p< 0.05$, with their result.

4.3.3. Viability assays of biofilm cells treated with MS07

We studied the anti-biofilm action of peptide MS07 upon *Pseudomonas aeruginosa* KCTC 1637 and *Escherichia coli* KCTC 1923 at sub-inhibitory strengths. As shown in Figure 6b, after employing biofilms with peptide MS07, it was detected that the count of biomass was diminished with rising strengths (1× MIC to 8× MIC). It was decreased by about 15% and 11% in MIC concentration with *P. aeruginosa* and *E. coli* biofilm. The viability of *Pseudomonas aeruginosa* and *Escherichia coli* biofilm was decreased after 24 h action at an elevated concentration of peptide MS07. A comparison of means compared to untreated control was presented using the ANOVA test, ***$p< 0.001$, p**< 0.01, *$p< 0.05$. Experiments were done in triplicate.

MS07 was checked for anti-biofilm activity against gram-positive strains (*M. luteus* ATCC 9341) as well as gram-negative strains (*E. coli* KCTC 1923 and *Pseudomonas aeruginosa* KCTC 1637). The MBECs (minimum biofilm eradication concentrations) of

the peptide MS07 showed higher anti-biofilm activity against gram-positive strain *Micrococcus luteus* (MBEC, 1.5 µM) which is consistent with its MIC concentrations (Table 4.1). MS07 exhibited minimum bactericidal concentration (MBCb) on biofilm of 6 µM and 8 µM respectively counter to the gram-negative strain *P. aeruginosa and E. coli* biofilm, respectively.

4.3.4. Anti-biofilm action of peptide MS07 over confocal microscopy

Consistent with antimicrobial activity, peptide MS07 reduced and interrupted the biofilm formation in a concentration dependent as established by a Backlight live/dead stain (Figure 4.4c). Confocal microscopy images explain the formation of the biofilm in slide after shaking for 3 days anaerobically. The plane surface of the image of *P. aeruginosa* biofilm exhibited distinct construction with green fluorescence. After treatment with MS07 significantly reduced biofilm formation concentration-dependently. It endorsed that peptide MS07 not only holds bactericidal activity but also could disrupt the biofilm.

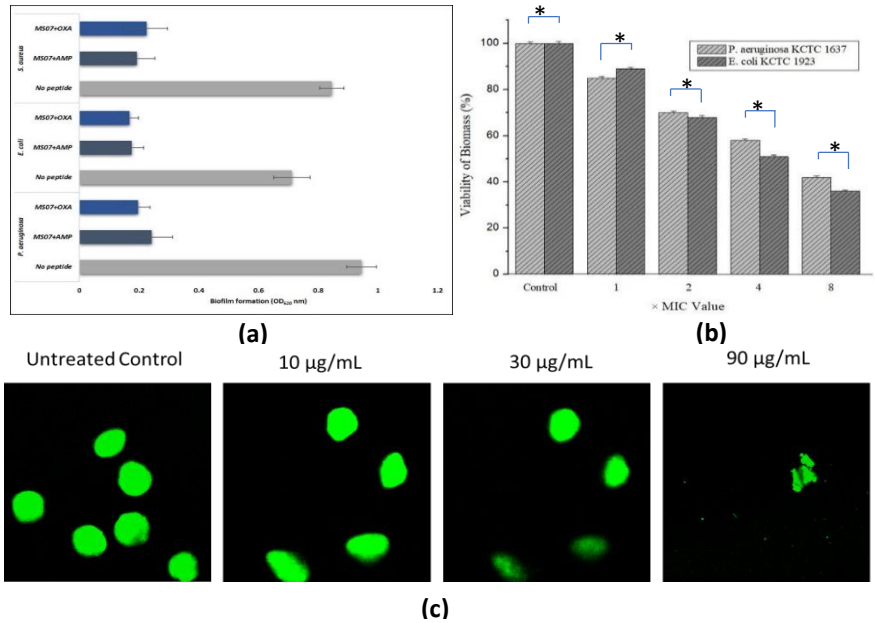


Figure 4.4: (a) Bacteria treated with combination of MS07 and Oxacillin (MS07+OXA); combination of MS07 and Ampicillin (MS07+AMP); To verify biofilm development, microbial cells in MH broth accompanied by 0.2% glucose were complemented to TCPs 96-well plate and incubated for 24h at 37°C. The results are presented as means± standard deviation. (b) The viability of *Pseudomonas aeruginosa* and *Escherichia coli* biofilm was decreased after 24 h action at a elevated concentration of peptide MS07. ANOVA test, ***p< 0.001, p**< 0.01, *p< 0.05. (c) Effect of MS07 on recognized *Pseudomonas aeruginosa* biofilm. Representative confocal microscopy images determined the formation of *Pseudomonas aeruginosa* biofilm after shaking for 72 h. The plane surface image of biofilm exhibited distinct structure with green fluorescence. Treatment with MS07 significantly reduced biofilm formation dose-dependently.

4.3.5. Time kill Assay

Our initial efforts were centered on the discovery of the peptide mechanism of MS07. Valuable information on the cellular process attacked by that compound can be given by calculating the time required by an antibiotic to exert its antimicrobial impact. Bacteriostatic agents interrupt cells' division but do not decrease the number of viable cells, and this group usually contains antibiotics that target nucleic acid and protein synthesis. Bactericidal agents decrease the amount of viable bacterial cells, and the time is taken for a bactericidal agent to kill bacteria gives more information on its target. We, therefore, controlled growth kinetics. MS07 exposed *E. coli* cells and many other antibiotics (Figure 4.5). Owing to the creation of large, unspecific pores in the bacterial membrane, lipopeptides such as polymyxin B are usually bactericidal within minutes of exposure, whereas many other antibiotics (like ampicillin) exert their killing effect over

many hours. Cell development is halted by bacteriostatic agents (like chloramphenicol), but cells are not destroyed. Measurements of optical density revealed that cells exposed to MS07 expanded for 38 minutes at a reduced rate, at which point a gradual decrease in cell count was observed. Polymyxin B instantly decreased the cell count, while ampicillin demonstrated an estimated 3-h lag, and optical density was not decreased by chloramphenicol. Complementary findings were obtained from a time-kill assay, which revealed that cells exposed to MS07 showed a small decrease in cell viability after 15 minutes of treatment, a significant reduction after 30 minutes, and total death after 60 minutes. Polymyxin B worked quicker, growing the viable cell population dramatically after 5 min and killing all cells by 30 min. These findings showed that MS07 is a peptide targeting the membrane, but does not function by a generic process of membrane lysis such as polymyxin B. The selectivity of MS07 against Gram-negative bacteria further reinforces this, as a peptide that acts by a generic lysis process will also attack Gram-positive species.

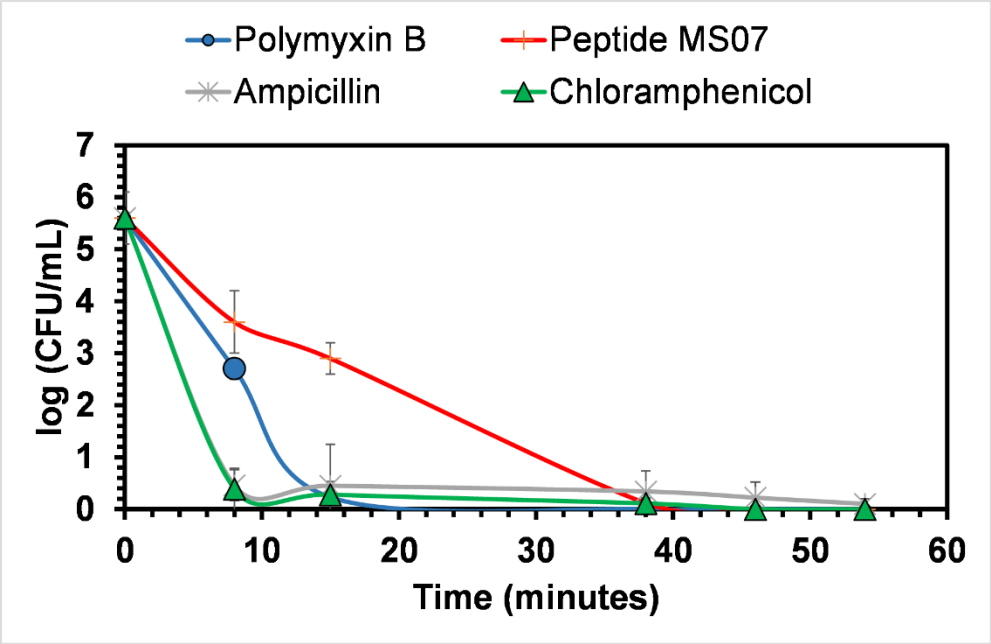


Figure 4.5. Time kill assay for MS07 and other antibiotics in the concentration of 10× MIC.

4.3.6. Lipopolysaccharides antagonize MS07 activity

Lipopolysaccharides (LPS) from *Escherichia coli* inhibited MS07's antimicrobial activity against *Escherichia coli* KCTC1923. LPS had slight impact at lower concentrations (2 to 25 μg/ml) on the antimicrobial activity of MS07, but LPS completely neutralized the bactericidal activity of MS07 at 100 μg/ml. Polymyxin B, an established outer membrane binding agent which was used as a positive control, became ineffective against the bacterium in the presence of 100 μg LPS/ml. Ampicillin act as negative control, that aim for cell wall biosynthesis did not affect LPS. The outcome suggests that MS07 has a high affinity for the LPS part of the outer membrane of Gram-negative bacteria. Therefore, the LPS section of live bacterial cells is perhaps the initial target of MS07.

4.3.7. MS07 binds to the LPS of the outer cell membrane

Preliminary data indicate that when this probe was attached to a purified LPS portion of live *Escherichia coli* cells, the polymyxin B conjugate's fluorescence was quenched. LPS affinity with compounds can attach to the binding sites on LPS with polymyxin B conjugate and thus displace the LPS conjugate, with a concomitant increase in fluorescence. A rise in fluorescence was caused by polymyxin B's addition to the polymyxin B conjugate-LPS complex, which revealed that the fluorescently labeled polymyxin B molecules were displaced and released from the LPS. The finding is consistent with previous reports that cationic polymyxin molecules can bind to and displace divalent cations (Mg^{2+} and Ca^{2+}), which stabilize the outer membrane's permeability barrier the negatively charged lipid A phosphates in LPS. Likewise, cationic MS07 molecules competed for binding on LPS with polymyxin B conjugate, resulting in the conjugate's release in a concentration-dependent manner. Moreover, the membrane-bound polymyxin molecules can also be displaced by MS07 from live *Escherichia coli* cells. These findings indicate that in the outer membrane of Gram-negative bacteria, MS07 and polymyxin molecules have the same binding sites on LPS. Therefore, the electrostatic interaction between MS07 and LPS can disturb the outer membrane's permeability and promote antibiotic uptake (Figure 4.6c).

