2014년 8월
박사학위논문

Purification and characterization of multi-functional peptide produced by *Bacillus* strain isolated from Korean food

조 선 대 학 교 대 학 원

약 학 과

성 정 헌

Purification and characterization of multi-functional peptide produced by *Bacillus* strain isolated from Korean food

국내 전통식품에서 분리한 *Bacillus* 속 세균이 생산하는 다기능성 펩타이드의 분리, 정제 및 특성 연구

2014 년 8 월 25 일

조 선 대 학 교 대 학 원

약 학 과

성 정 헌

Purification and characterization of multi-functional peptide produced by *Bacillus* strain isolated from Korean food

지도교수 유 진 철

이 논문을 약학 박사학위 논문으로 제출함

2014 년 4 월

조 선 대 학 교 대 학 원

약 학 과

성 정 헌

성정헌의 박사학위 논문을 인준함

위원장 고려대학교 교수 김 승 욱 (인)

위 원 선문대학교 교수 송 재 경 (인)

위 원 광주대학교 교수 이 효 정 (인)

위 원 조선대학교 교수 김 은 애 (인)

위 원 조선대학교 교수 유 진 철 (인)

2014 년 6 월

조 선 대 학 교 대 학 원

국문초록

국내 전통식품에서 분리한 *Bacillus* 속 세균이 생산하는 다기능성 펩타이드의 분리, 정제 및 특성 연구

성 정 헌

지도교수: 유 진 철

조선대학교 약학대학 약학과

국내 식품에서 분리한 바실러스 약 100 여종을 대상으로 항균, 항산화, 항염 펩타이드를 생산하는 균주를 스크리닝 하였다. 후보 미생물 중 MP40 발효물이 항균 및 항산화 효과가 있음을 확인하였다.

선별한 MP40 균주는 16S rRNA 염기서열분석법을 통하여 신규한 균주임을 확인하고 *Bacillus* sp. MP40 이라 명명하였다. 본 균주의 최적 배양 조건은 탄소원 sorbitol (1 % w/v), 질소원 peptone (1 % w/v),

tryptone (0.5 % w/v), soytone (0.5% w/v), 무기염류 calcium chloride (0.01 %), 37℃, 12 시간임을 확인하였다. 최적 배양 후 황산 암모늄 침전법과 Sepharose CL-6B, Sephadex G50 컬럼 크로마토 크래피를 이용하여 분리정제 후 펩타이드의 분자량이 1000Da 정도임을 확인하였다.

MP40 항균펩타이드는 그람 음성균인 *E.coli* 와 다약제 내성균인 반코마이신 저항성 장알균 (VRE)에 좋은 항균 활성을 나타내었다. 특히, 장알균(VRE)에 대한 항균 활성이 뛰어 났으며 VRE 의 항생제에 대한 최소 저해 농도값은 10-20 μg/ml 이었다.

MP40 펩타이드는 pH 와 온도 안정성 시험을 통해 pH 2.0-10.0, 20~100 ℃까지 항균 활성이 유지되었으며 단백질 분해효소인 lipase, proteinase K, α-chymotrypsin, tyrpsin 에 안정함을 확인하였다. 다양한 chemical 에 대한 안정성을 확인한 결과 다양한 유기용매, EDTA, TCA, 계면활성제 등에 활성이 저해되지 않고 유지되었다. MP40 항균 펩타이드의 항산화 활성은 DPPH 라디칼 소거능, 환원력, 총 페놀 함량 측정, 아질산 소거능 시험을 통해 분석하였다. 이 펩타이드의 DPPH 소거능과 환원력, 아질산 소거능은 대조군인 아스코르브산과 같은 농도에서 비교하였을 때 뛰어난 수준의 항산화 활성을 나타내었으며, 총 페놀 함량은 같은 농도에서 갈산보다 높은 수준의 페놀을 함유함을 확인하였다.

Murine macrophage 인 Raw264.7 대식세포는 LPS 에 의해 자극되면 일산화 탄소의 생성이 증가하는데 MP40 항균 펩타이드를 10~200 ug/ml 의 농도로 처리하였을 때 이의 생산을 억제하는 효과를 보였으며 이 농도에서의 세포독성도 나타내지 않았다.

본 연구를 통해 국내 식품에서 분리한 *Bacillus* sp. MP40 균주가 생산하는 펩타이드가 항생제 내성 세균에 항균 효과를 가지며, 높은 항산화 활성 및 NO 생성을 억제하는 것을 확인하였다.

