Thesis for the Degree of Doctor of Engineering

# Versatile novel antimicrobial peptides derived from purification process of *Mytilus coruscus* and genome analysis strategy of *Oplegnathus fasciatus*

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February 2020

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# 참담치 추출물과 돌돔 유전체 정보 기반 다기능성 항균 펩타이드 연구·개발

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by
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A dissertation
by
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# 오륜경의 공학박사 학위논문을 인준함

2020년 2월 21일

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# Chapter 1.

# General introduction

## 1. Background

In the aquaculture industry, antibiotics are effective agents for the prevention and cure of infectious diseases caused by pathogenic bacteria. However, mis-use and/or over-use of antibiotics can lead to the development of antibiotic-resistant strains and bio-accumulation [Y. Sun et al., 2015]. These risks pose not only a threat safety of sea foods but also a threat to human public health. Therefore, the use of antibiotics has been strictly restricted or prohibited, and alternatives to antibiotics with no side effects are demanded [P.C. Oyston et al.,]. Recently, diverse investigations about probiotics, bacteriophages, phytobiotics and antimicrobial peptides (AMPs) have gained great attention for the search of antibiotic substitutes. Among diverse antibiotic substitutes, we focused on AMPs as natural antibiotics and alternatives to antibiotics because they are produced by various aquaculture organisms to protect themselves against pathogenic microorganisms. Furthermore, many studies have contributed to the understanding and development of antimicrobial peptides from various organisms as alternatives to antibiotics [F.E. Ali et al., 2009; P. Joanne et al., 2009; J. Verdon et al., 2009].

## 2. Marine organism

Marine organisms are important source of bioactive molecules with industrial potential. Approximately 71% of Earth's surface was covered with the ocean, which has enormous resources [H.K. Kang et al., 2015]. Especially bioactive molecules from marine organisms have attracted a great deal of attention owing to their potential effect. The numerous compounds have been found in invertebrates and vertebrates of marine organisms for novel active substances.

Aquaculture provided about total of 66.6 million tons of seafood, including 175 species of maine invertebrates mainly crustaceans (Ecdysozosoa, Arthropoda) and mollusks (Lophotrochozoa, Mollusca) [D. Dsetoumieux-Garzón et al., 2016]. Among marine invertebrates, mollusk is one of the major phyla of invertebrates and about 23% of all the known marine organisms is constituted with majority of mollusk [L. Wang et al., 2013]. A number of investigations have been progressed; the major species include oysters (*Crassostrea gigas* and *Pinctada fucata*), hard-shell mussels (*Mytilus coruscus*), sea squirts (*Halocynthia roretzi*), Japanese carpet shells (*Ruditapes philippinarum*), ark shells (*Scapharca broughtonii*), and abalone (*Haliotis discus hannai*).

Mytilidae, mussels of the genus *Mytilus*, exhibit a typical distribution with five species, *Mytilus californianus*, *Mytilus trossulus*, *Mytilus edulis*, *Mytilus galloprovincialis*, and *M. coruscus* [J.D. Gaitan-Espitia et al., 2016]. The hard-shelled mussel, *Mytilus coruscus* is a marine bivalve mollusk and member of mytilidae family, which widely distributed in coast of Eastern Asia, including Korea, China and Japan. It has a thick and hard shell with a long egg shape and bent umbo. The surface is glossy black with about 12 to 17 cm in size and twice as big as the size of *M. edulis*. *M. coruscus* usually habits in the 20 m deep from intertidal region and attached on the rock using byssus in groups [S.K. Yoo, 1986].

Economically, *M. coruscus* is one of the most important marine shellfish species in South Korea because of its high nutritional value such as high content of taurine stimulates detoxification of the liver, has effects such as recovery of fatigue, reduction of blood cholesterol, and high inorganic contents such as calcium and iron, which is effective in maintaining bone health and preventing anemia. Despite its abundance, there have been only a few studies reporting [S.G Kim et al., 2013].

Among marine vertebrates, fish species are important and major resources for humans worldwide and have been studied a lot including olive flounder, black

rockfish, red seabream, and gray mellet. In particular, rock bream, *Oplegnathus fasciatus* is one of the most economically valuable fish species in the marine aquaculture industry of South Korea. The rock bream is a subtropical and carnivorous species and inhabits estuaries at various depths according to their growth stage.

The first draft genome sequence of *Oplegnathus fasciatus* was constructed in 2018 and used in various ways for molecular genetic applications including antimicrobial peptides and vaccine development [Y. Shin et al., 2004].

### 3. Immune system

Vertebrates and invertebrates have immune systems to defend themselves against invasive pathogens, and these systems consist of non-specific innate immunity and specific adaptive immunity as cell-mediated humoral component [J.A. Tincu and S.W. Taylor, 2004a]. The innate immune system in marine organism plays essential role and developed to survival because they are exposed to over $10^6$ bacteria/mL and $10^9$ virus/mL of seawater [J.W. Ammerman et al., 1984]. And the innate immune systems have been broadly studied in fish and mollusks and plays as a first line of defense against invading pathogens or virus. Innate immune system recognize by toll-like receptors (TLRs,) lectin, lipopolysaccharide, scavenger receptor via pattern recognition receptors (PRRs) and then immune effectors such as antimicrobial peptides (AMPs), lysozyme, and antioxidant enzyme are synthesized for elimination and neutralization of invaders [X. Wang et al., 2013, M. Zasloff, 2002]. Innate immune system is initiated rapidly within hours without immunological memory and long-lasting. Invading of microorganisms in blood or body fluids is controlled by phagocytosis. Because innate immunity does not have any specific properties, it first inhibits the growth in body fluids of various pathogenic microbes before the adaptive immune system or phagocytosis mechanism is activated after infection [J. Kasamatsu 2013].

Marine invertebrates such as marine mollusks are largely dependent on the innate immune system because acquired immune system is less developed, while innate

immunity is well established. As the first line of defense systems, marine mollusks rely solely on innate immunity, including both humoral and cellular immunity, and this is different with vertebrate immune system [J.A. Tincu and S.W. Taylor, 2004a]. Humoral immunity is mediated in the blood cells and plasma by antimicrobial agents, and cellular immunity is mediated by encapsulation, phagocytosis, and nodule formation [J.A. Tincu and S.W. Taylor, 2004a].

Among the innate immune components, antimicrobial peptides are the most dominant and well-developed constituent of the immune defense system in marine organism [J.A. Tincu and S.W. Taylor, 2004a]. Various antimicrobial peptides have been reported, as well as their various characteristics such as sequences, structures, and antibacterial activities [B.H. Nam et al., 2016].

**4. Antimicrobial peptides (AMPs)**

Increased and widespread of antibiotic-resistant bacteria is a serious issue for human health in the world. And appearance of multi-drug resistance microorganism, known as super bacteria, has caused the development of novel antibiotics substrates [ J.L Lee and Y. Park, 2014]. Antimicrobial peptides are present in all living organism from vertebrate to invertebrate such as mammals, insect, amphibians, echinoderms, plants, bacteria, fish, and mollusk. Antimicrobial peptides have become widely found in various species with different mechanism of conventional antibiotics, so many researchers in world wide are interested in antimicrobial peptides [M. Pushpanathan et al., 2013]. They are protein substances possess antimicrobial activity that remove invaders through naturalization and elimination in the event of microbial invasion without immunological memory [S.V. Sperstad et al., 2011]. Furthermore, some AMPs have other functions such as antifungal, antioxidant, antitumor, antiendotoxin, antiviral, antiparasitic, antiobesity, wound healing, and cell recruitment (Fig. 1) [M. Pasupuleti et al, 2012]. Antimicrobial peptides have similar characteristics such as short, amphipathicity and positive net charge. Therefore, antimicrobial peptides can be regard

as important substance for antibiotic substitute.

### 4.1 Structural features of AMPs

AMPs are made by forming a peptide bond of about less than 100 amino acids and have cation charge +2 to +9 by lysine and arginine and amphipathicity by hydrophobic and hydrophilic [A.A. Bahar and D. Ren, 2013]. The hydrophobic portion interacts with the hydrophobic components of the membrane and play a crucial role to determine the peptide specificity towards the target cells. The secondary structures of AMPs can be classified four parts; α-helical, β-sheet hairpin or loop, and extended [M. Pasupuleti et al., 2012]. α-helical peptides are common and widely studied among known AMPs and displayed amphiphilic characteristics with hydrophobic residues and β-sheet peptides have disulfide bond and/or cyclization of the peptide chain.

### 4.2 Mechanism of action of AMPs

The mechanism of action of membrane AMPs is divided in to two types; membrane active and non-membrane active AMPs (Fig. 2). Most antimicrobial peptides act on the membrane of microorganisms, leading to cell membrane destruction and working with nucleic acid after permeation into cells to inhibit microbial growth through protein synthesis degradation. Cationic peptides engage electrostatic with negatively charged molecules of bacterial cell membranes such as lipopolysaccharide or teichoic acid [M. Pasupuleti et al., 2012]. Then antimicrobial peptide enter into the lipid bilayer, which is initially attached parallel to the lipid bilayer, but as the peptide and lipid ratio increases, it sits perpendicular to the cell membrane and forms a pore through the cell membrane.

Membrane active AMPs proposed mechanisms of action include barrel-stave, toroidal pore, and carpet, which induces bacterial membrane destruction [M. pushpanathan et al., 2013]. In barrel-stave mechanism, a large number of alpha-helical

peptide with amphipathicity interrupts into the cell membrane and forms a hollow form of bundle to form the transmembrane pore [M. Pasupuleti et al., 2012]. The hydrophobic part of the peptide is combine with lipids, and the hydrophilic part forms the inner surface of the pore. In toroidal pore mechanism, peptides that interrupt into membrane form the pore, which causes the peptide to bend the lipid monolayer and the hydrophilic head of the peptide to form the pore. In carpet model, positively charged part of the peptide combine with phopholipid on the surface of the membrane, covering the surface of the cell membrane, and then destroying the cell membrane through micelle formation.

Cationic peptides is capable of forming transient pores on the membrane represent as permeabilizing peptides, whereas non-membrane permeabilizing peptides have the ability to translocate across the cell membrane without permeabilizing the membrane pores on the target cell membrane [M. pushpanathan et al., 2013]. Non-membrane permeabilizing peptides enter the cell and lead to death of bacteria through interaction with nucleic acid and inhibit the action of enzyme and synthesis protein or cell walls.

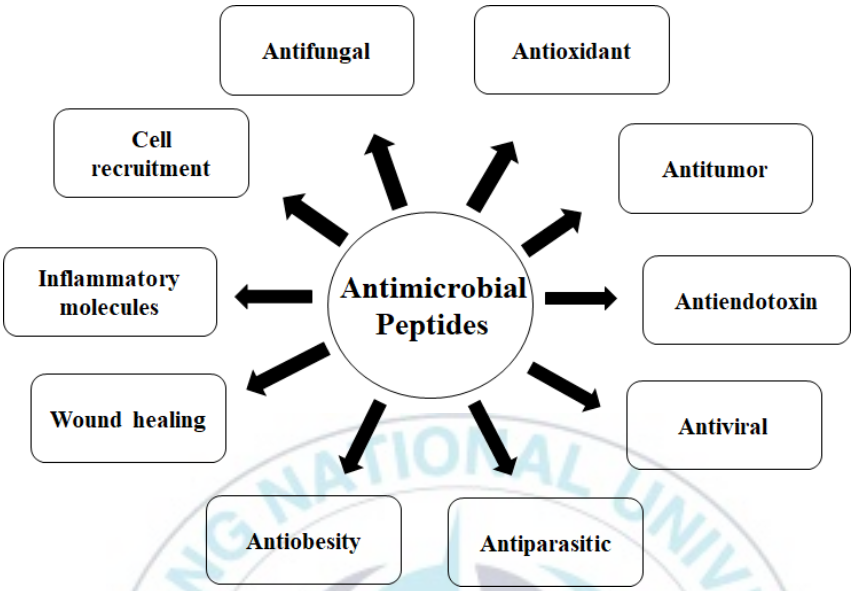
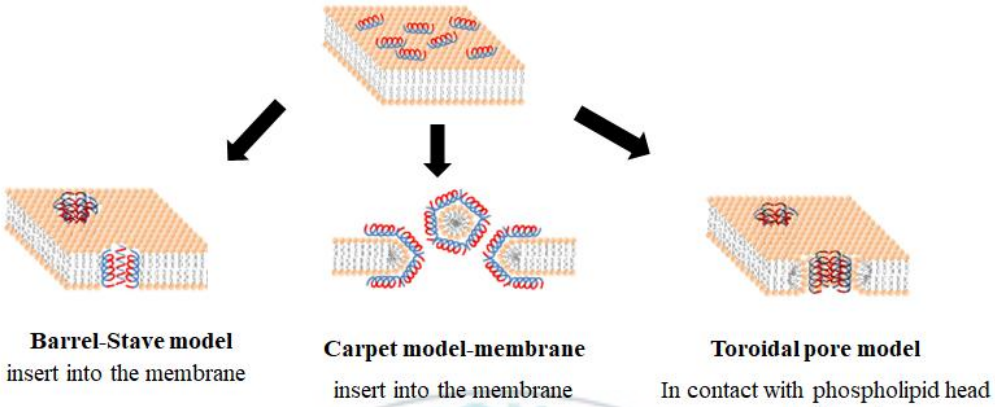



**Fig. 1. Versatile properties of AMPs**

**(A)**




**(B)**

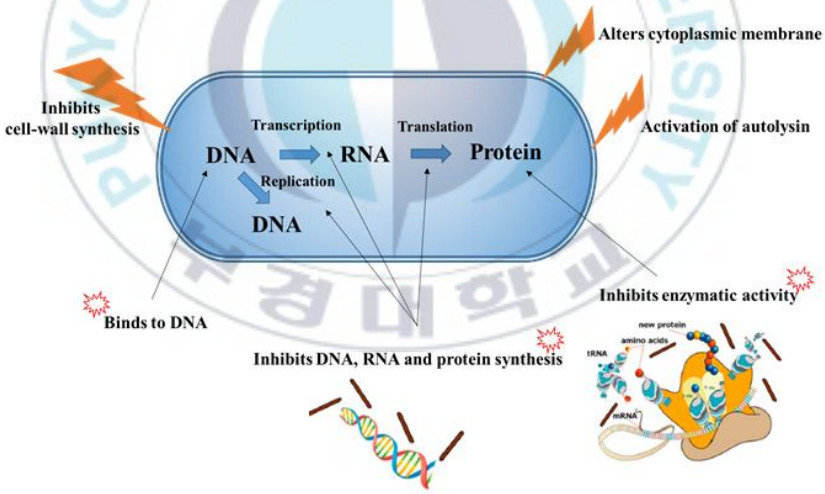



**Fig. 2. Different models for mechanism of membrane-active and non-membrane active AMPs (modified from Y.J. Jung and K.K Kang, 2013)**

### 4.3. Sources of Marine AMPs

Marine organisms are crucial sources of biologically active substance for the development of therapeutics. AMPs from marine organisms have been isolated in invertbrates, fish, microorganisms and algae. These are growing interest in field of research for the antibiotics substitute owing to broad spectrum activity against Gram-positive, Gram-negative bacteria, fungi and viruses with antiprotozoal, anticancer, and immunomodulatory [J.A. Tincu and S.W. Taylor, 2004b]. They have low possibility of resistance emergence and rapid onset of activity.

#### 4.3.1. Invertebrates AMPs

Several AMPs from marine invertebrate have been characterized in arthropoda, tunicata, mollusca, annelida, echinodermata, and cnidaria (Table 1). Defensin [M. Charlet et al., 1996], mytilins [G. Mitta et al., 2000c; M.J, Lee et al., 2018], myticins [G. Mitta et al., 1999], mytimycin [M. Charlet et al., 1996], big defensin [M. Gerdol et al, 2012], mytichitin-CBD [R. Oh et al., 2018; C.L. Qin et al., 2014] as examples, were have been reported in mollusca (Table. 2).

Studies on AMPs from marine mussels of the genus *Mytilus* have focused on *M. galloprovincialis* and *M. edulis*. However, AMPs from *M. coruscus* are far less well studied, and research is still in the early stages. Only a few AMPs have been characterized from *M. coruscus*, such as mytilin [R. Wang et al, 2010], myticusin-1 [Z. Liao et al., 2013] and mytichitin-1 [Q.C. Qin. 2014]. Mytilin family such as mytilin-1, mytilin-2 and mytilin-3 was isolated from *M. coruscus* serum [R.X. Wang et al., 2010]. Myticusin-1 displayed antimicrobial activity against Gram-positive bacteria more than Gram-negative bacteria and fungus [Z. Liao et al., 2013]. Mytichitin-1 with 55 amino acid residues was isolated from hemolymph of *M. coruscus*. This peptide had predominant activity against Gram-positive bacteria and fungi [C.L. Qin et al., 2014]. Myticusin-1 and mytichitin-1 have strong activity against Gram-positive bacteria than Gram-negative bacteria. So this study aims to

discuss antimicrobial peptides isolated from *M. coruscus*, emphasizing on antimicrobial spectrum, stability of purified antimicrobial peptides.

#### 4.3.2. Fish AMPs

AMPs from fish present a broad spectrum of antimicrobial activity, and even affect pathogens from other fish and humans [H.K. Kang et al., 2015]. Many AMPs from fish have been characterized (Table 3). Hepcidins [Shike et al., 2002], HSDF [Patrzykat et al., 2001], paradaxins [Oren and Shai., 1996], parasin-I [Park et al., 1998, Cho et al., 2002], piscidin [Lin et al., 2007, Bae et al., 2016], galectic-1 [Thulasitha et al., 2016], pleruocidins [Cole et al., 1997, Douglas et al., 2001] and misgurin [Park et al., 1997] were have been reported in fish (Table. 3). For example, hepcidin from *Oreochromis mossambicus* have been characterized with antibacterial activity against *Listeria monocytogenes*, *S. aureus* and *E. faecium* with 50-100 μg/mL and piscidin from hybrid striped bass and rock bream have been characterized with antimicrobial activity. The many compounds from fish have function as antibacterial antiviral, and antitumor agents.

#### 4.3.3. Marine microorganism AMPs

AMPs produced by marine microorganism have been the promising new source. Marine microorganisms can be survive in deep sea with lightless, high-pressure condition, and cold including bacteria and fungi. These stressful marine environment have resulted in the development of unique metabolism, which result in the production a wide variety of compounds including antimicrobial, antifungal, anti-protozoan, and antiviral properties. Although the proteobacterias are plentiful in marine environment, the study have still not been widely. The actinomycetes and cyanobacteria are the most studied while proteobacterias have still not been widely studied. For example, tauramamide from *Brevibacillus laterosporus* exhibited antimicrobial activity against Enterococcus sp. with 0.1 μg/mL (MIC) and weak

activity against methichillin resistant *S. aureus* with 200 μg/mL (MIC) [K. Desjardine et al., 2007]. Bacillistatins 1 isolated from *Bacillus silvestris* showed antimicrobial activity against *Streptococcus pneuminia* with 0.5-2 μg/mL (GI50) [G.R. Pettit et al, 2009].

#### 4.3.4. Marine algae AMPs

Algae are group of plants with biological importance with potential commercial value. Marine algae have been used as human food materials for source of bioactive natural substance and as sources of drug with antimicrobial, antifungal, anticancer, and antiviral activity. For example, the polyphenols, alkaloides, polysaccharides, terpenoids and fatty acids are produced by marine algae. They produce a cocktail of metabolites with interesting biological activities and their medical application have been investigated from several decades. [J. Garson, 1989 R. Nair et al., 2007 A. Raja et al., 2013, J.K. Nunnery et al., 2010, M.T. Cabrita et al., 2010, K. Desjardine et al., 2007, G.R. Pettit et al., 2009]

**Table 1. Summary of AMPs from marine invertebrates.**

| Phyla | Distribution | Antimicrobial peptides | Size | Antimicrobial spectrum | Tissue | Reference |
|---|---|---|---|---|---|---|
| Arthropoda | *Tachypleus tridentatus* | Big defensin | 79 aa | G+,G-,F | Hemocyte | Saito et al., 1995 |
| | *T. tridentatus* | Tachycitin | 73 aa | G+,G-,F | Hemocyte | Kawabata et al., 1996 |
| | *Scylla serrata* | Scygonadin | 102 aa | G+,G- | Hemocyte | Huang et al., 2006 |
| | *Homarus americanus* | Homarin | N.D. | G- | Hemocyte | Battison et al., 2008 |
| Tunicata | *Styela clava* | Clabanins | 23 aa | G+,G-,F | Hemocyte | Lee et al., 1997 |
| | *Halocynthia aurantium* | Dicynthaurin | 30 aa | G+,G- | Hemocyte | Lee et al., 2001 |
| | *Ciona intestinalis* | Ci-PAP-A | N.D. | G+,G-,F | Hemocyte | Fedder et al., 2008 |
| Mollusca | Pteriomorpha (subclass) | Defensins | 39-43 aa | G+,G- | Hemocyte/gill/mantle | Charlet et al., 1996 |
| | *Dolabella auricularia* | Dolabellanin-B2 | 33 aa | G+,G-,F | Body wall | Iijima et al., 2003 |
| | Bivalvia (class) | Mytilins | 32-34 aa | G+,G-,F | Hemocyte | Mitta et al., 2000 |
| Annelida | *Nereis diversicolor* | Hedisitin | 22 aa | G+,G- | Coelomocytes | Tasiemski et al., 2007 |
| | *Perinereis aibuhitensis Grube* | Perinerin | 51 aa | G+,G-,F | Homogenate | Pan et al., 2005 |
| Echinodermata | *Strongylocentrotus droebachiensis* | Strongylocins | 48-52 aa | G+,G-,F | Coelomocytes | Li et al., 2008 |
| Cnidaria | *Aurelia aurita* | Aurelin | 40 aa | G+,G-, | Ectoplasma | Ovchinnikova et al., 2006 |

G+: Gram-positive bacteria, G-: Gram-negative bacteria, F: Fungi, N.D.: Not determined

**Table 2. Summary of AMPs derived from *Mytilus* species.**

| Organism | Antimicrobial peptides | Size | Antimicrobial spectrum | Tissue | Reference |
|---|---|---|---|---|---|
| *Mytilus edulis* | Mytilin A, B | 34 AA | G+, G-, F | Hemocyte | Charlet et al., (1996) |
| *Mytilus edulis* | Defensin A, B | 35, 37AA | G+ | Hemocyte | Charlet et al., (1996) |
| *Mytilus edulis* | Mytimycin | 6.2 kDa | F | Hemocyte | Charlet et al., (1996) |
| *Mytilus edulis* | N.D. | N.D. | G+, G-, F | Gill | Mercado et al., (2005) |
| *Mytilus galloprovincialis* | Myticin | 40 AA | G+, G-, F | Hemocyte | Mitta et al., (1999) |
| *Mytilus galloprovincialis* | Defensins | 39 AA | Has not yet tested | Hemocyte | Mitta et al., (1999) |
| *Mytilus galloprovincialis* | Mytilin | 34 AA | G+, G-, F, P | Hemocyte | Mitta et al., (2000) |
| *Mytilus galloprovincialis* | Mytimycin | 54 AA | F | Hemocyte | Charlet et al., (1996) |
| *Mytilus galloprovincialis* | Mytimacin 1-5 | 61-78 AA | Has not yet tested | Hemocyte | Gerdol et al., (2012) |
| *Mytilus galloprovincialis* | Big defensin | 79-87 AA | Has not yet tested | Hemocyte | Gerdol et al., (2012) |
| *Mytilus coruscus* | Mytilin 1-8 | 34 AA | Has not yet tested | Hemocyte | Liao et al., (2010) |
| *Mytilus coruscus* | Myticin 1-9 | 40 AA | Has not yet tested | Hemocyte | Liao et al., (2010) |
| *Mytilus coruscus* | Myticusin | 104 AA | G+, G-, F | Hemocyte | Liao et al., (2013) |
| *Mytilus coruscus* | Protamine-like | 60 AA | G+, G-, F | Foot | Oh et al., (2016) |
| *Mytilus coruscus* | Sperm specific | 101 AA | G+, G-, F | Hemocyte | Oh et al., (2018) |
| *Mytilus coruscus* | Mytilin | 103 AA | G+, G-, F | Hemocyte | Lee et al., (2018) |
| *Mytilus coruscus* | Mytichitin-CB | 55 AA | G+, G-, F | Hemocyte | Qin et al., (2014) |
| *Mytilus coruscus* | Mytichitin-CBD | 55 AA | G+, G-, F | Mantle | Oh et al., (2018) |

G+: Gram-positive bacteria, G-: Gram-negative bacteria, F: Fungi, P: Prozoa, N.D.: Not determined

**Table 3. Summary of AMPs derived from fish**

| Organism | Antimicrobial peptides | Size | Antimicrobial spectrum | Tissue | Reference |
|---|---|---|---|---|---|
| White bass (*Morne chrysops*) | Hepcidins | 21 AA | *E. coli* | Gill | Shike et al., (2002) |
| Coho salmon (*Oncorhynchus kisutch*) | HSDF | 26 AA | G- | Mucus, blood | Patrzykat et al., (2001) |
| Red sea moses sole (*Pardachirus marmoratus*) | Paradaxins | 33 AA | G+, G- | Skin | Oren and Shai., (1996) |
| Asian catfish (*Parasilurus asotus*) | Parasin-I | 19 AA | G+, G-, F | Skin | Park et al., (1998)<br>Cho et al., (2002) |
| Hybrid striped bass (*Morone saxailis* x *Morone chrysops*) | Piscidin | 22 AA | G+, G- | Mast cell | Lin et al., (2012) |
| Rock bream (*Oplegnathus fasciatus*) | Galectin-1 | 135 AA | G+,G-, F, P, V | Blood | Thulasitha et al., (2016) |
| Rock bream (*Oplegnathus fasciatus*) | LEAP-2 | 103 AA | G+(*S. iniae*) G- | Blood | Hwang et al., (2019) |
| Rock bream (*Oplegnathus fasciatus*) | Piscidin | 70 AA | G+, G- | Gill | Bae et al., (2016) |
| Winter flounder (*Pleuronectes americanus*) | Pleruocidins | 25 AA | *E. coli* | Skin, intestine | Cole et al., (1997)<br>Douglas et al., (2001) |
| Loach (*Misgurnus anguillicaudatus*) | Misgurin | 21 AA | G+, G-, F | Whole fish | Park et al., (1997) |

G+: Gram-positive bacteria, G-: Gram-negative bacteria, F: Fungi, P: Prozozoa, V: Virus

## 5. Purpose of this study

AMPs are suitable substitution for antibiotics. We characterized the antimicrobial peptides based on extraction and purification, and genetic analysis.

First, we isolated, purified, and characterized the AMPs from the various tissues of *M. coruscus*. Our results support that AMPs from tissues of *M. coruscus* as an alternative material to antibiotics and an immune system-related peptide. Various researches about AMPs and study of *M. coruscus* is proceeding and required. Therefore this research is focused on AMPs derived from *M. coruscus*.

Second, we tried to designed and synthesized the antimicrobial peptide analogs of lipopolysaccharide binding protein (LBP) and catalytic domain of phospholipase (CDP) from rock bream *Oplegnathus fasciatus* based on native form by substitution amino acid sequence and modification. And peptides were evaluated multi-functional activities including antimicrobial activity, anti-cancer activity and anti-scuticociliates activity. Moreover, analogs were tested ability of DNA binding and DNA polymerase inhibition related to mechanism of action of antimicrobial peptides. The process of this present investigations were presented in Fig. 3. The main objectives of this study is focused on following:

Based on extraction and purification

(i) Isolation and purification antimicrobial peptides from tissue of *M. coruscus*.

(ii) Identification of antimicrobial peptides by HPLC, MALDI-TOF/MS and N-terminal sequencing

(iii) cDNA of antimicrobial peptides were cloned and sequenced using RACE method.

(iv) Cloning, expression purification of recombinant proteins and determination of antimicrobial and anti-parasitic activity.

(v) Quantitative analysis of gene expression by real-time qPCR.

Based on genetic analysis

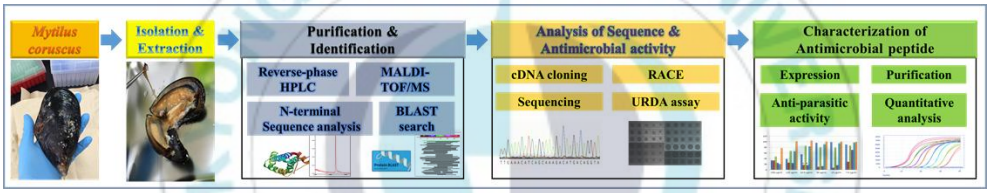
(i) Peptide design and synthesis with substitution and modification

(ii) Antimicrobial activity, anti-cancer activity and anti-scuticociliates activity with stability

(iii) Ability of DNA binding, membrane permeability and DNA polymerase inhibition related to mechanism of action of antimicrobial peptides.

(iv) Analysis of apoptosis in cancer cells

(A) Based on extraction and purification




(B) Based on genetic analysis

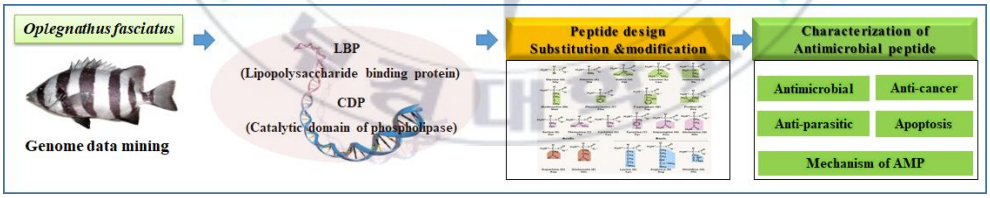



**Fig. 3. Scheme of the process of the research work**

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## II. Materials and methods

### 1. Tissue extraction

Hard-shell mussels were collected from a local fish market in Busan, Korea. Mussels were gently washed with tap water, and tissues were separated under aseptic conditions into seven organs: adductor muscle, hemocyte, gill, hepatopancreas, foot, excurrent siphon, and mantle. Tissues were homogenized and boiled for 5 min with four volumes of pre-heated 1% acetic acid (HAc) followed by cooling. The boiled tissues were centrifuged, and the supernatant was stored at −70°C until use. The supernatant was filtered through a 0.45-μm filter for purification.

### 2. Bacteria strains and culture conditions

Gram-positive, Gram-negative and fungi are given from KCTC, ATCC and KCCM for antimicrobial activity (Table 4). Antibiotic-resistant strains are given from CCARM (Table 4). All strains were grown overnight in the appropriate broth and temperature.

### 3. Test of antimicrobial activity by ultrasensitive radial diffusion assay (URDA)

The antimicrobial activities of specimens were tested against the bacteria and fungi listed in Table 4 using a modified ultrasensitive radial diffusion assay (URDA). All strains were grown overnight in the appropriate broth and temperature (25°C or 37°C). After incubation, bacteria and fungi were diluted to the McFarland turbidity standard of 0.5, corresponding to ~$10^8$colony forming units (CFU)/mL, and 0.5 mL of ~$10^6$CFU/mL diluted bacteria or *Candida albicans* was added to 9.5 mL of underlay gel containing 1% type I agarose and poured to 1-mm thickness in 2.2-mm-diameter wells. The samples were added to wells, and the bacterial or fungi suspension was overlaid with 10 mL of overlay gel containing 1% type I agarose and 10 mM phosphate buffer (pH 6.5). After incubation for 18 h at 25°C, 30°C, or 37°C, the diameters of clear zones were measured. The minimum effective concentration (MEC, μg/mL) of the synthesized peptide was calculated as the X-intercept of a plot of units

against the log10 of the peptide concentration.

**Table 4. Pathogenic strains used in this study.**

| | | Strain | | Pathogenicity |
|---|---|---|---|---|
| Microbes | Positive | *Bacillus cereus* | KCTC 1012 | Human |
| | | *Bacillus subtilis* | KCTC 1021 | Human |
| | | *Enterococcus faecalis* | KCTC 3206 | Human |
| | | *Staphylococcus aureus* | ATCC 6538 | Human |
| | | *Streptococcus mutans* | KCTC 3065 | Human |
| | | *Lactococcus garvieae* | KCTC 3772 | Human |
| | | *Streptococcus parauberis* | KCTC 3651 | Fish |
| | Negative | *Enterobacter cloacae* | KCTC 2361 | Human |
| | | *Escherichia coli* | ATCC 8739 | Human |
| | | *Escherichia coli* | ML35p | Human |
| | | *Klebsiella pneumoniae* | KCTC 12385 | Human |
| | | *Providencia stuartii* | KCTC 2568 | Human |
| | | *Pseudomonas aeruginosa* | ATCC 15522 | Human |
| | | *Vibrio parahaemolyticus* | KCCM 41664 | Fish and human |
| | | *Vibrio vulnificus* | KCCM 41665 | Fish and human |
| | | *Vibrio alginolyticus* | KCTC 2472 | Fish |
| | | *Vibrio anguillarum* | KCTC 2711 | Fish |
| | | *Vibrio harveyi* | KCCM 40866 | Fish |
| | Fungi | *Candida albicans* | ATCC 10231 | Human |
| Antibiotic resistant strains | Positive | *Staphylococcus aureus* | CCARM 0203 | Human |
| | | *Staphylococcus aureus* | CCARM 0204 | Human |
| | | *Staphylococcus aureus* | CCARM 3795 | Human |
| | | *Streptococcus pyogenes* | CCARM 0206 | Fish |
| | Negative | *Pseudomonas aeruginosa* | CCARM 0225 | Human |
| | | *Enterobacter cloacae* | CCARM 0252 | Human |
| | | *Escherichia coli* | CCARM 0238 | Human |
| | | *Escherichia coli* | CCARM 1A814 | Human |
| | | *Klebsiella erogenes* | CCARM 0249 | Human |
| | | *Klebsiella oxytoca* | CCARM 0248 | Human |
| | | *Salmonella typhimurium* | CCARM 0240 | Human |

**4. Purification by reversed phase high-performance liquid chromatography (HPLC)**

The purification step was carried out by reversed-phase HPLC with two types of reversed-phase columns: the CapCell-Pak $C_{18}$ reversed-phase column (5μm, 120Å, 4.6x250 mm; Shiseido, Japan) and the TSK-gel ODS-80$^{TM}$ $C_{18}$ reversed-phase column (4.6x150 mm; TOSOH, Japan). The eluted peaks were tested against *Streptococcus mutans* (KCTC 3065) by URDA for confirmation of antibacterial activity. First, acidified extract was injected to a CapCell-Pak $C_{18}$ reversed-phase column with a gradient of 5–65% acetonitrile (ACN) in 0.1% trifluoroaceticacid (TFA) over 60 min at a flow rate of 1.0 mL/min. Next, collected fractions were applied to TSK-gel ODS-80$^{TM}$ $C_{18}$ reversed-phase column with agradient of 5–65% ACN in 0.1% TFA over 120 min at a flow rate of 0.2 mL/min and 60 min at a flow rate of 1.0 mL/min. The eluted fractions were detected at 220 nm and vacuum dried for the further characterization.

**5. Proteolytic digestion of purified peptides**

Finally, a single purified peak was incubated with 2 μL (1,000 μg/mL) of trypsin at 37°C for 1 h to confirm the proteinaceous nature of the compound. Trypsin hydrolyzed the peptide bonds and digested the protein into peptides at the carboxyl-terminal side of arginine and lysine residues. After digestion, samples were incubated at 4°C to inactivate the trypsin. After protease reaction, the single purified peak was subjected to URDA testing and its antimicrobial activity was compared to that of the peptide without trypsin treatment.

**6. Determination of amino acid sequences and molecular weights of purified peptides**

The molecular weight of purified peptide was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrophotometry (MALDI-TOF/MS). The

purified peptide was dissolved in 1% TFA/50% $CH_3CN$ (1:1,v/v) mixed with α -cyano-4-hydroxycinnamic acid (CHCA) matrix solution (10 mg/mL CHCA in 0.1% TFA/50% $CH_3CN$, 1:1,v/v) and loaded onto MALDI plate Edman degradation was performed using pulse liquid automatic sequencer (model 473A; Applied Biosystems, USA) to obtain N-terminal amino acid sequence of target peptide.

**7. Stabilities on heat and salt**

*B. subtilis, B. cereus, E. coli, P. aeruginosa, S. mutans* and *C. albicans* were used as indicator strain of the URDA method to check antimicrobial activity of synthesized peptide after incubation at their optimal temperature. To check stability against salt concentration of antimicrobial peptide, NaCl of final concentration to be 0.5%, 1%, and 2%, was mixed with 0.01% acetic acid in final volume 10μl. The change in activity was measured after heating at 100°C for 10 minutes for thermal stability evaluation.

**8. Sequence analysis of cDNA and cloning**

To determine the complete sequence, cDNA from *M. coruscus* was constructed using a SMARTer® RACE 5'/3' Kit. RNA was extracted from foot tissue using the TRI Reagent. First-Strand cDNA was synthesized with poly (A) Polymerase to add a poly(A) tail. 5'-RACE-Ready cDNA synthesis reactions were performed in 11-μL final volumes containing 1.0 μL RNA and 1.0 μL 5'-cDNS Primer A. 3'-RACE-Ready cDNA synthesis reactions were performed in 12-μL final volumes containing 1.0 μL RNA and 1.0 μL 3'-cDNS Primer A for preparation of 3'-RACE-Ready cDNA. Tubes were incubated at 72°C for 3 minutes and then cooled to 42°C for 2 min. Oligonucleotide (1 μl) was added to the 5'-RACE cDNA synthesis reactions. Master mix was added to denatured RNA from 5'- and 3'-RACE-Ready cDNA synthesis reactions and incubated at 42°C for 90 min, followed by 70°C for 10 min. 3'- and

5'-RACE-Ready cDNA samples were stored at –20°C until use. Two gene-specific oligonucleotide primers were designed for sequence analysis (Table 5).

The full-length cDNA was cloned into pGEM® T-easy vector systems and transformed into competent *Escherichia coli* XL-1 blue. Plasmids were purified using the DNA-spin Plasmid Purification Kit, and sequencing was processed on a 3130XL Genetic Analyzer. Amino acid sequences were searched using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI). Sequences were aligned using BioEdit version 7.2.5 and GENETYX version 4.0 for comparison and analysis. Peptides were analyzed using the ExPASy-online tool.