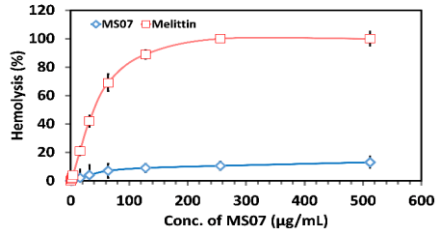
4.3.8. Effect of Lipopeptide on Outer Membrane Permeability N-Phenyl-1-naphthylamine (NPN) Assay

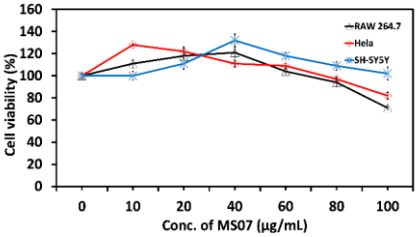
NPN is a low fluorescence quantum yield, uncharged lipophilic probe in an aqueous atmosphere that, when divided into a hydrophobic environment, becomes fluorescent, such as the one discovered in lipid membranes. The free hydrophobic solutes diffusion, including NPN, is constrained by the outer membrane (Figure 4.7a). However, embolism of NPN into the basic phospholipid inner leaflet and cytoplasmic membrane, once permeated, causes an increase in fluorescence emission, and can thus be used to analyze permeabilization of the bacterial outer membrane. The lipopeptide exposure resulted in permeabilized log-phase *E. Coli* cells, which improved NPN absorption after incubation with improved lipopeptide doses. Octapeptin C4 was less successful at membrane permeabilization, requiring a higher concentration for optimum NPN absorption than polymyxin B or MS07, possibly reflecting its decreased efficacy against strains susceptible to polymyxin.

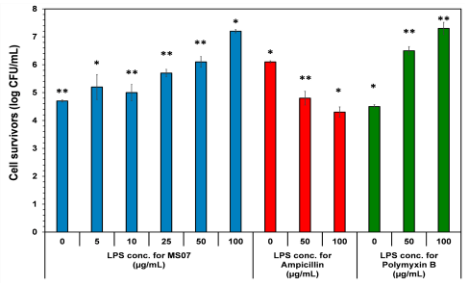
4.3.9. Membrane Depolarization 3,30-Dipropylthiadicarbocyanine iodide (diSC3-5) Assay

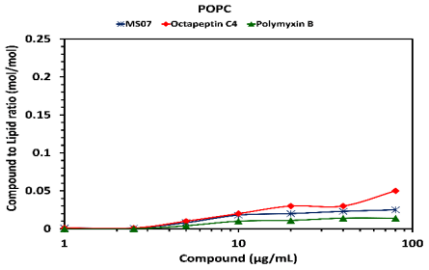
diSC3-5 is a carbocyanine potentiometric cationic probe that can be used to analyze changes in intact bacterial cells' capacity in the membrane. If the hyperpolarized indigenous state initiates in the bacterial membrane, the cationic dye combines in the membrane, and its fluorescence emission is self-quenched. However, if the membrane potential is disturbed, the dye lacks membrane affinity and is released into the aqueous atmosphere, leading to an increase in the collective emission of fluorescence. The figure shows that membrane depolarization in *E. coli* cells was initiated by octapeptin C4, polymyxin B, and MS07, in a dose-dependent way. Octapeptin C4 was less efficient than polymyxin B and MS07 at smaller doses (< 10 mg /mL) but was relatively more effective at higher concentrations (>100 mg/mL) (Figure 4.b). Fluorescence Inner Membrane Permeabilization- Enabled Cell Sorting SYTOX Green Staining Research Flow cytometry was accompanied by SYTOX green uptake. Our findings indicate that polymyxin B, MS07, and octapeptin C4 interacted with Gram-negative cells' outer and inner membranes. Interestingly, once performed treating with the compounds, two unlike cell numbers were discovered, containing of a number emitting mean fluorescence

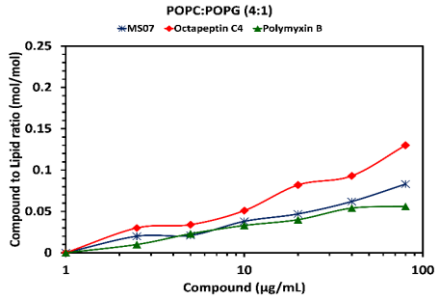
intensities of 10^4-10^5, resembling that of dead isopropanol-killed cells, along with an intermediate fluorescence intensity population of 10^3-10^4, much greater than live cells (10^1 to $<10^3$), but less than dead cells power. Cell populations with separate fluorescence emissions could either originate from varying degrees of green absorption of SYTOX or from cells with different concentrations of nucleic acids that arise from various cell division phases. Two species' existence implies that polymyxin B, MS07, and octapeptin C4 caused varying degrees of cell damage as only a single population was found in control cells permeated with isopropanol. Octapeptin C4 caused an analogous proportion of partial cell damage as MS07 and polymyxin, but not the same degree of total cell damage/death at the same concentration, which concurs with the comparative lipopeptide MIC values.











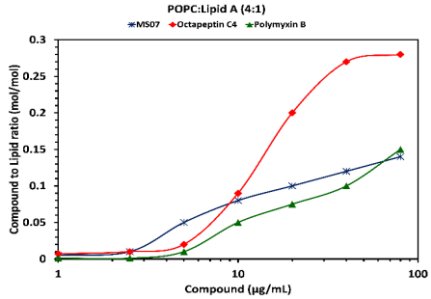


Figure 4.6: Hemolysis activity of MS07, Cytotoxicity assay of MS07 with RAW264.7 cell, SHSY5Y cell and HeLa cell, LPS binding activity of MS07, Binding of MS07, polymyxin B, and octapeptin C4 to phospholipid bilayers. Three lipid systems are compared: from top to bottom, POPC, POPC/POPG (4:1 molar ratio), and POPC/*E. coli* lipid A (4:1 molar ratio).

4.3.10. Leakage from Lipid Vesicles Induced by Lipopeptide

Interaction studies of Octapeptin C4, MS07, and Polymyxin B with the membrane that mimic the composition of numerous arrangements were induced from small unilamellar vesicles (SUVs) by calcein release. A suspension of SUVs encapsulated with calcein was applied to lipopeptide MS07 and other compounds at increasing concentrations, and membrane permeability was accompanied by fluorescence recovery monitoring. The degree of maximal leakage obtained as a function of the molar ratio of peptides to lipids, as shown in Fig. X, Y, Z for phosphatidylcholine (PC), phosphatidylcholine (POPC), lipid A, and phosphatidylglycerol (POPG). The dose-dependent binding of lipopeptides to POPC, POPC / POPG (4:1, wt: wt), and POPC / lipid A (4:1, wt: wt) was contrasted with two-fold concentrations of compounds varying from 64 to 1 mg / mL. Both assays were replicated at least three times, and all analyzed data was subtracted from the reference flow cell responses. As earlier stated for peptide-to-lipid binding affinities, the resulting fluorescence was stabilized for compound mass, lipid deposition, and interpreted as the compound-to-lipid ratio (C / L; mol/mol) (Figure 4.6d, 4.6e, 4.6f). Lipid permeabilization vesicles were assessed to analyze whether the permeabilization of a bacterial cell by polymyxin B and octapeptin C4 is a process dependent on the lipopeptide's capability to concede lipid bilayer integrity, regardless of the presence of lipid A (Figure 4.7c, 4.7d). Fluorescence emission de-quenching was quantified by leakage from lipid vesicles. Neither of the lipopeptides destabilized vesicles membrane consisting of POPC/POPG or POPC. Vesicle membrane containing lipid A was destabilized by MS07 and polymyxin B, whereas octapeptin C4 demonstrated minimal leakage (Figure 4.7e, 4.7f). This indicates that the capability of octapeptin C4 to combine to the lipid bilayer is not entirely lipid A dependent, unlike polymyxin B. Still, lipid A is expected to interrupt the integrity of the membrane.

4.3.11. Hemolytic Activity and Cytotoxicity

A desirable technique for developing antibacterial agents is to achieve the maximum possible antibacterial activity with minimum toxicity to the host. To assess their potential to handle bacterial cells selectively, the compounds with the most active antibacterial activity, like lipopeptide MS07, were evaluated for their hemolytic activity against red sheep blood cells in the range of 0-512 µg/mL. At 512 µg/mL (Figure 4.6a), the highest concentration tested, MS07, showed noticeable hemolytic activity. Interestingly, until the concentration of 256 µg/mL, the reference compound melittin nearly induced 100 percent hemolysis, MS07 showed less than 8 percent hemolysis. These findings also support MS07 's ability to use as an antibacterial therapeutic agent.

Cytotoxicity of peptides was assessed using Mammalian cells (HeLa and SH-SY5Y) and RAW 264.7 cells. MS07 presented no cytotoxicity at concentrations <60 µg/mL, while at 80 µg/mL and 100 µg/mL, respectively, they induced up to 14% and 46% cell death. Using MS07, cell viability was ≥80%, also at 80 µg / mL (Figure 4.6b).

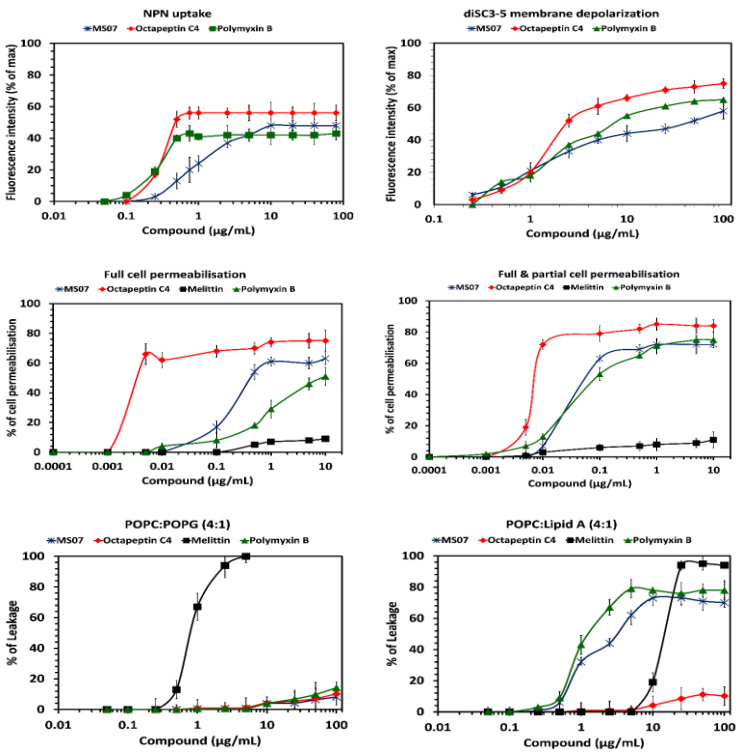


Figure 4.7: *E. coli* outer membrane permeabilization as measured with NPN fluorescence emission, Membrane depolarization of *E. coli* cells measured using fluorescence emission, Cell membrane permeabilization of *E. coli* as followed by SYTOX green fluorescence emission and flow cytometry, Leakage of lipid vesicles induced by lipopeptides as followed by calcein. Small unilamellar vesicles (SUVs)composed of POPC/POPG (4:1) (top) or POPC/*E. coli* lipid A (4:1)