ABSTRACT

Purification and characterization of multi-functional peptide produced by *Bacillus* strain isolated from Korean food

Seong, Jeong Heon

Advisor: Prof. Jin Cheol Yoo, Ph.D.

Department of Pharmacy,

Graduate School of Chosun University

Bacillus sp. MP40 was isolated from several hundreds of *Bacillus* strains preserved in our laboratory isolated from Korean traditional foods. Fermentation broth of MP40 showed antimicrobial, antioxidant activities. Therefore we selected this strain for the study. Based on the 16s rRNA sequencing, MP40 was found related to the *Bacillus amyliquefaciens*. Therefore the strain was classified as *Bacillus* sp. MP40. Antimicrobial peptide production from the strain was optimized by using several nutritional parameters. Sorbitol, peptone, tryptone, soytone, $CaCl_2$ were found

the best carbon, nitrogen and metal ion sources. After fermentation of MP40 strain under optimized condition for 12 h, broth was harvested. The antimicrobial peptide was precipitated using ammonium sulfate. MP40 AMP (ca. 1000Da) was purified to homogeneity by using sequential chromatographic steps. MP40 AMP performed antimicrobial activity against Gram negative bacteria and MDR bacteria such as *Escherichia coli*, vancomycin-resistant *enterococci* (VRE). Minimal inhibitory concentrations of MP40 AMP against VRE were in the range of 10–20 μg/ml. MP40 AMP was found to be stable at pH 2.0–10.0 and up to 100 ℃. The AMP activity was resistant to the proteolytic action of lipase, proteinase K and α-chymotrypsin, trypsin. The effect of several chemicals on the AMP was evaluated. The AMP was resistant to various organic solvents and detergents.

The antioxidative activities of the AMP were determined by using 2,2-diphenyl-a-picrylhydrazyl (DPPH), reducing antioxidant power, total phenol contents, nitrite scavenging activity methods. The MP40 AMP has good antiradical potency against the DPPH. The reducing power of MP40 AMP was increased in a concentration dependent manner. The DPPH scavenging, nitrite scavenging activity and reducing power of MP40 AMP was more effective than ascorbic acid (control). The total phenol contents of MP40 AMP were increased concentration dependent. Total phenol contents of peptide was significantly higher than gallic acid in same concentration

Bacillus sp. MP40 AMP was shown in this study to have potent inhibitory effects

on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 macrophages.

In conclusion, antimicrobial peptide producing MP40 strain was purified from a new *Bacillus* strain isolated from Korean foods. This AMP has antimicrobial, high antioxidant activities and inhibited nitric oxide production.

Contents

List of Tables

List of Figures

List of Abbreviations

AMP	Antimicrobial peptide
DMEM	Dulbecco's Modified Eagle's Medium
DPPH	2,2-diphenyl-1-picrylhydrazyl
ESBL	Extended spectrum beta lactamase
FBS	Fetal bovine serum
IMP	Imipenem resistance
LPS	Lipopolysaccharide
MDR	Multidrug-resistant
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTT	3-(4,5-dimethylthiazol-2-2,5-diphenyltetrazolium bromide)
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TPC	Total phenol contents
VRE	Vancomycin-resistant *enterococci*
VRSA	Vancomycin-resistant *Staphylococcus aureus*

I. Introduction

Antimicrobial peptides (AMPs) are ubiquitously secreted by a wide range of microorganisms, including bacteria, to protect themselves from other microbes. These peptides are composed of 10-40 residues polypeptides and affect the active element of the innate immune response. Also, AMPs have been confirmed to inhibit or kill Gram-negative and Gram-positive bacteria including important clinical pathogens, mycobacteria, protozoa, viruses, fungi, and cancer cell (Rana *et al*. 2006).

Antimicrobial peptides with biological activities contained large and diverse family of natural products, which include antibiotics, enzyme inhibitors. According to the way to synthesize, these peptides fall into two classes: nonribosomally synthesized peptides and ribosomally synthesized peptides (Hancock *et al*. 1999).

Nonribosomally synthesized peptides are class of peptide secondary metabolites, generally produced by microorganisms like bacteria and fungi. Nonribosoamal peptides are synthesized by nonribosomal peptide synthetases. These are independent of messenger RNA. Each nonribosomal peptide synthetase can synthesize only one type of peptide. These peptides are diverse family of products with a broad range of biological activities and pharmacological properties. Nonribosomal peptide antibiotics (actinomycin, bacitracin, daptomycin, vancomycin, gradicidin etc.), cytostatics and immunosuppressants drug are in commercial use.