**Table 5. Primer sequences used for RACE-PCR in this study**

| Primer name | Sequence (5'->3') |
|---|---|
| Chtinase_3RACE | 5'-GAT TAC GCC AAG CTT TGT GGC ATG AAT GGT AAA ATG CCA TGT-3' |
| Chitinase_5RACE | 5'-GAT TAC GCC AAG CTT GGA TTC CAT ACA AGA CCC CGA CCA C-3' |
| Protamine_3RACE | 5'-GAT TAC GCC AAG CTT CCA AGC CCA ACT CGT CGT TCA ACI TCC AGG-3' |
| Protamine_5RACE | 5'-GAT TAC GCC AAG CTT CCT GGA IGT TGA ACG ACG AGT TGG GCT TGG-3' |
| Sperm-GSP-3RACE | 5'-GAT TAC GCC AAG CTT CCA AGC CCA ACT CGT CGT TCA TCC AAG TCC AGG-3' |
| Sperm-GSP-5RACE | 5'-GAT TAC GCC AAG CTT GCT TTT GGA CCT GGA CTT GGA TGA ACG ACG AGT-3' |
| MytilinB–GSP-3'RACE | 5'-GAT TAC GCC AAG CTT CAA TTA TTC TGG CTA TTG CTC TTG CAG TA-3' |
| MytilinB–GSP-5'RACE | 5'-GAT TAC GCC AAG CTT CAG GGA ATT TCA TGG TAT GCT CAC TGG CAC-3' |
| Myticusin-3RACE | 5'-GAT TAC GCC AAG CTT GAC CAC CAG ATG GCA CAG TCG GCC TGC-3' |
| Myticusin-5RACE | 5'-GAT TAC GCC AAG CTT TAT TGC GGA TGC ATA TGC IGC GTC TTG IGC-3' |

**9. Construction of recombinant plasmid**

For construction of an *E. coli* expression vector, the target gene was amplified using a sense *Nde*I-linker primer and an antisense *Xho*I-linker primer and *Nde*I-linker primer and an antisense *Xho*I-linker primer. PCR was performed using primers for the target gene. PCR amplifications were carried out in 50-μL final volumes containing 1 μL DNA template, 1x PCR buffer containing $MgCl_2$, 0.2 mmol/l dNTPs, 0.6U *Taq* polymerase, and 1 pmol of each primer. The PCR conditions were denaturation at 94°C for 5 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 55-60°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min using a thermal Cycler. The amplified product was verified by electrophoresis on a 1.5% agarose gel. Products were purified using the QIAquick® PCR Purification Kit and cloned into pET-22b(+) expression vectors containing *Nde*I and *Xho*I sites and a C-terminal 6´ His-tag and cloned into pET-28a(+)-thioredoxin A (TrxA) fusion vector containing *BamH*I and *Xho*I sites, and the N-terminus was 6X His-tagged. The recombinant plasmid encoding the mytichitin-CBD and myticusin-beta gene was isolated and transformed into *E. coli* DH5α and BL21 (DE3) competent cells.

**10. Expression and purification of recombinant protein**

Cells were cultivated in 300 ml of Luria–Bertani (LB) liquid medium containing 100 μg/mL ampicillin at 37°C until the optical density at 600 nm reached 0.4. Isopropyl-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and growth was continued for 4 h. Cells were harvested and suspended in 50 mM Tris-HCl buffer (pH 8.0). Suspended cells were crushed by sonication in an ice-water bath and centrifuged at 12,000 rpm for 10 min at 4°C. The soluble and insoluble proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The supernatant was purified using Ni-NTA His

Bind Superflow™ resin. The proteins were eluted with a linear gradient of imidazole. After purification, collected fractions were dialyzed in 20 mM Tris-HCl buffer (pH 8.0) and analyzed by SDS-PAGE.

**11. Western blotting**

Samples were heated for 10 min at 100°C and separated with the EzWay-PAG gel system for western blots. Results were obtained using the WesternBreeze® chromogenic Immunodetection Kit (Invitrogen, USA) according to the manufacturer's protocol. Gels were washed and trans-blotted onto a nitrocellulose (NC) membrane at 15 V for 1 h. The NC membrane was incubated with anti-His primary antibody (1:5000, Invitrogen, USA) and then alkaline phosphatase-conjugated secondary antibodies for 1 h. Protein bands were developed using a chromogenic substrate.

**12. Hemolytic activity**

Hemolytic activity was determined using olive flounder (*Paralichthys olivaceus*) blood cells. Olive flounder blood cells were collected and diluted with sodium phosphate buffer (150 mM NaCl, 50 mM sodium phosphate pH 7.4) and then centrifuged at 5,000 rpm and 4°C for 1 min. Blood cells were washed to collect red blood cells, excluding the buffy coat and plasma. Next, 90 μL of 3% red blood cells in phosphate-buffered saline (PBS) was mixed with 10 μL of sample and incubated at 37°C for 1 h.

Then, 100 μL of 3% red blood cells in PBS and sample was centrifuged at 5,000 rpm and 4°C for 1 min, after which 70 μL of the supernatant was injected into a 96-well plate. Hemolysis was monitored by measuring the absorbance at 405 nm with a VICTOR3 1420 plate reader (PerkinElmer, USA). For this assay, 1% Triton X-100 and piscidin 1 (1 mg/mL) were used as positive controls, and 0.01% HAc and PBS were used as negative controls. The percentage of hemolytic activity was calculated as follows:

Hemolysis (%) = [($Abs_{405nm}$ test − $Abs_{405nm}$ buffer) / ($Abs_{405nm}$ 1% Triton X-100 − $Abs_{405nm}$ buffer)] × 100

**13. Anti-scuticociliate assay**

Anti-scuticociliate assay was determined in 96 well flat bottom plates containing $10^6$ cells/well at 20℃ by Premix WST-1 cell-proliferation assay kit (Takara, Japan). Peptides were diluted at concentrations of peptides and evaluated in a final volume of 200 μL/well, and L-15 media was used as negative control. Treated and untreated scuticociliates were performed in triplicates. After the treatment, plates were incubated at 20℃ for overnight. WST-1 solution was added to each well and absorbance was read in an ELISA reader at 450 nm. Values are expressed as percentage of viable scuticociliate after treated. The morphology and motility of scuticociliate was monitored through a digital microscope camera (DFC425C Leica, Germany).

**14. Quantitative analysis of gene expression**

The mRNA expression levels of target gene in tissues of *M. coruscus* after challenge with *Vibrio parahaemolyticus* and the temporal expression in foot tissue were determined by quantitative real-time PCR using Fast SYBR Green Master Mix and an ABI 7500 Real-time PCR System. Total RNA was extracted from each tissue (adductor muscle, hemocyte, gill, hepatopancreas, foot, excurrent siphon, and mantle) using the TRI Reagent. cDNA was synthesized with a Transcriptor First Strand cDNA Synthesis Kit and used as a template. Amplifications were performed in 20 μL final volumes containing 1 μl diluted cDNA, 10 μL 2 Fast SYBR Green Master Mix, 0.5 μL of each primer (10 pmol/μL), and 8 μL $H_2O$ using the following cycle profile: 1 cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 55°C for 1 min, and 72°C for 30 s. Melting curve analysis was performed to confirm specific amplification. Results were compared with the 18S rRNA levels using the $2^{-\Delta\Delta Ct}$ method to determine the differences; data are presented as relative expressions

(mean ± standard deviation).

**15. Peptide design and synthesis**

Peptides were designed and synthesized to maximize antimicrobial activity and susceptible to degradation by host protease by substitution and modification of amino acid residues. Peptides were designed based on the original amino acid sequence considering net charge, protein binding potential (Boman index), and hydrophobic ratio. The information of net charge, molecular weight, Boman index (potential protein interaction index), pI value and hydrophobicity were calculates by the Antimicrobial Peptide Database v2.34 (APD2; http://aps.unmc.edu/AP/main.php). All peptides was synthesized by Anygen Inc. (Korea) at a purity grade of > 95%. Peptides were purified using Shimadzu HPLC 10AVP system with SHIMADZU C18 analytical column. Elution was performed with water-acetonitrile linear gradient (5-60% of acetonitrile) containing 0.05% (v/v) trifluoroacetic acid. Mass analysis was performed with AXIMA CFR Kratos, Shimadzu. All synthesized peptides were dissolved in 0.01% acetic acid to 10 mg/mL.

**16. DNA-Binding assay**

In order to investigate the DNA-Binding assay, inhibition of the rate of migration of DNA bands was tested by electrophoresis on 1% agarose gel [B.H. Nam et al., 2014]. 5μL of λ-HindIII-digested DNA (100 ng) (Roche, Switzerland) was mixed with different concentration of peptide (0, 0.125, 0.25, 0.5, and 1 mg/mL) in 0.01% acetic acid, incubated at room temperature for 2 hr and then electrophoresed in 1% agarose gel in the TAE buffer containing ethidium bormide (EtBr).

**17. DNA polymerase inhibition assay**

The various concentration of analogs were used for PCR. gDNA of 100 ng was used as a template and 5μL of peptides (31.3, 62.5, 125, 250, and 500 μg/mL) were

added to the reaction mixture for PCR. We used following primer pairs: 16S F; AGA GTT TGA TCC TGG CTC AG and R; ACG GTT ACC TTG TTA CGA CTT and PCR compised 35 cycles each of 95°C for 30s, 55°C for 30s and 72°C for 3 min. After PCR, amplicons were electrophoresed in 1% agarose gels containing EtBr.

**18. Anti-cancer activity in cancer cells**

The colorimetric 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS dye assay was used to determine the cytotoxicity of HeLa cells in DMEM medium, supplemented with 10% FBS were placed into 96-well plates and incubated at 37℃ containing 5% $CO_2$. HeLa and A549 cells were seeded at a density of 5 x $10^4$/mL in a 96-well plates and peptide was added to the cells to final concentration of 20, 30, 40, 50 μg/mL. Melittin was added for positive control. After incubation for 24 h, 20 μL of MTS solution was added inth each well and the plates were incubated for 4 h at 37°C. The absorbance at 540 nm was measured by a microplate reader. The cell viability was determined the relatives as their percentage of the treated cells to one of the untreated cells by comparing their optical densities.

**19. Annexin V/Propidium iodide (PI) analysis**

Quantitative analysis of apoptotic cells was performed using an FITC Annexin V Apoptosis Detection Kit. HeLa and A549 cells were seeded at a density of 5 x $10^4$/ml in a 6 well culture dish and treated with peptides for 24 h. Then the cells were washed with phosphate buffered saline (PBS) and were collected. Harvested cells were mixed in 1X binding buffer and staied with an annexin V/PI at room temperature for 15 min. Than, the staining cells were detected by flow cytometry (FACS Calibur, Becton Dickinson, San Jose, Ca, USA), and analyzed by the CellQuest software (Becton Dickinson Co).

**20. Membrane permeabilization**

To check the lytic activity on the bacterial membrane, chromogenic bacteriolytic plate assay was tested using *E. coli* ML35p which contains the gene IPTG-induced β -galactosidase. *E. coli* ML35p was grown overnight in TSB broth at 37°C. After incubation, 50 μL of culture medium, 10 μL of IPTG 0.5 mM and 40 μL of X-gal 40 mg/mL was added to 9.5 mL of underlay gel containing 1% type I agarose and poured to 1-mm thickness in 2.2-mm-diameter wells. The 5 μL of samples were added to wells, and then overlaid with 10 mL of overlay gel containing 1% type I agarose and 10 mM phosphate buffer (pH 6.5). After incubation for 18 h at 37°C, the diameter of the cleaning zone and the presence of blue edge lines on the edge were checked.

## III. Results and Discussion

### Part I. Antimicrobial peptides derived from *Mytilus coruscus* by purification

# Chapter 1.

## Purification and characterization of an antimicrobial peptide mytichitin-chitin binding domain from hard-shelled mussel, *Mytilus coruscus*



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### 1.1. Screening of the acidified crude extract of *M. coruscus*

The acidified tissue extracts showed antibacterial activity against several bacterial species. Seven Gram-positive bacteria, nine Gram-negative bacteria, and one fungus, *C. albicans*, were selected for URDA. Five tissues, the adductor muscle, hemocyte, foot, mantle, and hepatopancreas, showed strong antibacterial activity against both Gram-positive and Gram-negative bacteria (Table 1.1). Notably, foot extract exhibited potent activity against Gram-positive bacteria including *Bacillus subtilis*, *S. mutans*, and *Staphylococcus aureus* and Gram-negative bacteria including *E. coli*, *Enterobacter cloacae, Klebsiella pneumonia, Providencia stuartii,* and P*seudomonas aeruginosa*. Adductor muscle and mantle showed lower activity than the other tissues against all tested strains.

### 1.2. Peptide purification

Acidified foot extracts had antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, and the fungus, *C. albicans*. Based on the initial screening results, *S. mutans*, which showed high susceptibility to the extract, was selected for the antibacterial assay after purification. In first step, most peaks eluted between 5 to 25 min from the CapCell-Pak $C_{18}$ reversed-phase column with a gradient of 5-65% ACN in 0.1% TFA. The active fraction was eluted at 18.4 min and exhibited antibacterial activity against *S. mutans* (data not shown). The fraction was collected, vacuum dried, and applied to a TSK-gel ODS-80$^{TM}$ $C_{18}$ reversed-phase column with gradient of 5-65% ACN in 0.1% TFA for further purification. The active fraction was eluted at 72 min. Finally, the target antibacterial peptide was purified by reversed-phase HPLC. The eluted fraction was treated with trypsin for 60 min at 37°C, which completely abolished all antibacterial activity against *S. mutans*. These results suggest that the extract contains a proteinaceous compound (Fig. 1.1. A).

**Table 1.1. Screening of the acidified crude extract of *M. coruscus***

| Organism | Tissue | | | | |
|---|---|---|---|---|---|
| | Adductor muscle | Hemocyte | Foot | Mantle | Hepatopancreas |
| **Gram positive** | | | | | |
| *B. cereus* | - | + | - | + | +++ |
| *B. subtilis* | +++ | +++ | +++ | +++ | +++ |
| *S. mutans* | - | + | +++ | +++ | - |
| *S. aureus* | - | + | +++ | | + |
| *S. haemolyticus* | - | - | - | - | - |
| **Gram negative** | | | | | |
| *E. coli* | +++ | ++ | +++ | +++ | +++ |
| *E. cloacae* | - | +++ | ++ | - | - |
| *K. pneumoniae* | - | ++ | ++ | - | - |
| *P. stuartii* | | ++ | +++ | | |
| *P. aeruginosa* | +++ | + | ++ | +++ | +++ |
| *P. mirabilis* | - | + | - | - | - |
| *V. alginolyticus* | - | - | - | - | - |
| *V. harveyi* | - | - | - | - | - |
| **Fungus** | | | | | |
| *C. albicans* | - | + | + | - | - |

Antimicrobial activity based on the diameter of inhibition zone (mm) follow this skim: : +++≥6mm, ++4-6mm, +≤4mm.

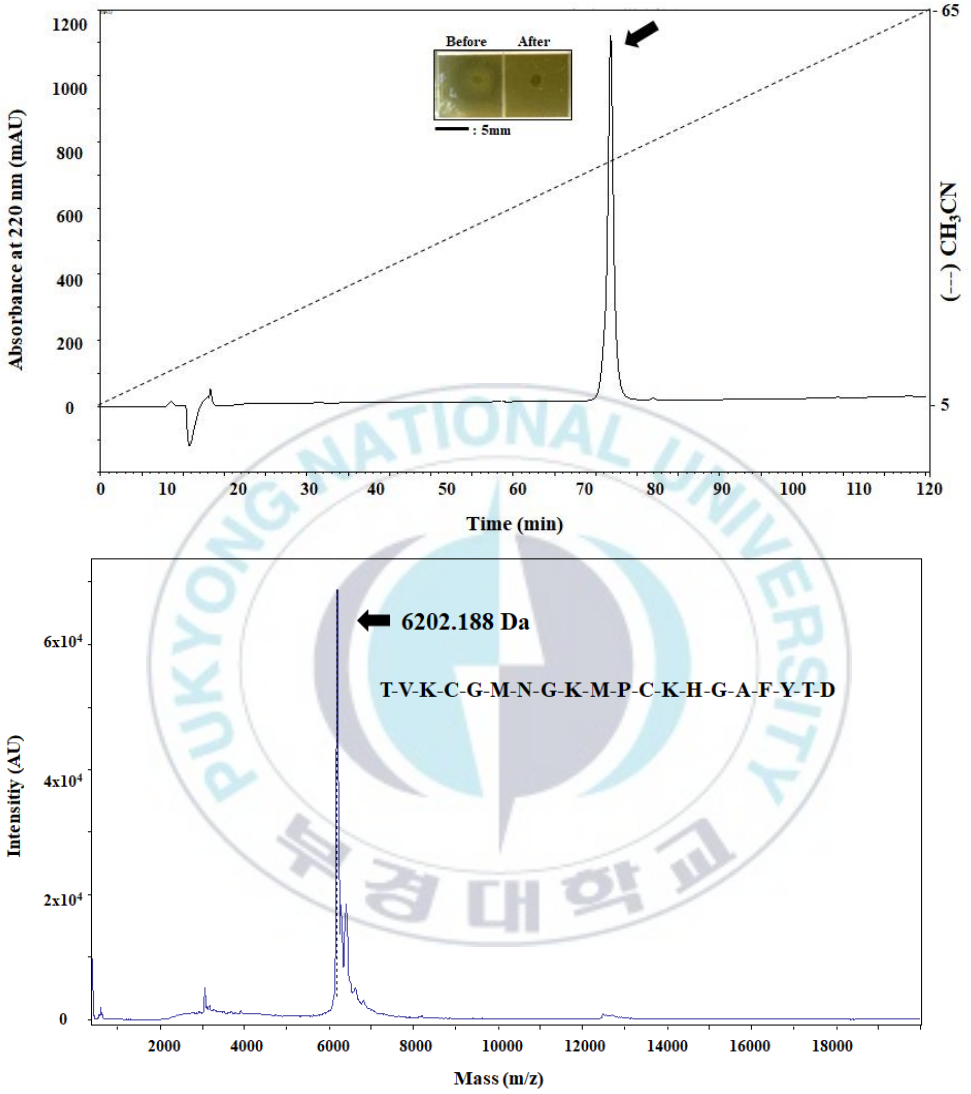



**Fig. 1.1. Purification and antimicrobial activity of the purified peptide from foot extract.** (A) Antimicrobial activity of the purified peak (before) and trypsin treated purified peak (after) against *S. mutans*. The active fraction was applied to a TSK-gel ODS-80™ $C_{18}$ reversed-phase column. Elution was performed with a linear gradient of 5–65% $CH_3CN$ (pH2.2) in 0.1% TFA for 120 min at a flow rate of 0.2 mL/min. The elution was monitored at 220 nm. The fraction of the absorbance peak (indicated

by the arrow) showed antimicrobial activity (before) and trypsin treated to purified extract (after) against *S. mutans*. The scale bar indicates 5 mm. (B) Mass spectrometry analysis of single mass with 6202.18 Da and N-terminal sequences (20 amino acid residues) was determined by Edman degradation.

### 1.3. Peptide identification

We obtained the molecular mass and N-terminal amino acid sequence of the purified peptide. The mass was 6202.188 Da, and the 20-amino acid sequence of purified peptide was identified by Edman degradation (Fig. 1.1b): TVKCGMNGKMPCKHGAFYTD. The $NH_2$-terminal 20-residues partial sequence of the purified peptide matched 100% with sequences in the *M. coruscus* expressed sequence tag (EST) database, EST mytichitin-1 (AHC08445.2), mytichitin-2 (AIF74556.1), mytichitin-3 (AIF74557.1), mytichitin-4 (AIF74558.1), and mytichitin-5 (AIF74559.1), and matched 95% with chitinase-like protein-1. Therefore, we designed specific primers to identify the 6.2 kDa purified antimicrobial peptide based on the partial N-terminus amino acid sequence and the EST database sequences of *M. coruscus*.

### 1.4. Cloning of cDNA encoding mytichitin-CBD

We designed specific primers to amplify cDNA of the AMP from *M. coruscus* and used the rapid amplification of cDNA ends (RACE) method to obtain the full-length cDNA sequence. The complete sequence from the purified AMP was 1,558 bp with an open reading frame of 1,341 bp encoding 446 amino acid residues; it matched six different identified sequences (Fig. 1.2). The cDNA sequence of the obtained AMP encodes a 25 amino-acid signal peptide and 421-amino-acid mature peptide. Signal peptides were determined using the SignalP 4.0 online tool. The 446 amino-acid sequence was compared with mytichitin-1 (Fig. 1.3). Five isoforms obtained from the purified AMP revealed high sequence similarity, at 97.7-99.1%, with the mytichitin-1

precursor of *M. coruscus*. Therefore, we named the identified cDNA of the purified antimicrobial peptide mytilin-A and called the 6.2 kDa isolated antimicrobial peptide mytilin-CBD. Multiple-sequence alignment analysis of mytichitin-1 and mytichitin-A with isoforms revealed that 25 signal peptides and catalytic sites (DGLDLDLE) were conserved, as shown in Fig. 3. Furthermore, the oligonucleotide sequence of the chitin-binding domain belonging to mytichitin-CBD was composed of 168 nucleotides encoding 55 amino acid residues. The chitin-binding domain was almost completely conserved at 96.3-98.1% with the mytichitin-1 precursor and 98.1-100% with chitinase-like protein-1. The theoretical molecular mass of mytichitin-CBD was 6,273.35 Da, and the acquired experimental mass was ~6.2 kDa. Purified mytichitin-CBD includes 12 basic residues (including six Lys, four Arg, and two His), and the theoretical isoelectric point (pI) was highly basic at 9.38.

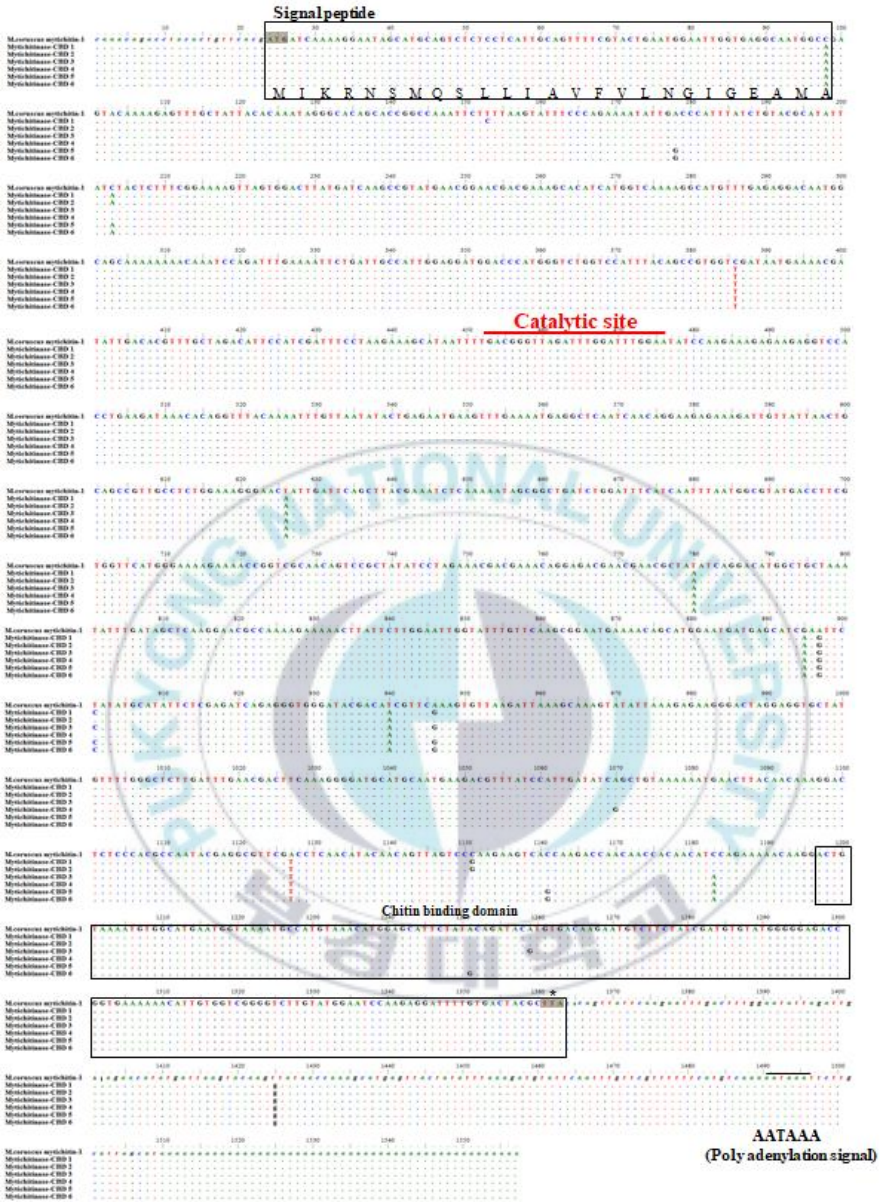



**Fig. 1.2. Multiple alignment of nucleotide sequences of mytichitin-1 and five isoforms. of mytichitin-A cDNA.** Conserved residues are shown in black boxes, the 5′ untranslated region (5′UTR) and 3′UTR are underlined, and the chitin-binding domain is outlined. The polyadenylation signal is indicated by a black line.

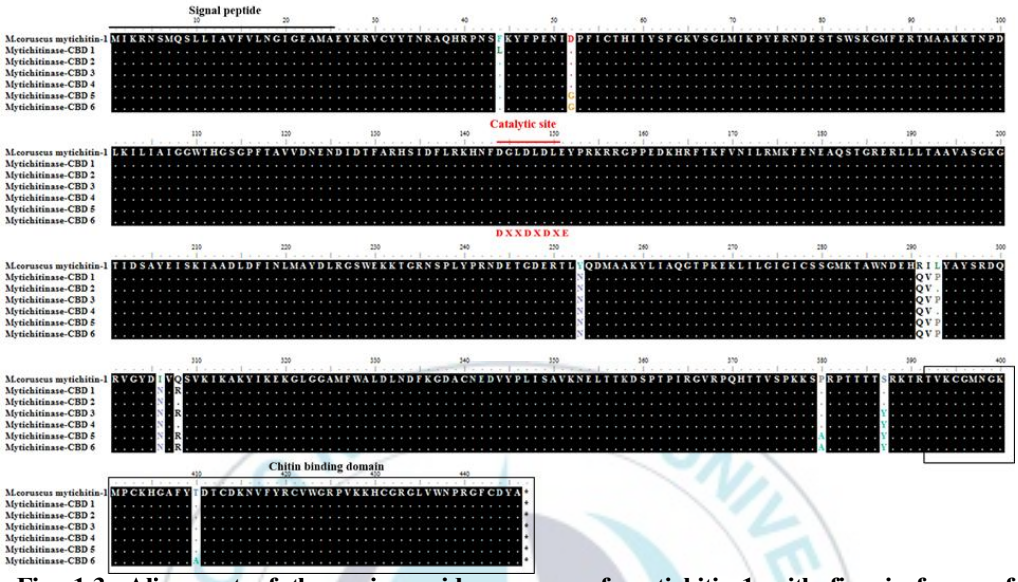



**Fig. 1.3. Alignment of the amino acid sequence of mytichitin-1 with five isoforms of mytichitin-A.** The catalytic site, DXXDXDXE, is indicated by a red line. Conserved residues are indicated by a black box. The signal peptide and chitin-binding domain are outlined.

**1.5. Expression and purification of protein**

We cloned mytichitin-CBD into expression vector pET-22b(+), and expression was induced by adding 1 mM IPTG. Bacterial lysates containing recombinant expression vector were analyzed on an ExWay-PAG gel with 10% Aspartate buffer. Recombinant proteins were detected in the supernatant as well as in the pellet. The purified mytichitin-CBD was observed at ~6 kDa (Fig. 1.4). The molecular weight appeared consistent with the theoretical molecular weight. Finally, the purified recombinant peptides were prepared for use by desalting and freeze-drying.

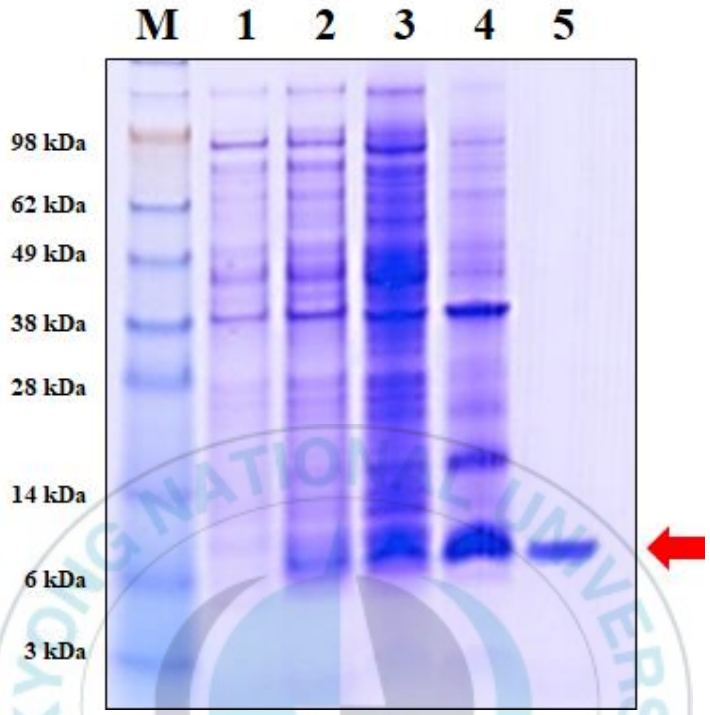



**Fig. 1.4. Overexpression and purification of the recombinant mytichitin-CBD.**

The 6×His-tagged recombinant protein was purified using Ni-NTA affinity chromatography. Lane M, molecular marker; lane 1, non-induced protein; lane 2, induced protein; lane 3, soluble protein; lane 4, inclusion body; lane 5, the purified mytichitin-CBD protein (denoted by the red arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

### 1.6. Antimicrobial activities

The antimicrobial activity of recombinant mytichitin-CBD was tested against a variety of bacterial strains using URDA method. The recombinant mytichitin-CBD had high antibacterial activity against Gram-negative strains including *E. coli*, *P. aeruginosa*, and *Vibrio harveyi*, Gram-positive strains including *B. subtilis* and *S. mutans*, and the fungus *C. albicans* (Table 1.2).

**Table 1.2. Antimicrobial activities and spectrum of purified recombinant mytichitin-CBD.**

| | Organism | Activity |
|---|---|---|
| **Gram-positive** | *B. cereus* | N/A* |
| | *B. subtilis* | +++ |
| | *S. mutans* | +++ |
| **Gram-negative** | *E. coli* | ++ |
| | *P. aeruginosa* | + |
| | *V. harveyi* | +++ |
| **Fungus** | *C. albicans* | ++ |

Antimicrobial activity based on the diameter of inhibition zone (mm) follow this skim: N/A; No Activity, +++≥6mm, ++4-6mm, +≤4mm.

### 1.7. Anti-scuticociliate activity

Purified recombinant protein was filtered and tested against the parasites. The motility and number of parasites were noted at 32, 64, 96, and 128 μM of recombinant protein. At 24 h after infection, 128 μM recombinant protein reduced parasites by approximately 50% (Fig. 1.5). Movement was observed with no or very little movement across the microscopic field. Treatment of the parasites with recombinant protein showed that the viability was lower than with untreated protein. Recombinant mytichitin-CBD significantly inhibited the parasites at a high concentration of protein (128 μM).

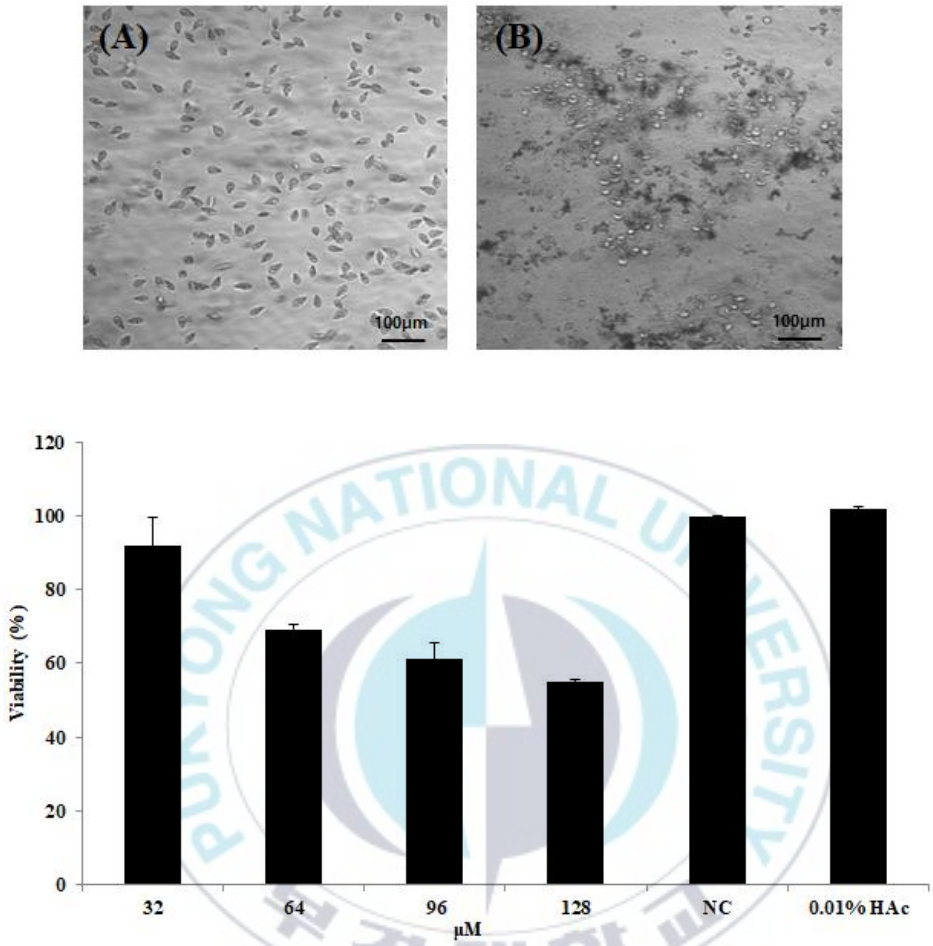
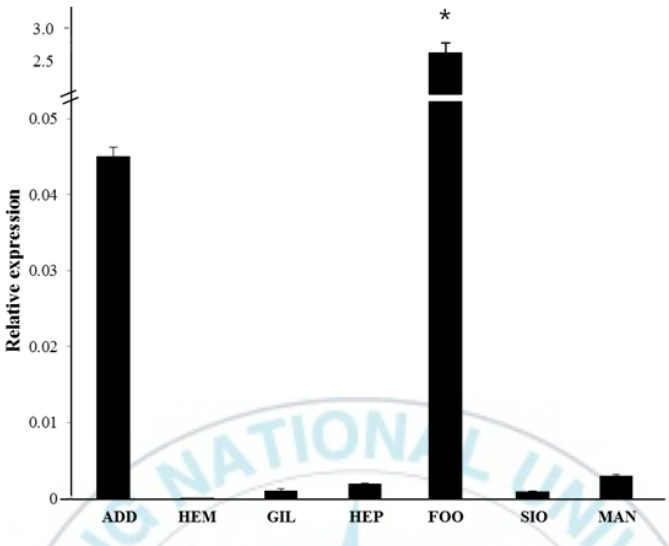



**Fig. 1.5. Anti-parasitic activity exhibited by recombinant mytichitin-CBD using WST-1 reagents.** (A) Negative control. (B) 128 μM of recombinant mytichitin-CBD. (C) Relative viability levels of parasites (scuticociliates). Error bars represent the mean ± SD of three technical replicates.

### 1.8. Quantitative analysis of mytichitin-CBD expression

As shown in Fig. 1.6A, mRNA of mytichitin-CBD was detected in tissues including the adductor muscle, hemocyte, gill, hepatopancreas, foot, mouth, and mantle by real-time PCR. Expression of mytichitin-A showed the highest level in the foot (2.5 fold), followed by adductor muscle (0.045 fold), while other tissues showed moderate responses. Temporal expression of mytichitin-A mRNA in foot tissue after microbial challenge is shown in Fig. 1.6B. Predominantly, the expression level of mytichitin-A increased 130 fold at 4 h.

**(A)**




**(B)**

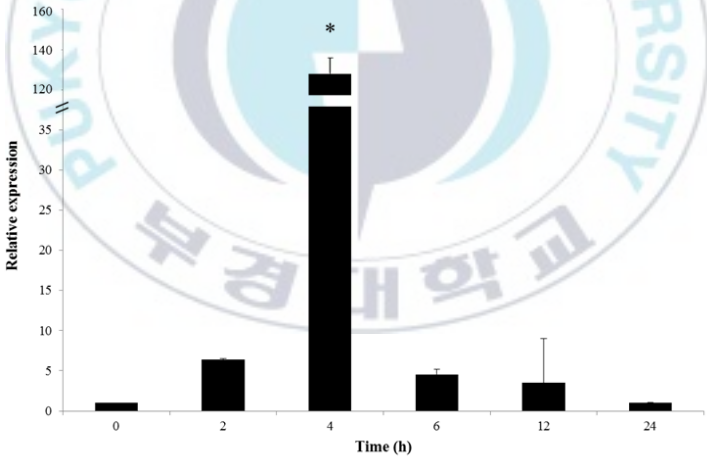



**Fig. 1.6. Quantitative analysis of mytichitin-CBD expression in the various tissues.** (A) Tissue-specific expression of mytichitin-CBD. Error bars represent the mean ± SD of three technical replicates. ADD, adductor muscle; HEM, hemocyte; GIL, gill; HEP, hepatopancreas; FOO, foot; SIO, siphon; MAN, mantle. (B) Temporal expression of mytichitin-A in foot tissue after challenge. Error bars represent the mean ± SD of three technical replicates.

**Discussion**

Sessile shellfishes have no motility, so they cannot avoid invaders or environmental stresses. Furthermore, due to their lack of an acquired immune system, an immunological characteristic of invertebrates, only the innate immune system protects them against various pathogenic microorganisms in the seawater to which they are exposed during filter feeding. Among the elements of the innate immune system, AMPs are the most important major defense factor, and various studies have reported that AMPs exhibit defense mechanisms against diverse pathogens including bacteria, viruses, fungi, and parasites. In addition, AMPs are known to have antiendotoxin, antitumor, antiobesity, vasculogenesis, cell differentiation, inflammatory molecules, cell recruitment, and wound healing activities, as well as defense abilities. For these reasons, researchers are examining the various applications of AMPs; we focused on the AMPs of the sessile hard-shell mussel *M. coruscus*.