4.3.12. Flow cytometry and confocal laser-scanning microscopy

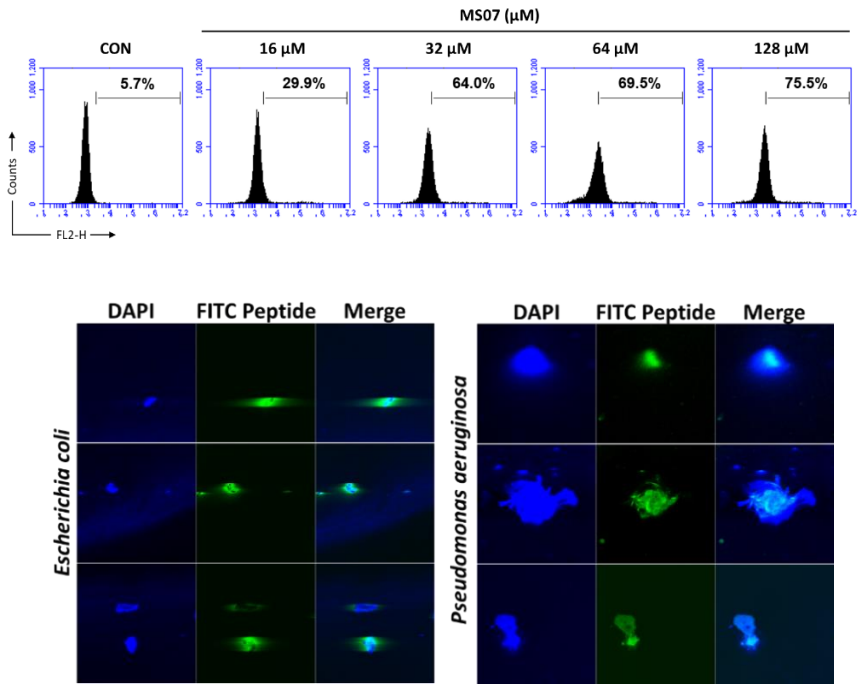
To further characterize the membrane integrity of *Escherichia coli* after MS07 treatment, flow cytometric analysis was performed. Since cytoplasmic membrane destruction, PI fluorescently stains nucleic acids. Only 5.7% of *Escherichia coli* exhibited a PI fluorescent signal in the absence of the peptide (control), indicating viable cell membranes. MS07 (membrane-targeting AMP) therapy at 4×MIC resulted in 75.5% of *Escherichia coli* cells, demonstrating PI uptake. In comparison, after treatment with MS07 at 1×MIC, the proportion of PI-positive *Escherichia coli* cells was just 29.9% (Figure 4.8a). For lipopeptides, identical findings were found, further suggesting that MS07 attacks bacterial cell membranes. Compared to the control, FITC-labeled MS07 displayed a significant change in the fluorescence intensity towards the lower right-hand area, showing 75.5% in *Escherichia coli* than the control can be due to the variations in the bacterial cell membrane.

In *Escherichia coli* KCTC 1923 and Pseudomonas aeruginosa KCTC 1637 cells, we conducted FITC-labeled MS07 uptake using confocal laser-scanning microscopy to validate the hypothesis further. There is a large improvement in fluorescence, as observed in the combined component (FITC/Nuclei) of both bacteria's photographs. This finding shows that MS07 labeled by FITC translocates within the bacterial cell by accumulating in the cell cytoplasm, indicating that MS07 exhibits an intracellular targeting mechanism by cell penetration (Figure 4.8b). However, due to the low permeability barrier of internal and external bacterial membranes, we still observed a few left-out FITC-MS07 in

Escherichia coli. This evidence further confirms that both *Escherichia coli* and Pseudomonas aeruginosa bacteria can translocate MS07 within them.

4.3.13. Transmission Electron Microscopy

To better understand the antibacterial mechanism of MS07, morphology, and intracellular alterations in bacterial cells after MS07 treatment were directly observed by TEM. Significant morphology changes occurred when the *Escherichia coli* KCTC 1923 cells were treated with MS07 for 1 h at 32 μg/mL to 128 μg/mL, as shown in the figure 4.8c. Conversely, the typical Gram-negative with intact membrane and high-density cytoplasm were displayed by the untreated *Escherichia coli* cells. Similarly, *Escherichia coli* cells treated with MS07 for 1 h at 32 μg/mL exhibited abnormalities, including cell wall disappearance, cell membrane disruption, and even intracellular content leakage. The untreated *Escherichia coli* cells, by comparison, preserved natural cell morphology, exhibiting intact cell walls, well-defined membranes, and cytoplasmic homogeneous electron density.



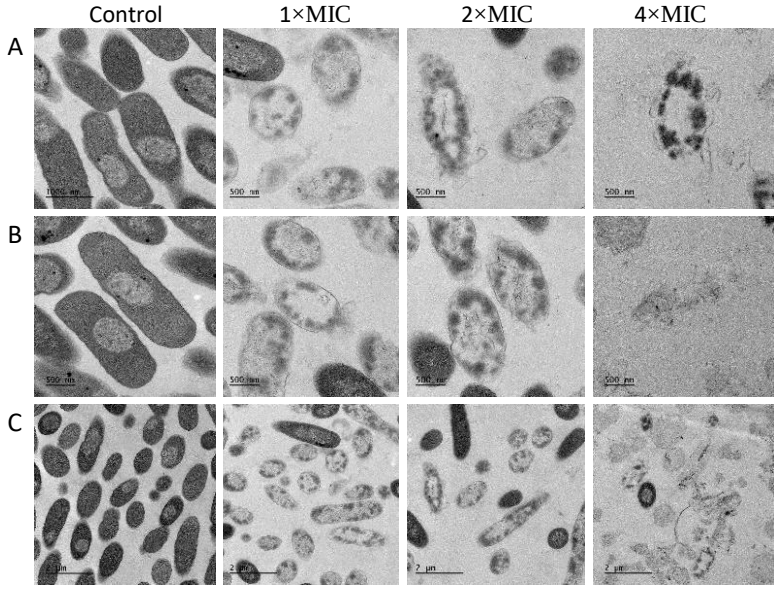


Figure 4.8: Flow cytometric analysis of *E. coli* treated with MS07. The increments of cellular fluorescence intensity of PI after treating with the peptides was analyzed by flow cytometry. Confocal laser-scanning microscopic images of peptide-treated *E. coli* and *P. aeruginosa* cells. Transmission electron micrographs (TEM) of *Escherichia coli* treated with peptide MS07.

4.4. DISCUSSION

Our study aimed to purify and characterize a novel peptide MS07 and study the prospect of their potential application for treatment. We performed an initial investigation of different purified AMP for antimicrobial and antibiofilm activity. *Bacillus* strain CBSMS07 was obtained from traditional Korean food (kimchi). The bacterial strain was detected based on morphological factors and 16S rRNA gene sequences. Purification of peptide MS07 was performed using sequential gel filtration, causing elution of peptide that was rid of undesirable proteins and retaining vigorous antimicrobial activity. Our

present study has checked culture medium composition on AMP production compared with available media in the market, MH, and MRS broth (data not shown). Here, we determined the optimized media by response surface methodology with three different independent variables to fix the growth media (1.773% mannitol, 1.249% peptone, and 0.012% NaCl) for large scale production.

Peptide MS07 was in the category that antimicrobial peptides are quite smaller in size (<10 kDa) and demonstrated a wide range of activities counter to several microorganisms. MS07 was lipophilic, and it moved towards lipophilic solution during solubility check (data not shown). The peptide was purified 27.77-fold, with a recovery of 7.71%. The purified MS07 has a lower molecular weight (6.099kDa), which was detected by MALDI-TOF that compared to other bacteriocin purified from *Bacillus* strains [80, 81]. Edman's degradation method was not effective in determining the N-terminal because N-terminal was blocked in the Edman reaction by modifying its cyclic structure. MS07 was displayed to be acting counter to both gram-negative and gram-positive bacteria, as shown in the table. The buffer's salinity was crucial because it was a measured factor when the peptide was used to make a pharmaceutical drug containing oral and systemic use. The peptide was very steady at pH 4-11 and up to 50°C, which is stable due to another peptide from different *Bacillus* strains [55]. The antimicrobial effect of MS07 counter to both gram-positive and gram-negative bacteria was comparable with the action of bacitracin and vancomycin, as presented in the table (Table 4.2). Although the lytic activity of peptide MS07 was seemingly more active against gram-negative bacteria than gram-positive bacteria. The result is more effective than other peptides produced from *Bacillus* sp. isolated from kimchi [53]. In vitro, the microdilution method showed comparable results

providing real-time data on bacterial growth by continuous recording on bacterial production. We observed regrowth of *E. coli* after 6 hr. of incubation until the concentration was 2×MIC but did not see regrowth on 4×MIC and 8×MIC concentration. The result of time-kill kinetics was compared to another antimicrobial peptide [82]. Investigation on the mode of action of peptide MS07 has shown its activity by developing transmembrane openings reliant on AMP and melittin both the peptide concentration as both peptides indicated a relationship between their mechanism of action [83]. Melittin was alone acted low activity rather than a combination of MS07 with melittin increase the activity against bacteria by disrupting the lipid membrane function throughout pore formation. Due to the probable similarities of mechanism, the synergy of action is found in combination with melittin.

Biofilms are significantly resistant to clinical medication by conventional antibiotics [84, 85] and are vital to the pathogenicity of bacterial pathogens associated with chronic diseases like respiratory infections. Besides, their susceptibility concerning antibiotics decreased when they are present in biofilms. The seek for new AMP that eliminated bacterial biofilms has, thus, turn out to be exceedingly pressing. In this study, the peptide MS07 was efficient at preventing P. aeruginosa and *Escherichia coli* biofilms' development and diminishing the viability of biofilms already developed. It was more effective when it applied a combination with oxacillin and ampicillin. The study exhibited that 256 µg/mL AMP MS07 drew a 58% reduction in *Pseudomonas aeruginosa* biofilm formation, a 64% decrease in *Escherichia coli* biofilm formation. The process is consistent with the earlier findings that its concentration increased the reduction of biofilm formation with peptide. The capacity of peptide MS07 to reduce biofilm

development was related to a reduced count of microbial cells getting the surface. Peptide MS07 could eradicate the accomplished biofilm cells at high concentration with potent antibacterial activity. The concentration of MS07 that destroys 99.9% of biofilm cells was tested counter to both Gram-positive and Gram-negative strains.

Moreover, MS07 has been shown to inhibit biofilm growth in vitro with its mechanism. In our study, we identified that the application of MS07 prompted a deficit of green fluorescence that was highly significant, accompanied by the cell cluster edge, which is consistent with other AMP [86]. In preventing P. aeruginosa and *Escherichia coli* biofilms' development by 58% and 64%, respectively, with the concentration of 8× MIC for, it found that cyclic lipopeptide MS07 was more than 50% effective at disrupting biofilm formation.