Ribosomally synthesized peptides (AMPs) are produced by mammals, birds, amphibians, insects, plants, and microorganisms and affect crucial components of their defense systems against microorganisms. These gene encoded peptides share some common physico-chemical properties. Such as being small, cationic, amphiphilic, and often being membrane active. Even though they vary in structure, they are cationic and amphiphilic, which indicates the fact that many of them attack target cells by permeabilizing the cell membrane (Papagianni *et al.* 2003). The action mechanisms have been investigated for some antimicrobial peptides, including defensin (Ganz *et al*. 2003), maganin (Ludtke SJ *et al.* 1996), nisin (Rollema *et al.* 1995), prophenin (Harwig *et al*. 1995), cecropin (Moore *et al*. 1996), cathelicidin and histatin (De Smet *et al*. 2005). Most AMPs do not target specific molecular receptors of pathogens but rather interact with each other and permeabilize microbial membrane (Park *et al*. 2009). The disruption of microbial cell structure is the main killing mechanism for antimicrobial peptides.

Antimicrobial peptides are diverse group of molecules, which are divided into 4 classes on the basis of their structure and amino acid composition. Antimicrobial peptides are small, positively charged, amphipathic molecules (which possess both hydrophobic and hydrophilic regions) of variable amino acid composition and length (six to 100 amino acids). These peptides include two or more positively charged residues provided by arginine, lysine, histidine and a large proportion (generally

>50 %) of hydrophobic residues. The secondary structures of these molecules follow 4 classes, including A) α-helical, B) β-stranded due to the presence of 2 or more disulfide bonds, C) β-hairpin or loop due to the presence of a single disulfide bond and/or cyclization of the peptide chain, and D) extended (Figure 1) (Powers *et al*. 2003; Peters *et al*. 2010).

Many of these peptides can fold into amphipathic or amphiphilic conformations, often induced by interaction with membranes. This trait of the antimicrobial peptides allows to partition into the membrane lipid bilayer. The ability to associate with membranes is a definitive feature of antimicrobial.

The action of antimicrobial peptides causes membrane defects such as pore formation, promotion of bilayer disruption, depending on the molecular properties of both peptide and lipid. Generally, the action mechanisms were suggested to describe the process of phospholipid membrane permeation by membrane-active peptides, the 'barrel-stave' and 'carpet' mechanisms (Figure 2) (Toke 2005; Bahar *et al.* 2013).

The 'barrel stave' mechanism describes the formation of transmembrane pores by peptide clusters. Combination of additional peptide monomers leads to a consistently increasing pore size. A decisive step in the barrel stave mechanism needs peptides to recognize one another in the membrane bound state. At that time, this mechanism is required a specific structures, such as amphipathic α-helix, β-sheet of both α-helix and β-sheet structures. Peptide assembly can occur on the surface or within the

hydrophobic centre of the membrane, because hydrophobic peptides can span membranes as monomers. In contrary for a single amphiphilic α-helix it is energetically unfavorable to transverse the membrane as a monomer. Therefore such monomers must associate on the surface of the membrane before the insertion.

According to the carpet model, peptides bind the phospholipid membrane surface up until a threshold concentration is reached, and then the peptides cause membrane permeation. High local concentration of the membrane surface is covered with peptide monomers, or alternatively, antimicrobial peptides that associate on the surface of the membrane can form a local carpet. At an intermediate stage wormhole formation has been suggested to occur. These Pores may allow the passage of low molecular weight molecules prior to complete membrane lysis. This formation called wormholes or toroidal pores.