The antimicrobial spectra of diverse acidified tissue extracts were analyzed against various pathogenic bacteria using the URDA method. Among the acidified tissue extracts, the adductor muscle, hemocyte, foot, mantle, and hepatopancreas revealed notable antimicrobial activities (Table 1.1). When starting this research, we hypothesized that extracts from mantle or hemocyte tissues would have strong antimicrobial activity because they first contact and/or respond to environmental stresses and invasive pathogens. Also, many antimicrobial peptides have been reported to be located and associated with epithelial or circulating cells. However, among the organs of *M. coruscus*, foot extract exhibited broad and strong activity against Gram-positive and Gram-negative bacteria, and even fungi (Table 1.1). Interestingly, various antimicrobial peptides also were isolated and identified in organs other than epithelial or circulating cells. Furthermore, involvement of AMPs in anti-infectious processes was different according to cells and tissues. Therefore, we chose the foot tissue extract for this AMP study. Selected foot extract fractions were purified and

analyzed, and finally ~6.2 kDa of a single AMP was isolated (Fig. 1.1). The N-terminal amino acid sequence determined with the Edman degradation method, cDNA cloning, and a BLAST search of the NCBI database indicated that the isolated AMP was very similar to mytichitin-CB, the chitin-binding domain of the C-terminal region of mytichitin-1 precursor. Furthermore, sequence analysis of the complete cDNA by RACE revealed that the isolated complete cDNA sequence was similar to the reported mytichitin-1 precursor protein; thus, we named the complete cDNA mytichitin-A precursor and identified the 55-amino-acid AMP as mytichitin-CBD.

Qin et al. reported the antimicrobial property of the chitin-binding domain (mytichitin-CB of mytichitin-1 precursor) from *M. coruscus*. They investigated the characteristics of 6297 Da of mytichitin-CB extracted from hemolymph (95 Da larger than mytichitin-CBD in this investigation), analyzed its antimicrobial activity spectrum, and performed sequence analysis and quantitative analysis of expression patterns in tissues. However, when we compared the results of mytichitin-CB (mytichitin-1) and mytichitin-CBD (mytichitin-A), antimicrobial activity of mytichitin-CB was recorded in Gram-positive bacteria and fungi, and few nucleotide and amino acid sequence differences were detected between mytichitin-1 and mytichitin-A. In addition, mytichitin-CB and mytichitin-CBD contain the same sequence of 55 amino acids and were isolated from the same species (*M. coruscus*), but they exhibited different molecular weights as well as inconsistent expression patterns and levels after bacterial challenge. According to the sequence analysis of mytichitin-CBD and the result of MALDI-TOF/MS, purified mytichitin-CBD comprised 55 amino acids including six cysteines, and the calculated molecular weight (6303.38 Da) did not corresponded with measured molecular weight (6202.188 Da). The calculated molecular mass was 6303.388 Da, 101.192 Da larger than the determined natural form of mytichitin-CBD. This phenomenon could not be explained even when considering disulfide bonds, similar isoforms, or reduced mytichitin-CBD length. However, many post-translational modifications such as allysine (lose 1.03 Da), amidation (lose 0.98 Da),

2,3-didehydroalanine (lose 18.02 Da), 2,3-didehydrobutyrine (lose 18.02 Da), (Z)-2,3-didehydrotyrosine (lose 2.02 Da), 3-oxoalanine cysteine (lose 18.08 Da), 3-oxoalanine serine (lose 2.02 Da), 2-oxobutanoic acid (lose 17.03 Da), pyrrolidone carboxylic acid (lose 18.02 Da), pyrrolidone carboxylic acid (lose 17.03 Da), and pyruvic acid (lose 33.10 Da) are known to reduce molecular mass. Therefore, the difference between the detected molecular weight and predicted mass could be valid. For this reason, more study is required to identify correlations between accurate molecular mass and post-translational modifications. Moreover, the variance in molecular weight between mytichitin-CBD and mytichitin-CB, which were isolated from the same species and have the same sequence, could be explained by differences in post-translational modification processes and the different habitat regions of *M. coruscus* from which specimens were collected for investigation. Various studies have reported similar findings of differing characteristics depending on habitat for identical genes or proteins from the same species.

In the present study, we found five mytichitin-A isoforms, and Qin et al. also reported four mytichitin-1 variants from *M. coruscus*, but the isoforms identified by the two studies had different sequences. Although AMPs are conserved during evolution, various isoforms have been reported for several AMPs such as defensins, mytilins, myticins, and mytimycin of mussels. In the case of the mytichitin-A and mytichitin-1 isoforms, conserved sequences, structures, and domains of chitotriosidase/chitinase, such as the signal peptide, chitin catalytic domain, chitin-bonding domain, and serine/threonine-rich linker, were preserved, but a high diversity of amino acid residues was observed in the variable regions depending on the species. Although structures and sequences of these isoforms of antimicrobial peptides are not completely different, it is known that the susceptibility and spectrum of antimicrobial activity differs. Mytilins, an AMP of mussel containing eight cysteines, was reported in five isoforms. Among mytilin isoforms, the mytilin B and C isoforms display a high degree of homology in their structure, but have different

activities against the fungus Fusarium oxysporum. The reason for the diversity of these AMPs and isoforms was reported to be the need for rapid and varied responses to a variety of intruders. Furthermore, various AMPs and isoforms of these AMPs are reported to have complementary properties that increase mussels antimicrobial capability.

The mytichitin-A protein and mytichitin-CBD peptide show high sequence similarity with chitotriosidases/chitinases and chitin-binding domains from other species. Chitinase is a member of the glycosyl hydrolase 18 superfamily (GH18), characterized for hydrolyzing the β-1,4 linkage of N-acetyl glucosamine present in chitin chains, and are present in a wide range of organisms. Antimicrobial activity of chitotriosidase/chitinase was initially shown in the field of plant microbial immunity by demonstrating that host-derived chitinases cleave chitin to protect against invading chitin-bearing pathogens. Furthermore, the antimicrobial activities of peptides containing chitin-binding domains were also described in arthropods such as shrimp and crabs. To confirm these results, mytichitin-CBD was cloned and expressed as a recombinant protein, and it demonstrated antimicrobial activities against pathogenic bacteria, fungi, and parasites. In the group of AMPs from mussels, mytichitin-CB, defensins, mytimycin, and myticins display specific antimicrobial activity against Gram-positive bacteria and/or exhibit anti-fungal activity. This limited antimicrobial activity spectrum was also discovered in other invertebrate AMPs. However, recombinant mytichitin-CBD exhibited antimicrobial activity against Gram-positive bacteria (*B. subtilis* and *S. mutans*), Gram-negative bacteria (*E. coli, P. aeruginosa, V. harveyi*), and fungi (*C. albicans*) (Table 1.3). Various mytilin isoforms identified from hemocytes of M. galloprovincialis also exhibited wide and varied antimicrobial activities according to isoforms. Mytilin B, C, and D inhibited both Gram-positive and Gram-negative bacteria, and mytilin B and D also obstructed the growth of fungus. In addition, similar to mytilin isoforms, mytichitin-CBD showed varied antimicrobial activity depending on the species of microorganism in the same group,

such as Gram-positive or Gram-negative bacteria. These varied antimicrobial activities can be explained by differences in compounds in the cell walls of Gram-positive and Gram-negative bacteria, but the distinction in activity between microbial species in the same group of bacteria is questionable and requires more research. Notably, antimicrobial activity as well as anti-parasitic activity were also observed from recombinant mytichitin-CBD with scuticociliates (Fig. 1.5). Scuticociliates are parasites that damage various aquaculture organisms, and economic loss caused by these parasites has increased rapidly. When the recombinant mytichitin-CBD was used as treatment, the movement of the parasites rapidly decreased. Furthermore, viability was decreased by 50% after treatment with 128 μM recombinant mytichitin-CBD, and viability increased dose dependently with treatments of <128 μM recombinant mytichitin-CBD (Fig. 1.5).

As with other AMPs derived from *M. coruscus*, the expression of mytichitin-A was induced immediately after bacterial challenge in isolated foot tissue. Therefore, it is known that mytichitin-A is expressed in a tissue-specific manner, and the process and mechanism of making mytilin-CBD antimicrobial peptide from mytichitin-A after expression must be determined. In addition, the temporal expression pattern of the mytichitin-A gene in foot tissue after challenge would contribute to our understanding of acute-phase proteins in anti-pathogenic responses. After bacterial infection, expression of several AMPs from *M. coruscus* was up-regulated, with the highest increase at 24-48 h. However, the transcript of mytichitin-A was up-regulated dramatically at 4 h, with a 130-fold increase compared to the control group in this study. After a peak at 4 h, the expression level recovered to normal over the following hours. These results reveal that *M. coruscus* might respond to invading bacteria rapidly and synthesize AMPs that participate in an immune defense. Similar expression patterns were observed in other bivalves.

In conclusion, we isolated, purified, and identified the mytichitin-A protein and derived 6.2 kDa of the mytilin-CBD peptide containing 55 amino acid residues from

acidified foot tissue extract of the mussel, *M. coruscus*, and this peptide exhibited antibacterial and anti-parasite activity. Expression levels and patterns were dependent on the tissue type and phase of infection. The results suggest that the identified mytichitin-CBD peptide might cooperate in the innate immune defense system of *M. coruscus*. Our results can be used to better understand the immune systems and responses to pathogens of marine invertebrates.

**Conclusion**

An antimicrobial peptide with 55 amino acid residues was purified by C18 reversed-phase high-performance liquid chromatography (HPLC) from foot extract of the hard-shelled mussel, *Mytilus coruscus*. This peptide showed strong antimicrobial activity against Gram-positive and Gram-negative bacteria, as well as fungi.

The purified peptide was determined to have a molecular mass of 6,202 Da by matrix-assisted laser desorption/ionization time-of-flight mass spectrophotometry (MALDI-TOF/MS). The N-terminal amino acid sequence of the purified peak was obtained by Edman degradation. The identified 20-amino acid sequence shared 100% identity with the N-terminal regions of mytichitin-1, mytichitin-2, mytichitin-3, mytichitin-4, mytichitin-5, and chitinase-like protein-1, and so was named mytichitin-CBD. The cDNA of mytichitin-CBD was cloned and sequenced by rapid amplification of cDNA ends (RACE). The mRNA transcripts were mainly detected in foot tissue, and they were up-regulated and peaked at 4 h after bacterial infection.

We constructed and expressed recombinant mytichitin-CBD protein. Recombinant mytichitin-CBD displayed antimicrobial activity against Gram-negative bacteria *Escherichia coli, Pseudomonas aeruginosa,* and *Vibrio harveyi*, Gram-positive bacteria *Bacillus subtilis* and *Streptococcus mutans*, and the fungus *Candida albicans*, as well as anti-parasitic activity against scuticociliates. The results of this study demonstrate that the peptide isolated from *M. coruscus* is related to the innate immune system of this marine invertebrate and is a possible alternative to antibiotics.

## Chapter 2.

## Isolation and purification of antimicrobial peptide, protamine-like from adductor muscle of *Mytilus coruscus*.



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### 2.1. Screening of the acidified crude extract of *M. coruscus*

Tissues isolated from *M. coruscus* were homogenized and process of extraction. Peptides were extracted and antimicrobial activity was analyzed. Each of the extracts showed activity against Gram-negative, Gram-positive strains and fungi, *C. albicans*. and foot extract showed the greatest antimicrobial activity against pathogenic strains (Table 2.1). The foot extract showed Gram-positive strains including *B. subtilis*, S. aureus and *S. mutans*, Gram-negative strain including *E. cloacae, E. coli, K. pneumoniaei, P. aeruginosa* but no activity against *V. alginolyticus* and *V. harveyi.* In addition, it displayed weak effect against *C. albicans*. These various ranges of antimicrobial activity showed strong activity against Gram-positive strains, such as antimicrobial peptide mytichitin and myticusin isolated from *M. coruscus* and needed to compare the activity by separation and purification as a single component. We need to test more different indicator strains but it had antibacterial activity to human and fish pathogen so this material can be used as medicine for human and animal. *S. mutans* was indicator strain for URDA because foot extracts strongly inhibited the growth of *S. mutans.*

**Table 2.1. Antibacterial activity of crude extracts against pathogenic strains.**

| Organism | Tissues | | | | |
|---|---|---|---|---|---|
| | Adductor muscle | Hemocyte | Foot | Mantle | Hepatopancreas |
| **Gram-positive** | | | | | |
| *B. cereus* | - | + | - | + | +++ |
| *B. subtilis* | +++ | +++ | +++ | +++ | +++ |
| *S. mutans* | - | + | +++ | +++ | - |
| *S. aureus* | - | + | +++ | | + |
| **Gram-negative** | | | | | |
| *E. coli* | +++ | ++ | +++ | +++ | +++ |
| *E. cloacae* | - | +++ | ++ | - | - |
| *K. pneumoniae* | - | ++ | ++ | - | - |
| *P. stuartii* | | ++ | +++ | | |
| *P. aeruginosa* | +++ | + | ++ | +++ | +++ |
| *P. mirabilis* | - | + | - | - | - |
| *V. harveyi* | - | - | - | - | - |
| **Fungus** | | | | | |
| *C. albicans* | - | + | + | - | - |

Antimicrobial activity based on the diameter of inhibition zone (mm) follow this skim: : +++≥6mm, ++4-6mm, +≤4mm.

### 2.2. Peptide purification

The extract of the foot was purified by HPLC with reversed-phase columns. Various fractions were identified using CapCell-Pak $C_{18}$ reversed-phase column, and each eluted fraction was measured with URDA method. As a result, strong antimicrobial activity was observed in the eluted fraction at approximately 26 min, recording a clear zone approximately 6.5 mm diameter (Fig. 2.1A). This fraction was purified by TSK-gel ODS-80$^{TM}$ $C_{18}$ column and a single pure fraction was acquired at 34.5 min, and the URDA confirmed the inhibition zone with a diameter of about 8.8 mm (Fig. 2.1B).

Antibacterial activity was also compared before and after the trypsin treatment to verify that the pure purified specimen is a protein-based antibacterial substance. As a result of cultivating the URDA reputation, we observed that antibacterial activity completely disappeared from the separated sample fraction after the trypsin treatment (Fig. 2.1B). The results of the complete loss of activity of the specimen purified with a typical protein breakdown enzyme were positive results showing that the extract was a protein-based antibacterial substance. The final purified substance was subjected to N-terminal sequence analysis through MALDI-TOF/MS molecular weight measurement and edman segmentation process for primary structure analysis.

### 2.3. Identification of peptide

N-terminal amino acid sequencing was performed by mass spectrometer and edman degradation method to investigate molecular weight and amino acid sequence of pepet ide purified from foot of *M. coruscus*. As a result, the molecular weight of about 6,7 01 Da was purified by MALDI-TOF mass spectrometer. Partial N-terminal amino acid s were determined 20 amino acids ; A-K-A-K-R-S-P-R-K-K-K-A-A-V-K-K-S-S-K-S an d searching GenBank database blast of NCBI showed 100% homology with sperm-spe cific protein and protamine-like PL-II/PL-IV precursor of *M. californianus*. The ORF was also analyzed using primers based on this information and was encoding 60 ami

no acids with 183 bp (Fig. 2.2). NCBI Gen has identified 60 sequences of amino aci ds. A search in the NCBI GenBank Database found 60 amino acid sequences matche d 100% of the protein-like PL-II/PL-IV precursors of *M. californianus*. Comparing am ino acids to other species, homology was 91.6% with sperm-specific PHI-2B/PHI-3 pr otein of *M. trossulus* and 70% with sperm-specific Phi-3 protein of *M. californianus*. (Fig. 2.2).

sperm-specific Phi-3 protein of *M. californianus* encodes 45 amino acids, smaller than 60 amino acids of peptide in this study and 39th, 41st, 42nd of amino acid sequences were inconsistent. And DNA sequence 183 bp of protamine like of *M. coruscus* had 97.2% homology with protamine-like PL-II/PL-IV precursor of *M. californianus* and 86.3% with histone h1-like protein of *M. trossulus* (Fig. 3.3).
In addition, 5' 3' UTR, domain, and isoform analyses are under way to characterize these genes, which can be used as data to increase understanding of the characteristics of the protamine-like protein. We also have a sequence of amino acids that we have in place of various antibacterial peptides.

Searching for the partial primary structure of the N-terminal in the Antimicrobial peptide database (http://aps.unmc.edu) showed very low 39.13% similarity with Piceain of *Picea sitchensis* [R. Liu et al., 2011]. Based on these results, it was assessed that the peptide isolated from this study was likely to be a novel antimicrobial peptide. Protein-like protein is a DNA condensation protein found in the sperm of various species and belonging to the sperm nuclear base protein (SNBPs) that replaces the plastic histone during sperm formation [J. Ausio 1999; Ausio et al., 2004;J. Ausio et al., 2006]. When sperm is formed in spermatocytes,, the somatic histone becomes a histone combined with DNA and passes through the intermediate stage protein-like protein [R.C. Richard, 2001]. The Protamine-like protein has the intermediate structure and function of the histone and the protamine and consists of a high rate of 35% to 50% of lysine and arginine [Ausio et al., 2004]. The somatic histone combines with DNA in a dynamic and plays an important role in gene expression control, but the

protamine and protamine-like combine tightly so that the genes are maximally compressed [Ausio et al., 2006]. Although many characteristics are reported at chemical levels, especially in marine invertebrates, specific structures resulting from interactions with DNA are not well known [Y. K Cha 2012]

The Protamine-like protein is divided into four groups: PL-I, PL-II, PK-III and PL-IV. PL-I has been studied in fish and amphibians as histone H1 related PL protein, but not much information in freshwater fish [J. Ausio et al., 2006]. Sperm specific protein; (PL-II, PL-III, PL-IV) have been reported to be related to sperm formation in the bile [J. Ausio 1999]. The PL-II protein of *Mytilus* sp. belongs to the histone H1 family and is similar to the winged helix motif of the histone H1 and has a spherical core consisting of 84 amino acids [J. Ausio et al., 2006]. PL-III protein is an intermediate product of histone and protamine, but has different characteristics from protamine [J. Ausio et al., 2006]. PL-IV protein, 6.5 kD, is a construct that is very similar to the C-terminal of the lysine-rich histone H1 and is a product of the post-translation modification process of PL-II/PLIV precursors [J. Ausio et al., 2006]. Although no systematic and evolutionary experiments have been conducted to reveal the function of isolated peptides in this study, antimicrobial activity has been reported in relation to the production of sperm in a variety of mussel, and antimicrobial activity in germ cells associated proteins has been partially known [Y.H. Kim et al., 2014]. It is not bivalvia but is known to have antibacterial activity in the protein known as arginine-rich polycationic protein separated from salmons germ cells [R.C. Richard, 2001]. Also, while the study of the protamine-like protein related to the generation of sperm in *M. californianus* has not been reported yet. It can also be regard to be unique in that it was found in the foot, not in the gut tissue containing the germ plasm of the *M. coruscus*, so the possibility of novel or multi-functional peptide is high, and further research is thought to provide a variety of data related to the immune system of invertebrates.

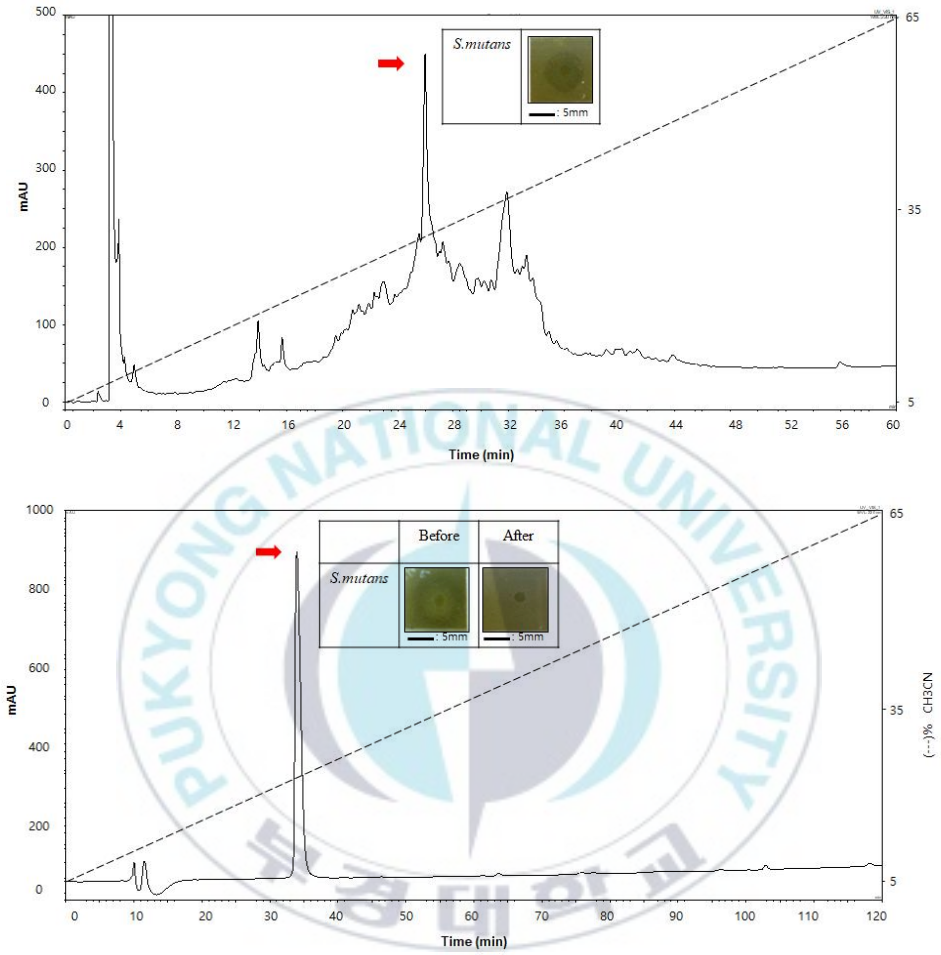



**Fig. 2.1. Purification and antimicrobial activity of crude extract. (**A) Purification of crude extracts from muscle of *M. coruscus.* The acidic extract was fractionated by the CapCell-Pak $C_{18}$ reversed-phase column. Elution was performed with a linear gradient of 5%→65% $CH_3CN$ (pH2.2) in 0.1% TFA for 60 min at a flow rate 1.0 mL/min. The eluate was monitored at 220 nm. Fraction of the absorbance peak showed antimicrobial activity against *S. mutans* (inset). (B) Antimicrobial activity of the purified peak (before) and trypsin treated purified peak (after) against *S. mutans*. Active fraction was applied to the TSK-gel ODS-80$^{TM}$ $C_{18}$ reversed-phase column

Elution was performed with a linear gradient of 5%→65% $CH_3CN$ (pH2.2) in 0.1% TFA for 120 min at a flow rate of 0.2 mL/min. The eluate was monitored at 220 nm. Fraction of the absorbance peak (indicated by the arrow) showed antimicrobial activity (before) and trypsin treated to purified extract (after) against *S. mutans*. Scale bar indicates 5 mm.

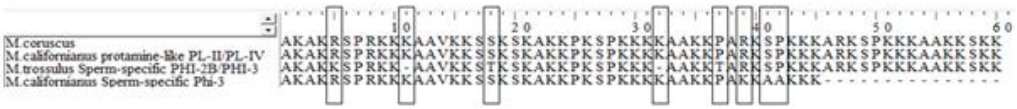



**Fig. 2.2. Alignment of the purified peptdie amino acid sequecne with the *Mytilus* species.** Unconserved amono acids are indicated by boxes. (ABC18200.1) *Mytilus californianus*, (Q05831.2) *Mytilus trossulus*, (P11860.1) *Mytilus californianus*.

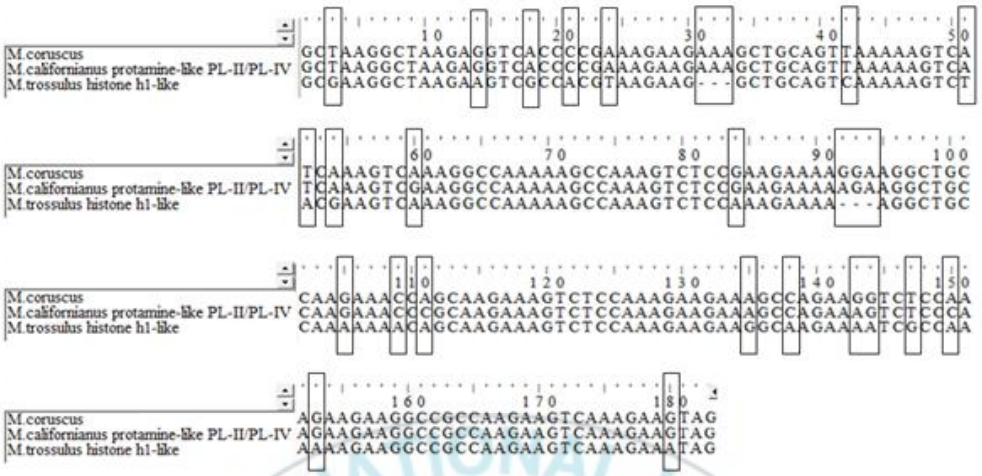



**Fig. 2.3. Alignment of the nucleotide sequence with the *Mytilus* species.** Unconserved sequences are indicated by boxes. (DQ305038.1) *Mytilus californianus* protamine-like PL-II/PL-IV, (L02876.1) *Mytilus trossulus* histone h1-like.

**2.4. Analysis of antimicrobial activity and stability**

Using degenerated primer and gene specific primer sets based on DNA sequence obtained, the ORF of the protamine-like II/IV precursors was revealed. Based on these sequences, the protein-like II/IV precursors of 60 amino acids were synthesized and antimicrobial activity was observed for various strains (Fig. 2.4). The synthesized peptides also showed antimicrobial activity against Gram-positive strains *B. cereus, B. subtilis, S. mutans*, Gram-negative strains *P. aeruginosa, E. coli*, and in the fungi *C. albicans* (Fig. 2.4). Different concentrations of peptides were tested antimicrobial activity against each strain and strength of activity present as the minimum effective concentration (MEC) (Table 2.2). Gram-positive strains *B. subtilis and S. mutans* showed the most sensitive antimicrobial activity (Table 2.2). Results showing high antimicrobial activity in *S. mutans* showed the match with results of activity in natural form extracted from tissue. Antimicrobial peptides found in various types of *Mytilus* spp. show antimicrobial activity in various microorganism, and in particular, they were more sensitive to Gram-positive strains, but found unique features that were less active only in *B. cereus*. There are 60 compositions based on the primary structure identified in this study.

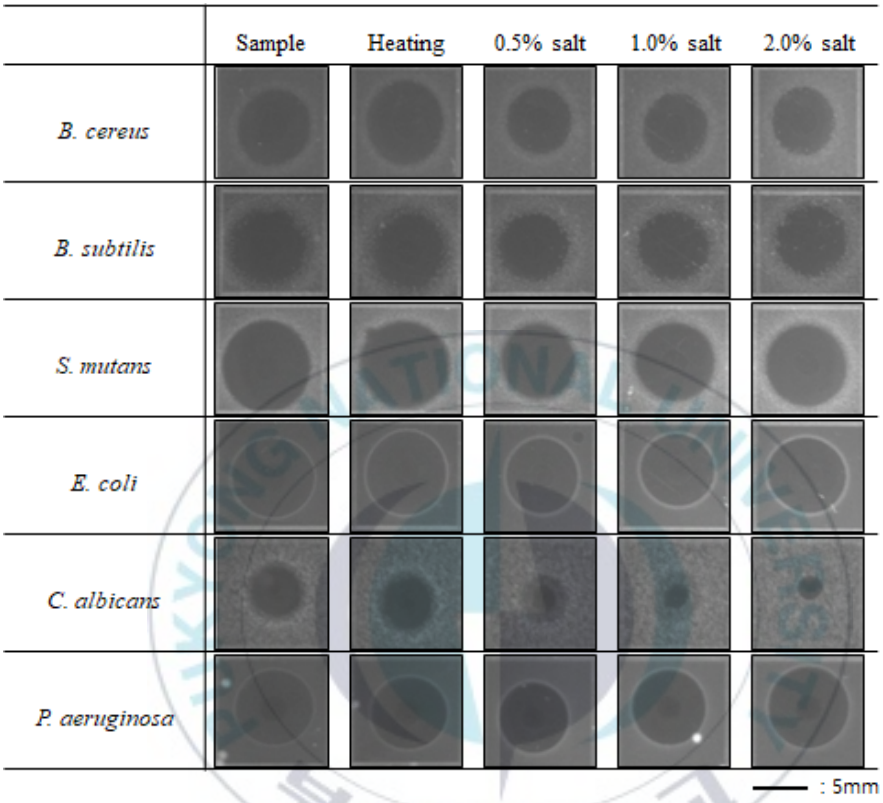
Synthetic peptides showed high thermal stability with no change in antimicrobial activity after 10 minutes of heating at 100°C and showed that activity was not inhibited by 2% of NaCl like other antimicrobial peptide from marine organism while antimicrobial activity derived from land-based organism were inhibited by NaCl. (Fig. 2.4). However antimicrobial activity was inhibited by NaCl against *C. albicans* only. This is expected to be caused by other mechanisms or structural differences in fungi, unlike ordinary microbes, and further studies of these mode of action are thought to provide important information for the various functional studies of antimicrobial peptide and the development of antibiotic replacements for fungi. Therefore, considering this wide range of antimicrobial activity and high thermal stability and salt stability, it was believed that the antimicrobial peptide from *M. coruscus* obtained

in this study would play a sufficiently stable, and it could be expected that there would be a high possibility as a candidate material for an antibiotic substitute for marine industry.

**Table 2.2. Minimal effective concentration (MEC) of peptide.**

| Microbe | Gram | MEC (μg/mL) |
|---|---|---|
| *B. cereus* | + | 20.8 |
| *B. subtilis* | + | 0.2 |
| *S. mutans* | + | 0.2 |
| *E. coli* | - | 2.6 |
| *P. aeruginosa* | - | 5.7 |
| *C. albicans* | fungi | 56.3 |

**Fig. 2.4. Antimicrobial activity and stability of synthesized peptide against 6 strains. Scale bar indicates 5 mm**



**2.5. Quantitative analysis of protamine-like expression**

Analysis of the expression at the mRNA level from each organ tissue separated from the *M. coruscus* showed that the incidence of antimicrobial peptide was the highest in foot and the gills, adductor muscle, hepatopancreas containing gonad, and blood in order (Fig. 2.5). The result was interesting that the expression in adductor muscle and gills was higher than expression in the hepatopancreas containing gonad and even in the case of defensin and molluscidin derived from oyster showed the highest levels of expression in gill. And the antimicrobial peptide in this study also tend to show higher levels of activity in gills after the foot, suggesting that each tissue is accumulating antibacterial materials.

The results of this study show that there are various antimicrobial substances in each of the tissues of *M. coruscus*, as in other marine vertebrates, and evaluated activity and characteristics of antimicrobial peptide separated from the foot muscle were extract. Various findings and properties have also been shown to have novel properties, although they tend to be similar to antimicrobial peptides in other marine animals. Characteristic analysis is carried out through UTR analysis and overexpression of recombined protein based on nucleotide sequence obtained by 5' and 3'-RACE PCR. In addition, it is currently producing various analogs with enhanced activity by modification or replacement of amino acid sequence in peptide.

It will evaluate and conduct research as an antimicrobial peptide with various functions such as anticancer, antioxidant and anti-parasite activity. Applicability of antimicrobial peptide was evaluated through study of mechanism of antimicrobial peptide and further conduct research on the immune system of marine invertebrate. And research of production and purification of antimicrobial peptide will be carried out to developantimicrobial peptide as antibiotic substitute, which is thought to be economically viable in the production and industrialization of marine antibiotic substitute. It is believed to be able to provide a lot of information on mechanism of innate immune system of marine invertebrate and development of antibiotic

replacement materials.

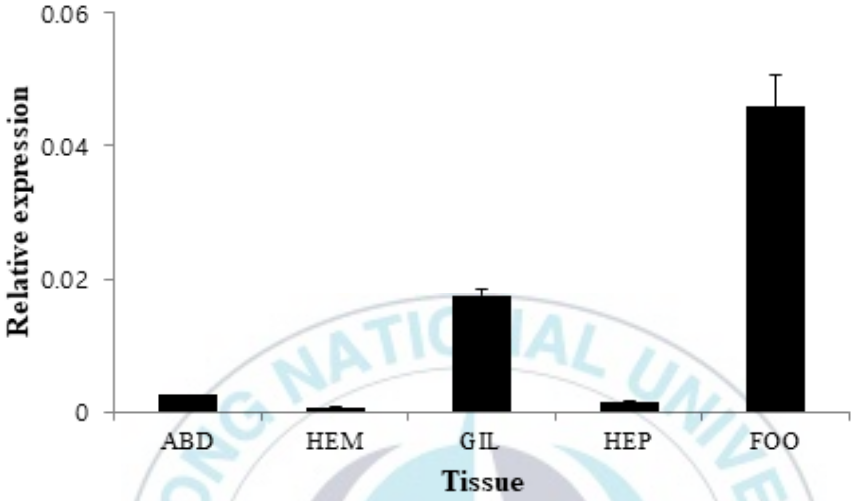



**Fig. 2.5. Quantitative analysis of protamine-like expression in the various tissues.**

Tissue-specific expression of protamine-like. Error bars represent the mean ± SD of three technical replicates. ABD: abductor muscle, HEM: Hemocyte, GIL: gills, HEP: Hepatopancrea, FOO: Foot.

**Conclusion**

In this study, we investigated antimicrobial peptide from the acidified muscle extract of *Mytilus coruscus*, which mostly inhabits China, Japan, and Korea, to develop a natural product-derived antibiotics substitution in terms of its abuse and restriction. Antimicrobial peptide was purified by $C_{18}$ reversed-phase high-performance liquid chromatography and was detected as having a molecular mass of 6,701 Da by MALDI-TOF/MS. The N-terminal amino acid sequence of the purified peak was obtained from edman degradation, and 20 identified residues shown 100% identity with the N-terminus region of sperm-specific protein and protamine-like PL-II/PL-IV precursor of *Mytilus californianus*. We also identified 60 open-reading frame (ORF) encoding amino acids with 183 bp of purified peptide based on the obtained amino acid residues. The amino acid sequence of ORF showed 100% and the nucleotide sequence revealed 97.2% identity with the protamine-like PL-II/PL-IV precursor of *Mytilus californianus*. Synthesized antimicrobial peptide showed antimicrobial activity against Gram-positive bacteria, including *Bacillus cereus* (minimal effective concentration [MEC], 20.8 μg/mL), *Bacillus subtilis* (MEC, 0.2 μg/mL), *Streptococcus mutans* (MEC, 0.2 μg/mL), Gram-negative bacteria including *Pseudomonas aeruginosa* (MEC, 5.7 μg/mL), *Escherichia coli* (MEC, 2.6 μg/mL) and fungi, *Candida albicans* (MEC, 56.3 μg/mL). In addition, synthesized peptide showed stable activities under heat and salt conditions against Gram-positive and Gram-negative bacteria, but was inhibited by salt against only *C. albicans*. With these results, isolated peptide from *M. coruscus* could be an alternative agent to antibiotics for defending against pathogenic microorganisms, and helpful information to understand the innate immune system of marine invertebrates.

## Chapter 3.

## The antimicrobial characteristics of McSSP-31 purified from the hemocyte of *Mytilus coruscus*



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### 3.1. Screening of the acidified crude extract of *M. coruscus*

Antimicrobial substances were isolated from blood of *M. coruscus* by hydrothermal extraction with acetic acid. The antimicrobial activity of the obtained extract was analyzed using the 9 strains of Gram-positive bacteria, 14 strains of Gram-negative bacteria, and the 1 species of fungi. Blood cell extracts showed strong activity against Gram-positive strains including *B. subtilis* and *Streptococcus parauberis* and Gram- negative strains including *Enterobacter cloacae* and *Vibrio parahamolycus* and low activity for fungi *C. albicans* and antibiotic-resistant strains (Table 3.1). Among them, *B. subtilis* was used as an indicator strain in the URDA experiment for verifying antimicrobial activity during purification process.

Antimicrobial peptides in freshwater fish were reported with defensin, mytilin, myticin and myymycin, and many studies were conducted mainly in the *Mytilus galloprovincials* and *Mytilus edulis* [G. Mitta et al, 2000a; G. Mitta et al., 2000b; G. Mitta et al., 2000c]. However, the antimicrobial activity spectrum of these antimicrobial peptides is reported to be limited in activity to specific Gram-positive, Gram-negative or fungi depending on the type, and there are not many results that the activity varies in both Gram-negative bacteria, Gram-positive bacteria and fungi, as in the blood extract in this study [G. Mitta et al, 2000a; G. Mitta et al., 2000b; G. Mitta et al., 2000c]. Defensin and myticin are known to have strong antimicrobial activity in Gram-positive bacteria, low antimicrobial activity in Gram-negative bacteria and fungi, and myticin is known to be only strong in fungi [G. Mitta et al, 2000a; G. Mitta et al., 2000b; G. Mitta et al., 2000c]. However, it has been reported that there are many different isoforms in the mytilin, which vary in antimicrobial spectrum [G. Mitta et al, 2000a; G. Mitta et al., 2000b; G. Mitta et al., 2000c]. Among them, mytilin B, C and D are reported to have antimicrobial activity in Gram-positive bacteria and G1 type only in Gram-positive bacteria. Thus, the blood cell-derived extract of *M. coruscus* extracted from this study could be determined to have a wide antimicrobial spectrum showing antimicrobial activity in Gram-positive bacteria,

Gram-negative bacteria and fungi because of a mixture of various ingredients other than pure single peptides, and intended to separate pure antimicrobial peptide using HPLC. It could be various treatments and preventive drugs because they show antimicrobial activity against strains such as *B. cereus*, *B. subtilis, E. coli*, *S. parauberis*, *Pseudomonas aeruginosa*, and *V. parahamolyticus*.