4.5. APPLICATION OF CYCLIC PEPTIDE

In chronic skin disorders such as psoriasis and dermatitis, antimicrobial peptides, and those involved in inflammatory processes provide options for the creation of new therapeutic orientations. It is necessary for these peptides to be delivered to their site of action in an active form and in sufficient amounts to have the desired effect in order to maximize their potential. The skin is poorly penetrated by many polymers and susceptible to enzymatic degradation. Cyclic peptide derivative synthesis will dramatically modify the peptide's physicochemical characteristics with the potential to increase the permeation of the peptide's skin. Cyclization will, besides, stabilize the structure of the peptide and thus improve its stability.

4.6. CONCLUSION

Cyclic lipopeptide MS07 has potent antimicrobial and antibiofilm activities, purified from a *Bacillus* bacterial strain that was freshly obtained from Korean fermented food Kimchi. It mostly acted against gram-negative bacteria, but it has a substantial value against gram-positive bacteria also. In summary, the result presented here demonstrated the activity of a cyclic lipopeptide MS07, which was effective against broad spectrum pathogenic bacteria and maintained more significant antibiofilm activity. Our work collectively suggests that peptide MS07 serves as a favorable contender for improving therapeutic molecules for bacterial diseases and treatment caused by biofilm-covered bacteria.

CHAPTER FIVE

ANTIOXIDANT MECHANISM STUDY OF MS15

5.1. INTRODUCTION

In the last few years, the arena of free radical science has undergone a remarkable progression. The in vivo production of free radicals is mainly caused by the ingestion of molecular oxygen, which is itself a radical species due to its composition because oxygen is the ultimate acceptor of electrons in the electron flow mechanism that generates ATP energy. When the electron flow is unpaired (free radical), however, problems can take place. Denham Harma first invented the free-radical principle. A free radical, having one or more unpaired electrons in a molecule, is an animal capable of life. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) that play a critical role in the aging process include free radical species [87]. In aerobic organisms, a normal by-product of oxygen (O2) is continuously produced. Like metabolism, reactive oxygen species (ROS) are involved in many complex cellular processes, such as gene expression, mitogenesis of signal transduction, and cell proliferation regulation. High ROS is associated with various pathological conditions such as aging, cancer, inflammation, and hepatic diseases. Therefore, interest has recently increased in the use of antioxidants to maintain human health and the prevention and treatment of certain diseases [88].

Pro-oxidants' formation in the form of RNS and ROS is effectively reserved for the numerous antioxidant defense levels in the normal state of the healthy human body. ROS and RNS contain radicals such as superoxide ($O_2\cdot-$), hydroxyl ($OH\cdot$), peroxyl ($RO_2\cdot$), hydroperoxyl ($HO_2\cdot$), alcoxyl ($RO\cdot$), peroxyl ($ROO\cdot$), nitric oxide ($NO\cdot$), nitrogen dioxide ($NO_2\cdot$), and lipid peroxyl ($LOO\cdot$), as well as non-radicals such as hydrogen peroxide (H_2O_2), hypochloric acid (HOCl), ozone (O_3), singlet oxygen ($1O_2$),

peroxynitrate (ONOO−), nitrogen oxide (HNO_2), trioxide of dinitrogen (N_2O_3) and lipid oxide (N_2O_3) [89].

5.1.1. Environmental stress and factors

In livestock, plants, and humans, environmental pressures may induce metabolic changes that either increase the output of reactive oxygen and nitrogen species (RONS) or decrease antioxidants' production. In livestock, plants, and humans, environmental stresses may trigger metabolic changes that either increase the output of reactive oxygen and nitrogen (RONS) species or decrease antioxidants' production.

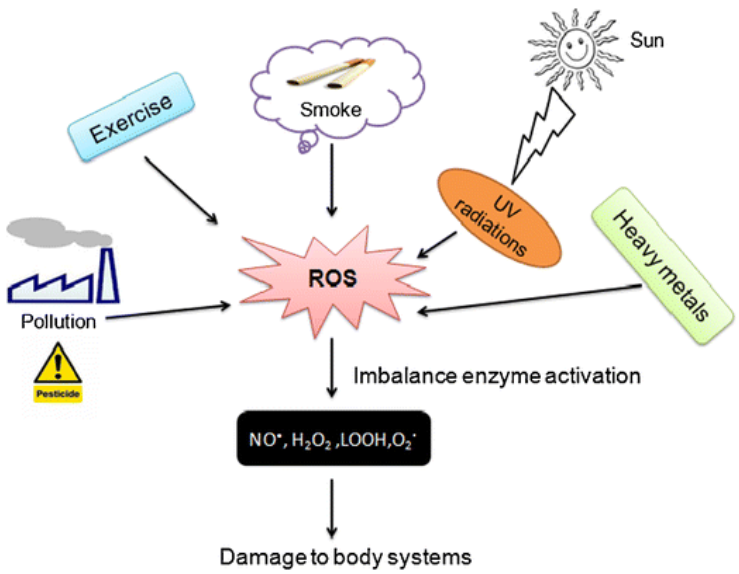


Figure 5.1: Overview of generation of ROS by various factors.

5.1.2. ROS and its complication in humans

Exposure to environmental or pathological factors, such as air toxins, tobacco smoking, UV rays, radiation, and hazardous chemicals, results in an imbalance that leads to oxidative stress between pro-oxidants and antioxidants. If the formation of oxygen species is significantly increased or the number of antioxidants is decreased, the pro-oxidant antioxidant equilibrium in the cell is changed toward the pro-oxidants. Oxidative stress is considered this condition. In the case of chronic inflammation, oxidative stress is ultimately caused by the following two primary mechanisms : (1) a decline in the concentration of antioxidants due to mutant antioxidant enzymes, toxins, or decreased consumption of natural antioxidants; (2) an increase in the amount of oxygen-, nitrogen-, or carbon-based reactive species extracted from activated phagocytes [90]. Essential biomolecules such as lipids, nucleic acids, proteins, polyunsaturated fatty acids, and carbohydrates may be affected by oxidative stress and DNA harm that can contribute to mutations. Cellular constituents do not effectively scavenge these ROS; they can stimulate free radical chain reactions, damage cell biomolecules, and eventually lead to disease conditions such as Alzheimer's disease, chronic degenerative disorders, and stroke, rheumatoid arthritis, diabetes, and cancer [91].

Antioxidants have also been shown, based on their function, to provide cellular defense against oxidative stress by direct or indirect pathways. In natural ways, by intake or chemical modification, antioxidants scavenge the reactive oxygen and nitrogen species. In comparison, indirect mechanisms include the upregulation of step II detoxifying and antioxidant enzymes. Aerobic species have successful antioxidant networks containing primary enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and inducible step II detoxifying enzymes (heme oxygenase-1 (HO-1)

and NAD(P)H-quinone dehydrogenase 1 (NQO1) by activating NF-E2-related factor-2 (Nrf-2) to protect against oxidative stress [92]. Besides, certain antioxidants, both specifically and indirectly, show their behavior and are thus called bifunctional antioxidants.

In the treatment of oxidative damage, synthetic antioxidants are widely used. Their long-term use and heavy doses, however, are related to side effects, including cancer. Scientists have also continued to express an interest in screening novel biological compounds from diverse organic origins. Peptides have various antioxidant functions, which vary depending on the proteinase used and their sequences' amino acid structure. Different amino acids have been reported to show radical-scavenging behaviors, such as tryptophan, histidine, leucine, and cysteine. Also, the amino and carboxylic groups in the divisions of acidic (aspartic acid and glutamic acid) and simple (arginine, lysine, and histidine) amino acids have been shown to improve metal chelation by their charging properties [93]. There is an extensive tradition of using fermented vegetables as a source of a beneficial bioactive compound.

5.1.3. Role of exogenous antioxidants in oxidative stress

Exogenous antioxidants are antioxidants that, by eating antioxidant-rich foods, we get through our diet. Exogenous antioxidants include carotenoids, tocopherols, ascorbates, anthocyanidins, phenolic acids, bioflavonoids, etc. The main plant-origin antioxidant compounds are phenolics and flavonoids. When they oxidize and combine with proteins, phenolic compounds are expected to protect plants against tissue damage. Similarly, by acting as hydrogen donors or chelating metals, flavonoids can serve as antioxidant agents.

There is no doubt, given all these considerations, that exogenous antioxidants are going to become part of the food system. On the other hand, excess ingestion of polyphenols also results in severe damage as follows exogenous antioxidants are antioxidants that, by eating antioxidant-rich foods, we get through our diet. Exogenous antioxidants include carotenoids, tocopherols, ascorbates, anthocyanidins, phenolic acids, bioflavonoids, etc. The main plant-origin antioxidant compounds are phenolics and flavonoids [94]. When they oxidize and combine with proteins, phenolic compounds are expected to protect plants against tissue damage.

Similarly, by acting as hydrogen donors or chelating metals, flavonoids can serve as antioxidant agents. There is no doubt, given all these considerations, that exogenous antioxidants will become part of the food system. Excess ingestion of polyphenols, on the other hand, also results in severe damage as follows.

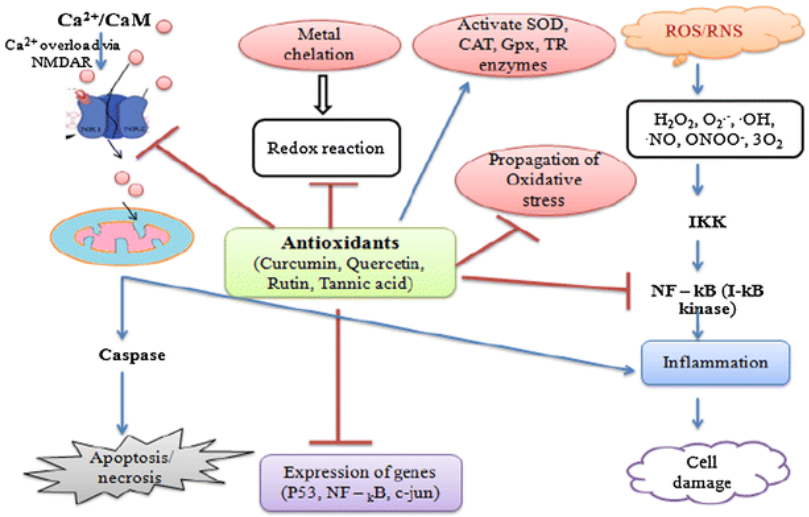


Figure 5.2: The antioxidant defense mechanism against harm caused by free radicals in humans. Polyphenols or antioxidants affect the influx of glutamate-mediated Ca^{2+} by

NMDAR, thus reducing the influx of glutamate-induced Ca^{2+} into mitochondria; antioxidants interact with the caspase pathway and prevent cell damage; antioxidants prevent the spread of oxidative stress; enable the expression of CAT , SOD, GPx, TR antioxidant enzymes; interact with metal chelation and inhibit redox reaction; Catalase (CAT) , superoxide dismutase (SOD) , glutathione peroxidase (GPx), thioredoxine reductase (TR), calcium (Ca^{2+}), calmodulin (CaM), IKK (I-kB kinase), NF-kB (nuclear factor), reactive oxygen (ROS), reactive nitrogen (RNS), hydroxyl radical (·HO), superoxide anion (O_2·-), hydrogen peroxide (H_2O_2), N-Methyl-d aspartate receptors (NMDAR) [95].