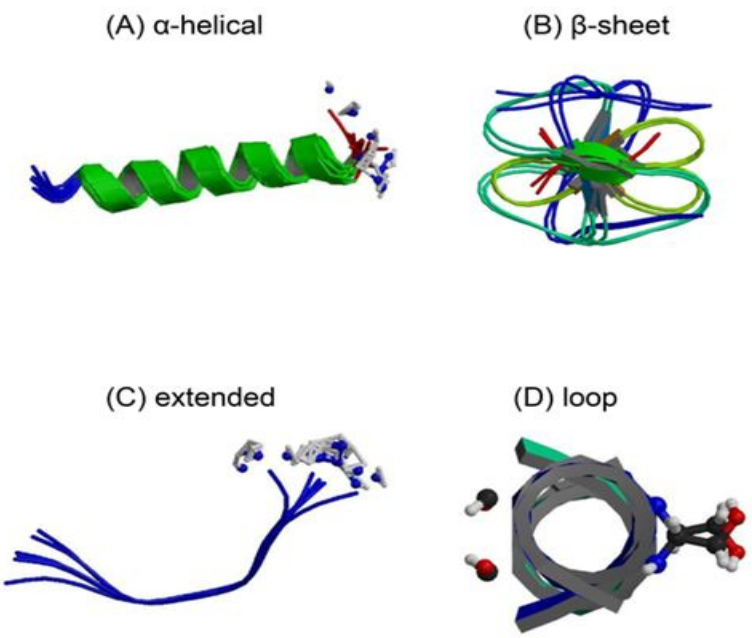


Figure 1. Structure classes of antimicrobial peptides

Antimicrobial peptides can be grouped into four major classes based on their secondary structures, including the (A) α-helical, magainin-2; (B) β-sheet, β–defensin 1; (C) extended, indolicin; (D) loop, Gramicidin (Peters *et al*. 2010)

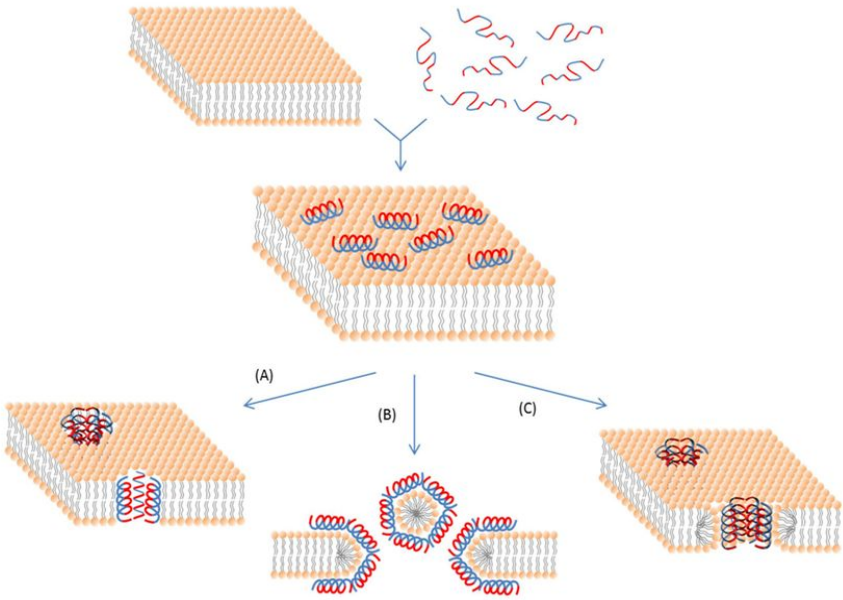


Figure 2. Antimicrobial peptide mechanisms of action

(A) In the barrel-stave model, the peptides span the membrane and form a pore with the hydrophilic portion lining the pore. (B) The carpet model is characterized by the spanning of the membrane by the peptide followed by a detergent-like action that disrupts the membrane structure. (C) The toroidal model differs from the barrel-stave mechanism as the hydrophilic portion of the peptide is associated with the lipid head group. The hydrophilic and hydrophobic faces of the peptides are colored in blue and gray, respectively (Bahar *et al*. 2013).

AMPs produced by microbes such as *Bacillus* spp. play a major role in therapeutic applications. The *Bacillus* genus is a genus of Gram-positive, rod-shaped, endospore-forming bacteria spread into the environment. *Bacillus* species can be obligate aerobes of facultative anaerobes. Production of antimicrobial peptides by Bacillus strains has been increasingly characterization in the recent past and many peptides produced by this group of bacteria found to be suitable for various applications (Baindara *et al.* 2013).

Also, *Bacillus* has been widely used in the fermentation industry for the production of antibiotics as well as several extracellular enzymes. A large number of peptides with biological activities have been increasingly reported from this group and have become a centre of attention for antimicrobial study (Wu *et.al.* 2005; Ahern *et al*. 2003; Oscariz *et al*. 1999; Cherif *et al*. 2001; Dischinger *et al*. 2009). Most of the peptides produced by *Bacillus* are antibacterial and a few are antifungal, antitumor, fibrinolysis-promoting, immunosuppressive, amylases, lipases and proteases.

Inflammation is part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. The inflammatory process is a protective response that occurs in response to infection, tissue injury or noxious stimuli. In these process, activated inflammatory cells (neutrophils, eosinophils, mononuclear phagocytes and macrophages) secrete increased amounts of nitric oxide (NO) and cytokines, such as interleukin IL-1ß, IL-6, and tumor necrosis

factor TNF-α (Yoon *et al*. 2012).