**Table 3.1. Screening of the acidified hemocyte extract of *M. coruscus*.**

| Strains | Activity | Strains | Activity | Strains | Activity |
|---|---|---|---|---|---|
| **Gram positive** | | **Gram negative** | | **Fugus** | |
| *B. cereus* KCTC 1012 | ++ | *E. coli* CCARM 1A814 * | - | *C. albicans* KCCM 11282 | + |
| *B. subtilis* KCTC 1021 | +++ | *E. coli* KCCM 40271 | ++ | | |
| *S.aureus* KCCM 1133 | - | *E. cloacae* CCARM 0252 * | - | | |
| *S.aureus* CCARM 0203* | - | *E. cloacae* KCTC 2361 | +++ | | |
| *S.aureus* CCARM 0204* | +++ | *K. pneumoniae* KCTC 12385 | ++ | | |
| *S.aureus* CCARM 3795* | - | *P. mirabilis* KCTC 2510 | - | | |
| *S.mutans* KCTC 3065 | - | *P. stuartii* KCTC 2569 | ++ | | |
| *S. parauberis* KCTC 3651 | +++ | *P. aeruginosa* KCTC 1636 | ++ | | |
| | | *V. parahaemolyticus* KCCM 41664 | +++ | | |

Antimicrobial activity based on the diameter of inhibition zone (mm) follow this skim: +++ ≥7 mm, ++ 4-7 mm, + ≤4 mm
Asterisk (*) show antimicrobial activity against antibiotic resistant bacteria.

### 3.2. Peptide purification

After centrifugation, various peaks were obtained and refined through the retrograde column by removing solids by passing the upper solution through a 0.45 μm filter (Milipore, Darmstadt, Germany). In the first stage of refining, more than 30 subdivisions were secured using CapCell-Pak $C_{18}$ reversed-phase column, and each separated fraction was checked for antimicrobial activity against *B. subtilis* by URDA method. The results showed antimicrobial activity in some subdivisions. It was shown that the 16 minute elution from the 220 nm absorption wavelength of the various segments was very low in detection for wavelengths of 254 nm and 280 nm and that the clear zone diameter on the URDA had strong antimicrobial activity with a clear zone diameter of about 7.7 mm (Fig. 3.1A). This division was further refined to ensure the final three subdivisions, of which antimicrobial activity was found in a single peak eluted at 13.8 minutes (Fig. 3.1B). Antimicrobial activity was compared before and after the trypsin treatment to check the proteinicity of the final purified antimicrobial peptide. The trypsin used in the experiment breaks down the peptide bonds after lysine and arginine amino acid residues, which are often held by antimicrobial peptides known as representative protein breakdown enzymes. The URDA method showed that the antimicrobial activity after the trypsin treatment was completely lost (Fig. 3.1B). These results showed that the candidate material that was isolated in this study was a protein antimicrobial substance and convinced that it was an antimicrobial peptide that contained one or more lysine or arginine residues in the sequence of amino acids, the primary structure [M. Charlet et al., 1996; A. Kesarcodi-Watson et al., 2008;Y. Shai 2002;H. H.W. Van't et al., 2001;S. S. Yechiel 2000]. The final purified antimicrobial peptide candidate material was freeze-dried for the sequence of amino acids, which is the primary structure, and N-terminated amino acid sequencing by MALDI-TOF/MS molecular weight measurement and Edman decomposition (Fig. 3.1C).

(A)

(B)

(C)

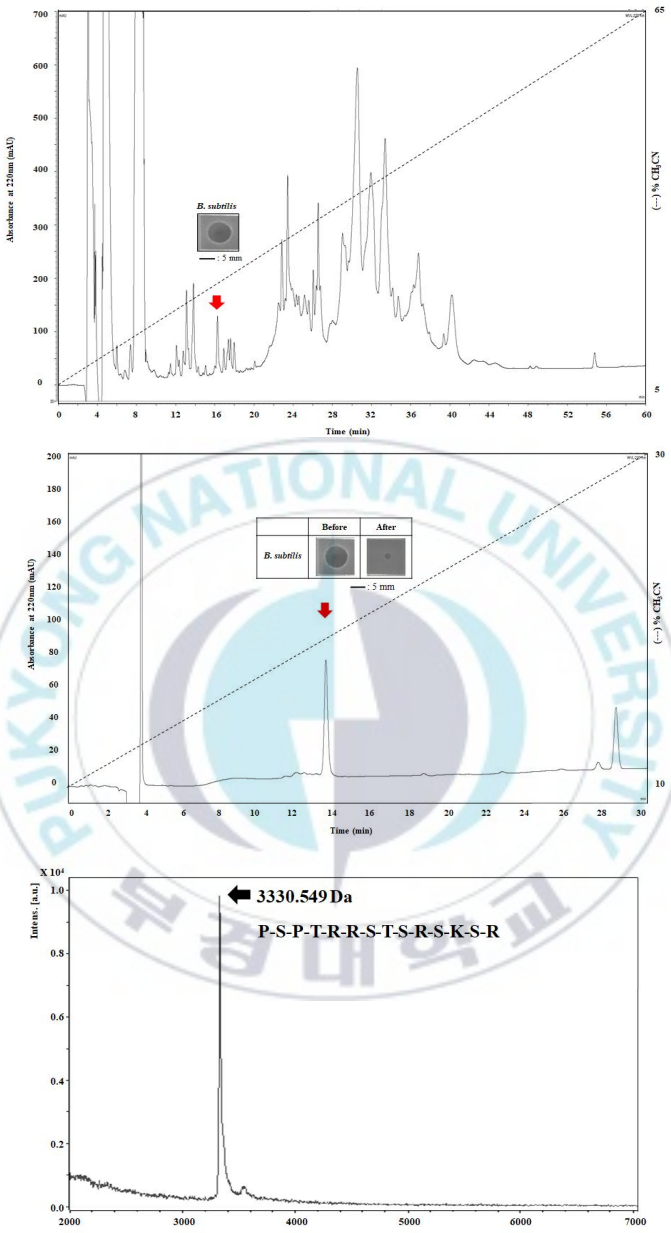



**Fig. 3.1. Purification and antimicrobial activity of purified peptide and mass spectrometry analysis with N-terminal sequences determined by Edman degradation.**

(A) Antimicrobial activity of the extract against *B. subtils*. The acidic extract was

fractionated by the CapCell-Pak $C_{18}$ reversed-phase column. Elution was performed with a linear gradient of 5%→65% $CH_3CN$ (pH2.2) in 0.1% TFA for 60 min at a flow rate 1.0 mL/min. The eluate was monitored at 220 nm. Fraction of the absorbance peak showed antimicrobial activity against *B. subtils* (inset).

(B) Antimicrobial activity of the purified peak (before) and trypsin treated purified peak (after) against *B. subtils*. Active fraction was applied to the TSK-gel ODS-80$^{TM}$ $C_{18}$ reversed-phase column. Elution was performed with a linear gradient of 10%→ 30% $CH_3CN$ (pH2.2) in 0.1% TFA for 30 min at a flow rate of 1.0 mL/min. The eluate was monitored at 220 nm. Fraction of the absorbance peak (indicated by the arrow) showed antimicrobial activity (before) and trypsin treated to purified extract (after) against *B. subtils*. Scale bar indicates 5 mm. (C) Mass spectrometry analysis of single mass with 3330.549 Da and N-terminal sequences (14AA) determined by Edman degradation.

### 3.3. Peptide identification

MALDI-TOF/MS analysis was conducted to check the molecular weight of the final purified tuna blood cell derived antimicrobial peptide and the degree of separation after refining it and confirming that it is a single substance with a molecular weight of 3,330.549 Da (Fig. 3.1C). In order to identify the primary structure of the N-terminal of the pure separated fraction, the sequence of 14 amino acids was identified and the sequence of amino acids found was P-S-P-T-R-S-R-S-S-K-S-R (Fig. 4.1C).

### 3.4. Cloning and sequence analysis of cDNA

14 N-terminal sequences found by Edman degradation were searched in GenBank database of NCBI and the results exhibited that 93% (13 of 14 residues) matched with sperm-specific protein Phi-1 of *M. californianus,* 87% matched with protein-like PL-III protein (13 of 14 residues), and 87% matched with sperm-specific protein Phi-1 of *M. edulis* (12 of 14 residues) (Fig. 3.2B). Unusually, the most similar of known antimicrobial proteins from *M. coruscus* was mytilin-5 precursors and showed a low similarity of 35% (5 of 14 residues). As such, antimicrobial peptides and related studies and reports from *M. coruscus* are few in comparison to other *Mytilus* species, and it is believed that the character analysis of sperm-specific protein from *M. coruscus* obtained in this study may later be important research materials related to antimicrobial activity and characteristics of marine invertebrates.

Based on the amino acid sequence of obtained N-terminal amino acids and sperm-specific protein of other *Mytilus* species, the gene specific primers and degenerated primers were designed to analyze UTR sequences of 5' and 3' along with the ORF (Fig. 3.2). An analysis of the sequence after cloning the products obtained by the PCR reaction using each primer found that the antimicrobial peptide that was separated consisted of 306 bp of ORF coding 101 amino acids, 5'UTR of 15 bp and 3'UTR of 54 bp (Fig. 3.2A). The molecular weight of the antimicrobial peptide by

MALDI-TOF mass spectrometers was about 3,330 Da, and based on this molecular weight information and a sequence of amino acids found, the antimicrobial peptide derived from *M. coruscus* in this study was 31 amino acids from proline (Pro, P) following start codon, methionine (Met, M) (Fig. 3.2). In addition, obtained full amino acid sequence of antimicrobial peptide from *M. coruscus* was compared and analyzed with other antimicrobial peptides from *Mytilus* spp (Fig. 3.3B). Amino acid sequence of peptide in this study showed 93.5% homology with sperm-specific protein and 76.4% homology with protamine-like PL-III from *M. californianus,* and protamine-like PL-III 84.3% homology with *M. trossulus* 79.4% homology with sperm-specific protein from *M. edulis*. Therefore, the peptide of 3.3 kDa derived from hemocytes of *M. coruscus* obtained in this study was confirmed to be an antimicrobial peptide derived from sperm-specific protein Phi-1 (PL-III) of *M. coruscus,* reported for the first time related to antimicrobial activity and named sperm-specific protein-31 (McSSP-31). As we are currently looking for a variety of McSSP-31 isoforms, and we are conducting research on low-molecular analogs that enhance the activity of peptides, we expect to be able to provide a variety of information related to the immune system of marine.

Sperm nuclear basic proteins (SNBPs) are attached to nuclear DNA at the time of sperm formation, engaged in structural stability and package, divided into histone (H) types, protamine (P) types, and protamine-like (PL) types. PL-II, PL-III(Ø1), and PL-IV(Ø3) belonging to sperm-specific protein, which is a representative protein of SNBPs, have the intermediate structure and function of the protamine and histone. The PL type has a relatively small percentage of histone compared to other species of bivalve and has a high content of arginine, a basic amino acid. And in mussel, somatic histone is replaced by SNBPs in response to DNA, which is reported to be related to sperm growth [D.D Heath and T.J. Hilbish, 1998;S. Ruiz-Lara et al., 1993;A. Santel eet al., 1997;]. Studies of these characteristics have been studied a lot in marine invertebrates, but little information on genes and frequent gene sequence

variation in very close species. Therefore, it is believed that the results of this study and subsequent research will reveal the proteins properties related to the reproduction and generation of the *M. coruscus*, and the results of the research on the antimicrobial activity and properties of antimicrobial peptide involved in the generation process will provide a variety of data in terms of phylogenesis, evolutionary biology, and immunology.

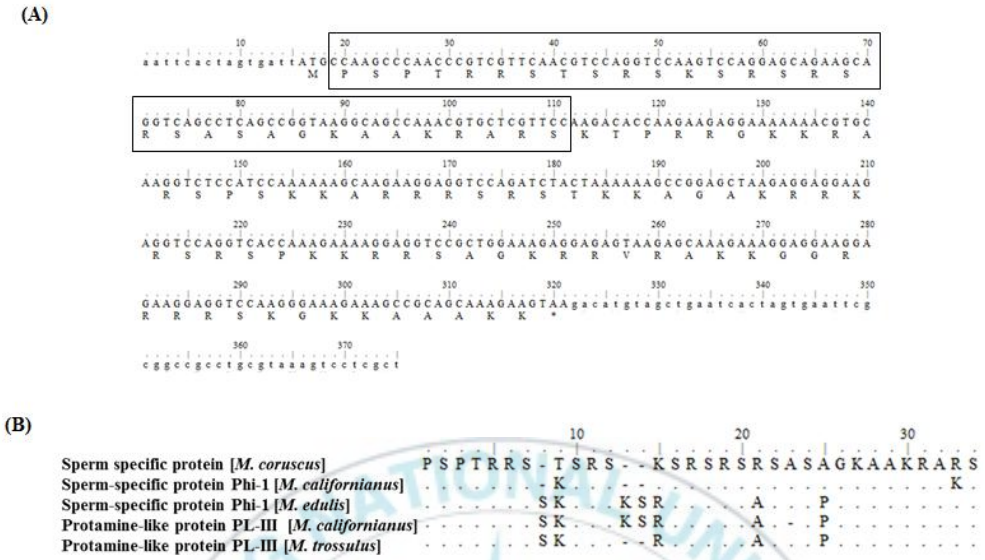



**Fig. 3.2. Sequence analysis of sperm-specific protein**

(A) Full cDNA sequence of sperm-specific protein from *M. coruscus*. (B) Sequence comparison of the N-terminal region of protamine-like protein and sperm-specific protein from *M. californianus, M. trossulus* and *M. edulis.*

### 3.5. Antimicrobial activities and stability of McSSP-31

Based on the base sequence obtained through cDNA cloning and RACE analysis, McSSP-31 consisting of 31 amino acids was synthesized to verify antimicrobial activity and evaluate the properties. The synthesized antimicrobial peptide showed activity against Gram-positive strains *B. subtilis*, *Streptococcus mutans*, *Staphylococcus aureus*, and showed activity against Gram-negative strains *E. coli*, *Klebsiella pneumoniae, Proteus mirabilis* and *P. aeruginosa* and weak antimicrobial activity against fungus *C. albicans* (Fig. 3.3A). The synthesized antimicrobial peptide showed higher antimicrobial activity against the Gram-negative strains than against the Gram-positive strains, with the strongest activity in *E. coli* and *P. aeruginosa* among the Gram-negative strains. McSSP-31 also confirmed that there is a similar trend of antimicrobial activity in Gram-negative strains with high activity and low activity in Gram positive strains and fungi, rather than showing an antimicrobial spectrum for a wide range of strains, such as defensin and myticin, which are antimicrobial peptides separated from other *Mytilus* sp. (Fig. 3.3A).

The thermal and salt stability of separated and synthesized McSSP-31 was measured using the URDA method (Fig. 3.3A). When the active change in McSSP-31 was measured by heating at 100°C for 10 minutes, it was not possible to observe the change in antimicrobial activity in strains other than *C. albicans*, thereby providing very good thermal stability (Fig. 3.3A). In addition, 0.5-2% NaCl was added to assess the bacterial activations by saline. However, when stability through NaCl addition was evaluated, McSSP-31 retained antimicrobial activity in the gram negative strains only, and in the gram positive strains, the higher the NaCl concentration, the less antimicrobial activity was achieved (Fig. 3.3A). And for the fungal species *C. albicans*, the antimicrobial activity was confirmed to disappear due to the addition of 0.5% NaCl. Activity of antimicrobial peptides separated from terrestrial animal are reported to be inhibited by NaCl, but antimicrobial peptides derived from marine organism are known to have no effect on antimicrobial activity by NaCl [R, Oh et

al., 2016]. However, McSSP-31 separated from the blood cells was able to observe the results of antimicrobial activity inhibited by NaCl, even though it was isolated from mussel. These changes in antimicrobial activity were only found in Gram-positive bacteria and fungi, so they could be expected as a result of differences in action techniques due to structural differences in cell walls and cell membranes different from Gram-negative bacteria, and by analyzing amino acid sequences, structures, and antimicrobial activity characteristics, they will provide important information for the development of antimicrobial peptide for control of Gram-positive bacteria and fungi. It also predicted that Gram-negative strains will not be subject to active salt damage, so they will be developed and utilized as an additive material for control of Gram-negative bacteria. The synthesized McSSP-31 was measured for antimicrobial activity against antibiotic-resistant of 11 strains in 8 species (Table 4.2). Antimicrobial activity for antibiotic-resistant strains was weak, but especially activity was sensitive against *S. aureus* CCARM 0203, *S. aureus* CCARM 0204 and *E. coli* CCARM (Fig. 4.3B). Analysis of thermal stability by heating at 100℃ shows that synthetic peptides has greater activity against *S. aureus* CCARM 0204 after heating, there has been no change in activity in the rest of the strains, and antimicrobial activity against *S. aureus* CCARM 3795 has disappeared (Fig. 3.3).

In addition, the stability of antimicrobial activity after NaCl treatment for synthetic peptides was analyzed, and there was no noticeable change in activity in the other strains except for the *S. aureus* CCARM 3795, but the strength of activity was reduced similar to that of general indicator strains (Fig. 3.3B). Antimicrobial activity against *S. aureus* CCARM 3795 was nearly lost after heating and NaCl processing (Fig. 3.3B). They belong to Gram-positive strain, but its sensitivity varies against antibiotic-resistant strains. In addition, these characteristics are thought to be related to antibiotic resistance mechanism, so further research is needed to develop materials for the control of antibiotic resistant strains.

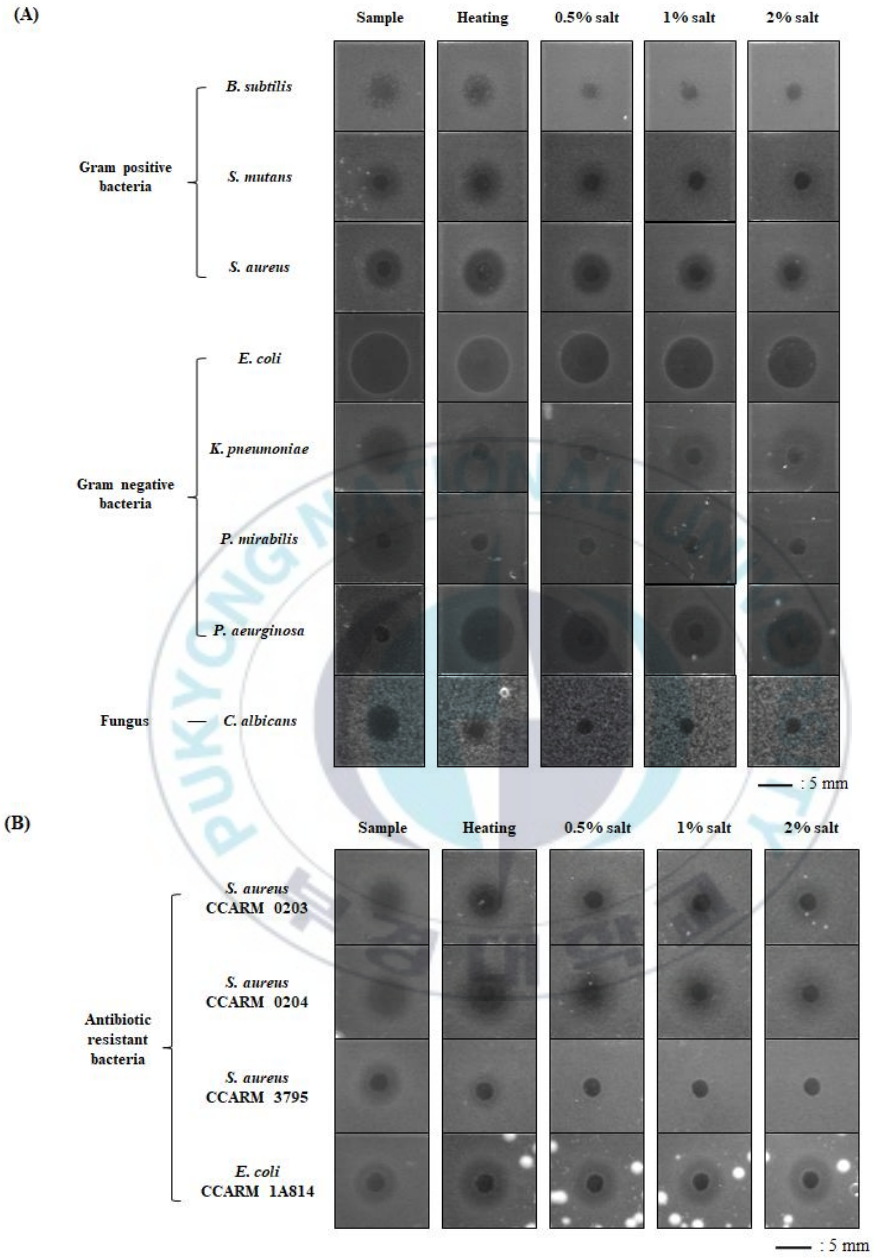



**Fig. 3.3. Antimicrobial activity and stability of synthesized peptide against Gram positive, Gram negative bacteria, fungi (A), and antibiotic resistant bacteria (B). Scale bar indicates 5 mm.**

### 3.6. Hemolytic activity

The hemolytic activity of McSSP-31 was analyzed in the red blood cells of olive flounder. Piscidin 1 and 1% Triton X-100, used as a positive control, showed very strong hemolytic activity at 100 μg/mL, the synthetic peptide showed very low hemolytic activity at the same concentration compared to the two controls (Fig. 3.4A). In addition, after measuring the hemolytic activity of McSSP-31 by concentration, the positive control, piscidin 1, showed 100% hemolytic activity at high concentration 100 μg/mL and rapidly reduced hemolytic activity to 11.6% at low concentration 6.5 μg/mL. On the other hand, McSSP-31 showed 15.2% hemolytic activity at high concentrations of 100 μg/mL and approximately 5.0% hemolytic activity at low concentrations of 6.5 μg/mL (Fig. 3.4B). Therefore, it was possible to expect that McSSP-31 derived from *M. coruscus* in this study would be utilized as an additive to treat bacterial diseases.

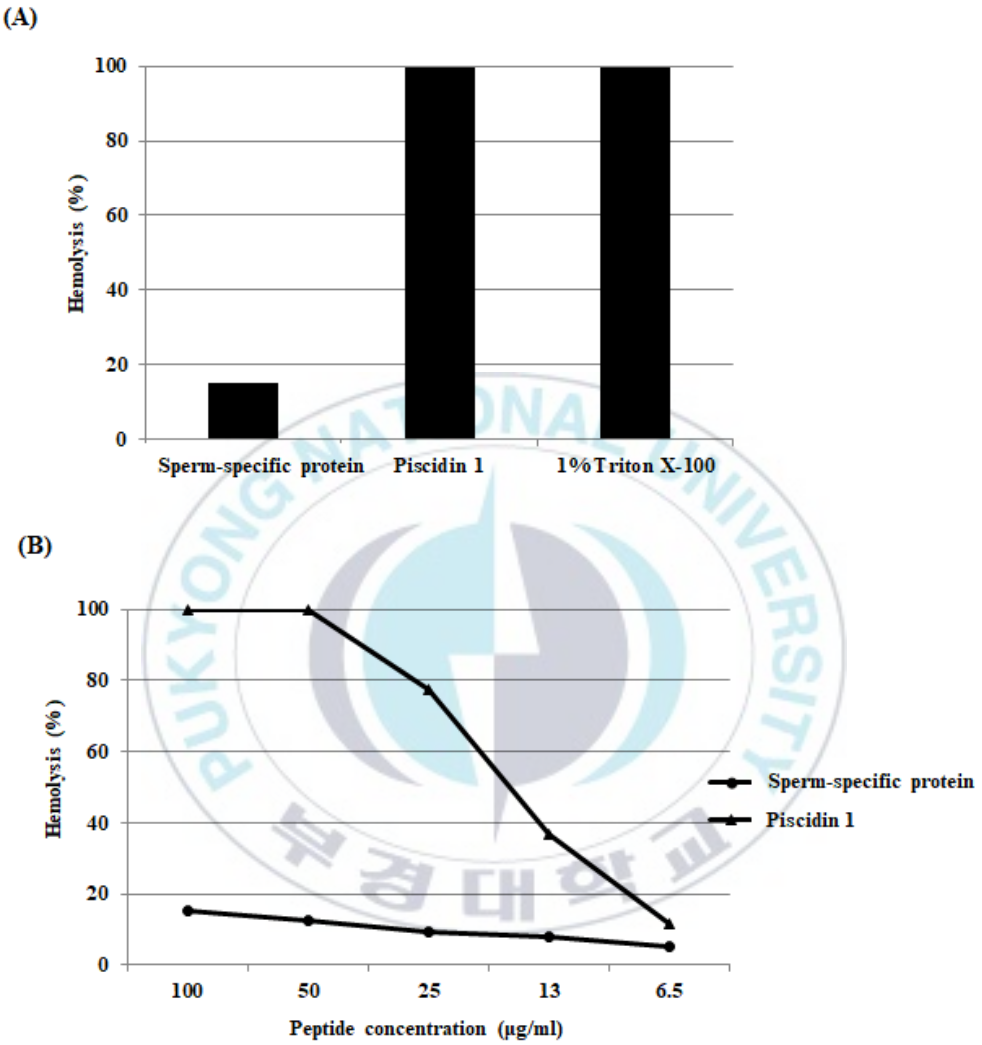



**Fig. 3.4. Hemolytic activity of sperm-specific protein and piscidin 1 against erythrocytes of flounder (*Paralichthys olivaceus*).** (A) The concentration of each sample was 100 μg/mL, (B) Effect of hemolysis depending on concentrations (100, 50, 25, 13, 6.5 μg/mL).

### 3.7. Cytotoxicity of McSSP-31

To check toxicity to HUVEC cells, the cell toxicity was compared and evaluated using a positive control of 50 μg/mL concentration of McSSP-31 and melittin found in the bee venom consisting of 26 amino acids has excellent antimicrobial and anti-cancer activity reported in Fig 3.5 [D. Wade et al., 1992;H. Zhu et al., 2014]. The survival rate of the HUVEC cells was verified by treating each sample and the MTS solution after incubation for 24 hours. The degree of cell toxicity was analyzed by measuring cell viability using the MTS solution. When McSSP-31 was treated, the higher cell survival rate was found at 98.2%. There was no morphological change, but the lower survival rate of 11.1% was observed with melittin was treated. However, with these activity, the strong cytotoxicity limits its use in areas that require antimicrobial peptide, but McSSP-31 with 31 amino acids found in this experiment is expected to have low cytotoxicity and be available as an antimicrobial material in a variety of areas.

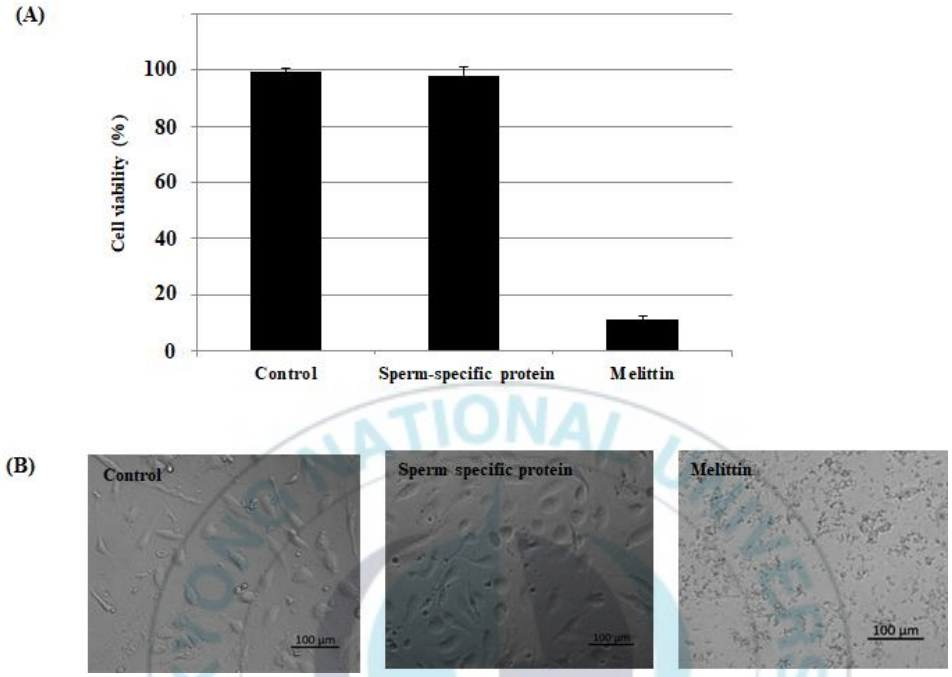


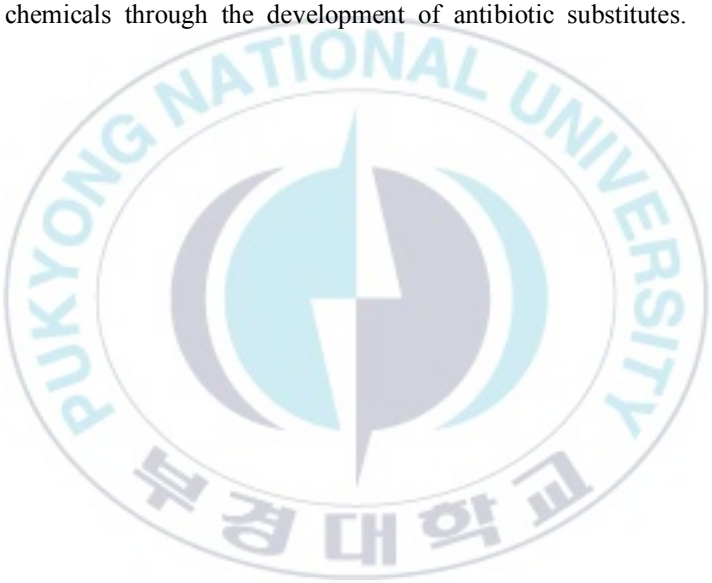

**Fig. 3.5. Cell viability on HUVEC cell by MTS assay. (A) Determination of the cell viability after treatment of 50 μg/mL of peptide and melittin for positive control on HUVEC cell.** Error bars represent the mean ± SD of three technical replicates. (B) Differentiation of HUVEC cell mophology after treatment of peptide and melittin for 24hr.

### 3.8. Quantitative analysis of McSSP-31 expression

The expression at the mRNA level from the seven tissues separated from the *M. coruscus* were analyzed as qPCR. The expression of McSSP-31 was the highest in the adductor muscle, followed by hemocyte, foot muscles, mantle, hepatopancreas, gill, and siphon (Fig. 3.6). Therefore, the study found that McSSP-31 is not a tissue specific protein that is expressed only in certain tissues, but rather a gene that is highly expressed in all tissues of *M. coruscus*. Although the main functions of the group of sperm-specific proteins and protein-like proteins in mussel are sperm cell formation and DNA stabilization, similar results were found in this study as they are known to be genes that distribute not only in reproductive cells of living organisms but also in high copy rate in tissues [D.D. Heath and T. J. Hilbish, 1998].

Various marine invertebrates are reported to be highly dependent on the innate immune system due to poor acquired immune system and to have a large number of antimicrobial-related substances in the blood cells and plasma components of various tissues. However, in this study, it was found that only blood cell extracts of body fluid had high antimicrobial activity, and that there was one or more different antimicrobial substances in purification step. Among them, a single separation of antimicrobial peptide measuring approximately 3.3 kDa was purified, and studies such as N-terminal analysis and cDNA cloning showed that it was a fragment of sperm-specific protein derived from *M. coruscus*. Antimicrobial peptide, consisting of 31 amino acids, from proline$^{2}$ to the serine$^{32}$ of sperm-specific protein, had no hemolytic activity on the blood cells, and it was also shown that there was no hemolysis to human HUVEC cells. Antimicrobial peptide separated from some marine organisms, such as piscidin 1, is reported to have excellent antimicrobial activity but have limited industrial use due to hemolyisis [J.K. Seo et al., 2017]. McSSP-31 separated from in this study had antimicrobial activity against Gram-positive, negative strains and fungi. Since there is little hemolytic activity and cytotoxicity, it is thought that it will be highly likely to be used as a substitute for antibiotics such as feed

additives and treatments for fisheries products. Based on the sequences and secondary structure of amino acids, analogs are designed with increased antimicrobial activity through substitution and amidation of amino acids and evaluate various functions such as anti-parasite activity, antioxidant activity, and anti-inflammatory activity. In addition, mass production conditions are being tested for industrial use by evaluating the expression and activity of recombinant proteins. Therefore, if these results are analyzed and utilized, it will provide a lot of information on the antimicrobial peptide and innate immune system of marine invertebrates, further contributing greatly to the reduction of chemicals through the development of antibiotic substitutes.



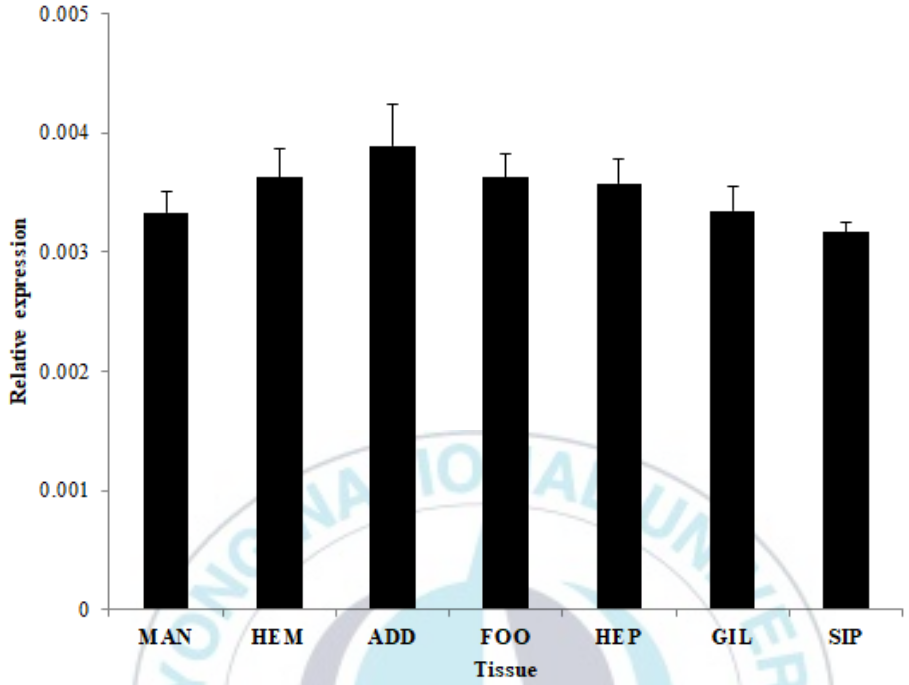



**Fig. 3.6. Quantitative analysis of sperm-specific protein expression in the various tissues.**

Tissue-specific expression of sperm-specific protein. Error bars represent the mean ± SD of three technical replicates. MAN: mantle, HEM; hemocyte, ADD; adductor muscle, FOO; foot, HEP; hepatopancreas, GIL; gill, SIP; siphone.

**Conclusion**

We isolated and purified antimicrobial peptide from acidified hemocyte extract of *Mytilus coruscus*. Antimicrobial peptide was purified by $C_{18}$ reversed-phase high performance liquid chromatography (HPLC) and determined as 3330.549 Da by matrix-assisted laser desorptionionization time-of-flight mass spectrophotometer (MALDI-TOF/MS). N-terminus 14 amino acid sequence was identified as P-S-P-T-R-R-S-T-S-R-S-K-S-R by edman degradation method. The acquired sequence showed 93% similarity with sperm-specific protein Phi-1 from *M. californianus*. The identified open-reading frame (ORF) of peptide was 306 bp encoding 101 amino acids by rapid amplification of cDNA ends (RACE), cloning and sequencing analysis. We compared the full length sequence with other known proteins that revealed the high identity with sperm-specific protein Phi-1 (93.5%) of *M. californianus*.

We synthesized antimicrobial peptide (McSSP-31), which showed antimicrobial activity against Gram-positive bacteria including *B. subtilis*, *S. mutans*, *S. aureus* and Gram-negative bacteria including *E. coli*, *K. pneumoniae*, *P. mirabilis*, *P. aeruginosa* and fungi, *C. albicans*. Also, synthesized antimicrobial peptide showed strong antimicrobial activity against antibiotic-resistant strains including *S. aureus*. The cytotoxicity of peptide was determined with the HUVEC human cell line. The peptide did not exhibit any significant cytotoxic effects on normal human cell line, and showed very low hemolytic activity to flounder hemoglobin. The results of this research demonstrate that purified peptide from hemocyte of *M. coruscus* have antimicrobial activity against various bacteria and potential as alternative agent of antibiotics.

## Chapter 4.

## Mytilin B, Antimicrobial peptide from the hemocyte of *Mytilus coruscus* : Isolation, purification and characterization



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### 4.1. Screening of the acidified crude extract of *M. coruscus*

Antimicrobial substances separated from blood cell of *M. coruscus* were extracted by hydrothermal extraction with added acetic acid, and a total of 25 strains, including 10 strains, 6 species of Gram-positive strains, 14 strains, 8 species of Gram-negative, and 1 fungus, were evaluated as representative bacteria. The blood cell extract showed strong antimicrobial activity in Gram-positive strains including *B. subtilis* and *Streptococcus parauberis* and Gram-negative strains including *Enterobacter cloacae* and *V. parahamolycus*. It also showed anti-bacterial activity in the fungus *Candida albicans* (Table 4.1). Among them, *B. subtilis* was used as an indicator strain of the URDA method for verifying its activity during the purification, since it showed the strongest antimicrobial activity in *B. subtilis*.

Antimicrobial peptides have been reported that possess activity against a wide range of harmful microorganisms, such as Gram-positive strains, Gram-negative strains, fungi, viruses and parasites, but the range of antimicrobial activity in most antimicrobial peptides is known to vary widely depending on the Gram-positive or Gram-negative strains [I.N. Hsieh and K. L. Hartshorm 2016]. It has been shown that most organism produce different kinds of peptides to effectively respond to pathogens invading from the outside, and that various antimicrobial peptides work together to have a wide spectrum through complementary effects [G. Yu et al., 2016]. However, it was found that the blood cell extracts derived from *M. cocuscus* used in this study and the tissue extracts from the blood cells, foot, gills, adductor muscle and hepatopancrea in previous studies had a very wide range of antimicrobial activity [R. Oh et al., 2016; R. Oh et al., 2017]. This may be achieved due to the superior antimicrobial activity of peptides contained in tissue extracts. And when compared with other tissue extracts in *M. cocuscus*, activities were recorded in the case of blood cell extracts a wide range of antimicrobial peptides indicating strong activity against Gram-positive strains, Gram-negative strains and fungi, and therefore determined that more diverse and more antimicrobial than other tissues [R. Oh et al.,

2016]. Based on these results, blood cell extracts were selected from *M. coruscus* and isolated and purified using HPLC.