5.1.4. Antioxidant peptides from fermented food kimchi

Koreans have been consuming salted and fermented vegetables such as kimchi for more than 2,000 years, providing high amounts of vitamins, minerals, dietary fibers, and other functional ingredients. Studies have shown that kimchi, among others, has an anticancer, antimicrobial, antioxidant, antiatherosclerotic, antidiabetic, and anti-obesity impact. One of the most essential sources of proteolytic enzymes that can efficiently hydrolyze proteins to prepare bioactive peptides has long been microbial fermentation [96]. *Bacillus amyloliquefaciens* is not a probiotic bacterium, and few probiotic bacteria are used for human medical and dietary supplementation, such as *Bacillus subtills*, *Bacillus clausii*, *Bacillus cereus*, *Bacillus coagulans*, and *Bacillus licheniformis*.

The probiotic-like *Bacillus* strain from kimchi was identified in the current study, and an antioxidant peptide was purified. After verifying the innovation of purified MS15, we

characterized the antioxidant peptide designated as MS15 and examined the antioxidant mechanism of action.

5.2. MATERIALS AND METHODS

5.2.1. Evaluation of Antimicrobial Susceptibility

The agar dilution method utilized to test the minimum inhibiting concentration (MIC) of peptide MS15 was used to assess the antimicrobial inhibitory range [62]. Vancomycin and Bacitracin were carried as standard antibiotics comparing MS15 MIC value. Pathogenic microorganisms that contain specific concentrations of peptide MS15 and reference antibiotics were added to the MH-agar medium, and incubation was performed at 37 °C. MIC was distinguished according to the concentration of the dilution, which could diminish selected microorganism's visible growth (1.0×10^7 CFU/mL). Gram-negative bacteria: *Salmonella typhimurium* KCTC 1925, *Pseudomonas aeruginosa* KCTC 1637, *Extended-spectrum beta-lactamase* W_1, *Alcaligenes faecalis* ATCC 1004, ESBL 31, *Escherichia coli* KCTC 1923, ESBL V4 *(Escherichia coli)*; Gram-positive bacteria: VRE 4, VER 89, VRE98, *Staphylococcus aureus* KCTC 1928, *Mycobacterium smegmatis* ATCC 9341, *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Micrococcus luteus* ATCC 9341, MRSA B15, VRSA, MRSA 5-3, MRSA B15. The broth dilution method was employed to get the minimum bactericidal concentration (MBC) value. MBC has been rated as the lowest concentration to inhibit bacterial growth fully (≥ 99.9%) [63].

5.2.2. Bacterial Killing Kinetics Assay of Peptide MS15

Bacteriocin activity of MS15 was studied in the logarithmic growth phase (Log_{10}), in time-kill kinetics findings analyzed with *Escherichia coli* cells (5.5×10^5 CFU/mL), which was prepared in LB broth medium [64]. Various concentrations of MS15 (half of MIC to four times of MIC; 1 mL) was inserted into 10 mL LB medium and incubated at 37 °C with different time intervals of 0 to 36 h. The CFU (colony-forming unit) of *Escherichia coli* cells was established. The GC (growth control) study was presented without peptide for the species. The logarithm graph (CFU/mL) was designed alongside the time of growth.

5.2.3. DPPH (2,2-Diphenyl-1-Picrylhydrazyl) Radical Scavenging Capability Assay

The scavenging capacity of DPPH radical was evaluated, applying the procedure portrayed by Nanjo et al. with a minor modification [97]. A 100 µL various concentration (2.5–20 µM) of peptide MS15 sample solution (ethanol itself acted as a control) was vigorously mixed to DPPH (100 µL; 50 µM) in ethanol. Before the assay, the combination was left to keep in the dark place for 30 min at a normal temperature. Hereafter, the optical density of the final solution was assessed at 517 nm. Furthermore, ascorbic acid (2–16 µM) was evaluated as a reference antioxidant compound. The aptitude toward scavenging the DPPH radical was analyzed as:

$$\text{Scavenging activity of DPPH radical } (\%) = \left[\frac{(A_{cont} - A_{samp})}{A_{cont}}\right] \times 100,$$

where A_{samp} is the optical density of the test sample and A_{cont} is the optical density of the blank (control). All analyses were studied in triplicate.

5.2.4. ABTS (2,2'-Azino-bis (3-Ethylbenzthiazoline-6-Sulfonic Acid)) Radical Scavenging Capability Assay

The $ABTS^{\bullet+}$ radical decoloration action method was applied to determine the $ABTS^{\bullet+}$ radical scavenging capability of peptide MS15 with a slight modification of the method portrayed by Carrasco-Castilla et al. [98]. The stock solution was formulated with ABTS (7 mM) and 2.45 mM potassium persulphate, which was made in water and left in the dark for 14 h to proceed with radical growth. Furthermore, the $ABTS^{\bullet+}$ solution was resuspended in ethanol and equilibrated to offer an absorbance of 0.705 ± 0.04, which was achieved at 734 nm. Variable concentrations (2.5–20 µM) of the peptide fraction (10 µL) were let to mix with 990 µL of the $ABTS^{\bullet+}$ solution for 5 min and the optical density was noted at 734 nm. Ascorbic acid (2–16 µM) was analyzed as a reference standard antioxidant molecule. The % (percentage) inhibition of the $ABTS^{\bullet+}$ radical was evaluated as:

$$\text{Scavenging activity of ABTS }^{\bullet+}\text{ radicle (\%)} = \left[\frac{(A_{cont} - A_{samp})}{A_{cont}}\right] \times 100,$$

where A_{samp} is the optical density of the sample and A_{cont} is the optical density of the control. All analyses were studied in triplicate.

5.2.5. Superoxide Radical Scavenging Capability Assay

The capability assay of superoxide anion radicals was checked in the riboflavin/EDTA system by UV exposure [99]. The reaction combination consisting of DMPO (0.1 M), EDTA (5.0 mM), riboflavin (0.3 mM), and various concentration of MS15 dilutions were exposed under a UV lamp for 60 s at 365 nm. A mixture of the reaction was transported

through a sealed capillary tube into the ESR spectrometer cavity, and the spin adduct was reported.

5.2.6. FRAP (Ferric Reducing Antioxidant Power) Activity Assay

The FRAP assay was brought with a minor alteration for the measurement of reduction power, as previously described by Benzie et al. [100]. In brief, the FRAP reagent included, 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) solution in HCl (40 mM), ferric chloride solutions ($FeCl_3 \cdot 6H_2O$; 20 mM), and acetate buffer (pH 3.5; 300 mM) with a ratio of 1:1:10. A 10 µL aqueous sample was mixed at different concentrations and 990 µL of FRAP mixture and incubated at 37 °C for half an hour. The optical density was assessed at 595 nm. Besides, ascorbic acid as a standard antioxidant compound was also studied.

5.2.7. CUPRAC (Cupric Reducing Antioxidant Capacity) Activity Assay

CUPRAC assay of MS15 was established using the process explained by Apak et al. [101], with minor modifications. A solution of ammonium acetate buffer (1 M; pH 7.2), neocuproine (7.5 mM), and $CuCl_2$ (10 mM), and the aliquots were combined to the ultimate solution. After one hour of incubation at 37 °C, the optical density was measured at 450 nm. Moreover, ascorbic acid was employed as a reference antioxidant compound.

5.2.8. ORAC (Oxygen Radicle Absorbance Capacity) Assay

Following the previous article, the ORAC assay was executed [102]. Trolox was employed as a positive control, which was an analog of vitamin E that was a water-soluble compound. After adding 20 mM AAPH with an emission wavelength of 520 nm and

excitation wavelength of 480 nm, the analyzer was designed to take absorbance of the fluorescence of 200 nM fluorescein in a minute. The test was performed under pH 7.5 conditions at 37 °C with a blank sample in similar. The findings were determined in the areas under the fluorescence decay curves, applying experimental and blank sample variations stated as the Net AUC (area under the curve) values.

5.2.9. Cellular NO (Nitric Oxide) Measurement in RAW 264.7 Cells

Griess reagent was utilized to calculate the concentration of nitric oxide (NO) in the medium as an indicator of NO output as our previous report [103]. At a count of 1.8×10^5 cells per well, RAW 264.7 cells were inoculated into a 96-well microplate and incubated at 37 °C for 1 h. At different concentrations of MS15 (2.5–20 µM), the cells were then treated with 1 µg/mL LPS (lipopolysaccharide). NO concentration of the supernatants was assessed with the addition of Griess reagent after 24 h incubation. The absorbance of the mixtures was assessed at a wavelength of 520 nm, employing a fluorescence microplate scanner.

5.2.10. Intracellular ROS (Reactive Oxygen Species) Measurement in RAW 246.7 Cells

The DCF-DA method was applied to determine the cellular oxidative pressure produced by LPS because of reactive oxygen species (ROS) [104]. The amount of ROS formed was associated with fluorescence intensity. RAW 264.7 cells were first inoculated with DMEM in 96-well plates (1.8×10^5 cells/well) for 24 h. Various concentrations of MS15 (2.5–20 µM) subsequently pretreated in the cells after incubation and incubated for one hour. Moreover, cells were activated by LPS (1 µg/mL) and set for an extra 24 h. The

cells were resolute two times with phosphate-buffered saline (PBS) buffer and treated with DCF-DA (25 μM) in a dark place at 37 °C for 30 min. Gallic acid (1.5–18 μM) was analyzed as a reference standard antioxidant compound. The fluorescence intensity was assessed employing a fluorescence microplate reader at an emission and excitation wavelength of 520 nm and 485 nm, correspondingly.

5.2.11. Cell Culture and Cell Cytotoxicity Assay

Cells (RAW 264.7 cells and HeLa cells) were cultured in DMEM medium supplied with FBS (10%) and penicillin/streptomycin (100 μg/mL) at 37 °C under CO_2 as our earlier report [80]. For cytotoxicity assay, cell seeding was performed into 96-well microplate at a density of 5.5×10^5 cells/well (Nunc, Roskilde, Denmark). It was cultured and treated with various concentrations of MS15 according to the treatment plan. After incubation, 20 μL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide**)** reagent (5mg/mL) was put in to each well. After two hours of incubation, 100 μL of DMSO was dissolved and incubated for 12 h. Absorbance was noted using a microplate reader (KisanBio, Seoul, South Korea) at 570 nm.