Nitric oxide (NO) is produced by many different cell types, and it is an important regulator and mediator of various processes including smooth muscle relaxation, neurotransmission, and murine macrophage-mediated cytotoxicity for microbes and tumor cells. NO is a major product and its production is controlled by the nitric oxide synthases (iNOS), iNOS is highly expressed in macrophages; its activation leads to organ destruction in some inflammatory and autoimmune diseases. During inflammation, macrophages play a central role in managing many different immune-pathological phenomena, including the overproduction of pro-inflammatory cytokines and inflammatory mediators such as iNOS, COX-2 and TNF- α, IL-1ß, IL- 6 (Yoon *et al*. 2009).

This study describes the identification of an AMP-producing strain, *Bacillus* sp. MP40, isolated from Korean foods as well as purification and biochemical characterization. And I examined the antioxidant activity and the anti-inflammatory effects of antimicrobial peptides from *Bacillus* sp. by measuring the production in murine macrophage Raw 264.7 cells.

II. *Bacillus* sp. MP40

A. Materials & Methods

1. Materials

Sepharose CL-6B and Sephadex G-50 were obtained from Pharmacia (Uppsala, Sweden). All other chemicals and reagent were of analytical grade.

Trypsin, Proteinase K, α-chymotrypsin, lipase were purchased from Sigma (St. Louis, USA). Lipopolysaccharide (LPS), dimethyl sulfoxide (DMSO), Griess reagent and 3-(4,5-dimethylthiazol-2-2,5-diohenyltetrazolium bromide (MTT) were also from Sigma. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin solution were from Invitrogen (Grand Island, NY, USA). 2, 2-Diphenyl picryl hydrazyl (DPPH), Ascorbic acid, Foline-Ciocalteau reagent and Gallic acid were from Sigma (St. Louis, USA).

2. Isolation and production of biologically active antimicrobial peptide from *Bacillus* sp. MP40

a. *In vitro* screening and isolation

Korean food samples were collected from various locations in chonnam province. The Korean food samples were suspended in distilled water. After dilution, samples were inoculated on the surface of *Bacillus* isolation agar (MRS and Muller Hinton) plates. The plate were incubated at 37 ℃ and each colony was transferred to new plate. Pure cultures were obtained from selected colonies for repeated culture. The isolated *Bacillus* strains were maintained as suspensions in 20 % glycerol (v/v) at -80 ℃.

b. Screening for antimicrobial activity

Among the several hundreds of microbial strains stocked in our laboratory, a strain MP40 was found to possess antimicrobial activity and was selected. *In vitro*, antimicrobial activity was primarily determined by the paper disk method using paper disk (8 mm, Toyo) against *Mycobacterium smegmatis* and activity was determined by the diameter of clear zone.

MIC (minimal inhibitory concentration) value was determined by agar dilution method using Muller Hinton Broth for bacteria. Observation was made after 18 h for bacteria at 37 ℃ following inoculation of test organisms.

c. Sequence similarities

The BLAST program (www.ncbi.nlm.nih.gov/blst) was employed in order to assess the degree of DNA similarity.

d. Optimization of fermentation process

Antimicrobial peptide production of the strain was optimized by using several culture parameters such as carbon, nitrogen sources and metal ions.

The influence of various carbon sources on peptide production was determined using media supplemented with 1 % yeast extract combined with 1 % supplements such as glucose, mannitol, starch, lactose, fructose, sorbitol, sucrose, maltose. Fermentation was carried out in 250 ml Erlenmeyer flasks containing 50 ml media with constant shaking at 37 ℃, 180 rpm. Afterwards, the influence of nitrogen source on peptide production was determined using medium containing 1 % sorbitol (best carbon source) combined with 1 % supplements such as beef extract, malt extract, tryptone, yeast extract, oat meal, soytone, peptone, dried yeast. Furthermore, the effect of metal ions on peptide production was evaluated in media containing the best carbon source, the best nitrogen source and 0.01 % supplements such as Na_2HPO_4, NaH_2PO_4, $MgSO_4$, $ZnSO_4$, $MgCl_2$, KH_2PO_4, $FeSO_4$, NaCl, $CaCl_2$.