**Table 4.1. Antimicrobial activity of hemocyte extract of *M. coruscus* against pathogenic bacteria**

| | Strains | Activity |
|---|---|---|
| Gram positive bacteria | *B. cereus* | ** |
| | *B. subtilis* | *** |
| | *S. aureus* | T |
| | *S. mutans* | * |
| | *S. parauberis* | *** |
| Gram negative bacteria | *E. cloacae* | *** |
| | *E. coli* | ** |
| | *K. pneumoniae* | * |
| | *P. mirabilis* | T |
| | *P. stuartii* | ** |
| | *P. aeruginosa* | ** |
| | *V. parahaemolyticus* | *** |
| Fungi | *C. albicans* | * |

Asterisk represent the diameter of clear zone. *: 3.0-4.9mm, **: 5.0-6.9mm, ***: more than 7.0mm, T: Trace

### 4.2. Isolation and purification of peptide by HPLC

Hemocyte extracts of *M. coruscus* were isolated and purified using HPLC to obtain pure single fractionation. Various fractions obtained through CapCell-Pak $C_{18}$ reverse column used in the first purification were verified for antimicrobial activity against *B. subtilis* by URDA method. At 220 nm wavelengths and at the rest of the wavelengths, low-variable peaks were selected for antimicrobial activity, and activity was shown in the eluted peak at 16 minutes, 22-28 minutes and 29-35 minutes, showing highly sensitive antimicrobial activity with a clear zone of 5.5 mm in the eluted fraction at approximately 26 min of these (Fig. 4.1). This fraction was finally purified by performing one more purification process using PEPTIDE XB-$C_{18}$ reverse column (Fig. 4.2). To determine the protein material of this antimicrobial material, the URDA method confirmed the change in antimicrobial activity against *B. subtilis* by treating and adding the proteinase K, which breaks down the C-terminal of fatty or aromatic amino acids that are high in antimicrobial peptide. After observing antimicrobial activity, the size of clear zone was 6.3 mm prior to the treatment of proteinase K, but the antimicrobial activity was completely lost (Fig. 4.2). This confirms that the final purified peptide is an antimicrobial peptide containing one or more of the fatty or aromatic amino acids and has lost antimicrobial activity due to decomposition by proteinase K.

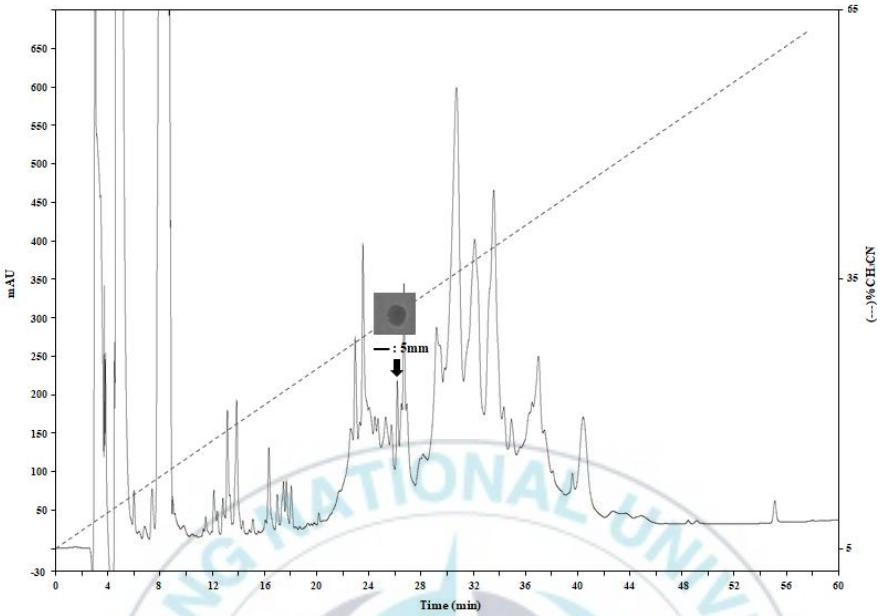



**Fig. 4.1. Purification and antimicrobial activity of hemocyte extract of *M. coruscus*. The extract was fractionated by the CapCell-Pak $C_{18}$ reversed-phase column.** Elution was performed with a linear gradient of 5-65% $CH_3CN$ in 0.1% TFA for 60 min at a flow rate of 1mL/min. The eluate was monitored at 220 nm. Fraction of the absorbance peak (indicated by the arrow) showed antimicrobial activity against B. subtilis (inset). The elution point of the active peak was at 29% $CH_3CN$. Scale bar indicates 5mm.

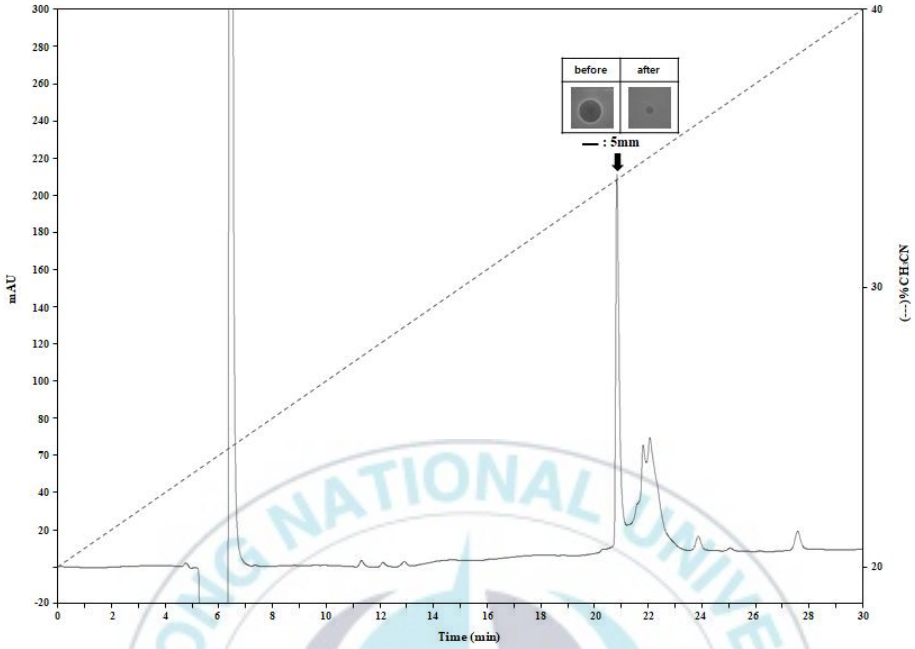



**Fig. 4.2. Final purification of pooled active fraction and antimicrobial activity. The active fraction was applied to a PEPTIDE XB-$C_{18}$ reversed-phase column.** Elution was performed with a linear gradient of 20-40% $CH_3CN$ in 0.1% TFA for 30 min at a flow rate of 0.5 mL/min. The eluate was monitored at 220 nm. The elution point of the active peak was at 33% $CH_3CN$ (indicated by the arrow). Antimicrobial activity of the purified peak (before) and proteinase K treated purified peak (after) against *B. subtlis*. Scale bar indicates 5mm.

**4.3. Peptide identification**

MALDI-TOF/MS analysis was conducted to measure the purity and molecular weight of the final purfied peptide separated by HPLC from hemocyte extract of the *M. coruscus,* and the antimicrobial peptide that checked purity and molecular weight was performed by Edman degradation to analyze the N-terminal sequence. MALDI-TOF/MS results confirm the molecular weight of 4041.866 Da (Fig. 4.3). Edman degradation performed to analyze the amino acid sequence at the N-terminal confirmed the sequence of up to 25 amino acids, and identified the sequence of S-C-A-S-V-C-K-A-R-C-R-A-R-R-C-G-Y-Y-V-S-V-F-Y-R-G, including four cysteine. The found antimicrobial peptide contained a number of cysteine, and these cysteine-rich peptides were reported in a variety of organisms, as well as in bivalvia. For example, there are drosomycin separated from *Drosophila,* penaeidin separated from the whiteleg shrimp, tachycitin separated from horseshoe crabs, hepcidin separated from peciformes, and defensin, myticin, myticusin, mytilin from *Mytilus* [8, 10-11, 35]. These include 6-14 residues of cysteine, which together form a number of disulfide bond. This is a chemical bond between the cysteine residues, which closely folds the peptide to stabilize it structurally [R. Oh et al., 2016].

Based on a sequence of 25 amino acids obtained from the N-terminal sequence, results for GenBank database blasts of NCBI showed that 100% identity with mytilin B precursor, 96% (24 of 25 residues) identity with mytilin-8 precursor and mytilin-4 precursor, 92% (23 of 25 residues) identity with mytilin 6 precursor and mytilin 7 precursor, 83% (15 of 18 residues) identity with mytilin 3 precursor, 78% (14 of 18 residues) identity with mytilin 5 precursor. Mytilin is recently known an antimicrobial peptide of mussels, most mytilins are reported to be presegment composed of 22 amino acids, mature peptide consisting of 34 amino acids, and C-terminal composed of 48 amino acids containing a lot of acidic amino acid residues [G. Mitta et al., 2000a; G. Mitta et al., 2000b; Mitta et al., 2000c, P. Rosh et al., 2008]. Although it is assumed that the section of the presegment will be converted to a signal sequence

for transport into the inner membrane space of endoplasmic reticulum, the structural importance of the acidic C-terminal has not been accurately identified [G. Mitta et al., 2000a; G. Mitta et al., 2000b; Mitta et al., 2000c]. It is expected that C-terminal will interact with the cation portion of the peptide to help stabilize the structure or protect cells produced from interaction with the membrane. Therefore, the results in this study and further study provide a variety information the structural importance and the characteristics of in terms of the immunological aspects of mytilin.

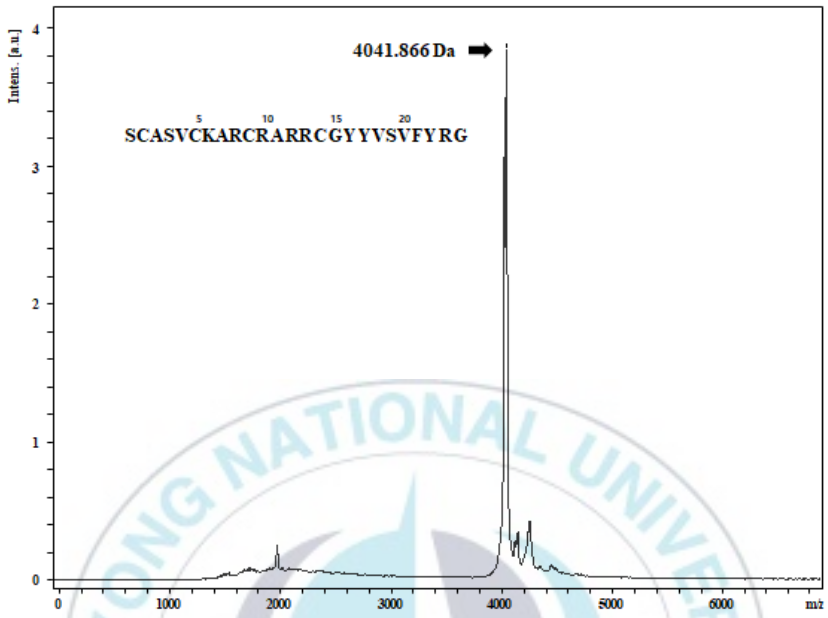



**Fig. 4.3. The molecular weight of the purified peptide was determined using an ultraflleXtreme™ MALDI TOF/MS spectrometer equipped with a pulsed smart beam II in linear mode.** The molecular weight of the purified peptide is 4041.866 Da and N-terminal sequences (24 amino acids) were determined by edman degradation.

**4.4. Analysis of rapid amplification cDNA ends (3' RACE, 5' RACE PCR)**

In order to identify the cDNA of antimicrobial peptide from hemocyte of *M. coruscus*, the RACE method was used to analyze 5'UTR, 3'UTR, and ORF of antimicrobial peptide based on the residual N-terminal amino acid obtained by Edman degradation method and the NCBI gene information.

Based on the sequence information of the 25 N-terminal amino acid residues obtained and the NCBI genetic information, a set of gene-specific primers was produced to secure and analyze 5'UTR, 3'UTR, and ORF sequences using RACE method. The results revealed 312 bp of ORF encoding 103 amino acid residues, and revealed that it consists of 66 bp signal peptide encoding 22 amino acid residues through SIGNALP 4.0 online tool (http://www.cds.dtu.dk/services/ signalP) and 102 bp mature peptide coding 34 amino acid residues (Fig. 4.4). In addition, the polyadenylation signal (AATAA) was verified within the 3'UTR with 5'UTR of 75 bp and 3'UTR of 136 bp (Fig. 4.4). The 103 amino acid sequences of reported mytilin B precursors (FJ973154.1) and mytilin B isoform 1 found in this study were 100% identity, while isoform 2 was 98% (101 of 103 matches), isoform 3 and isoform 4 were 99% (102 of 103 matches). The molecular weight of the antimicrobial peptide by MALDI-TOF mass spectrometers was 4041.866 Da and, antimicrobial peptide, mytilin isolated from hemocyte of *M. coruscus* was able to identify 34 amino acids of mature peptide through N-terminal analysis results (Fig. 4.5).

Mytilin B isofom 1 had 100% identity with mytilin B precursor in 34 amino acids with antimicrobial activity of mature peptide and theoretical molecular weight was 4019.81 Da and value of isoelectric point was 9.63 with 9 positive net charged residues (1 lysine, 8 arginine). Isoform 2, 3, and 4 had 97% (33 of 34 matches) identity with 4000.77 Da, pI 9.45 and 9 positive net charged residues (1 histidine, 1 lysine, 7 arginine). Until recently, mytilin from *M. coruscus* in NCBI database have registered in a total of eight (mytilin A, B, 3, 4, 5, 6, 7 and 8) and all research were progressed by team of Lian, Z and Liu, M in China. Amino acid and DNA

sequence were only registered but the data for characteristics of antimicrobial activity could not be found. So it is probably only study of the cDNA library and registered sequence information.

We are collecting a variety of antimicrobial peptides and isoforms that reduces the size and lower molecular size from hemocyte of *M. coruscus*. In addition, various physiological functions are assessed and considered can be applied to antimicrobial peptides. Antibiotics to replace the alternative materials for the development of antibiotics that can be expected to provide a lot of information. And activity, functional and biochemical characteristics of antimicrobial peptides would be helpful to understand the innate immune system of marine invertebrates.

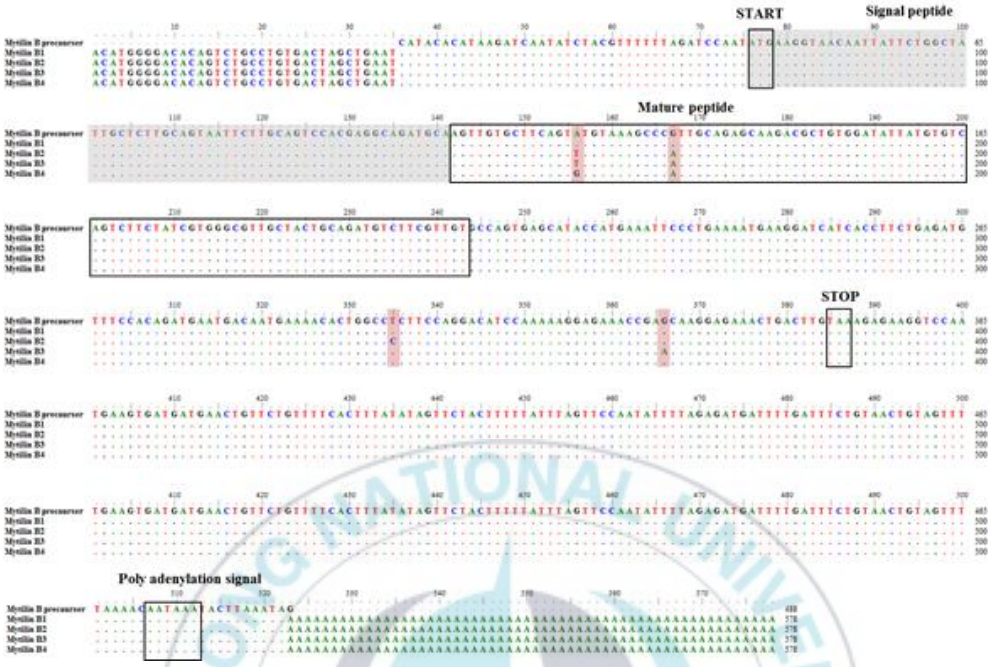



**Fig. 4.4. Multiple alignment of nucleotide sequences of mytilin B precursor and mytilin B isoforms.** Signal peptide is indicated by grey box. The mature peptide and polyadenylation signal is indicated by black outlined. Conserved residues are indicated by dot.

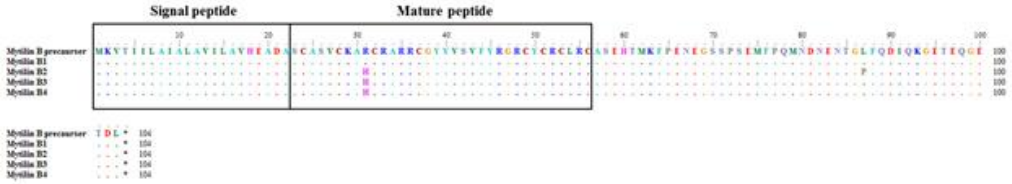



**Fig. 4.5. Amino acid sequence alignment of mytilin B precursor with 4 isoform of mytilin B.** Signal peptide and mature peptide regions were outlined by black boxes. Conserved residues of amino acids are indicated by dot.

**4.5. Synthesis of antimicrobial peptide**

The sequence obtained using the gene-specific primer set was analyzed and the ORF of mytilin B was revealed based on this analysis. The molecular weight of 34 amino acid sequences found to be cDNA cloning was 4019.8 Da, but the molecular weight of the purified peptide measured with MALDI-TOF/MS was 4041.866 Da, a difference of about 49 Da. This is thought to be the difference in molecular weight due to post-translational modification including allysine (-1.03 Da), amidation (-0.98 Da), 2, 3-didehydroalanine (-18.02 Da), 2, 3-didehydrobutyrine (- 18.02 Da), (Z)-2, 3-didehydrotyrosine (-2.02 Da), 3-oxoalanine cysteine (-18.08 Da), 3-oxoalanine serine (-2.02 Da), 2-oxobutanoic acid (-17.03 Da), and pyruvic acid (-33.10 Da). Also, mytilin B derived from *M. edulis*, was a peptide that was found to have no alanine residue at the C-terminal, and two peptides were synthesized according to the presence of the alanine residues at the C-terminal, and antimicrobial activity was observed and compared to various strains [P. Rosh et al., 2008]. Without the alanine residue at the C-terminal of the synthesized antimicrobial peptide, the molecular weight of about 22 Da is less than the molecular weight measured by MALDI-TOF/MS, and the molecular weight of about 49 Da if the alanine residues are present. This is expected to be the molecular weight difference due to post-translational modification and further research is needed on the causes of molecular weight differences later on. The molecular weight of the antimicrobial peptide, synthesized based on the sequence and molecular weight, is 4090.9 Da and a total of 35 amino acids (S-C-A-S-V-C-K-A-R-C-R-A-R-R-C-G-Y-Y-V-S-V-F-Y-R-G-R-C-Y-C-R-C-L-R-C-A) is named mytilin B1, the molecular weight is 4019.8 Da, and by removing the alanine at the C-terminal end of mytilin B1, a total of 34 amino acids (S-C-A-S-V-C-K-A-R-C-R-A-R-R-C-G-Y-Y-V-S-V-F-Y-R-G-R-C-Y-C-R-C-L-R-C) is named mytilin B1.

**4.6. Antimicrobial activities and stability**

The two synthesized peptides were measured antimicrobial activity by URDA method according to the concentration of antimicrobial peptide against various strains shown in Table 5.4. The degree of activity was expressed in MEC (Table 4.3) In addition, the stability of peptides due to heat treatment and salt was measured in the same way (Table 4.2). Antimicrobial activity assessment in Mytilin B1 showed activity in the Gram positive strains, *Bacillus cereus* (4.9 mm) and *S. parauberis* (6.8 mm), in the Gram negative strains, *E. cloacae* (4.7 mm), *E. coli* (6.1 mm), *Klebsiella pneumonia* (5.9 mm), *Proteus mirabilis* (4.7 mm), *Providencia stuartii* (5.6 mm), *Pseudomonas aeruginosa* (5.3 mm), and *Vibrio ichthyoenteri* (6.9 mm). In addition, the antimicrobial activity was also strong in fungi *C. albicans* (6.4 mm). The results of the antimicrobial activity assessment in mytilin B2 showed antimicrobial activity in *B. cereus* (5.2 mm) and *S. parauberis* (6.2 mm) in Gram negative strains, *E. cloacae* (5.2 mm), *E. coli* (6.3 mm), *K. pneumonia* (6.2 mm), *P. mirabilis* (5.5 mm), *P. stuartii* (5.8 mm), *P. aeruginosa* (5.3 mm), and *V. ichthyoenteri* (7.0 mm). Antimicrobial activity was also verified in fungi *C. albicans* (6.3 mm).

The strains that showed antimicrobial activity were similar to those of mytilin B1 and mytilin B2, but the antimicrobial activity of mytilin B2 with no alanine residue at the end of C-terminal was stronger (Table 4.2). From this, it was determined that the alanine residue at the C-terminal were related to the reduction of antimicrobial activity. The thermal treatment and the evaluation of antimicrobial activity by salinity in Mytilin B1 showed a particular increase in antimicrobial activity after heat treatment against all strains except *E. coli* and *C. albicans*. Antimicrobial activity was increased when the concentration of salt was increased by 1-2%. There was a slight salt effect against *P. stuartii* and no change in antimicrobial activity was seen in the rest of the strains due to heat treatment or salt concentration. And as a result of heat treatment and the evaluation of antimicrobial activity by salinity in mytilin B2, antimicrobial activity increased after heat treatment against *S. parauberis* strains, and

antimicrobial activity increased when salt concentration was increased from 1 to 2% against *E. coli*. There was a slight effect by salt against *P. mirabilis* and no changes in activity were seen in other strains due to heat treatment or salt concentration (Table 4.2). Peptides that show changes in antimicrobial activity due to heat treatment or changes in salinity such as mytilin B1 and B2, have been reported in previous studies and have very high stability in heat and salt [R. Oh et al., 2017; J.K. Soe et al., 2014]. In addition, when MECs were calculated based on antimicrobial activity, the mytilin B1 was 46.6 to 89.7 μg/mL for Gram positive strains, 7.4 to 39.5 μg/mL for Gram negative strains, and 31.8 μg/mL for fungal *C. albicans*. Mytilin B2 was 41.6 to 86.2 μg/mL for Gram positive strains, 11.2 to 29.8 μg/mL for Gram negative strains, and 26.0 μg/mL for fungi *C. albicans*. Both mytilin B1 and B2 were the lowest in the *E. cloacae* strains at 7.4 to 11.2 μg/mL (Table 4.3).

Antimicrobial peptides from hemocyte were known including a number of mytilin isoforms, myticin A and myticin B of *M. galloprovincialis*, defensin A and defensin B of *M. edulis*, mythichitin-CB and myticusin of *M. coruscus* [G. Leoni et al., 2017; Z. Liao et al., 2013; C.L. Qin et al., 2014]. Among them, myticalin A8, C9 were acitve against Gram negative strains *E. coli*, *P. aeruginosa*, *Acinetobacter bamanani* (Minimum Inhibitory componentization, MIC 1-8 μM), and Gram-positive strain *Staphylococcus aureus, B. subtilis* (MIC 2-16 μM). In particular, myticusin-1 derived from *M. coruscus* was more active against the Gram-positive strain *S. aureus* (MIC < 5 μM) than the Gram-negative strain *E. coli* (MIC > 10 μM) [33]. And myichitin-CB showed strong activity against Gram-positive strains *B. subtilis, S. aureus, Sarcina luteus, Bacillus megaterium* (MIC < 5 μM) and also showed activity against fungi *C. albicans* and *Monila albicans* (MIC 6.25 - 25 μM) [C.L. Qin et al., 2014].

The results confirm that the activity of the antimicrobial peptide derived from blood cells is active not only in Gram positive strains, Gram negative strains, but also in fungi, so the range of antimicrobial spectrum varies widely [G. Mitta et al., 2000a; G. Mitta et al., 2000b; Mitta et al., 2000c]. It is also reported that the

antimicrobial peptide derived from terrestrial animal is inhibited by NaCl, but the antimicrobial peptide derived from marine animal is known to have little effect on the antimicrobial activity by NaCl [R. Oh et al., 2016; J.A. Tincu and S. W. Taylor 2004]. Therefore, considering the wide range of antimicrobial activity and stability against high heat and salt, it is believed that mytilin B antimicrobial peptide derived from *M. coruscus* in this study will play an efficient and stable role as an antibiotic replacement as well as a variety of treatment and prevention agents.

**Table 4.2. Antimicrobial activity and stability of mytilin B1 and mytilin B2 against pathogenic bacteria under heat & salt conditions**

| | Strains | Mytilin B 1 | | | | | Mytilin B 2 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Sample | Heating | 0.5% NaCl | 1% NaCl | 2% NaCl | Sample | Heating | 0.5% NaCl | 1% NaCl | 2% NaCl |
| Gram Positive bacteria | *B. cereus* | * | ** | ** | ** | * | ** | ** | ** | ** | * |
| | *S. parauberis* | ** | *** | ** | ** | ** | ** | *** | ** | ** | ** |
| Gram Negative bacteria | *E. cloacae* | * | ** | ** | ** | ** | ** | ** | ** | ** | ** |
| | *E. coli* | ** | ** | ** | *** | *** | ** | ** | ** | *** | *** |
| | *K.pneumoniae* | * | ** | ** | ** | ** | ** | ** | ** | ** | ** |
| | *P. mirabilis* | * | ** | * | * | * | ** | ** | * | * | * |
| | *P. stuartii* | ** | *** | * | * | * | ** | ** | ** | ** | ** |
| | *P. aeruginosa* | ** | *** | ** | ** | ** | ** | ** | ** | ** | ** |
| | *V. ichthyoenteri* | ** | *** | ** | ** | ** | *** | *** | ** | ** | ** |
| Fungi | *C. albicans* | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** |

**Table 4.3. Minimal effect concentration (MEC, μg/mL) of peptide**

| | Strains | Mytilin B 1 | Mytilin B 2 |
|---|---|---|---|
| Gram positive | *B. cereus* | 46.6 | 41.6 |
| | *S. parauberis* | 89.7 | 86.2 |
| Gram negative | *E. cloacae* | 7.4 | 11.2 |
| | *E. coli* | 13.4 | 12.3 |
| | *K. pneumoniae* | 35.7 | 29.0 |
| | *P. mirabilis* | 12.5 | 25.6 |
| | *P. stuartii* | 39.5 | 21.2 |
| | *P. aeruginosa* | 35.2 | 29.8 |
| | *V. ichthyoenteri* | 24.2 | 20.9 |
| Fungi | *C. albicans* | 31.8 | 26.0 |

### 4.7. Hemolytic activity

Hemolytic activity of peptide against erythrocytes of olive flounder was measured to test systemic toxicity and 1 % triton x-100 and piscidin from hybrid striped bass typical used for positive control. Piscidin 1 and 1 % triton x-100 exhibited 100% hemolysis at concentration of 100 μg/mL but mytilin B1 and mytilin B2 caused 6% and 15% of hemolysis (Fig 4.6B).

Concentration hemolysis of peptides were tested and piscidin 1 exhibited 77% hemolysis at 25 μg/mL and 12% hemolysis at a low concentration of 6.5 μg/mL. mytilin B1 and mytilin B2 showed lower toxicity compared to piscidin 1. In contrast, mytilin B1 showed 6% hemolysis at a high concentration of 100 μg/mL, 10% hemolysis at concentration of 25 μg/mL and 12% hemolysis at concentration of 6.5 μ g/mL, and mytilin B displayed 15% hemolysis at a high concentration of 100 μg/mL, 9% hemolysis at concentration of 25 μg/mL and 5% hemolysis at concentration of 6.5 μg/mL, respectively (Fig. 4.6A). Different level of hemolytic activity is considered due to hydrophobicity and structural difference by alanine of C-terminal. Mytilins with low hemolytic activity are expected as antimicrobial materials in various fields.

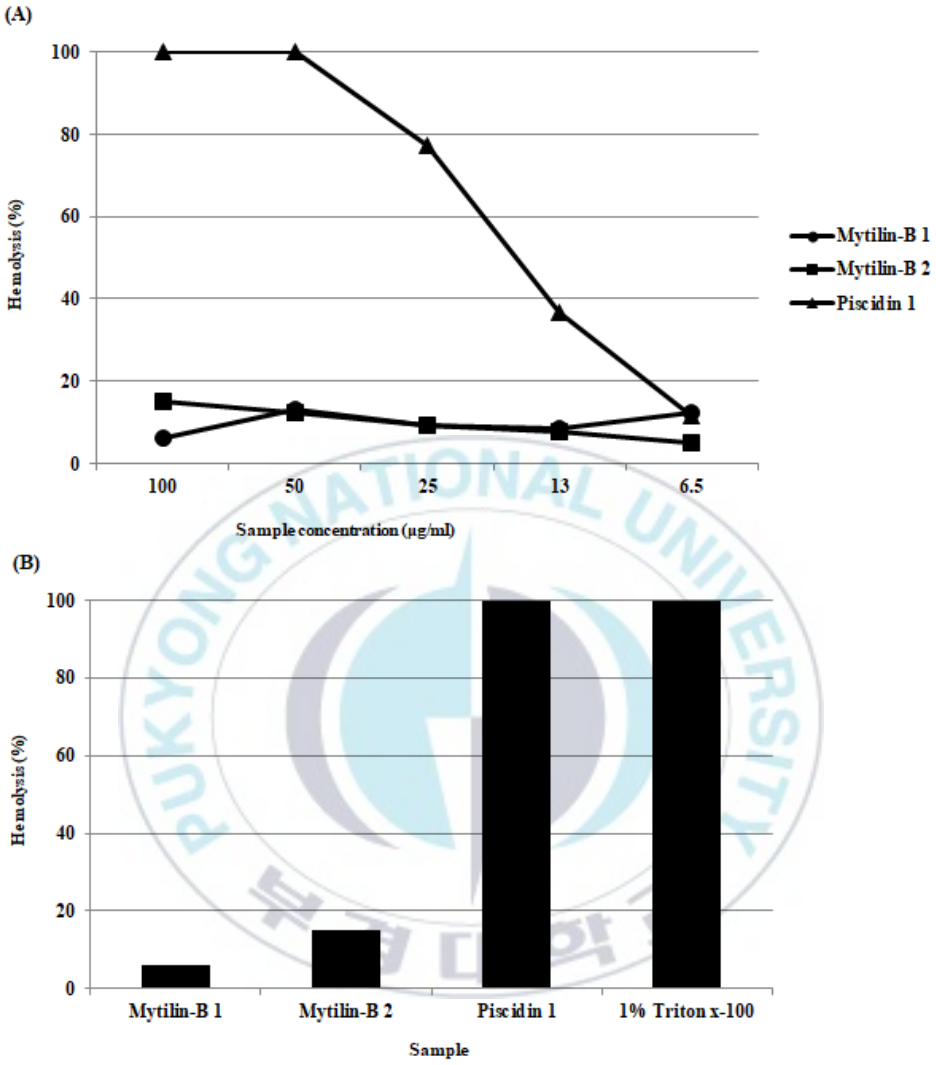



**Fig. 4.6. Hemolytic activity of mytilin B1, mytilin B2, and piscidin 1 against erythrocytes of flounder (*Paralichthys olivaceus*).** (A) The concentration of each sample was 100 μg/mL. (B) Effect of hemolytic activity depending on concertration of samples (100, 50, 25, 12.5, 6.5 μg/mL).

**4.8. Cell cytotoxicity by MTS assay**

To check cytotoxicity to HUVEC, melittin, an antimicrobial peptide consisting of 50 μg/mL concentration of mytilin B1, B2 compounds and 26 amino acids separated from bee venom as a positive contrast medium, was used. Sample and positive controls were processed and MTS solutions were added to HUVEC to determine the survival rate of living cells after 4 hours (Fig. 4.7-4.8). when 35 residues of mytilin B1 and 34 residues of mytilin B2 compounds were treated on HUVEC, they were found to have a high cell viability of 100% and no morphological change was observed, but in the treatment of the control, melittin, a very low cell survival rate of about 1% (Fig. 4.7-4.8). Test of cytotoxicity on the previously found melittin (10 μg/mL) treatment on HUVEC showed a cell survival rate of about 40% after 5 minutes and a cell survival rate of nearly 0% after 60 minutes. Therefore, the mytilin B1 and mytilin B2 in this study are thought to be hardly cytotoxic and regarded as an antimicrobial active material.

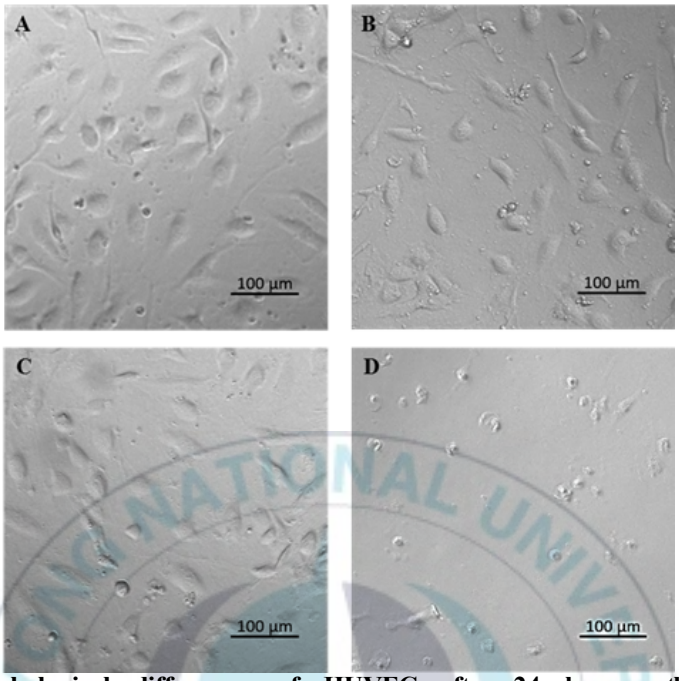



**Fig. 4.7. Morphological differences of HUVEC after 24 hr growth with each specimens.** (A) HUVEC control (B) HUVEC treated with mytilin B1 (C) HUVEC treated with mytilin B2 (D) HUVEC treated with melittin

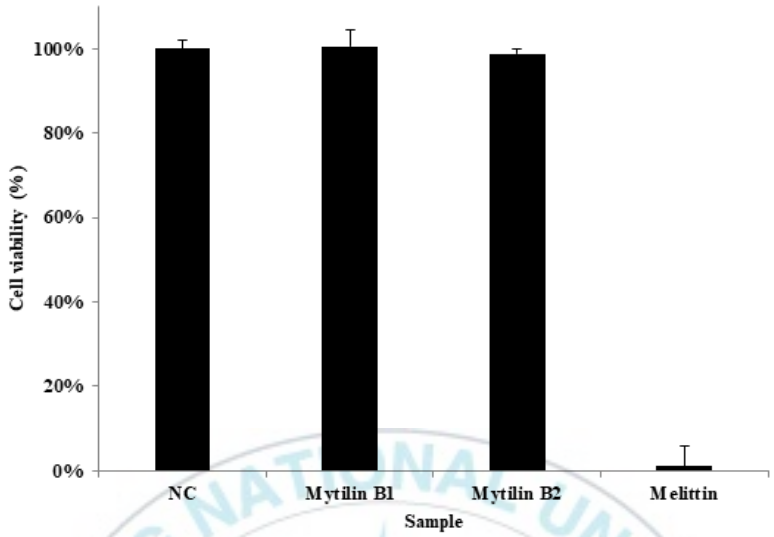



**Fig. 4.8. Cell viability of HUVEC treated with mytilin B after 24 hr growth.** Error bars represent the mean ± SD of three technical replicates.

**4.9. Quantitative analysis of mytiln B expression**

The expression of mytilin B was analyzed by qPCR in tissue (Hepatopancreas, siphon, gills, adductor muscle, foot, hemocyte, and mantle) from *M. coruscus.* The level of gene expression was verified by melting curve. The analysis of the variation in expression of mytilin B gene in each tissue showed that it was the highest in siphon, followed by the foot muscles and gills (Fig. 4.9). In unusual, mytilin B was expressed in isolated blood cells at a similar level to the hepatopancreas, adductor muscle and mantle. Thus, mytilin B was separated from the blood cells but was found to have no tissue specificity (Fig. 4.9).

Previous studies have shown that many of the various antimicrobial peptide in various tissues of marine invertebrates are known to exist in the blood or plasma components of the animals, but this study shows that they are highly present in other tissues, such as siphon and foot as well as in the blood cells, which are circulating [J. K. Seo et al., 2017]. As such, studies have been reported on the tissue non-specific expression analysis of antimicrobial peptide, for example, mytichitin-CB, separated from *M. coruscus*, showed high levels of expression in the blood cell-derived antimicrobial peptide cgmoluscidin from *Crassostrea gigas*, which is derived from gill but high levels in mantle [C. L. Qin et al., 2014; J. K. Seo et al., 2013].

In this study, antimicrobial substance is isolated and purified in the blood cells of *M. coruscus*, which is proved peptide based on the treatment of protease K. The molecular weight of 4041.866 Da and sequence of 25 amino acid was determined by MALDI-TOF/MS and Edman degradation methods. In addition, it was confirmed as mytilin B by cDNA cloninig and two antimicrobial peptides were synthesized depending on the presence or absence of the alanine residues at the end of C-terminal to evaluate antimicrobial activity. The results showed strong antimicrobial activity not only in Gram-positive, and Gram-negative strains, but also in fungi, with high stability in heat and salt, but little hemolysis and cytotoxicity. On the other

hand, in the case of positive control piscidin 1, antimicrobial activity was strongly shown, but there was a report that high hemolytic activity and cytotoxicity limited industrial use [T. Yuan et al., 2012], so mytilin B in this study, is believed to present various data in terms of the immunology of the trench, showing the possibility of becoming a functional feed additive and an antibiotic substitute.