5.2.12. Western Blot Analysis with Cell Lysates

Cell lysates of RAW 264.7 cell were added with sample buffer (Tris-HCl (250 mM; pH 6.8), 2-mercaptoethanol (5%), glycerol (50%), bromophenol blue (0.5%), SDS (10%), DTT (0.5 M)), and boiled at 100 °C for 5 min to denature the mixture portrayed in the earlier study [105]. ReadyPrep™ protein extraction kit (cytoplasmic/nuclear) from Bio-Rad (Bio-Rad Lab., Hercules, CA, USA) was used to extract nuclear protein. Protein aliquots (20 μg) were separated using SDS-PAGE (10%) and electro transfer to

polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Hereafter, the membranes were incubated with primary antibodies in skim milk (5%). A series of primary antibodies named β-actin, anti-Nrf2, anti-HO-1, and lamin B were utilized. Horseradish peroxidase (HRP) (Santa Cruz Biotech. Inc., Santa Cruz, CA, USA) was used as secondary antibodies. The ECL (electrochemiluminescence) solution system (Perkin Elmer) was applied to discover the antibody–antigen reaction.

5.2.13. RT-PCR (Reverse Transcription-Polymerase Chain Reaction) Assay

TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA) was used to extract total RNA from RAW 264.7 cells by following our prior study [103]. Total RNA (5 μg) was transcribed employing RT& GO Mastermix (MP Biomedicals, Seoul, Republic of Korea) into cDNA, and the product was applied to the PCR thermal cycler. Oligonucleotide primers (*SOD1*, *CAT*, *Nrf-2*, *GPx-1*, *GAPDH*, *HO-1*) were amplified with a denaturation step.

Sequences of the primer designed for RT-PCR were stated as *SOD1 (*NM_011434.2*)*: forward, 5'- CAG CAT GGG TTC CAC GTC CA -3'; reverse, 5'- CAC ATT GGC CAC ACC GTC CT -3'; *CAT (*NM_001752.4*)*: forward, 5'- AG GCT CAG CTG ACA CAG TTC -3'; reverse, 5'- GC CAT TCA TGT GCC GAT GTC -3'; *Nrf-2 (*NM_010902.4*)*: forward, 5'- CTT TAG TCA GCG ACA GAA GGAC -3'; reverse, 5'- TCC AGA GAG CTA TTG AGG GACT -3'; *GPx-1 (*NM_001329503.2*)*: forward, 5'- TTC CCG TGC AAC CAG TTTG -3'; reverse, 5'- GGA CGT ACT TGA GGG AAT TCA GA -3'; *GAPDH (*NM_001357943.2*)*: forward, 5'- GCG AGA TCC CGC TAA CAT CA -3'; reverse, 5'- AGT GAT GGC ATG GAC TGT GG -3'; *HO-1 (*XM_002743721.5*)*: forward, 5'- CCA GAA AGT GGG CAT CAG CT -3'; reverse, 5'- GTC ACA TTT ATG

CTC GGC GG -3'. Ethidium bromide staining was used to visualize the PCR products after electrophoresis.

5.2.14. Statistical Analysis

The experimental data were presented with three replications per inspection, and every single point signifies the mean± SD (standard deviation). Statistical analysis was completed employing one-way predictive analysis ANOVA (IBM SPSS statistics software; version 20.0). Difference was measured significant in statistics among the treatment represent as, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.3. RESULTS

5.3.1. Assessment of Antimicrobial Susceptibility and Time-kill Kinetics Analyses

The MICs of peptide MS15 were assessed against different pathogenic bacteria, such as MIC of Gram-positive pathogens (2.5–160 µg/mL) and MIC of Gram-negative pathogens (MIC of 40–160 µg/mL). The concentration of MS15 concerning MIC was contrasted to the value of MIC, which represents the standard antibiotic of bacitracin and vancomycin (Table 5.1). Antimicrobial activity of peptide MS15 was more potent rather than the standard antibiotic counter to a broad array of pathogenic bacteria, which is different from Gram-positive pathogen, for instance, Vancomycin-resistant *Enterococci* 98, Vancomycin-resistant *Staphylococcus aureus,* Methicillin-resistant *Staphylococcus aureus* 5-3, *Staphylococcus aureus*, Vancomycin-resistant *Enterococci* 89, Methicillin-resistant *Staphylococcus aureus* B15, and Gram-negative pathogens, for instance, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Extended-*

spectrum beta-lactamase (W1, 31). The bactericidal activity was assessed by using the broth dilution method, and it kills bacteria in a range concentration of 5–320 μg/mL.

Table 5.1. Antimicrobial field of activity for peptide MS15 against various pathogens.

Microorganisms	MIC of MS15 (μg/mL)			MBC of MS15 (μg/mL)
	MS15	Bacitracin	Vancomycin	
Gram-negative bacteria				
Escherichia coli KCTC 1923	40	160	80	80
Pseudomonas aeruginosa KCTC 1637	160	> 160	> 160	320
Salmonella typhimurium KCTC 1925	40	80	40	80
Alcaligenes faecalis ATCC 1004	160	160	80	320
Extended-spectrum beta-lactamase V4 *(Escherichia coli)*	80	160	80	80
Extended-spectrum beta-lactamase W1	40	80	40	80
Extended-spectrum beta-lactamase 31	40	80	40	80
Gram-positive bacteria				
Vancomycin-resistant Enterococci 4	160	80	160	320
Vancomycin-resistant Enterococci 89	80	> 160	> 160	160
Vancomycin-resistant Enterococci 98	80	> 160	> 160	160
Staphylococcus aureus KCTC 1928	160	> 160	> 160	160
Methicillin-resistant *Staphylococcus aureus* 5-3	2.5	5	2.5	5
Methicillin-resistant *Staphylococcus aureus* B15	40	160	80	120
Mycobacterium smegmatis ATCC 9341	20	40	2.5	40

Micrococcus luteus ATCC 9341	40	40	2.5	40
Enterococcus faecalis ATCC 29212	20	5	2.5	20
Bacillus subtilis ATCC 6633	10	20	0.8	20
Vancomycin-resistant Staphylococcus aureus	80	160	> 160	160

MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration.

The time-killing kinetics of peptide MS15 at different MIC concentrations indicated a decline in the total of viable cells around different time intervals, subsequently up to the 36 h against the reported organism *Escherichia coli*. A logarithm of viable colony count was analyzed with time intervals on multiple concentrations. Peptide MS15 decreased *Escherichia coli* count at 40 µg/mL (concentration of MIC) up to 12 h. An initial decrease in the amount of surviving *Escherichia coli*, at 40 µg/mL peptide concentrations was detected. *E. coli* cells recovered from the peptide activity after 12 h remaining and began to develop again. Mostly regrowth was noticed up to 120 µg/mL, although 160 µg/mL demonstrated a total decline of the *E. coli* count.

5.3.2. Radical Scavenging Activity Assay

ABTS $^{\bullet+}$and DPPH radical-scavenging activity was significantly influenced (* $p < 0.05$) by the concentration variation of peptide MS15 and reference standard ascorbic acid. Peptide MS15 exhibited concentration-dependent antioxidant activity in diverse in vitro antioxidant assay, containing scavenging of ABTS $^{\bullet+}$ and DPPH radicles. The results of the scavenging of DPPH and ABTS $^{\bullet+}$ were compared with reference standard ascorbic acid. In contrast, the constant free DPPH radical receives hydrogen radical or an electron from antioxidant and diminishes to develop a steady non-radical form DPPH-H, converted to diphenyl-picrylhydrazine molecule through resulting yellow in color and

decline in absorbance. As displayed in Figure 5.3a, peptide MS15 exhibited excellent DPPH radical scavenging activity towards higher concentration, which was near to that of ascorbic acid. Our peptide showed a DPPH radical scavenging range of 17%–69% in a dosages range of 2.5–20 µM. In contrast, ascorbic acid showed a DPPH radical scavenging range of 32%–85% in a dosages range of 2–16 µM.

The $ABTS^{\bullet+}$ discoloration assay directly associates, including the significance of the scavenging capability of an antioxidant compound involving its hydrogen donating capacity, which was profoundly colored and was concluded by evaluating absorbance at 734 nm. Furthermore, in $ABTS^{\bullet+}$ scavenging assay, the $ABTS^{\bullet+}$ radicle was pre-formed by the oxidation process along with potassium persulfate, which could be decreased in the existence of hydrogen-providing antioxidants. Peptide MS15 showed $ABTS^{\bullet+}$ radical scavenging up to 68% in a concentration of 20 µM (Figure 5.3b). In contrast, ascorbic acid showed $ABTS^{\bullet+}$ radical scavenging up to 77% in a concentration of 16 µM. On the other hand, our peptide MS15 showed a superoxide radical scavenging range of 6%–67% in a dosages range of 3–18 µM (Figure 5.3c). In contrast, ascorbic acid showed a superoxide radical scavenging range of 29%–82% in a dosages range of 4–20 µM. Remarkably, peptide MS15 considerably scavenged both the $ABTS^{\bullet+}$ and DPPH superoxide radical in a concentration reliant approach.

5.3.3. Reducing Power Measurement Assay

We checked the electron-donating capacity by utilizing the method of FRAP and CUPRAC. The mainly FRAP method is a complete electron transfer process where antioxidants diminish ferric ions. We found great activity through the FRAP process,

whereas the FRAP value is equivalent to ascorbic acid found up to 72.73% ($p < 0.001$) in a concentration of 20 µM (Figure 5.3d). Otherwise, the CUPRAC assay is used to check the transfer of n-electron reductant antioxidants. The CUPRAC value equivalent to ascorbic acid found up to 71.34% ($p < 0.001$) in a concentration of 20 µM (Figure 5.3e). Trolox value (net AUC) and dose-dependent MS15 have increased the findings suggested that MS15 (18 µM) showed similar antioxidant activity to 16 µM Trolox (Figure 5.3f).

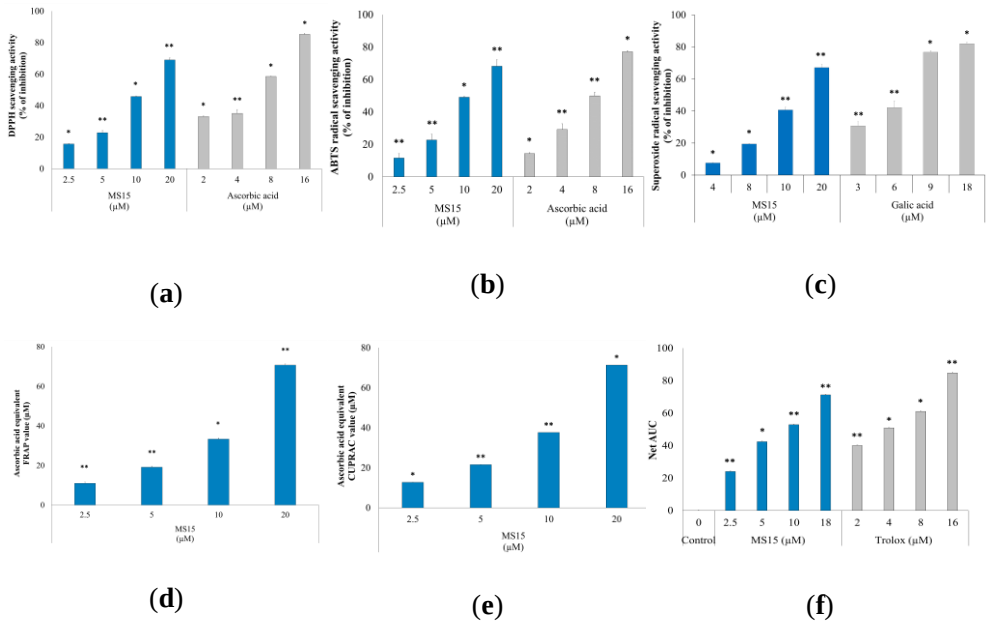
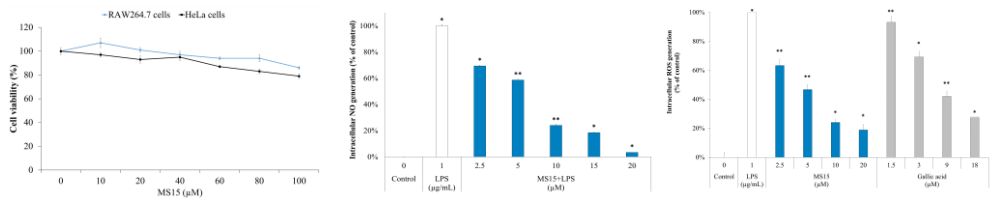


Figure 5.3. (**a**) The DPPH, (**b**) $ABTS^{\bullet+}$, and (**c**) SOD radical scavenging assay were performed with several concentrations. (**d**) FRAP assay and (**e**) CUPRAC assay were conducted with various concentrations of MS15, where ascorbic acid and gallic acid were assessed as a standard antioxidant compound. (**f**) The net area under the curve (Net AUC) was obtained by subtracting the area under the blank curve from the area under the sample curve, which showed the ORAC properties of the samples. Applying the student's t-test, significantly different from the control denoted as, ** $p < 0.01$, * $p < 0.05$.

5.3.4. Cell Viability Assay

The MTT assay with various concentrations (10–100 µM) of MS15 was measured in RAW 246.7 cells, and human epithelial HeLa cells were thoroughly examined. The outcome demonstrated no cytotoxic effects at a concentration up to 40 µM, whereas the significantly less cytotoxic impact of up to 100 µM (Figure 5.4a).



(**a**) (**b**) (**c**)

Figure 5.4. (**a**) Cytotoxicity assay, cell seeding (RAW 264.7 cells, and HeLa cells) was performed into 96-well microplate at a density of 5.5×10^5 cells/well. (**b**) Generation of NO and (**c**) generation of ROS were evaluated in RAW 264.7 cells. Applying the student's t-test, a significant difference from the control denoted as, ** $p < 0.01$, * $p < 0.05$.

5.3.5. ROS Measurement and Nitric Oxide Inhibitory Assay

ROS measurement and NO inhibitory assay were investigated in macrophage cell lines RAW 264.7, and results were compared with a reference standard antioxidant Gallic acid. The intracellular ROS level in macrophages proliferated in cells inspired through LPS or other inducements quickly increased, initiating oxidative stress. Hereafter, we attempted to assess MS15 antioxidant activity by analyzing the generation of NO and ROS.

Stimulation of the LPS potentially enhanced the growth of the medium associated with control cells, which was untreated. In contrast, pretreatment with MS15 remarkably increased NO scavenging activity by 13.6% (*** $p < 0.001$), 33.2% (*** $p < 0.001$), 45.1% (*** $p < 0.001$), and 65.4% (*** $p < 0.001$) at dosages of 2.5, 5, 10, and 20 µM, correspondingly (Figure 5.4b). RAW 246.7 cells were evaluated by observing cell morphology to investigate other effects of MS15 on the ROS generation. The findings displayed that pretreatment with MS15 significantly reduced ROS generation by increasing concentration (Figure 5.4c). Gallic acid was presented as a positive control that demonstrated being active in reducing cellular ROS generation. These findings imply that both MS15 reduced the generation of NO and the generation of intracellular ROS deprived of any cellular toxicity.

5.3.6. Effects of MS15 on Antioxidant Enzyme in RAW 264.7 Cells

In order to explore the impact of MS15 on antioxidant enzymes (GPx-1, CAT, SOD1) and detoxifying enzymes of phase II (HO-1), RT-PCR analysis was used to assess a concentration-dependent rise in the mRNA expression of GPx-1, CAT, and SOD1 (Figure 5.5a). RAW 264.7 cells were used to treat with MS15 at 2, 5, 10, and 20 µM for 24 h. Western blot assessments were used to confirm the protein levels of HO-1 (Figure 5.5b). Furthermore, the Western blot assessment (time reliant) showed substantially increased protein expression of HO-1 after the treatment of MS15 (Figure 5.5c). Nrf2 is typically attached to the Keap1 protein, and the stimulation of antioxidant enzymes (phase II) can be accomplished while Nrf2 was left off, and the nucleus was translocated. It was noted that treatment with MS15 increased the amount of Nrf2 mRNA in a dose reliant way (Figure 5.5d). The mechanism of activation of Nrf2 by MS15, along with the protein

levels of Nrf2 translocation in the cytosol, was evaluated in a time-dependent enhancement process to explain after 24 h treatment (Figure 5.5e). MS15 therapy has been able to increase HO-1 transcriptional and translation rates. The transcription level of the Nrf2 was evaluated to verify that MS15 stimulates phase II enzymes via Nrf2. It was discovered that MS15 considerably enhanced Nrf2 mRNA expression (Figure 5.5f).

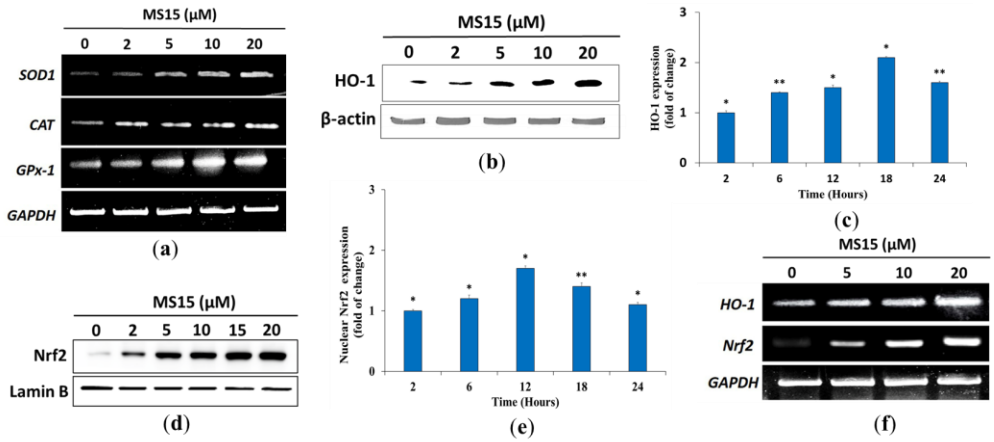


Figure 5.5. Effects of MS15 on the expression mRNA levels were checked on the detoxifying enzyme, primary and Phase II antioxidant in RAW 264.7 cells. RAW 264.7 cells with the required concentration of MS15 were pretreated for 24 h. (**a**) RT-PCR was used to test the mRNA expression of Phase II antioxidants, and primary antioxidant enzymes (GPx-1, CAT, SOD1) were assessed in a concentration reliant way. The expression of HO-1 was evaluated by Western blot analysis in (**b**) a concentration reliant and (**c**) time reliant (the fold of change) way. Impact of MS15 on Nrf2 activity, RAW264.7 cells with the required concentration of MS15 were pretreated for 24 h. Western blot analysis was used to calculate the nuclear translocation of Nrf2 in (**d**) a concentration reliant and (**e**) a time reliant (fold of change) way. (**f**) The mRNA of the

detoxifying enzyme (HO-1) and Nrf2 were assessed by RT-PCR in a concentration reliant way.

5.4. DISCUSSION

The principal goal of the study was to purify a potential antioxidant peptide that has potent antimicrobial activity and evaluate its antioxidant activity pathway. In the current study, we described the impact of the different components of carbon, nitrogen, and metal-ion sources of several culture conditions and growth media to get optimum bacteriocin activity. Growth media composition, temperature, and pH of dissolved peptide played a crucial part in the production and stability of bacteriocin activity. Peptide MS15 imposed cyclic structure so that the amino acid sequence could not be detected by using the Edman degradation method, although purification was detected with MALDI TOF/MS. The peptide was purified by two-step column chromatography with the 24.49-fold and recovered activity of 5.92%, that of total bacteriocin activity was found at 16000 AU, which was consistent with our previous report [53]. Primarily, we investigated the antimicrobial spectrum of peptide MS15 against different pathogens that were significantly higher than our previous report. In contrast, it showed better MBC value, produced high bacteriocidal activity, which can be compared with our previous article [52]. Time-kill kinetics findings showed a rapid decline and regrowth of *Escherichia coli* cells after 12 h, which is consistent with the kinetics of other antimicrobial peptide reports [77, 106].

Oxidative stress is characterized as a discrepancy among free radical development and its removal by mechanisms of cellular defense. ROS consists of hydroxyl radical ($OH^{\bullet}$),

superoxide anion ($O_2^{\bullet}$), and hydrogen peroxide (H_2O_2) and are by-products of cellular metabolic effects, and pathways are intracellular, toxic organisms, partially formed by oxygen reduction (O_2) [107]. ROS is well known to be chemically further reactive than $O_2^{\bullet}$ and thus, ROS was assumed to perform primarily as cellular damaging agents, responding extensively to lipids and proteins [108]. From the study, antioxidant activity was assessed using different methods and the fundamental mechanism by which MS15 mitigated oxidative stress throughout the translation and transcriptional in RAW 264.7 cells via the Nrf2-Keap pathway. A compound is an antioxidant when it can stabilize, has an electron or hydrogen donating capability, and transfer the unpaired electron, and has the power to chelate metals [109]. The most common spectrophotometric are $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ methods for evaluating the antioxidant potential of any molecule under study. Peptide MS15 exhibited a high $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging activity. The finding revealed that scavenging activity was performed by transferring hydrogen reaction (DPPH assay) or electron transfer reaction (ABTS assay). As presented in Figure 5.3a,b, peptide MS15 demonstrated more active radical scavenging activity than its precursor, in contrast with earlier reports [110]. MS15 displayed a considerably superior quench of SOD radical as a dose reliant rise in inhibition percentage.

The variation of color evaluated antioxidant power in the FRAP from the reduction of the yellow ferric complex to the blue ferrous complex. Otherwise, due to the smaller redox potential of CUPRAC reactive, citric acid was not oxidized, and sugar decline with the CUPRAC reactive. CUPRAC and FRAP are significant measures of reducing the ability of an antioxidant that is correlated with the earlier report [111] with the involvement of samples liable for separating the free radical chain by hydrogen atom donation. The

findings showed the visible antioxidant ability of MS15, which was rising slowly with improving sample strength compared to ascorbic acid equivalent. Besides, the ORAC assay uses a peroxyl radical derived from LPS, which mimics the peroxyl radical of lipid that participating in the in-vivo chain reaction of lipid peroxidation. An antioxidant has a protecting impact which is evaluated by an assessment of the region covered by the curve of fluorescence decay (AUC) that had higher reduction capability [102].