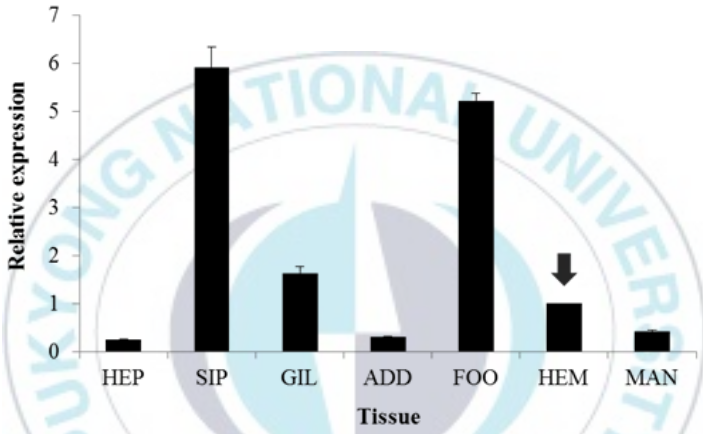



**Fig. 4.9. Quantitative analysis of the mytilin B gene expression level from the various tissues**. HEP; hepatopancreas, SIP; siphon, GIL; gill, ADD; adductor muscle, FOO; foot, HEM; hemocyte, MAN: mantle. Error bars represent the mean ± SD of three technical replicates.

**Conclusion**

We purified antimicrobial peptide from the acidified hemocyte extract of *Mytilus coruscus* by $C_{18}$ reversed-phase high-performance liquid chromatography (RP-HPLC). The peptide was determined to be 4041.866 Da by matrix-assisted laser desorption ionization time-of-flight mass spectrophotometer (MALDI-TOF/MS) and N-terminus of 25 amino-acids sequence was identified. Comparison with N-terminal amino acid sequence of the purified peptide and other antimicrobial peptides revealed 100% identity with the mytilin B precursor of *Mytilus coruscus*. We also identified 312 bp open-reading frame (ORF) encoding 103 amino acids based on the obtained amino acid residues. The nucleotide sequence of ORF and amino acid sequence revealed 100% identity with the mytilin B precursor of *Mytilus coruscus*. We synthesized two antimicrobial peptides defending on existence of alanine residue of C-terminus, and named mytilin B1 and B2. Synthesized antimicrobial peptides showed antimicrobial activity against Gram-positive bacteria including *Bacillus cereus* and *Streptococcus parauberis* [minimal effective concentrations, MECs 41.6 – 89.7 μg/mL], Gram-negative bacteria including *Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Providencia stuartii, Pseudomonas aeruginosa* and Vibrio ichthyoenteri [MECs 7.4 – 39.5 μg/mL] and fungi, Candida albicans [MECs 26.0 – 31.8 μg/mL]. And antimicrobial activity was stable under heat and salt conditions. Furthermore, peptides did not exhibit significant hemolytic activity and cytotoxic effect. These results suggest that mytilin B could be applied as alternative antibiotic agent, and helpful information to understand the innate immunity of hard-shelled mussel.

## Chapter 5.

## Myticusin-beta, antimicrobial peptide from mantle of *Mytilus coruscus*

**5.1. Isolation of the peptide from the acidified crude extract of *M. coruscus***

Mantle tissue from *Mytilus coruscus* was collected, homogenized, and acidified for purification. Acidified mantle extracts were subjected to URDA and showed antimicrobial activity against Gram-positive bacteria including *Bacillus cereus*, *Bacillus subtilis*, and *Streptococcus mutans*, as well as Gram-negative bacteria including *Escherichia coli* and *Pseudomonas aeruginosa* (data not shown). Based on the initial screening results, *B. subtilis*, which showed high susceptibility to the extract, was selected as the reference strain for the antimicrobial assay.

Acidified mantle extract was subjected to RP-HPLC for isolation and purification. Several peaks exhibited antimicrobial activity against *B. subtilis*. The most active fraction was eluted at 30–32 min from the CapCell-Pak $C_{18}$RP column (Fig. 5.1A). This fraction was collected, concentrated, and applied to the TSK-gel ODS-80$^{TM}$ $C_{18}$ RP column for further purification. Finally, the purified active fraction was eluted at 31–33 min and treated with trypsin to confirm its proteinaceous nature. Antimicrobial activity against *B. subtilis* disappeared completely after trypsin reaction (Fig. 5.1B). These results suggest that the purified peak represented a proteinaceous component with antimicrobial activity.

Antimicrobial peptides found in *Mytilus* species have mainly been isolated from hemocytes and hemolymph. Defensin from *Mytilus edulis* and *Mytilus galloprovincialis*, mytilin isoforms A and B from *M. edulis*, mytilin isoforms C, D, and G1 from *M. galloprovincialis*, myticins A and B and mytimycin from *M. galloprovincialis*, mytichitin-CBD from *M. coruscus* and myticusin-1 from *M. coruscus* have been reported [M. Gerdol et al., 2012; C.L Qin et al., 2014; H,H Liu et al., 2019; R. Oh et al., 2018; Z. Liao et al., 2013]. Uniquely, the AMP found in this investigation was purified from the mantle of *M. coruscus,* and the eluted fraction exhibited antimicrobial activity against Gram-positive bacteria, such as *B. subtilis*, in contrast to myticusin-1 of *M. coruscus*.

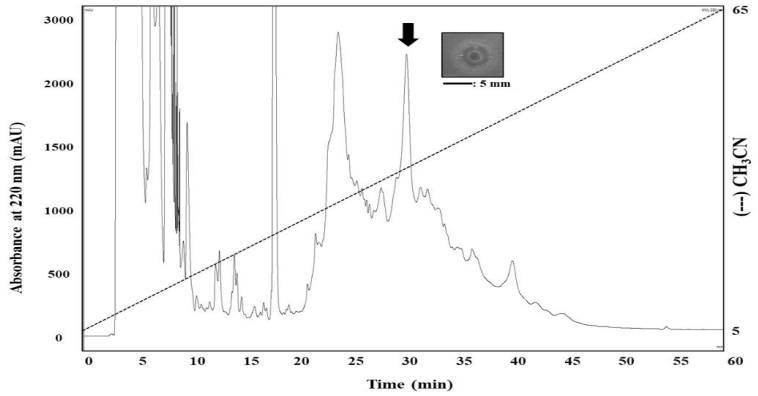
**5.2. Identification of peptide**

The molecular weight and N-terminal amino acid sequence of the purified peptide were determined to enable identification. The molecular mass of the purified peptide was 11,182.003 Da based on MALDI-TOF/MS analysis, and 23 amino acid residues of the purified peptide were obtained through Edman degradation: S-D-H-Q-M-A-Q-S-A-C-M-G-L-A-Q-D-A-A-Y-A-S-A-I (Fig. 5.1C).

The identified partial sequence of 23 N-terminal residues matched 91% with a hypothetical protein partial (OPL33655) sequence from *Mytilus galloprovincialis* and 82% with the myticusin alpha precursor (AFJ04410) of *Mytilus coruscus*. For these reasons, we proposed the name of myticusin-beta for the purified peptide.

Diverse AMPs of various of phyla contain amino-terminal copper and nickel (ATCUN)-containing peptides (ATCUN-AMPs), which have a tripeptide with the consensus sequence Xaa-Xaa-His (where Xaa is an amino acid), have been reported [M.D.J. Libardo et al., 2015]. AMPs in mollusks that contain the ATCUN motif are considered to show antimicrobial activity, and the identification of threonine-aspartic acid-histidine (TDH), histidine-proline-histidine (HPH), and histidine-serine-histidine (HSH) motifs in mollusks can be expected. For example, the ATCUN motif region sequence of myticusin-1 from *M. coruscus* is Thr-Asp-His (TDH), that of myticin B from *M. galloprovincialis* is His-Pro-His (HPH), and that of myticin A from *M. galloprovincialis* is His-Ser-His (HSH). From the results of this study, myticusin-beta has serine-aspartic acid-histidine (SDH) in the N-terminus of the peptide. As reported for ATCUN-AMPs, this peptide has a conserved sequence of three amino acid residues including histidine at the N-terminus, whereas the hypothetical partial (OPL33655) protein sequence from *M. galloprovincialis* contains serine-aspartic acid-histidine (SDH) in its N-terminus. The spectrum of antimicrobial activity may be dictated by the ATCUN motif, and further research on the correlation between the sequence of this motif and peptide activity is required.

**(A)**

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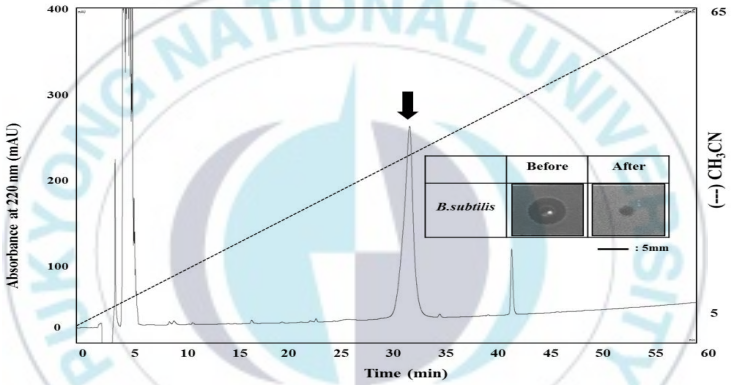



**(C)**

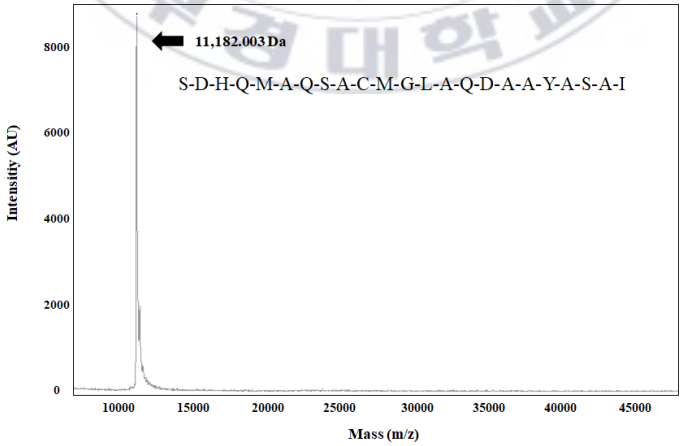



**Fig. 5.1. Purification and antimicrobial activity of purified peptide from mantle extract of *M. coruscus*.**

(A) Antimicrobial activity of the purified peak (30-32 min) against *B. subtilis*. Acidified extract was injected to CapCell-Pak $C_{18}$ reversed-phase column with gradient of 5-65% ACN in 0.1% TFA over 60 min at a flow rate of 1.0 mL/min. (B) Antimicrobial activity of the purified peak (31-33 min) and trypsin treated purified peak against *B. subtilis*. Active fraction was applied to the TSK-gel ODS-80$^{TM}$ $C_{18}$ reversed-phase column. Elution was performed with a linear gradient of 5%→65% ACN (pH 2.2) in 0.1% TFA for 60 min at a flow rate of 1.0 mL/min. The elute was monitored at 220 nm. Fraction of the absorbance peak (indicated by the arrow) showed antimicrobial activity before and after trypsin treatment against *B. subtilis*. Scale bar indicates 5 mm. (C) Mass spectrometry analysis of single mass with 11,182.003 Da and N-terminal sequences (23 amino acids) determined by Edman degradation.

**5.3. cDNA analysis and cloning of cDNA encoding myticusin-beta**

We screened the cDNA clones to determine the sequence of myticusin-beta. Specific primers were designed to amplify the cDNA of myticusin-beta from *Mytilus coruscus* and the full cDNA sequence.

The complete sequence of myticusin-beta was 574 bp in length and contained a 387-bp ORF sequence (Fig. 5.2). The purified peptide encoding 128 amino acids begins with 24 amino acids of signal peptide, which was determined using the SIGNALP 4.0 online tool (http://www.cbs.dtu.dk/services/SignalP). The mature peptide contains 10 basic residues, 4 Lys, 4 Arg and 2 His, and its total hydrophobic ratio was 39%. The cleavage site for release of the signal peptide is between glycine24 and serine25. Mature peptides of 104 amino acids have 87% identity with myticusin-1 of *M. coruscus* and 88% identity with the hypothetical protein of *Mytilus galloprovincialis*.

Furthermore, we identified two other isoforms of myticusin-beta from the mantle of *M. coruscus* and compared their amino acid sequences. We compared 128 amino acid sequences of myticusin-beta and two isoforms, myticusin-beta 2 and myticusin-beta 3 were compared with that of myticusin-1, revealing 74.2% amino acid identity (Fig. 6.3). Myticusin-beta had very high amino acid sequence similarity with the two isoforms, showing 98.4% and 99.2% identity with myticusin-beta 2 and myticusin-beta 3, respectively.

According to a comparison of 128 amino acid sequences of myticusin-1 with those of myticusin-beta and its isoforms, threonine74 in myticusin-1 was substituted with alanine74 and glycine74, whereas histidine118 in myticusin-1 was replaced with serine118 and asparagine118. Consequently, two types of isoforms were identified for myticusin-beta. Differences in the amino acid sequence can lead to diverse changes in protein functions; therefore, additional analysis of the antimicrobial activity and structural properties of the synthetic peptide is necessary.

A calculated molecular weight of 11,082.53 Da was obtained for the 104 amino

acids of the mature peptide, and the detected molecular weight was 11,182.003 Da based on mass spectrometric analysis (Fig. 6.1C). The Calculated molecular weight was 99.473 Da smaller than the detected molecular weight and this phenomenon could be caused by various types of post-translational modification, such as allysine (loss of 1.03 Da), amidation (loss of 0.98 Da), 2,3-didehydroalanine (loss of 18.02 Da), 2,3-didehydrobutyrine (loss of 18.02 Da), (Z)-2,3-didehydrotyrosine (loss of 2.02 Da), 3-oxoalanine cysteine (loss of 18.08 Da), 3-oxoalanine serine (loss of 2.02 Da), 2-oxobutanoic acid (loss of 17.03 Da), pyrrolidone carboxylic acid (loss of 18.02 Da), pyrrolidone carboxylic acid (loss of 17.03 Da), and pyruvic acid (loss of 33.10 Da), as well as modifications that increase molecular weight.


**Fig. 5.2. cDNA sequence and derived amino acids sequence of myticusin-beta**

Deduced amino acid sequences of the ORF are shown under the nucleotide sequences. Asterisk indicates the stop codons and outline indicates the signal peptide of myticusin-beta.

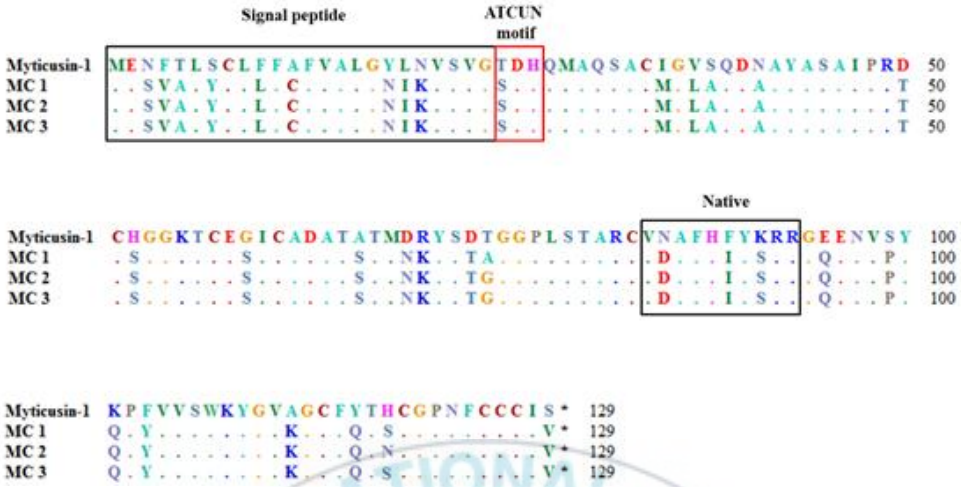



**Fig. 5.3. Multiple sequence alignment of myticusin-1 with myticusin-beta and isoforms.** Myticusin-beta indicates MC1 and two isoforms indicates MC2, MC3. Myticusin-beta and two isoforms of myticusin-beta are identified and compared to myticuisn-1. Residues are shaded base on degree of conservation.

**5.4. Expression and purification of protein**

Based on the cDNA sequence determined using the RACE method, the myticusin-beta gene was amplified and cloned into the TrxA-fused pET-28a(+) vector . The pET-28a(+)-TrxA fusion vector comprising an N-terminal 6X His-tag fused with TrxA protein from *Escherichia coli* was used as the expression vector, because it is useful for high-level production of soluble protein in the *E. coli* cytoplasm [C.H Kim et al., 2017]. The constructed plasmid was transformed into *E. coli* BL21 for expression.

Induced recombinant protein expression was analyzed using an EzWay-PAG gel and protein expression was detected in the supernatant as well as in the pellet. The target protein with 12 kDa of Trx was purified, and the result was in agreement with the calculated molecular weight of the target protein with Trx (Fig. 5.4). Western blot analysis was performed to confirm expression of the recombinant protein and a single clear band corresponding to the molecular weight of the expected protein was observed. Finally, the purified recombinant peptide was desalted and freeze-dried for evaluation of its antimicrobial activity.

Expression systems using *E. coli* as the host pose several obstacles to the expression of small cationic peptides. The target recombinant protein gene may be recognized as a harmful or unnecessary protein introduced from the outside, and thus would be unproduced or expressed as an insoluble aggregate. Insoluble aggregation leads to the need for refolding into the correct form through a refolding process to obtain activity [Y. Shan et al., 2015]. Various methods have been investigated to overcome the shortcomings of the *E. coli* expression method, among which fusion protein tactics have been reported as the most suitable for high-yield expression of target proteins. Therefore, we ligated myticusin-beta into the pET-28a(+)-TrxA fusion vector to make it suitable for cationic peptide expression [C.H Kim et al., 2017; K.L. Piers et al., 1993].

Several fusion protein methods have been developed using glutathione S-transferase

(GST), N-utilization substance A (NusA), maltose-binding protein (MBP), and Trx protein [S. Costa et al., 2014]. These fusion partners improved the production and solubility of target

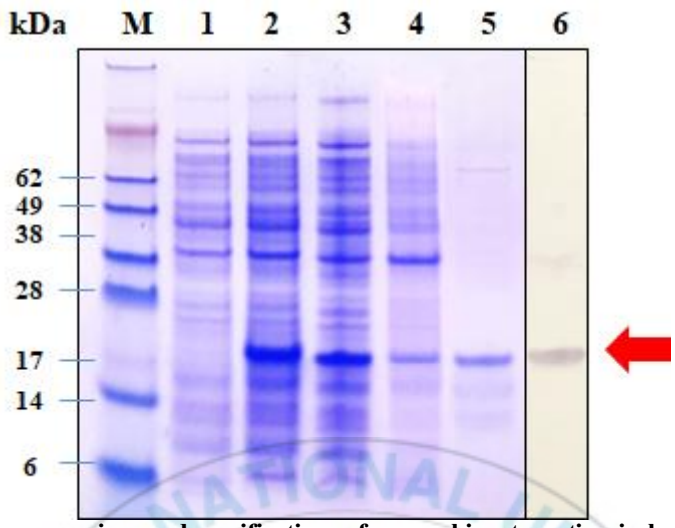



**Fig. 5.4. Overexpression and purification of recombinant myticusin-beta. TrxA fused recombinant protein was purified using Ni-NTA affinity chromatography.** Lane M, molecular marker; lane 1, non-induced protein; lane 2, induced protein; lane 3, soluble protein; lane 4, inclusion body; lane 5, purified myticusin-beta protein (denoted by red arrow), lane 6, result of western blot with purified myticusin-beta protein.

proteins. Among these fusion partners, GST (20 kDa), NusA (55 kDa), and MBP (43 kDa) are large tags and their co-expression may lead to erroneous assessment. However, TrxA protein is desirable due to its small size of only 12 kDa and strong solubility-enhancing effects [S. Costa et al., 2014].

In this study, myticusin-beta was produced through induced expression, and the *trx*A fusion expression system was used for expression of a soluble form of the AMP in the cytoplasm of *E. coli*, which was suitable for subsequent purification steps.

### 5.5. Hemolysis activity

To determine the toxicity of the recombinant peptide, hemolytic activity was measured against erythrocytes from flounder (*Paralichthys olivaceus*). Piscidin 1 and Triton X-100, used as positive controls, showed strong hemolytic activity, but recombinant myticusin-beta peptide did not show hemolytic activity (Fig. 5.5A). Furthermore, Piscidin 1 showed very high hemolytic activity at a high concentration that decreased sharply at a low concentration, whereas myticusin-beta peptide showed no dose-dependent hemolytic activity (Fig. 5.5B). These results demonstrate that the recombinant peptide has no hemolytic activity.

Several AMPs have disadvantages, including hemolytic activity toward host cells. AMPs bind indirectly to bacterial surfaces via electrostatic interactions or interact directly with host cells, increasing the permeability of the cell membrane and causing membrane disruption [G. Diamond et al., 2009]. Therefore, the non-hemolytic property is necessary for application of AMPs as alternative antibiotics. In this investigation, we showed that the recombinant protein myticusin-beta showed low hemolytic activity, and thus could be applied as a substitute for antibiotics.

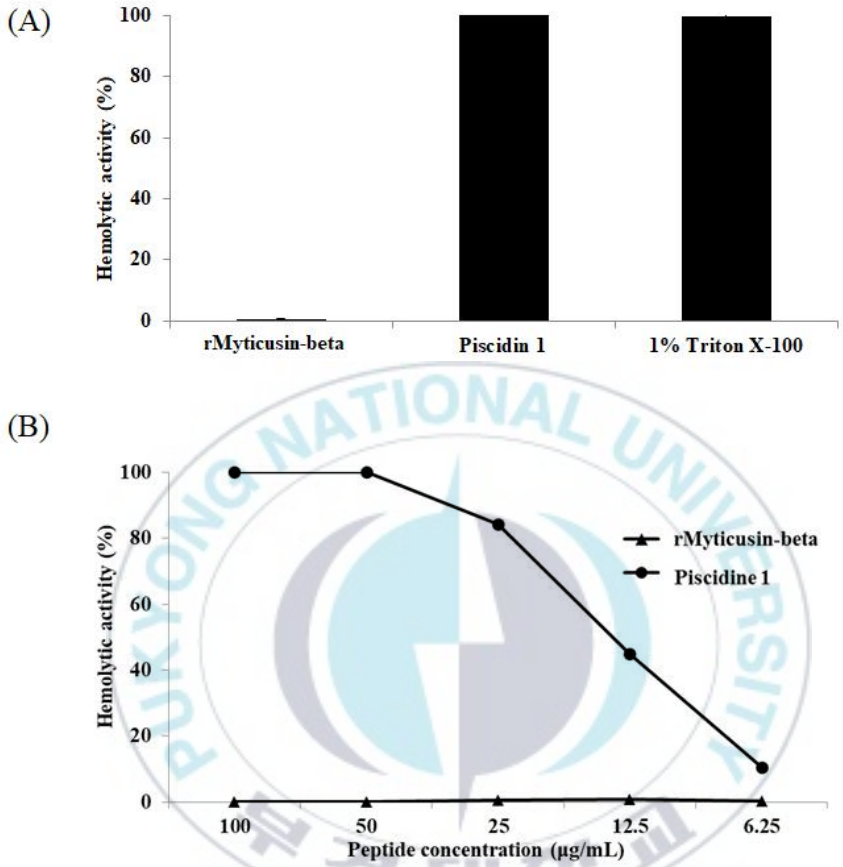



**Fig. 5.5. Hemolytic activity of myticusin-beta and piscidin 1 against erythrocytes of flounder (*P. olivaceus*).**

(A) The concentration of each sample was 100 μg/mL, (B) Effect of hemolysis depending on concentration (100, 50, 25, 12.5, 6.25 μg/mL).

### 5.6. Anti-scuticociliate activity

Scuticociliates are parasites regarded as the causative pathogen of the disease scuticociliatosis in the marine aquaculture industry [M.C. Piazzon et al., 2013]. They cause mass mortality and significant economic losses in aquaculture. Frequent outbreaks of scuticociliatosis in aquaculture fish have been increasing worldwide, and development of effective anti-scuticociliate technologies is required to solve this problem. Some marine AMPs show activity against protozoa. For example, the antimicrobial cationic peptide Pc-Pis from *Psuedosciaena corcea* is genetically related to the piscidin family and shows antimicrobial activity against bacteria, fungi, and parasites [S.F. Niu et al., 2013].

We investigated the anti-scuticociliate activity of the recombinant protein against a scuticociliate using the WST-1 solution assay [M. Ginouves et al., 2014]. The anti-scuticociliate activity of the recombinant protein was evaluated based on the mortality, morphology, and number of living parasites. The highest concentration of recombinant protein, 200 μg/mL, diminished parasite numbers by approximately 50% after 24 h (Fig. 5.6). Damaged scuticociliates were observed under a microscope, and the spindle shape of scuticociliate cells were swollen or burst (Fig. 5.6A). In addition, the fast-moving parasites slowed or stopped their movement. Scuticociliates treated with recombinant protein had lower viability than untreated scuticociliates. In this investigation, the viability of scuticociliates decreased with increasing concentration of the recombinant protein through the WST-1 cell proliferation assay (Fig. 5.6B). These data indicate that recombinant myticusin-beta has antiparasitic activity against scuticociliates.

### 5.7. Antimicrobial activity

In the present study, we verified the antimicrobial activity of the expressed myticusin-beta protein using the URDA method with various pathogenic reference strains. Recombinant myticusin-beta showed antimicrobial activity against both

Gram-positive and Gram-negative bacteria (Fig. 5.7). Myticusin-beta displayed strong activity against Gram-positive strains, including *Bacillus subtilis*, *Clostridium perfringens*, *Staphylococcus aureus*, and *Streptococcus mutans*, as well as Gram-negative strains including *Pseudomonas aeruginosa* and *Vibrio alginolyticus*, moderate activity against *Bacillus cereus* and *Klebsiella pneumoniae*, and weak activity against *Streptococcus iniae* and *Escherichia coli*. Interestingly, Gram-positive strains showed greater susceptibility to myticusin-beta than Gram-negative bacteria.

The antimicrobial spectrum of an AMP describes whether it has a wide range of activity or narrow specificity to certain strains. Various AMPs such as defensins, mytilins, mytichitin-CBD, myticusin-1, and myticins from *Mytilus* species have activity against bacteria and fungi, but mytimycin has activity only against fungi. Among reported AMPs, myticin, mytichitin-CBD, and myticusin-1 are much less active against Gram-negative bacteria and fungi than other AMPs [B.H. Nam et al., 2014]. Myticusin-beta also showed antimicrobial activity against Gram-positive and Gram-negative bacteria, but no activity against fungi. Activity that was stronger against Gram-positive than Gram-negative bacteria has been reported for most mollusk AMPs.

Multiple modes of action of AMPs against Gram-positive and Gram-negative bacteria and fungi have been described in recent years. Differences in membrane composition have also been well investigated in Gram-positive and Gram-negative bacteria and fungi. Gram-positive bacteria contain several layers of peptidoglycan in the cell wall, whereas the bacterial membrane of Gram-negative bacteria includes a single layer of peptidoglycan surrounded by an outer membrane containing lipopolysaccharides, which protects bacteria as a permeability barrier [B.H. Nam et al., 2014]. AMPs may interact with or pass through the cell envelope to reach their cellular target. Fungal cell walls contain complex polysaccharides, such as chitin and glucans, that inhibit passage through the cytoplasmic membrane. Therefore, a broad range of antimicrobial activity of AMPs can be explained based on functional

mechanisms and microbial cellular structures.

As noted above, AMPs may have broad or specific antimicrobial activity spectra, and must be carefully considered for use alone or in combination with other AMPs for their intended purpose. Therefore, continuing research to identify novel AMPs is necessary for industrial application of AMPs to replace antibiotics.

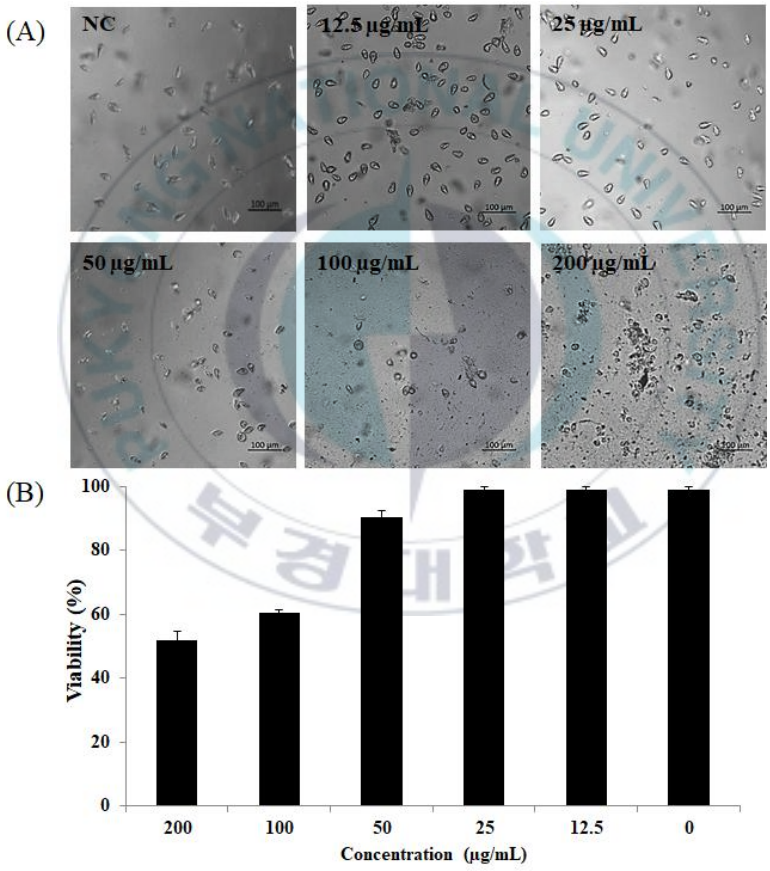
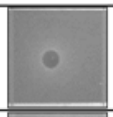
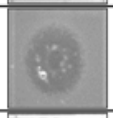
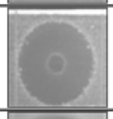
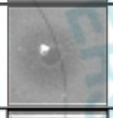
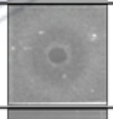
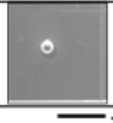



**Fig. 5.6. Anti-scuticociliate activity of recombinant myticusin-beta using WST-1 reagent.**

(A) Agglutination of parasites treated with different concentration of recombinant myticusin-beta (0-200 μg/mL) (B) Viability percent of parasites (scutiociliates) with

concentration of recombinant myticusin-beta (0-200 μg/mL). Error bars represent the mean± SD of three technical replicates.

| Strain | | Clearing zone (mm) | Activity |
|---|---|---|---|
| Gram positive | *B. cereus* | 6.4 |  |
| | *B. subtilis* | 7.6 |  |
| | *C. perfringens* | 10.8 |  |
| | *S. aureus* | 8.8 |  |
| | *S. iniae* | Trace |  |
| | *S. mutans* | 7.0 |  |
| Gram negative | *E. coli* | 4.7 |  |
| | *P. aeruginosa* | 7.5 |  |
| | *V. alginolyticus* | 7.1 |  |
| | *K. pneumoniae* | 7.2 |  |

: 5 mm

**Fig. 5.7. Antimicrobial activities and spectrum of purified recombinant myticusin-beta. Effect of antimicrobial activity of recombinant myticusin-beta against strains.**

The value of antimicrobial activity was shown as a diameter of the clearing zone. (Diameter of well ; 2.2 mm)

### 5.8. Quantitative analysis of myticusin-beta expression

As shown in Fig. 5.8, mRNA expression of myticusin-beta was measured via real-time quantitative PCR as the transcriptional levels of myticusin-beta gene in various tissues, including the adductor muscle, foot, gill, hemocytes, hepatopancreas, mantle, and excurrent siphon. The mRNA levels were quantified after normalization to the internal reference gene β-actin, and the amplification specificity for myticusin-beta and β-actin was determined and analyzed from the melting curve. Gene expression of myticusin-beta was detected in all tissues (Fig. 5.8). Myticusin-beta mRNA expression was highest in the mantle, followed by hemocytes, moderate in the adductor muscle and gill, and low in the foot, hepatopancreas, and excurrent siphon.

*Mytilus* species have an open circulatory system characteristic of mollusks, and AMPs are produced and transported through the hemolymph to all tissues. Furthermore, abundant myticusin-1 from *Mytilus coruscus* was isolated from hemocytes [J.A. Tincu and S.W. Taylor, 2004]. However, the greatest enhancement of myticusin-beta mRNA expression was detected in mantle tissue after bacterial infection (Fig. 5.8). These results indicate that myticusin-beta is produced in a tissue-specific manner and is related to the innate immune system of *M. coruscus*

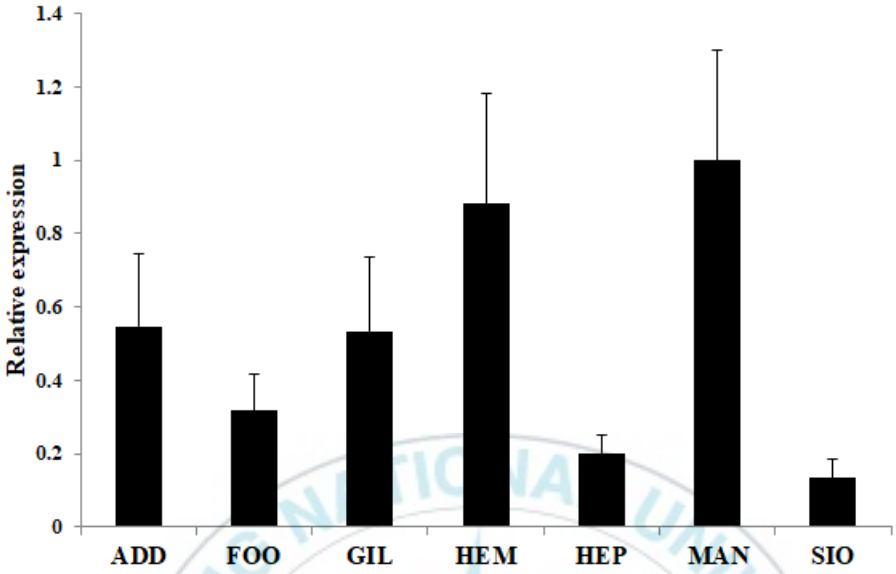



**Fig. 5.8. Quantitative analysis of myticusin-beta expression in the tissues.**

Tissue-specific expression of myticusin-beta. Error bars represent the mean ±SD of three technical replicates. ADD; adductor muscle, FOO; foot, GIL; gill, HEM; hemocyte, HEP; hepatopancreas, MAN: mantle, SIO; excurrent siphon

**5.9. Antimicrobial activity of peptide analogs of myticusin-beta**

In this study, we considered the length of the peptide, charge, hydrophobicity, Boman index, and amino acid side chain in development of a short antimicrobial peptide as a substitute for antibiotics. We selected and synthesized several analog peptides, and selected the peptide fragment VDAFHIYSRR in the mature peptide of myticusin-beta (Table 5.2). Based on these results, amino acids in the selected region were replaced and rearranged to increase antimicrobial activity. The net charge, molecular weight, Boman Index, and pI were predicted using APD2 and the ExPASy online tool (Table 5.2). Schiffer–Edmundson helical wheel projections were performed to predict hydrophobic and hydrophilic regions in the secondary structures of synthetic peptides (Fig. 5.9).

The EMBOSS Pepwheel program was used to identify hydrophobic and hydrophilic regions in the secondary structure of the native and analog peptides. With this program, we could predict interactions and effects between adjacent amino acids and side chains. We considered amino acids not only at the front and back, but also at the top and bottom of the helical structure, and designed cationic AMPs to increase antimicrobial activity. Arginine (Arg), tyrosine (Tyr), aspartic acid (Asp), and serine (Ser) residues in the native form of myticusin-beta were modified with Arg or Leu in the analog peptide (Fig. 5.9). Re-modeling of the analog peptide showed opposite positions of hydrophobic and hydrophilic regions, contributing to the antimicrobial activity. Cationic AMPs are amphipathic peptides with an overall positive charge that interact with negatively charged bacterial membranes and form transmembrane pores. Given these characteristics, we attempted to improve the related values of various factors. The antimicrobial activities of the native and analog peptides are shown in Table 6.3. Interestingly, increased net charge (+1 to +4), basic pI value (> 10), reduced Boman index value (≤ 1), and converted structure increased the spectrum of antimicrobial activity (Table 6.3). The analog showed increased antimicrobial activity against all strains tested (nine Gram-positive bacteria, nine Gram-negative bacteria,

and one fungus, *Candida albicans*).

The development of AMPs with increased activity in this manner was previously reported as a valid method, and the synthesized analog peptide showed increased antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi due to its low Boman index, increased net charge, and total hydrophobic ratio. This study of synthetic analog peptides shows that AMPs can be activated and can contribute greatly to research into new antibiotic substitutes.

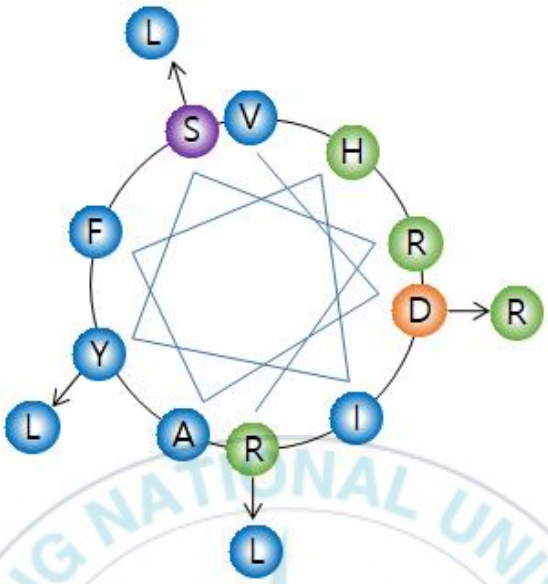



**Fig. 5.9. Helical wheel depiction of myticusin-beta mimetic peptide.**

**The arrows indicate the amino acid residues substituted in the peptide. Amino acid residues were substituted increased net charge and hydrophobic rate.** Hydrophobic residues are blue, acidic residue is red, basic residues are orange and uncharged residues are purple.

**Table 5.1. Sequences and physicochemical properties of the peptide variants used in this study.**

| | Sequence | Length | Charge | MW (Da) | Boman index (kcal/mol) | pI | hydrophobic ratio (%) | Hydrophobicity (kcal/mol) | Region | Modification |
|---|---|---|---|---|---|---|---|---|---|---|
| **Native** | VDAFHIYSRR | 10 | +1 | 1,263.42 | 3.3 | 8.72 | 40 | 1.01 | 84-93 | |
| **Analog** | VRAFHILLRL | 10 | +2 | 1,237.56 | 0.59 | 11.8 | 70 | -1.09 | | D→R, Y→L, S→L, R→L |

**Table 5.2. Antimicrobial activities of peptides against pathogenic bacteria based on the URDA method.**

| | Native | Analog |
|---|---|---|
| **Diameter of zone of inhibition (mm)** | | |
| **Gram positive** | | |
| *Bacillus cereus* | - | 4.9 |
| *Bacillus subtilis* | 4.5 | 7.9 |
| *Enterococcus faecalis* | - | 5.1 |
| *Streptococcus mutans* | - | 8.0 |
| *Streptococcus iniae* | - | 5.1 |
| *Staphylococcus aureus* | 2.5 | 7.2 |
| *Streptococcus vestibularis* | - | 2.5 |
| *Streptococcus parauberis* | - | 13.1 |
| *Lactococcus garvieae* | - | 7.9 |
| **Gram negative** | | |
| *Vibrio alginolyticus* | - | 6.6 |
| *Vibrio parahaemolyticus* | - | 7.6 |
| *Escherichia coli* | - | 11.7 |
| *Enterobacter cloacae* | - | 8.6 |
| *Klebsiella pneumoniae* | - | 9.9 |
| *Edwardsiella tarda* | - | 4.3 |
| *Proteus mirabilis* | - | 6.6 |
| *Providencia stuartii* | - | 9.1 |
| *Pseudomonas aeruginosa* | - | 9.2 |
| **Fungus** | | |
| *Candida albicans* | 2.5 | 9.3 |

Well diameter: 2.2 mm

**Conclusion**

We isolated and purified 11,182 Da of a novel AMP from acidified mantle tissue extract of *Mytilus coruscus*. The purified AMP was composed of 24 amino acids of signal peptide and 104 amino acids of mature peptide based on the complete sequence of the target protein. The sequence identity was similar to the reported myticusin-1 of *M. coruscus*. Hence, we proposed naming the purified peptide myticusin-beta and identified two other isoforms based on the sequence.

Constructed TrxA-fused recombinant myticusin-beta was expressed in a soluble form, and the purified single peptide exhibited a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative strains. Furthermore, recombinant myticusin-beta revealed anti-scuticociliate activity in a dose-dependent manner without causing hemolysis. We designed a short AMP analog derived from myticusin-beta based on its amino acid sequence, secondary structure, charge, hydrophobicity, and correlation of the amino acid side chain. The analog was rearranged with substituted amino acids and displayed strongly increased activity on the URDA plate.

The highest transcriptional level of the myticusin-beta gene was detected in mantle tissue after infection. These results suggest that myticusin-beta is an immune-related AMP of *M. coruscus* and a promising template for the development of novel AMPs as a substitute for antibiotics. This report contributes to elucidating AMPs and the immune system of mollusks, although more studies are needed on the structural features and functional mechanisms of AMPs.

**Part II. Novel antimicrobial peptides from *Oplegnathus fasciatus* based on genetic analysis**

# Chapter 1.

## Multi-functional activities of catalytic domain of phospholipase (CDP) from rock bream *Oplegnathus fasciatus*

### 1.1. Peptide design and synthesis

The CDP series of peptides were designed and synthesized considering net charge, protein binding potential (Boman index), and hydrophobic ratio and α-helical domains were analyzed (Table 1.2). We obtained the sequences from CDP-N (Native form) as template, which provided 13 residues sequence. We chose to place Proline (Pro) at position 10 in CDP-N to Leucine (Leu), Methionine (Met), Lysine (Lys), Valine (Val), and Isoleucine (Iso), and Tryptophan (Trp) at position 4 in CDP-N to Phenylalanine (Phe). The peptides showed positive net charge (+4 to +5), lower Boman Index (<2), similar or higher hydrophobic ratio by substitution of amino acid.

We synthesized peptides have N-terminal acetylation and C-terminal amidation chemically. These modifications reduce the overall charge thus overall solubility might decrease and increase the metabolic stability enhancing the interaction between peptide and membrane and increase membrane permeability.

### 1.2. Antimicrobial effect of CDP peptides

The antimicrobial activities of analogs were tested by URDA against several bacterial species and yeast *C. albicans* (Fig. 1.1). CDP-N (native form) and CDP A2 (analog form) were tested antimicrobial activity against 18 strains. CDP-N and CDP A2 had similar antimicrobial activity against 8 strains but activities increased against *B. cereus, V. alginolyticus, V. parahaemolyticus, S. iniae, S. aureus, K. pneumoniae, E. cloacae, S. parauberis, P. mirabilis*, and *P. stuartii* (Fig. 1.1).

The five analogs were tested antimicrobial activity against 18 strains, fungi (*C. albicans*) and 11 antibiotic resistant strains (Table 3 and Table 4). CDP A2-5 have strong activity against 11 strains than CDP A2. The activity of CDP A2-3 and CDP A2-4 was stronger than CDP A2 against 10 strains. All analogs have antimicrobial activity showed strong activity against Gram-positive bacteria including *B. subtilis. S. aureus* (ATCC 6538), *S. aureus* (CCARM 0204), *S. aureus* (CCARM 0238) *S. parauberis*, and fungi, *C. albicans* (MECs 1.01-10.44 μg/mL) and also against

antibiotic resistant strains.

### 1.3. Hemolytic activities

The hemolytic activity of the analogs were determined using blood cells of olive flounder (Fig. 1.2). Piscidin 1 and Triton X-100 as positive control showed strong hemolytic activity and showed a very high hemolytic activity at a high concentration and decreased sharply at a low concentration. CDP A2-2, CDP A2-3, CDP A2-4, and CDP A2-5 did not hemolysis at 6 μg/mL but CDP A2-1 had hemolysis slightly. These results suggested that CDP analogs have lower hemolytic activity than CDP A2.

Several AMPs have disadvantages such as hemolytic activity toward host cells. AMPs bind to indirectly bacterial surfaces via electrostatic interactions or directly interact with host cells, act by increasing the permeability of the cell membrane and lead to membrane disruption. Therefore, non-hemolytic AMP is necessary for application of AMPs as alternative antibiotics. In this investigation, we proved that CDP A2 analog peptides showed lower hemolysis activity than CDP A2 and applicability as substitute of antibiotics.

**Table 1.1. Summary of design of CDP analogs**

| Name | Amino acid sequence | Molecular weight (Da) | Length (AA) | Net charge | Boman index (kcal/mol) | Hydrophobic ratio (%) |
|---|---|---|---|---|---|---|
| CDP-N | VKLWV KLVTP LIK | 1536.9 | 13 | 3 | -1.14 | 61 |
| CDP-A2 | VKLWV KLVKL LIK | 1580.1 | 13 | 4 | -1.29 | 69 |
| CDP-A2-1 | VKLWV KLVKM LIK | 1598.14 | 13 | 4 | -1.09 | 69 |
| CDP-A2-2 | VKLWV KLVKK LIK | 1595.1 | 13 | 5 | -0.49 | 61 |
| CDP-A2-3 | VKLWV KLVKV LIK | 1566 | 13 | 4 | -1.22 | 69 |
| CDP-A2-4 | VKLWV KLVKI LIK | 1580.1 | 13 | 4 | -1.29 | 69 |
| CDP-A2-5 | VKLFV KLVKL LIK | 1580.1 | 13 | 4 | -1.29 | 69 |

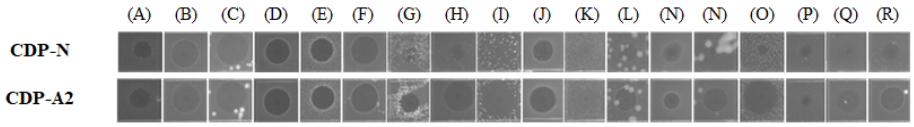



**Fig. 1.1. Antimicrobial activity of CDP (native form) and CDP A2 (analog)**

**Table. 1.2. MEC for antimicrobial activities of CDP derived synthetic peptide against microbes.**

| | | Strain | | CDP-I-A2 | CDP-A2-1 | CDP-A2-2 | CDP-A2-3 | CDP-A2-4 | CDP-A2-5 | Piscidin |
|---|---|---|---|---|---|---|---|---|---|---|
| Microbes | Positive | *Bacillus cereus* | KCTC 1012 | 14.4 | 17.5 | 20.9 | 10.4 | 9.0 | 17.9 | 11.7 |
| | | *Bacillus subtilis* | KCTC 1021 | 5.5 | 5.0 | 4.9 | 1.9 | 5.9 | 4.15 | 5.0 |
| | | *Enterococcus faecalis* | KCTC 3206 | 41.9 | 24.3 | 15.8 | 13.4 | 31.8 | 26.81 | 10.9 |
| | | *Staphylococcus aureus* | ATCC 6538 | 9.7 | 8.6 | 4.7 | 9.4 | 7.1 | 8.76 | 4.8 |
| | | *Streptococcus mutans* | KCTC 3065 | 15.8 | 11.8 | 5.0 | 11.8 | 16.4 | 14.61 | 13.4 |
| | | *Lactococcus garvieae* | KCTC 3772 | 49.1 | 37.1 | >250 | >250 | 49.1 | 24.1 | >250 |
| | | *Streptococcus parauberis* | KCTC 3651 | 4.9 | 6.6 | 5.1 | 1.2 | 4.4 | 1.66 | 10.2 |
| | Negative | *Enterobacter cloacae* | KCTC 2361 | 6.5 | 7.1 | 15.4 | 5.9 | 6.2 | 8 | 21.2 |
| | | *Escherichia coli* | ATCC 8739 | 5.6 | 13.2 | 12.8 | 11.9 | 9.7 | 6.62 | 4.5 |
| | | *Escherichia coli* ML35p | ML35p | 13.5 | 17.4 | 9.9 | 9.9 | 13.8 | 26.27 | 7.8 |
| | | *Klebsiella pneumoniae* | KCTC 12385 | 4.2 | 5.6 | 12.1 | 8.2 | 9.7 | 8.19 | 20.0 |
| | | *Providencia stuartii* | KCTC 2568 | 13.4 | 13.1 | 7.0 | 5.6 | 4.8 | 2.71 | 28.0 |
| | | *Pseudomonas aeruginosa* | ATCC 15522 | 15.0 | 7.0 | 12.2 | 9.0 | 8.0 | 10.51 | 22.4 |
| | | *Vibrio parahaemolyticus* | KCCM 41664 | 10.4 | 20.1 | 82.4 | 28.1 | 10.3 | 10.15 | >250 |
| | | *Vibrio vulnificus* | KCCM 41665 | 7.8 | 12.7 | 18.3 | 20.9 | 14.3 | 6.9 | 19.9 |
| | | *Vibrio alginolyticus* | KCTC 2472 | 13.9 | 15.5 | 8.3 | 7.7 | 14.8 | 5.19 | 7.9 |
| | | *Vibrio anguillarum* | KCTC 2711 | 11.1 | 12.6 | 15.9 | 17.7 | 13.7 | 1.01 | 15.4 |
| | | *Vibrio harveyi* | KCCM 40866 | 7.5 | 12.6 | 15.0 | 21.8 | 6.9 | >250 | >250 |
| | Fungi | *Candida albicans* | ATCC 10231 | 6.6 | 5.6 | 8.0 | 3.7 | 4.0 | 9.51 | 11.2 |

**Table. 1.3. MEC for antimicrobial activities of CDP derived synthetic peptide against antibiotic resistant strains.**

| | | Strain | | CDP-I-A2 | CDP-A2-1 | CDP-A2-2 | CDP-A2-3 | CDP-A2-4 | CDP-A2-5 | Piscidin |
|---|---|---|---|---|---|---|---|---|---|---|
| Antibiotic resistant strains | Positive | *Staphylococcus aureus* | CCARM 0203 | 7.2 | 5.8 | 7.1 | 3.0 | 6.6 | 11.21 | 7.0 |
| | | *Staphylococcus aureus* | CCARM 0204 | 3.3 | 3.7 | 4.5 | 4.0 | 1.8 | 6.12 | 6.9 |
| | | *Staphylococcus aureus* | CCARM 3795 | 20.6 | 19.7 | 6.2 | 12.9 | 19.5 | 9.32 | 4.2 |
| | | *Streptococcus pyogenes* | CCARM 0206 | 11.7 | 2.3 | 8.8 | 6.1 | 6.7 | 4.38 | 14.1 |
| | Negative | *Pseudomonas aeruginosa* | CCARM 0225 | 56.0 | 18.8 | 19.6 | 11.0 | 7.1 | 36.81 | 16.6 |
| | | *Enterobacter cloacae* | CCARM 0252 | 11.0 | 15.2 | 12.3 | 15.0 | 40.7 | >250 | 9.8 |
| | | *Escherichia coli* | CCARM 0238 | 13.6 | 6.5 | 4.1 | 8.2 | 6.1 | 10.44 | 8.5 |
| | | *Escherichia coli* | CCARM 1A814 | 27.2 | 12.4 | 8.2 | 10.1 | 21.6 | 30.16 | 7.0 |
| | | *Klebsiellaaerogenes* | CCARM 0249 | 63.8 | 15.4 | 12.1 | 12.0 | 25.9 | >250 | 27.5 |
| | | *Klebsiellaoxytoca* | CCARM 0248 | 20.5 | 18.2 | 19.5 | 34.0 | 42.7 | >250 | 7.2 |
| | | *Salmonella typhimurium* | CCARM 0240 | 45.8 | 12.7 | 5.7 | 9.2 | 5.6 | 15.64 | 19.6 |

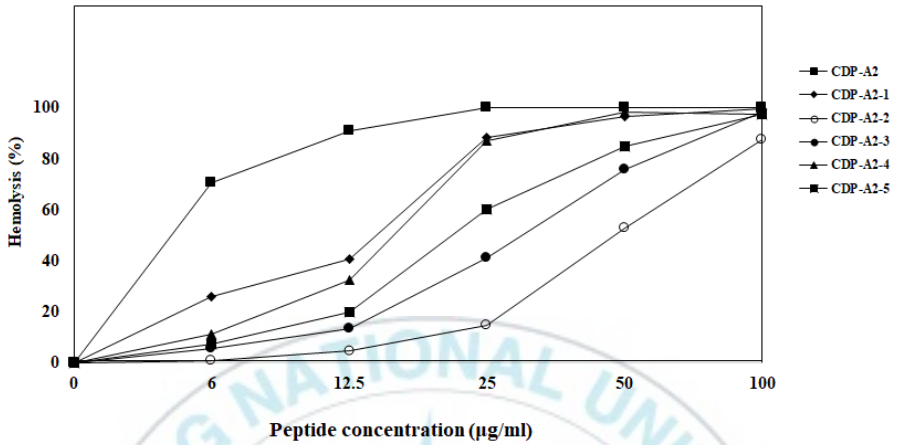



**Fig. 1.2. Hemolytic activity of CDP analogs peptides against blood cell of olive flounder.**

### 1.4. DNA binding ability of CDP analogs

DNA-binding is commonly proposed to be an action of the antimicrobial peptide. DNA-binding assay was investigated using electrophoretic mobility shift assay (EMSA) (Fig. 1.3). Assay for ability of DNA binding was performed using 0.156, 0.313, 0.625 and 1.25 μg of peptide. The electrophoretic mobility of the DNA was almost completely inhibited with 0.313 μg of peptide except for 0.156 μg of CDP A2-3 and CDP A2-4. The series of CDP have strong DNA binding ability. This implies that CDP A2 analogs exhibited high nucleic acid binding affinity and inhibits cellular processes by interrupting DNA and RNA metabolisms. And directly DNA binding suggest the multiple interrupting roles against various DNA associated enzymes.

### 1.5. DNA polymerase inhibition assay

To investigate whether the peptides inhibit DNA polymerase activity, 0.156, 0.313, 0.625, 1.25 and 2.5 μg of CDP A2 peptides were used for test of inhibition DNA polymerase activity (Fig. 1.4). As shown in Fig 4, the series of CDP A2 showed completely inhibited the DNA polymerase activity at all tested concentrations. Inhibition of DNA polymerase is one of mechanism of action of antimicrobial peptide. This peptides kill cells by targeting specific cellular activities such as synthesis of DNA, protein or cell wall.

### 1.6. Anti-scuticociliate test

We attempted to determine the anti-scuticociliate activity of peptides was determined by WST-1 solution assay (Fig. 1.5). The anti-scuticociliate activity of peptide was evaluated with mortality, morphology and number of parasites.

CDP A2 analogs peptides were tested against the parasites. The motility and number of parasites were noted at 0, 1, 5, 10, and 15 μg/mL of synthesized peptides (Fig. 1.5). At 24 h after infection, CDPA2-2, CDP A2-3, CDP A2-4, and CDP A2-5

analogs have low anti-scuticociliate activity. However 10 μg/mL of CDP A2 and CDP A2-1 reduced parasites by approximately 15% and 5%. Movement was observed with no or very little movement across the microscopic field. Treatment of the parasites with synthesized peptides showed that the viability was lower than with untreated peptide. In this investigation, we found that the viability of scuticociliate is decreasing due to the concentration of peptides by WST-1 cell proliferation assay. Thus, these data provided CDP A2 and CDP A2-1 has strong anti-parasitic activity against scuticociliate

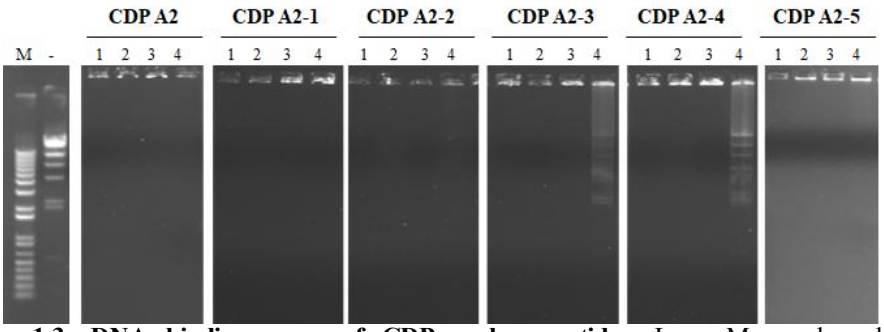



**Fig. 1.3. DNA binding assay of CDP analog peptides.** Lane M; marker, lane (-); negative control, lane 1; 1.25 μg of peptide, lane 2; 0.625 μg of peptide, lane 3; 0.313 μg of peptide, lane 4; 0.156 μg of peptide.

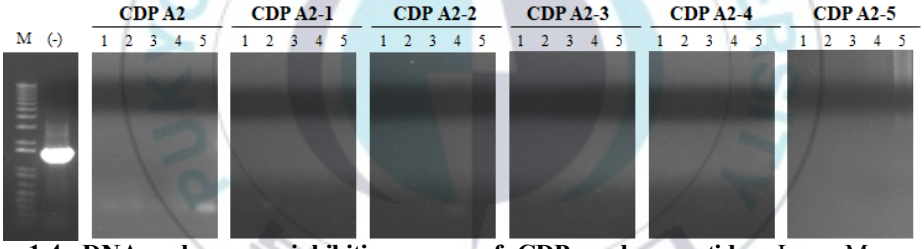



**Fig. 1.4. DNA polymerase inhibition assay of CDP analog peptides**. Lane M; marker, lane (-); negative control, lane 1; 2.5 μg of peptide, lane 2; 1.25 μg of peptide, lane 3; 0.625 μg of peptide, lane 4; 0.313 μg of peptide, lane 5; 0.156 μg of peptide.

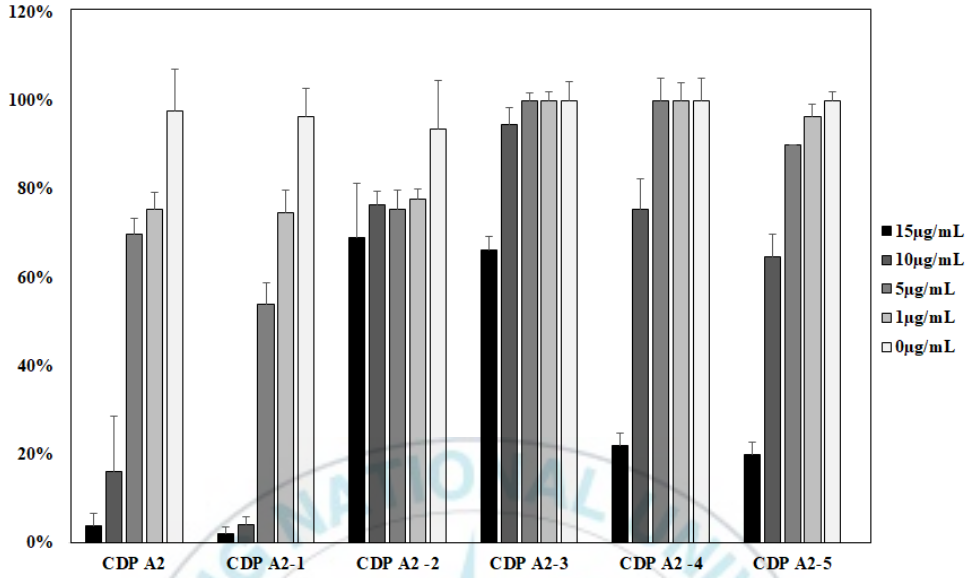


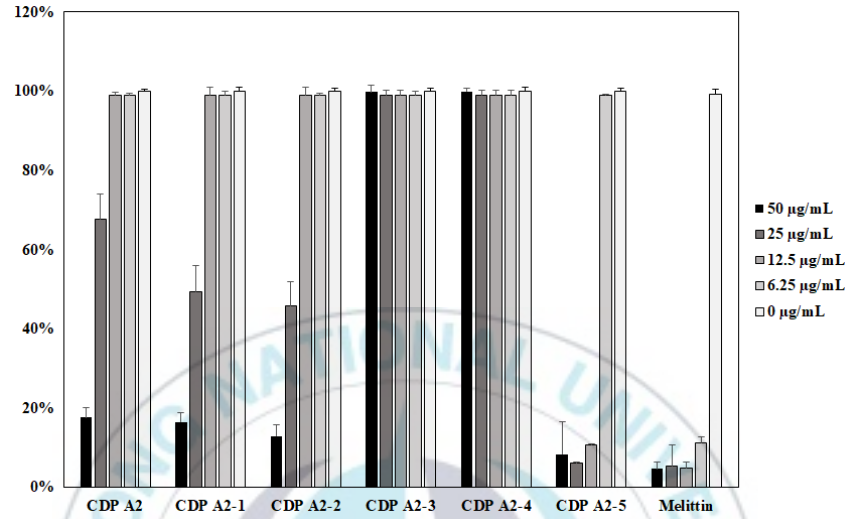

**Fig. 1.5. Anti-scutica activity. Effect of CDP A2 on scuticocliates viability assayed by WST-1**. Data represent the average of three replicates and error bars represent the standard deviation.

### 1.7. Cytotoxicity of CDP A2 on cancer cells

We investigated anti-cancer activity of CDP peptides on cancer cell lines (HeLa and A549 cells) using MTS assay (Fig. 1.6). HeLa and A549 cells were treated with 0, 6.25, 12.5, 25, and 50 μg/mL of CDP A2 analogs. CDP A2-5 have inhibit the growth of HeLa cell on 12.5 μg/mL and CDP A2-2 and CDP A2-3 showed approximately 50% of viability in HeLa cell on 25 μg/mL (Fig. 1.6A). CDP A2-4 and CDP A2-5 did not exhibit anti-cancer activity. 50 μg/mL of CDP A2-1 effected growth of HeLa cell with about 18% but viability of HeLa cell was 65% at 12.5 μg/mL of CDP A2-1. 50 μg/mL of CDP A2, CDP A2-1 and CDP A2-2 had below 50 % of viability of A549 cells (Fig.6B). CDP A2-5 showed viability from 70% to 80% 12.5 and 25 μg/mL of peptides while CDP A2-3 and CDP A2-4 had no activity in A549 cells.

The cell morphology was observed after treatment of CDP A2 (Fig. 1.7). was altered with CDP A2 (Fig. 1.7). HeLa and A549 cells were cultured with CDP A2 for 24 h and observed using a microscope. The morphology of the cells treated with CDP A2 presented shrinkage and detachment from the plate surface, which are representative features of apoptosis.

(A)




(B)

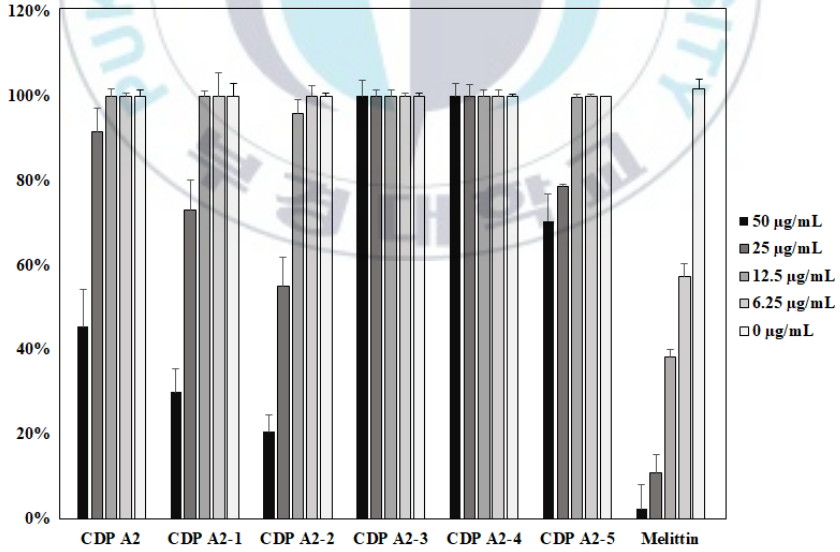



**Fig. 1.6. Anti-cancer activity in cancer cell.** (A) The viability of HeLa cell with various concentrations of series of CDP A2. (B) The viability of A549 cell with various concentrations of series of CDP A2.

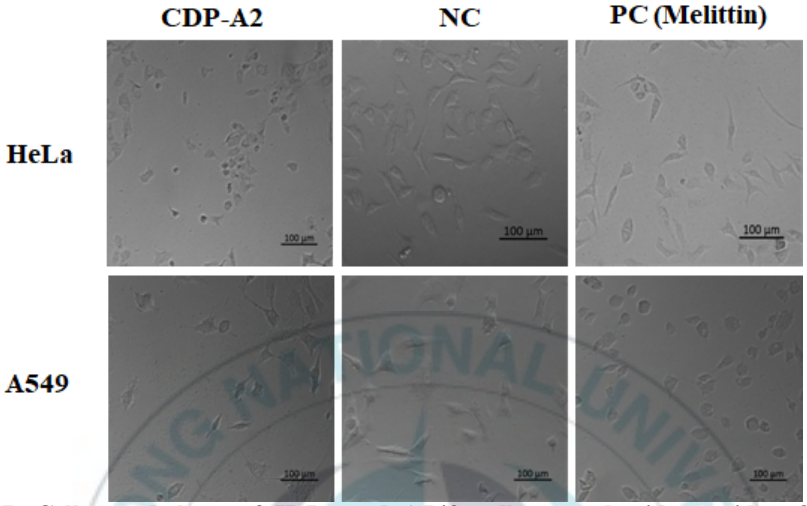



**Fig. 1.7. Cell morphology of HeLa and A549 cells treated with peptides. Scal bar is 100 μm.**

### 1.8. Effect of CDP A2 on cancer cell membrane

Annexin V FITC-A and Propidium Iodide-A plots from the gated cells show the populations corresponding to viable and non-apoptotic (Annexin V-PI-), earlt (Annexin V+PI-), and late (Annexin V+PI+) apoptotic cells (Fig. 1.8). In the untreated samples with HeLa cell as control, viable and non-apoptotic cells were 99.5%. After cells were treated samples of 10 μg/mL, 3% of cells were observed in Annexin V-PI- section and early apoptotic cell population was increased 14.1% in Annexin V+PI-). The population of late apoptotic or dead cell was increased up to 94.1% in the late apoptotic cell. The total population of apoptotic cells were 94.4% and 98 % with 20 μg/mL and 30 μg/mL of CDP A2 .

In the A549 cells, the majority of A 549 cells were viable and non-apoptotic at 97.7% (Fig. 1.9). After 30 μg/mL of CDP A2 was treated to A 549 cells, slight increase in the Annexin V+PI+ population was observed up to 27.8%. The early apoptotic cells were 53% and total population of apoptotic cells were 80.8 %.

### 1.9. Membrane permeabilization of CDP A2 analogs

To investigate permeability of bacterial membrane against *E. coli* ML35p, CDP A2 analogs were used for chromogenic bacteriolytic plate assay (Fig.1.10) [G. Mardones and A. Venegas, 2000]. The inhibition zones with color formation along the edge of spots indicate that the series of CDP A2 would be expected to inhibit the replication of the bacteria in-vitro (bacteriostatic) or kill the bacteria in-vitro (bactericidal). As shown in Fig. 10, As a result, CDP A2 showed inhibition clear zone with weak blue halo at the edge but other CDP A2 series no color for peptides against *E. coli* ML35p. These pattern proposed the series of CDP A2 has bacteriostatic activity.

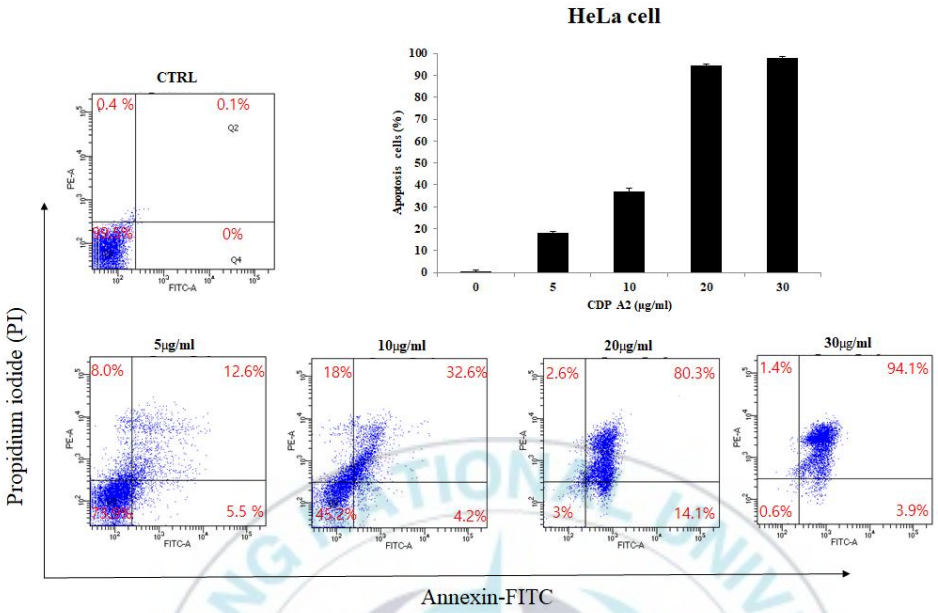



**Fig. 1.8. Quantitative analysis of HeLa cells apoptosis induced by treatments with CDP A2.**

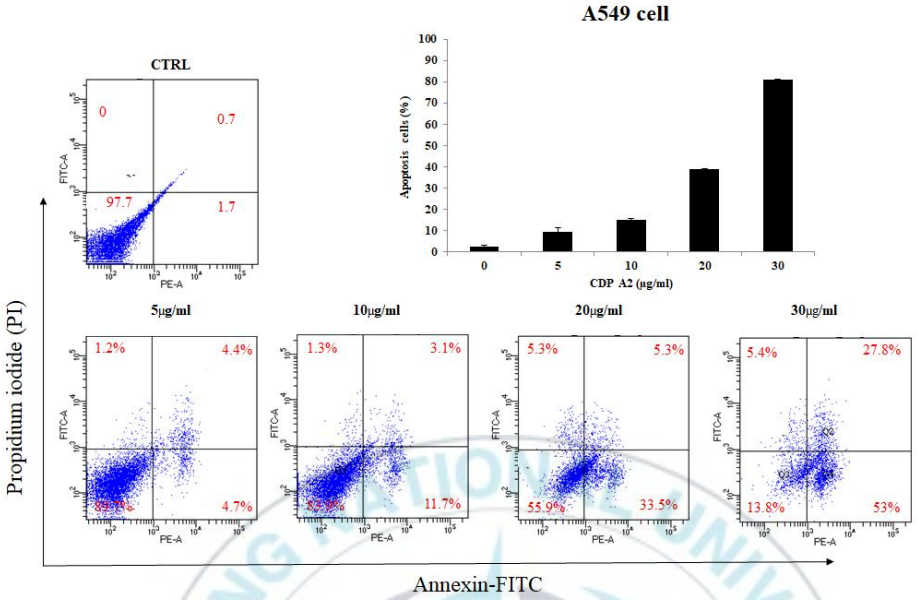



**Fig. 1.9. Quantitative analysis of A549 cells apoptosis and necrosis induced by treatments with CDP A2.**

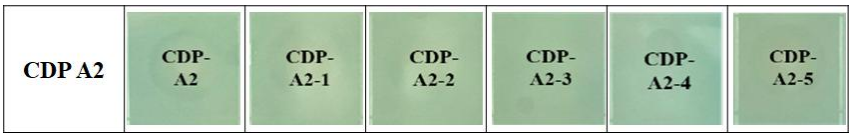



**Fig. 1.10. Membrane permeabilization of *Escherichia coli* ML 35p by CDP analog peptides**

**Conclusion**

Using the results of *Oplegnathus fasciatus* genetic analysis, catalytic domain of phospholipase (CDP) was selected. We decided to design peptide analogs in the specific region in amino acid sequence of CDP. The part of sequence of CDP and analog was synthesized as CDP A2 which showed significant antimicrobial activity. Five analogs of CDP A2 were synthesized based on amino acid sequence of CDP A2 derived from rock bream *Oplegnathus fasciatus* by substitution amino acid sequence. Multi-functional activities of CDP A2 were evaluated including antimicrobial activity, viability in cancer cells including HeLa and A549 cells and scuticociliates treated with peptides. The designed analogs exhibited more improved antimicrobial and anti-scuticociliates activity. Analogs of CDP A2 were improved the antimicrobial activity and reduced hemolysis while activity of anti-scuticociliates was similar. CDP A2-3 and CDP A2-4 did not show anticancer activity and CDP A2-2 exhibited enhanced anticancer activity at 25 μg/mL than CDP A2. In addition, CDP A2 analogs had ability of DNA binding and DNA polymerase inhibition related to mechanism of action of antimicrobial peptides. CDP has bacteriostatic activity

Fluorescence-activated cell sorter (FACS) analysis showed that CDP A2 peptide inhibited cell viability and induced apoptosis of cancer cell lines by Fluorescence-activated cell sorter (FACS) analysis. The population of late apoptotic cells was increased in HeLa cells at 94.1% and population of early apoptotic cells and late apoptotic cells was 53% and 27.8% in A 549 cells. We have identified shorter AMP fragments that have multi-functional activity and stability with minimal synthesis cost and non-toxicity.

# Chapter 2.

## Multi-functional activities of lipopolysaccharide binding protein (LBP) from rock bream *Oplegnathus fasciatus*

**2.1. Peptide design and synthesis**

Peptides were designed based on the original LBP 5N sequence (Table 2.1). All peptide were designed and synthesized considering structure, net charge, protein binding potential (Boman index), and hydrophobic ratio. To increase helicity and amphipathicity, amino acids residues were substituted Proline (Pro) at position 3 in LBP 5N to Leucine, and Glycine (Gly) at position 4 in LBP 5N to Lysine (Lys) as LBP 5A. After then, we substitutes Leucine (Leu) and Lysine (Lys) with other amino acid residues. Leucine (Leu) at position 3 in LBP 5A were substitutes with Methionine (Met), Isoleucine (Iso), Lysine (Lys) and Valine (Val), and Lysine (Lys) at position 4 in LBP 5A to Glycine (Gly).

The eight peptides of LBP series showed positive net charge (+3 to +5), lower Boman Index (<2), higher hydrophobic ratio by substitution of amino acid, N-terminal acetylation and C-terminal amidation, thus enhancing the interaction between peptide and membrane and increase membrane permeability. The EMBOSS Pepwheel program was used to identify hydrophobic and hydrophilic regions in the secondary structure of the native and analog peptides (Fig. 2.1).