The power of cellular antioxidants derives from their immediate power, described as the ability to quench free radicals, with either electrons or hydrogen contributing to ROS. Simultaneously, indirect capability includes defending alongside oxidant stress by stimulating detoxify the expression and antioxidant enzymes in Phase II [112]. During cell proliferation and maintenance, the scavenging activity of ROS performs a crucial part in cell homeostasis. A variety of SOD, GPx, and CAT enzymes are related to eliminating these free radical species in cells. If multiple occurrences of oxidative stress damage these enzymes, then progressive diseases may result. An electron O_2 reduction forms the superoxide of cytosol (O_2^-) through the slipping of electrons from the electron transport chain of the mitochondrial electron carriers. It was entirely proven that SOD promptly transforms O_2^- into H_2O_2. Besides, the antioxidant enzymes, GPx, and CAT, will detoxify H_2O_2. Those enzymes function collectively as free radicals in the metabolic pathway [113, 114]. In this analysis, together with mRNA and protein levels of antioxidant enzymes, for instance, GPx-1, CAT, and SOD1 in RAW 264.7 cells, MS15 treatment enhanced radically, demonstrating the ability of MS15 to preserve the state of cell homeostasis and defend the cell against oxidative stress.

Under normal conditions, the Keap1 protein binds Nrf-2 in the cytoplasm and performs a crucial part in Phase II enzyme stimulation when released and translocated to the nucleus [115]. MS15 treatment has been competent to enhance the translational and transcriptional levels of HO-1 and, in line with previous therapies, facilitates the Nrf-2 translocation in a concentration-reliant way into the nucleus. The process suggests that the antioxidant properties of MS15 are partly due to the concentration-dependent initiation of HO-1 in RAW 264.7 cells, controlled by dose-reliant activation of Nrf-2 [116]. However, the inhibition of the HO-1 role by HO-1 inhibitor considerably hindered those protective impacts of MS15 against H_2O_2. Such findings are in the substantial arrangement among other latest results indicating that HO-1 reestablished cell persistence by preventing apoptosis induced by oxidative damage. The antioxidant role of HO-1 performs by turning heme into the strong biliverdin prooxidant and, ultimately, bilirubin, which is a powerful antioxidant [105]. The interface among MS15 and Nrf-2 that imitates the activity of other Nrf-2 inducers, such as caffeoylquinic acid and 5-O- sulforaphane, that antioxidant response element (ARE)-dependent gene expression in HT29 cells and modulates nuclear translocation on objectives, for instance, NQO-1, Nrf-2, and HO-1 [117]. The antioxidant mode of action of MS15 might be powered by ARE-dependent gene expression and nuclear translocation, given numerous suggested molecular procedures controlled by antioxidant peptides (Figure 5.6).

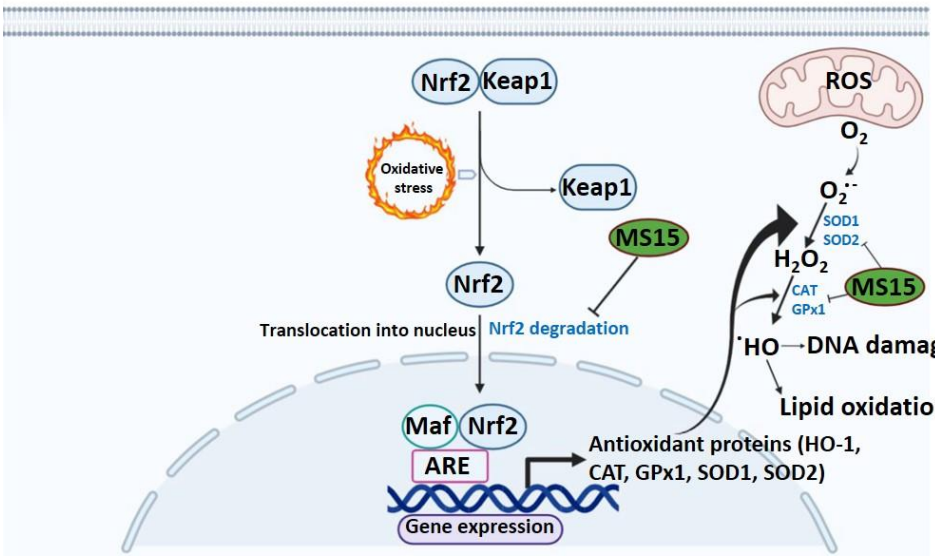


Figure 5.6. Illustration of hypothetical antioxidant mechanism for peptide MS15 against oxidative stress.

Different synthesized antioxidant peptide analogs will investigate the antioxidant mode of action after elucidating the completed structural details of MS15 and assessing the subsequent findings, for instance, the phosphorylation of PI3K/Akt and MAPKs, and the luciferase assays. Thus, while further studies should be undertaken on the significance of several GSH-dependent enzymes, the present findings imply that MS15 's cellular protecting capacity against oxidative stress depends on, however, on the signaling pathway of Nrf2 / HO-1 in RAW 264.7 cells.

5.5. CONCLUSION

The identification of novel antimicrobial and antioxidant peptide MS15 against oxidant damage, throughout its potential effects, might assist well in understanding its action against the various pathophysiological situations related to oxidant stress. This analysis clearly shows that MS15 has a presence of antioxidant operation, the capability to move

hydrogen atoms, and the capacity to avoid oxidative stress caused by H_2O_2 in RAW 264.7 cells. It was done through increasing the amount of cellular thiol, GST activity, and antioxidant enzyme expression and redox-sensitive transcription factor Nrf-2 expression. In conclusion, the present study showed that peptide MS15, which was purified from *Bacillus velezensis*, had significant and dose-dependent antioxidant capacity. The proposed underlying mechanism through which MS15 mitigates oxidative stress via translational and transcriptional control of oxidoreductase (phase I) and detoxifying enzyme (phase II).

6. FUTURE DIRECTIONS

Antimicrobial peptides are widely analyzed for their composition, function, and mode of action. Despite this research, there are many protected fields of study where new expertise, including new insights into the manufacture of drugs for multiple therapeutic applications, can be acquired. Below are some of the crucial topics that need to be discussed in the near future.

• Latest findings show that D-amino acids containing peptides retain the same biological role as L-amino acids containing peptides. Thus, the design of D-amino acids containing antimicrobial peptides may render them immune to endogenous proteases and, therefore, more stable.

• The self-assembling of small peptides into nanostructures is a developing topic in nanobiotechnology. These self-assembled peptides show striking features that may have

broad applications. Core-shell nanoparticles created by self-assembly of amphiphilic peptides show strong antimicrobial properties against many bacteria, fungi, and yeasts. Develop new nanoparticles with useful therapeutic agents. There is a need for more research.

• Development of novel bioinformatics tools for the identification and screening of antimicrobial peptides.

• Further study is needed to design non-toxic, shorter, and potent peptidomimetic antimicrobials that should be highly stable and have necessary antimicrobial efficacy.

• The biological activity, toxicity studies, and pharmacokinetics of antimicrobial peptides should be checked in animal models.

• Further research is required to classify or identify possible amino acids that play an essential role in the antimicrobial and cytotoxic action of anti-microbial peptides to allow for the design of a more significant number of antimicrobial peptides in the foreseeable future.

• Toxicity is the primary barrier to the conversion of antimicrobial peptides into active drugs. It is, therefore, important to extensively explore the nature of cell-selective and useful peptides.

• Further analysis is needed to clarify the structure-function relationship, secondary structure, aggregation or oligomerization, and membrane activity of antimicrobial peptides. Such studies will help in the design of novel peptides in the near term.

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LIST OF PUBLICATION

1. **Md Maruf Khan**, Young Kyun Kim, Suzia Aktar Suchi, Ying-Yu Jin, Joo-Won Suh, and Jin Cheol Yoo. Elucidation of membrane targeting mechanism of lipopeptide MS07 from *Bacillus siamentis*. ***Frontiers in Microbiology***. (Submitted)

2. **Md Maruf Khan**, Young Kyun Kim, Tahmina Bilkis, Joo-Won Suh, Dae Young Lee, and Jin Cheol Yoo. (2020) Reduction of Oxidative Stress through Activating the Nrf2 mediated HO-1 Antioxidant Efficacy Signaling Pathway by MS15, an Antimicrobial Peptide from *Bacillus velezensis*. ***Antioxidants***, 9(10), pp.934.

3. Md. Saifur Rahman, Young Kyun Kim, **Md Maruf Khan**, Sang Hun Lee, Yun Hee Choi, Seung Sik Cho, Chulhwan Park, and Jin Cheol Yoo. (2020) Purification and identification of novel alkaline pectinases PNs31 and its immobilization for bioindustrial applications. ***Korean J. Chem. Eng.*** *DOI: 10.1007/s11814-020-0648-5.*

4. **Md Maruf Khan**, Young Kyun Kim, Seung Sik Cho, Ying-Yu Jin, Joo-Won Suh, Dae Young Lee, and Jin Cheol Yoo. (2020) Response Surface Optimization of Culture Conditions for Cyclic Lipopeptide MS07 from *Bacillus siamensis* Reveals Diverse Insights Targeting Antimicrobial and Antibiofilm Activity. ***Processes***, 8(6), pp.744.

5. Sudip Regmi, Yoon Seok Choi, Young Kyun Kim, **Md Maruf Khan**, Sang Hun Lee, Seung Sik Cho, Ying-Yu Jin, Dae Young Lee, Jin Cheol Yoo, and Joo-Won Suh. (2020) Endoglucanase Produced by *Bacillus subtilis* Strain CBS31: Biochemical Characterization, Thermodynamic Study, Enzymatic Hydrolysis, and Bio-industrial Applications. ***Biotechnology and Bioprocess Engineering***, 25(1), pp.104-116.

6. Sudip Regmi, Yoon Seok Choi, Young Kyun Kim, **Md Maruf Khan**, Sang Hun Lee, Yun Hee Choi, Seung Sik Cho, Ying-Yu Jin, Jin Cheol Yoo, and Joo-Won Suh. (2020) Industrial attributes of β-glucanase produced by *Bacillus sp.* CSB55 and its potential application as bio-industrial catalyst. ***Bioprocess and Biosystems Engineering***, 43(2), pp.249-259.

7. **Md Maruf Khan**, Yoon Seok Choi, Young Kyun Kim, and Jin Cheol Yoo. (2018) Immobilization of an alkaline endopolygalacturonase purified from *Bacillus paralicheniformis* exhibits bioscouring of cotton fabrics. ***Bioprocess and biosystems engineering***, 41(10), pp.1425-1436.

8. Miri Kim, **Md Maruf Khan**, and Jin Cheol Yoo. (2017) Antimicrobial and antioxidant peptide from *Bacillus* strain CBS73 isolated from Korean Food. ***J. Chosun Natural Sci***, 10(3), pp.154-161.

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