**Table. 2.1. Summary of design of LBP analogs**

| Name | Amino acid sequence | Molecular weight (Da) | Length (AA) | Net charge | Boman index (kcal/mol) | Hydrophobic ratio (%) |
|---|---|---|---|---|---|---|
| LBP-5N | RFPGL MMKLL | 1205.59 | 10 | 2 | -0.29 | 60 |
| LBP-5A | RFLKL MMKLL | 1292.2 | 10 | 3 | -0.13 | 70 |
| LBP-5A-1 | RFMKL MMKLL | 1309.79 | 10 | 3 | 0.12 | 70 |
| LBP-5A-2 | RFIKL MMKLL | 1291.76 | 10 | 3 | -0.13 | 70 |
| LBP-5A-3 | RFLKL MKKLL | 1288.73 | 10 | 4 | 0.65 | 60 |
| LBP-5A-4 | RFLGL MMKLL | 1220.63 | 10 | 3 | 0 | 60 |
| LBP-5A-5 | RFPKL MMKLL | 1275.71 | 10 | 3 | 0.35 | 60 |
| LBP-5A-6 | RFKKL MKKLL | 1303.75 | 10 | 5 | 1.7 | 50 |
| LBP-5A-7 | RFVKL MMKLL | 1277.73 | 10 | 3 | -0.04 | 70 |

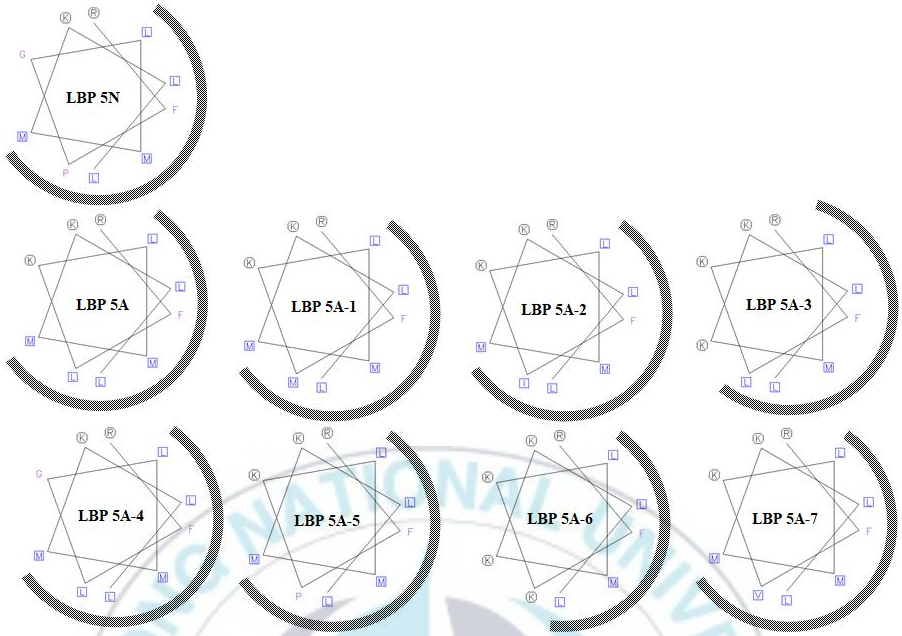



**Fig. 2.1. Helical wheel of LBP and derivative sequences.** Square box represent hydrophobic residues, circle represent hydrophilic residues and The hydrophobic face is indicated as a grey line. Projections were made uisng EMBOSS pepwheel.

**2.2. Antimicrobial activities of LBP 5A analogs**

The antimicrobial activities of LBP 5 (native form) and LBP 5A (analog) were tested by URDA against several bacterial species and yest *C. albicans*. Activity of LBP 5A was similar or improved than native form against most of strains (Fig. 2.2). Based on structure of LBP 5A, amino acids were substituted and terminal modification. After than, antimicrobial activities of LBP derived synthetic peptides were determined by measuring minimal effective concentrations (MECs) by URDA (Table 2.2). All analogs have antimicrobial activity showed strong activity against Gram-positive bacteria *S. aureus* CCARM 0203 (MECs 1.01-10.44 μg/mL). Most analogs lower activity against *L. garvieae*, *P. stuartii*, and *V. harveyi*. LBP 5A-3, LBP 5A-5 and LBA 5A-6 showed much less activity than other 5 analogs, excluding *B. subilis E. cloacae*, *S. aureus* CCARM 0203. LBP-5A and LBP 5A-2 exhibited potent activity against 15 strains (MECs 4.3-10.8 μg/mL) and 18 strains (MECs 3.1-10.5 μg/mL). LBP 5A-4 and LBP 5A-7 showed strong activity against 14 strains (MECs 2.7-10.7 μg/mL) and 13 strains (MECs 5.3-10.3 μg/mL). LBP 5A-1 have strong antimicrobial activity against 10 strains (MECs 2.6-9.0 μg/mL).

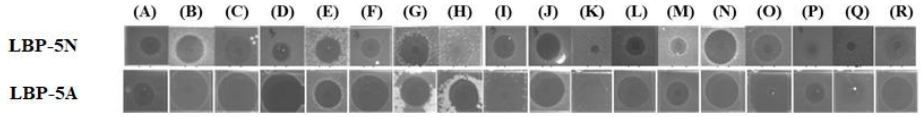



**Fig. 2.2. Antimicrobial activity of LBP native form and analog.** (A) *B. cereus* (B) *B. subtilis* (C) *E. coli* (D) *S. mutans* (E) *C. albicans* (F) *P. aeruginosa* (G) *V. alginolyticus* (H) *V. parahaemolyticus* (I) *S. iniae* (J) *S. aureus* (K) *L. garvieae* (L) *E. cloacae* (M) *E. faecalis* (N) *K. pneumoniae* (O) *S. parauberis* (P) *E. tarda* (Q) *P. mirabilis* (R) *P. stuartii*

**Table. 2.2. MEC for antimicrobial activities of LBP 5A derived synthetic peptide.**

| | | Strain | | LBP-5A | LBP-5A-1 | LBP-5A-2 | LBP-5A-3 | LBP-5A-4 | LBP-5A-5 | LBP-5A-6 | LBP-5A-7 | Piscidin |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Microbes | Positive | *Bacillus cereus* | KCTC 1012 | 17.2 | 20.4 | 17.5 | 37.2 | 19.0 | >250 | >250 | 14.2 | 11.7 |
| | | *Bacillus subtilis* | KCTC 1021 | 6.5 | 12.7 | 7.8 | 12.6 | 8.8 | 6.4 | 7.4 | 8.2 | 5.0 |
| | | *Enterococcus faecalis* | KCTC 3206 | 11.6 | 8.4 | 4.6 | 26.0 | 6.6 | >70 | >70 | 26.0 | 10.9 |
| | | *Staphylococcus aureus* | ATCC 6538 | 4.6 | 3.3 | 4.1 | 12.6 | 7.4 | 17.1 | 13.7 | 6.1 | 4.8 |
| | | *Streptococcus mutans* | KCTC 3065 | 9.4 | 13.4 | 12.4 | 11.7 | 11.1 | 13.6 | 15.6 | 4.7 | 13.4 |
| | | *Lactococcus garvieae* | KCTC 3772 | >250 | 16.0 | 16.5 | >250 | >250 | >250 | >250 | >250 | >250 |
| | | *Streptococcus parauberis* | KCTC 3651 | 8.1 | 14.5 | 10.5 | 12.5 | 11.1 | 33.8 | 19.2 | 7.3 | 10.2 |
| | Negative | *Enterobacter cloacae* | KCTC 2361 | 10.8 | 6.5 | 7.9 | 10.3 | 11.5 | 16.6 | >70 | >70 | 21.2 |
| | | *Escherichia coli* | ATCC 8739 | 4.3 | 6.5 | 4.2 | 7.1 | 10.7 | 12.1 | 13.4 | 8.0 | 4.5 |
| | | *Escherichia coli* | ML35p | 5.9 | 2.6 | 3.1 | 1.2 | 5.0 | 6.5 | 13.8 | 6.1 | 7.8 |
| | | *Klebsiella pneumoniae* | KCTC 12385 | 7.0 | 11.7 | 10.1 | 12.0 | 18.4 | 27.3 | >250 | 10.3 | 20.0 |
| | | *Providencia stuartii* | KCTC 2568 | 24.1 | >250 | >250 | >250 | >250 | >250 | >250 | 33.5 | 28.0 |
| | | *Pseudomonas aeruginosa* | ATCC 15522 | 11.8 | 17.2 | 7.2 | 11.3 | 15.2 | >250 | >250 | 12.4 | 22.4 |
| | | *Vibrio parahaemolyticus* | KCCM 41664 | 14.4 | 34.5 | 19.9 | >70 | 28.0 | >250 | >250 | 49.5 | >250 |
| | | *Vibrio vulnificus* | KCCM 41665 | 15.0 | 20.8 | 12.6 | 14.5 | 11.7 | 26.5 | 10.1 | >250 | 19.9 |
| | | *Vibrio alginolyticus* | KCTC 2472 | 11.9 | 22.4 | 16.1 | 30.3 | 24.2 | >70 | >70 | 22.2 | 7.9 |
| | | *Vibrio anguillarum* | KCTC 2711 | 13.0 | 24.5 | 15.9 | 27.6 | 6.5 | 20.7 | 24.8 | 20.1 | 15.4 |
| | | *Vibrio harveyi* | KCCM 40866 | 17.2 | >250 | 51.7 | >250 | 48.8 | >250 | >250 | 50.4 | >250 |
| | Fungi | *Candida albicans* | ATCC 10231 | 5.7 | 11.2 | 8.3 | 11.0 | 12.7 | 12.0 | 9.8 | 8.4 | 11.2 |
| Antibiotic resistant strains | Positive | *Staphylococcus aureus* | CCARM 0203 | 5.7 | 7.6 | 7.5 | 5.2 | 8.4 | 6.2 | 6.8 | 5.3 | 7.0 |
| | | *Staphylococcus aureus* | CCARM 0204 | 4.7 | 8.0 | 5.3 | 5.5 | 8.5 | 7.4 | 18.5 | 6.2 | 6.9 |
| | | *Staphylococcus aureus* | CCARM 3795 | 12.8 | 13.8 | 5.4 | 14.1 | 19.5 | 38.1 | >250 | 28.1 | 4.2 |
| | | *Streptococcus pyogenes* | CCARM 0206 | 13.3 | 12.0 | 7.8 | 18.3 | 12.2 | >250 | >250 | 8.1 | 14.1 |
| | Negative | *Pseudomonas aeruginosa* | CCARM 0225 | 11.2 | 23.1 | 17.1 | 22.4 | >250 | >250 | 63.0 | 20.8 | 16.6 |
| | | *Enterobacter cloacae* | CCARM 0252 | 5.5 | 7.4 | 5.5 | 7.4 | 3.5 | 5.5 | 5.4 | 24.8 | 9.8 |
| | | *Escherichia coli* | CCARM 0238 | 7.7 | 8.8 | 6.9 | 14.5 | 2.7 | 5.3 | 12.5 | 8.1 | 8.5 |
| | | *Escherichia coli* | CCARM 1A814 | 6.0 | 13.8 | 7.4 | 12.8 | 7.6 | 16.3 | 45.1 | 16.0 | 7.0 |
| | | *Klebsiellaaerogenes* | CCARM 0249 | 19.6 | 21.6 | 11.7 | 15.7 | 9.4 | 14.9 | >250 | 19.5 | 27.5 |
| | | *Klebsiellaoxytoca* | CCARM 0248 | 9.8 | 9.0 | 4.8 | 13.9 | 3.0 | 16.5 | >250 | 23.7 | 7.2 |
| | | *Salmonella typhimurium* | CCARM 0240 | 14.0 | 19.1 | 9.0 | 13.1 | 6.3 | 17.8 | 27.7 | 7.0 | 19.6 |

### 2.3. Hemolytic activities

The hemolytic activity of the analogs were determined using blood cells of olive flounder for test cytotocixity (Fig 2.3). LBP 5A-3, LBP 5A-5, LBP 5A-6, and LBP 5A-7 did not showed hemolytic activity up to 50 μg/mL but LBP 5A-7 caused hemolysis at 100 μg/mL of peptide. LBP 5A-1, LBP 5A-2, and LBP 5A-4 did not exhibited hemolysis up to 25 μg/mL. These results suggested that LBP analogs have lower hemolytic activity than LBP 5A.

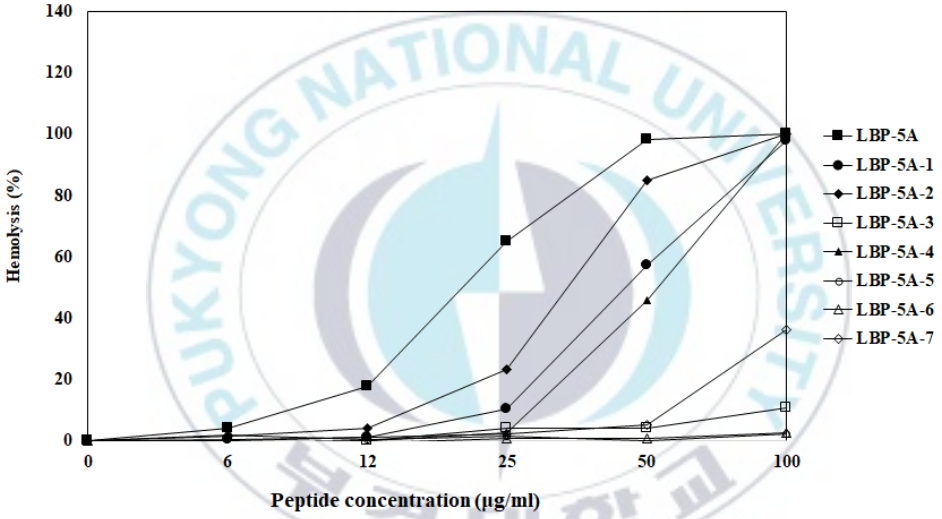



**Fig. 2.3. Hemolytic activity of LBP 5A analogs peptides against blood cell of olive flounder.**

**2.4. DNA-Binding assay of LBP 5A analogs**

Binding ability of LBP 5A analogs to DNA were investigated using electrophoretic mobility shift assay (EMSA). The electrophoretic mobility of the DNA was almost completely inhibited by peptides. LBP 5A and LBP 5A-2 almost inhibited electrophoretic mobility of the DNA at 0.313 μg of peptide and LBP 5A-1, LBP 5A-3, and LBP 5A-7 slightly inhibited at 0.313 μg of peptide. LBP 5A-4 and LBP 5A-5 inhibited at 1.25 μg of peptide compared to non-complexed DNA (Fig. 2.4).

**2.5. DNA polymerase inhibition assay**

To determine whether the peptides inhibit DNA polymerase activity, various concentrations of peptides were added to the PCR reaction mixtures. The series of LBP peptides showed inhibitory activity and LBP 5A-2 has strong DNA polymerase inhibitory ability among the series of LBP (Fig. 2.5). LBP 5A-1, LBP 5A-3, LBP 5A-5 and LBP 5A-7 inhibit DNA polymerase activity with 1.25 μg of peptide and LBP 5A and LBP 5A-4 inhibit DNA polymerase activity with 2.5 μg of peptide but LBP 5A-6 showed weak DNA polymerase inhibitory ability.

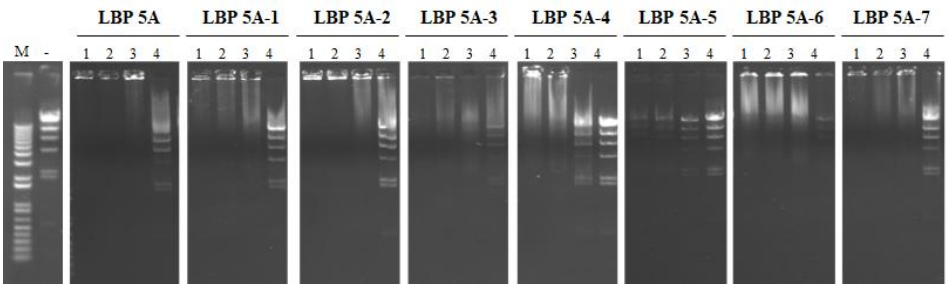



**Fig. 2.4. DNA binding assay of LBP 5A analog peptides**. Lane M; marker, lane (-); negative control, lane 1; 1.25 μg of peptide, lane 2; 0.625 μg of peptide, lane 3; 0.313 μg of peptide, lane 4; 0.156 μg of peptide.

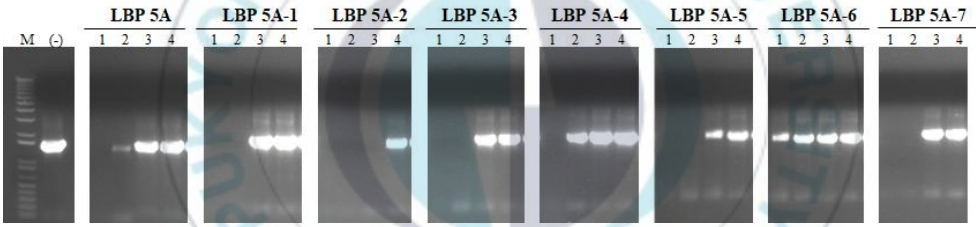



**Fig. 2.5. DNA polymerase inhibition assay of LBP 5A analog peptides.** Lane M; marker, lane (-); negative control, lane 1; 2.5 μg of peptide, lane 2; 1.25 μg of peptide, lane 3; 0.625 μg of peptide, lane 4; 0.313 μg of peptide, lane 5; 0.156 μg of peptide.

**2.6. Anti-scuticociliate activity**

LBP 5A peptide were tested against the parasites. The motility and number of parasites were noted at 25, 50, 100, and 200 μg/mL of synthesized peptides. At 24 h after infection, 50 μg/mL of LBP 5A reduced parasites by approximately 35% (Fig. 2.6). LBP 5A-2, LBP 5A-3, and LBP 5A-6 inhibited 50% of viable suticotiliates at 100 μg/mL of synthesized peptides and LBP 5A-1 and LBP 5A-4 inhibited 50% of viable suticotiliates at 200 μg/mL of synthesized peptides while LBP 5A-5 and LBP 5A-7 did not have anti- suticotiliates activity.

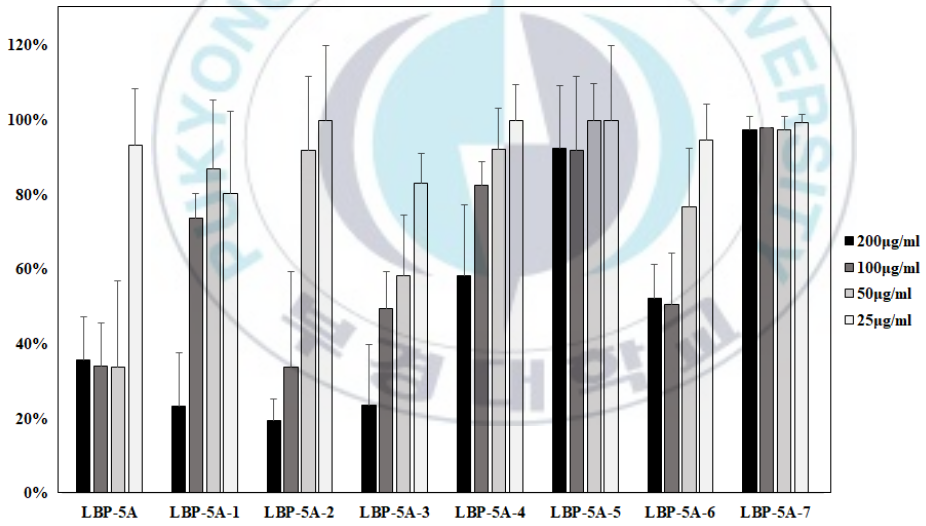


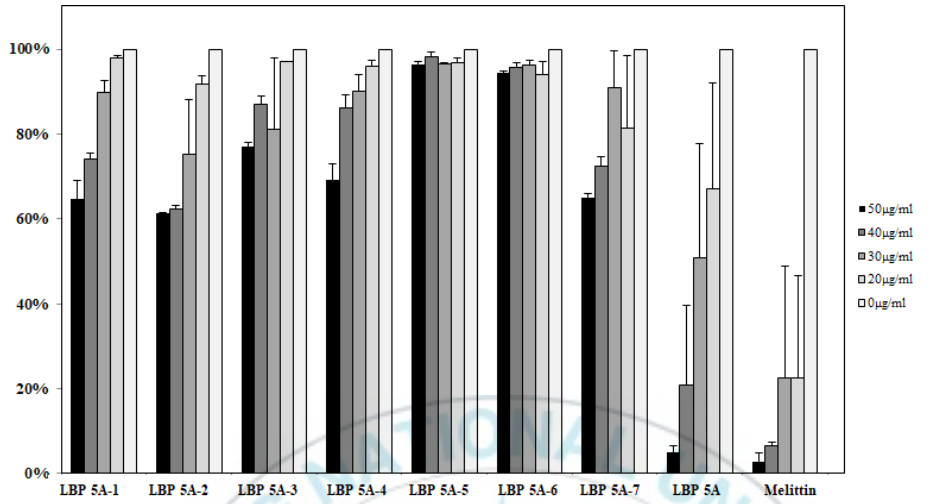

**Fig. 2.6. Viability of scuticoliates with different concentration of LBP 5A and analogs assayed by WST-1**. Data represent the average of three replicates and error bars represent the standard deviation.

**2.7. Anti-cancer activity in cancer cells**

To examine anti-cancer activity, various concentrations of LBP 5A analogs were incubated with cancer cells, HeLa and A549 cells at 37 ℃ and 5% $CO_2$ atmosphere for 24h. We investigated 30 μg/mL of LBP 5A showed about 50% viability of HeLa cell and analogs exhibited above 60% viability with 50 μg/mL of peptides (Fig 2.7A). The viability of A549 cells treated with 50 μg/mL of LBP 5A-1, LBP 5A-2, LBP and 5A was below 50% and 50 μg/mL of LBP 5A-4 and LBP 5A-7 was about 50% (Fig 2.7B).

The cell morphology was altered with peptides (Fig. 8). HeLa and A549 cells were cultured with LBP 5A for 24 h and observed using a microscope. The morphology of the cells treated with LBP 5A presented shrinkage and detachment from the plate surface, which are representative features of apoptosis.

(A)




(B)

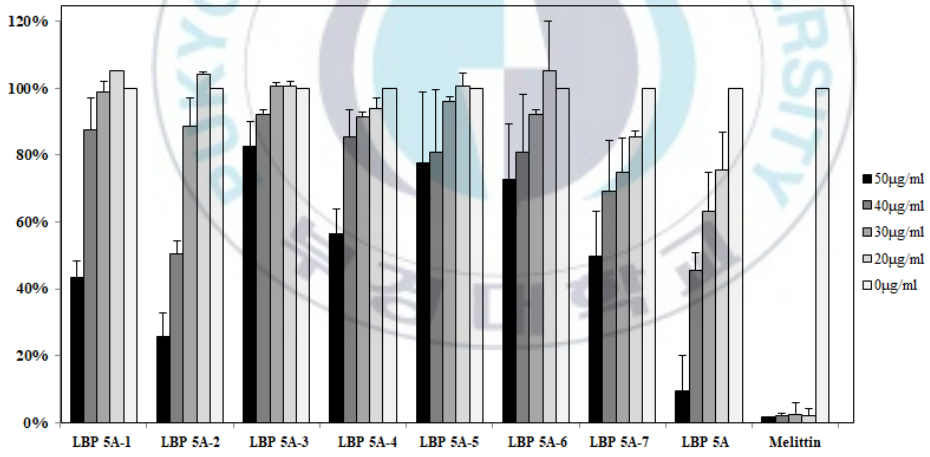



**Fig. 2.7. Anti-cancer activity in cancer cell.** (A) The viability of HeLa cell with various concentrations of series of LBP 5A. (B) The viability of A549 cell with various concentrations of series of LBP 5A.

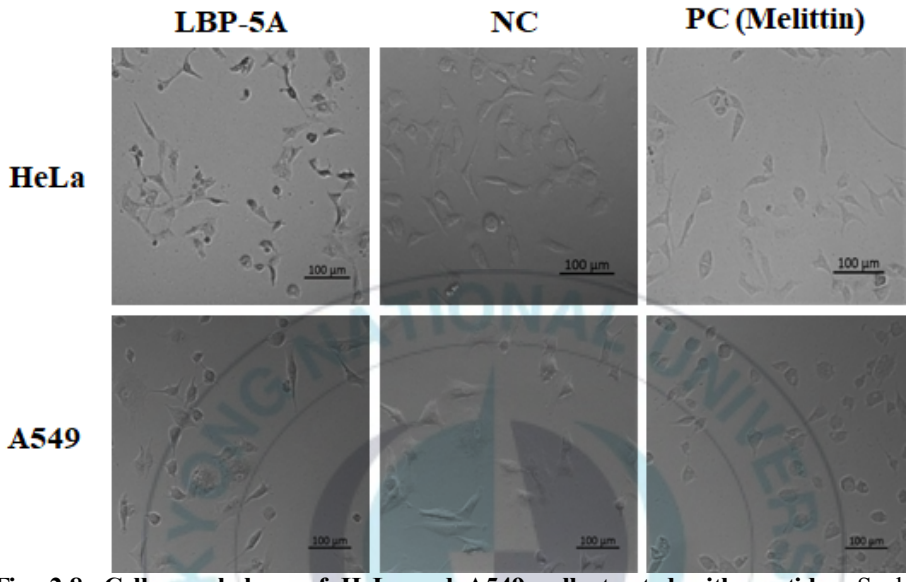



**Fig. 2.8. Cell morphology of HeLa and A549 cells treated with peptides.** Scale bar is 100 μm.

**2.8. Apoptosis in cancer cell**

To investigate the apoptosis induced by LBP 5A, cervical cancer cell, HeLa is treated with various concentrations of LBP 5A for 24 h (Fig. 2.9). Quantification of apoptosis by LBP 5A was measured by annexin-V-FITC/PI staining. We observed that LBP 5A significantly increased the number of apoptotic cells in HeLa cells. The positive cells of annexin-V-FITC staining were approximately 27.7% and 46.7% at 20 μg/mL and 30 μg/mL of LBP 5A for 24h in HeLa cells.

**2.9. Antimicrobial activity of modified LBP 5A by N-terminal acetylation, C-terminal amidation and D-form.**

We tried to develop an active enhanced model through N-, C-terminal modification and D-from, and test how it affects antimicrobial activity (Fig. 2.10). The peptide concentrations of 1000, 500, 250, 125, 62.5, and 31.3 μg/mL were used for test with N-terminal acetylated LBP, C-terminal amidated LBP, N-, C-terminal modificated LBP and D-form of LBP. Antimicrobial activity of N-terminal acetylated LBP was similar to L-form of LBP but C-terminal amidated LBP and N-, C-terminal modificated LBP was stronger than L-form of LBP.

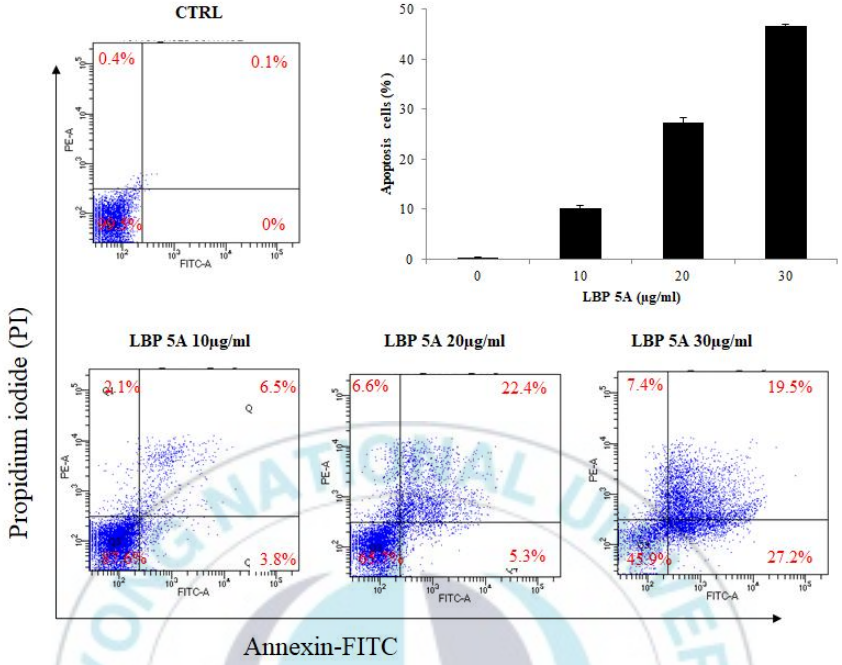



**Fig. 2.9. Quantitative analysis of HeLa cells apoptosis induced by treatments with LBP 5A.** Cells were treated with various concentration of LBP 5A for 24h and then harvedsted cells were stained with annexin-V-PI staining solution at room temperature for 15 min.


**Fig. 2.10. Antimicrobial activity of modified LBP 5A.**

**2.10. Membrane permeabilization ability**

In order to check ability of permeabilize bacterial membrane against *E. coli* ML35p, series of LBP 5A was used for chromogenic bacteriolytic plate assay (Fig. 2.11). As a result, LBP 5A showed strong membrane permeability for *E. coli* ML35p and LBP 5A-1, LBP 5A-2, and LBP 5A-7 showed mild membrane permeability. LBP 5A-3, LBP 5A-4, LBP 5A-5, and LBP 5A-6 showed weak membrane permability. These results suggest that the LBP 5A analogs may be directly permeate the membrane of bacteria to indicate antimicrobial activity.


**Fig. 2.11. Membrane permeabilization of *Escherichia coli* ML35p by LBP 5A**

**Conclusion**

Lipopolysaccharide binding protein (LBP) was selected in the results of *Oplegnathus fasciatus* genetic analysis. We decided to design and synthesis peptide analogs in the particular region in amino acid sequence of LBP. The section of sequence of LBP (native form) and LBP 5A (analog form) were synthesized and LBP 5A showed developed antimicrobial activity clearly. Seven analogs of LBP 5A were designed and synthesized based on amino acid sequence of LBP 5A by substitution amino acid sequence, D-form and N, C-terminal modification.

The antimicrobial activity, anti-cancer activity and anti-scuticociliates activity was tested with peptides. The LBP 5A analogs were demonstrated have improved antimicrobial with reduced hemolysis while only LBP 5A was strong anticancer activity. LBP 5A with strong antimicrobial activity were modified N-terminal and C-terminal and resulted in increased membrane permeability. LBP 5A Analogs exhibited that peptides bind to DNA or polymerase and inhibit polymerase chain reaction related to mechanism of action of antimicrobial peptides. In addition LBP 5A and analogs showed strong and mild membrane permeability for *E. coli* ML35p suggesting LBP 5A series directly permeate the membrane of bacteria. LBP has bactericidal activity.

Fluorescence-activated cell sorter (FACS) analysis showed that LBP 5A inhibited cell viability and induced apoptosis of cancer cell lines by Fluorescence-activated cell sorter (FACS) analysis. We have identified shorter AMP fragments that have multi-functional activity and stability with minimal synthesis cost and non-toxicity.

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# 참담치 추출물과 돌돔 유전체 정보 기반 다기능성 항균 펩타이드 연구·개발

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요 약

본 연구는 항생제 남용의 문제점 및 사용제한에 따른 천연물 유래 항생제 대체재 개발을 위하여 우리나라와 중국, 일본에서만 서식하는 고유종인 참담치 (*Mytilus coruscus*)의 각 조직으로부터 항균 펩타이드를 분리 및 정제하였고 N-말단 서열을 확보 후 서열정보를 바탕으로 전체 아미노산 및 염기서열 분석, 펩타이드의 합성 및 재조합 단백질 제작을 통해 활성기반의 항균 펩타이드의 특성 분석에 대한 연구이다. 그리고 유전체 분석을 통해 돌돔 LBP (Lipopolysaccharide binding protein) 과 CDP (Catalytic domain of phopholipase)의 아미노산 서열을 기반으로 안전성과 항균활성이 증가된 유도체를 개발하여 다기능성 분석에 대한 연구이다.

첫 번째 연구에서 참담치의 족부 근육 추출물로부터 항균 펩타이드 mytichitin-CBD를 분리하였으며 이는 55개의 아미노산으로 구성된 6.2 kDa 의 펩타이드로 그람 양성, 음성, 진균류에서 강한 항균활성을 보였다. 그리고 조직별 시간별 정량 분석을 통해 mRNA가 족부근육에서 4시간째에 주로 발현이 되는 것을 확인하였다. 또한 재조합 단백질 제작으로 항균활성 뿐만 아니라 항기생충 활성도 확인하였다.

두 번째 연구에서 참담치의 족부 근육 추출물에서 분자량 6.7 kDa 의 항균 펩타이드 protamine-like protein을 분리 및 정제하였다. N-말단 아미노산 서열 분석을 통하여 20개의 아미노산 서열을 밝혔으며 이는 캘리포니아 홍합 (*Mytilus californianus*)의 sperm-specific protein 또는 protamine-like PL-II/PL-IV precursor와 100% 상동성을 지님을 밝혀냈다. 이러한 정보를 바탕으로 open-reading frame (ORF)를 분석한 결과 60개의 아미노산을 코딩하는 183 bp로 캘리포니아 홍합의 protamine-like PL-II/PL-IV precursor와 아미노산 서열이 100% 일치하였고 유전자 염기서열은 97.2%의 상동성을 나타냈다. 합성된 항균 펩타이드는 그람 양성 균주 *Bacillus cereus* (MEC, 20.8 μg/mL), *Bacillus subtilis* (MEC, 0.2 μg/mL), *Streptococcus mutans* (MEC, 0.2 μg/mL), 그람 음성 균주 *Pseudomonas aeruginosa* (MEC, 5.7 μg/mL), *Escherichia coli* (MEC, 2.6 μg/mL) 그리고 진균류 *Candida albicans* (MEC, 56.3 μg/mL)의 항균활성을 나타냈다. 또한 높은 열안정성을 지녔으며, *C. albicans*를 제외하고 염안정성을 지닌 항균 펩타이드로 확인되었다.

세 번째 연구에서 참담치 hemocyte에 존재하는 항균 펩타이드를 분리 및 정제하였으며 이는 3.3 kDa이며, Edman 분해법을 통해 14개의 N-말단 아미노산 서열을 확보하였다. 분석한 N-말단 서열은 *M. californianus*의 sperm-specific protein Phi-1과 protamine-like PL-Ⅲ protein과 각각 93%와 87%의 유사도를 나타냈으며, *M. edulis*의 sperm-specific protein Phi-1과 87% 일치함을 확인하였다. 또한

ORF는 306 bp의 길이에 101개의 아미노산을 코딩하고 있음을 밝혔으며, 이는 *M. californianus*의 sperm-specific protein Phi-1와 93.5% 유사하였다. 분자량과 아미노산 서열에 근거하여 31개 아미노산으로 구성된 펩타이드를 합성하였으며 이는 그람 양성균인 *B. subtilis, S. mutans, S. aureus*와 그람 음성균인 *E. coli, Klebsiella. pneumoniae, P. mirabilis, P. aeruginosa* 그리고 진균류인 *C. albicans*에 항균 활성을 보였다. 합성한 펩타이드는 항생제 내성균주인 *S. aureus* CCARM 0203와 *S. aureus* CCARM 0204에 항균 활성을 보였다. 합성 항균 펩타이드는 넙치 혈장에 대한 용혈현상은 없었고, 세포독성을 확인한 결과 HUVEC cell line에 전혀 독성을 보이지 않았다. 본 연구 결과, 참담치의 혈구로부터 분리 및 정제한 sperm-specific protein 유래 항균 펩타이드는 다양한 균주에 항균 활성을 보였고 낮은 세포독성을 가졌으며, 이러한 특성은 본 실험에서 분리한 항균 펩타이드가 항생제 대체재로서 개발 가능성을 제시하고 있다.

네 번째 연구에서 참담치의 혈구 유래의 4 kDa 의 항균 펩타이드, mytilin을 분리 및 정제하였다. 25개의 N-말단 서열을 확보로 이는 참담치의 mytilin B precursor와 100%, mytilin 8 precursor, mytilin 4 precursor와 96% 일치하였다. 또한 103개의 아미노산 서열을 코딩하고 있는 312 bp의 ORF을 밝혔으며 이는 참담치의 mytilin B precursor와 100% 일치하였다. 밝혀진 분자량과 아미노산 서열을 바탕으로 C-말단 alanine 잔기의 유무에 따라 2개의 펩타이드를 합성하였으며 이는 mytilin B1과 B2라고 명명하였다. 이들은 그람 양성 균주 *B. cereus, Streptococcus parauberis* [minimal effective concentrations, MEC 41.6 - 89.7 μg/mL], 그람 음성 균주 *Enterobacter cloacae, E. coli, K. pneumoniae, Proteus mirabilis, Providencia stuartii, P. aeruginosa, Vibrio ichthyoenteri* [MEC 7.4 - 39.5 μg/mL] 그리고 진균류인 *Candida albicans* [MEC 26.0 - 31.8 μg/mL]에 항균활성을 나타냈다. 본 연구 결과, 참담치 혈구 유래 mytilin B1과 mytilin B2는 넓은 항균 스펙트럼을 가지고 열과 염분에 대한 안정성이 높으며 용혈현상과 세포독성은 나타나지 않았다.

다섯 번째 연구에서 참담치의 외투막에서 분리된 myticusin-beta는 11 kDa의 24개 아미노산 잔기의 signal peptide와 104개의 mature peptide로 구성된 574bp, 128개 아미노산 잔기로 밝혀졌으며 재조합 단백질 제작으로 그람 양성균주 (*B. cereus, B. subtilis, Clostridium perfringens, S. aureus, Streptococcus iniae, S. mutans*) 와 그람 음성균주 (*E. coli, P. aeruginosa, Vibrio alginolyticus, K. pneumoniae*) 에 대해서 항균 활성을 보임을 확인하였다. 뿐만아니라 정제된 재조합 단백질은 항 기생충 활성을 보이기도 하였다. 서열정보를 바탕으로 항균 활성이 강화된 유도체를 제작하여 항균 활성 및 안정성을 확인하였으며 조직적 정량적 분석을 통해 외투막에서 가장 높게 발현됨을 밝혔다.

여섯 번째 연구에서 돌돔의 CDP, LBP 유전자의 native form을 기반으로 하여 항균활성과 기능이 증가되고 독성이 감소한 유도체를 제작하기 위하여 아미노산 잔기 치환, 펩타이드 말단 변형 방법을 사용하였다. 제작한 유도체들의 항세균, 항기생충 활성은 증가하거나 유지되었으며 LBP 5A 유도체들은 항균활성은 증가하고 용혈활성은 감소하였지만 항기생충, 항암활성은 감소하였다. CDP A2의 경우 유도체들은 항균활성 이외 활성은 낮거나 비슷하였다. LBP 5A 는 항균 활성이 강하여 N말단의 acetylation과 C말단의 amidation으로 활성과 안정성이 증가한 항균 펩타이드를 개발하였다.

본 연구 결과들을 통해 참담치에서 분리한 항균 펩타이드 mytichitin-CBD, protamine-like protein, McSSP-31, mytilin B1, mytilin B2, myticusin 들은 넓은 항균 스펙트럼과 열과 염분에 대한 높은 안정

성으로 기능성 사료첨가제 및 항생제 대체제로써 충분히 안정적인 역할을 할 뿐만 아니라 추후 항균 펩타이드의 구조적 중요성과 참담치의 면역학적 측면에서 해양무척추동물의 선천성 면역 기작에 대한 정보를 제시할 것으로 사료된다. 그리고 돌돔의 CDP, LBP 펩타이드 유도체개발을 통해 분자량과 독성은 낮고 항균활성, 투과성, 다기능성, 안정성을 증가된 물질로 새로운 항균 기능성 소재로 사료첨가제로 활용 가능한 것으로 보인다.

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