e. Purification of *Bacillus* sp. MP40 producing antimicrobial peptide

The culture broth of strain MP40 was centrifuged at 6,000 rpm for 1 h at 4 ℃. In order to precipitate proteins, solid $(NH_4)_2SO_4$ was added to the supernatant to the final concentration of 30-80 % saturation. Precipitate was collected by centrifugation at 6000 rpm for 1 h at 4 ℃, dissolved in 10 mM Tris–HCl buffer (pH 7.0) and cut-off using amicon 1~10 kDa filter. The sample was then loaded on Sepharose CL-6B column (2.2 cm×116 cm) equilibrated with 10 mM Tris–HCl buffer (pH 7.0). All fractions were assayed for protein content and antimicrobial activity. Fractions showing antimicrobial activity were pooled, concentrated using 1,000 Da centricon (Amicon, USA), and then loaded on Sephadex G50 column (1.5 cm×70 cm) pre-equilibrated with 10 mM Tris–HCl buffer (pH 7.0). Finally, fractions containing high antimicrobial activity were concentrated using 1,000 Da centricon. To assess antimicrobial activity conveniently, the disk diffusion method was employed with the indicator strain *Mycobacterium smegmatis*.

f. Polyacrylamide gel electrophoresis

To determine the purity and molecular weight, the purified peptide was subjected to Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine SDS-PAGE). Following electrophoresis, the gel was stained with Coomassie Brilliant Blue

R-250 and destained with a solution containing methanol:glacial acetic acid:distilled water (1:1:8 by volume). A low-range protein marker (Fermentas, USA) was used for the calibration.

For in situ detection of inhibitory activity, the gel was washed with 50 mM Tris-HCl buffer (pH 7.9) containing 2.5 % Triton X-100. The gel was then overlaid with soft agar containing the indicator strain (10^6 colony-forming units) was incubated overnight at 37 ℃.

g. Stability of *Bacillus* sp. MP40 producing antimicrobial peptide

The thermal stability of the MP40 AMP was assessed by exposing aliquots of the peptide solution to different temperatures (20, 40, 60, 80, 100 ℃ for 30 min) and 121 °C for 15 min, then tested for residual antimicrobial activity. The effect of pH on the antimicrobial activity was tested by aliquots of peptide solution to pH from 2.0-10.0 (at increment of two pH unit) various pH buffer systems [citric acid–sodium phosphate buffer (pH 2.0–6.0), Tris–HCl buffer (pH 7.0–9.0), and sodium bicarbonate–sodium hydroxide buffer (pH 10.0)]. Samples were incubated at room temperature (25 °C) for 1 h then assayed for residual antimicrobial activity.

MP40 AMP was treated with different types of proteolytic enzymes at a final concentration of 1 mg/ml; lipase (50 mM Tris-HCl, pH 7.5), protease K (50 mM Tris-

HCl, pH 7.5), α-chymotrypsin (50 mM Tris-HCl, pH 7.5), trypsin (50 mM Tris-HCl, pH 8.0). Chemicals (working concentrations in Table 5.) were added to the AMP and the samples were incubated for 1 h at room temperature before being tested for residual antimicrobial activity. After the treatment, the samples were tested for residual antimicrobial activity against test organism.

3. Biological activities of antimicrobial peptide

a. Antimicrobial activity

Antimicrobial activity in terms of minimal inhibitory concentration (MIC) was determined according to Muller Hinton agar dilution method. Various microorganisms, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *S.aureus* (VRSA) and Vancomycin-resistant *enterococci* (VRE), IMP (Imipenem resistance *Pseudomonas aureus*), ESBL (Extended spectrum beta lactamase *Escherichia coli*) were used as test organisms. Bacitracin and vancomycin were used as reference antibiotics. Following inoculation of test organisms, plates were incubated at 37 ℃ and the results were observed after 12 h.

b. Antioxidant activity

(1) DPPH radical scavenging activity

DPPH is a stable free radical containing an odd electron in its structure and usually utilized for detection of the radical scavenging activity in chemical analysis. The DPPH radical scavenging activity was measured using the method described by Zhan *et al.* with some modification. Various concentrations of peptide solution 100 µl were added to 100 µl of DPPH (0.3 mM) in methanol solution in 96 well plates. The mixture was kept at room temperature for 30 min and the absorbance at 517 nm

measured on spectrophotometer. Methanol was used for the blank and distilled water was used for the negative control. Ascorbic acid was used as the positive control.

The DPPH radical scavenging ability was calculated by following equation:

$\% = \{1-[(A_{Sample}-A_{blank})/A_{Control}]\}*100$

Where A_{Sample}, A_{blank} and $A_{control}$ are the absorbance of sample, blank and control, respectively.

(2) Reducing power

The reducing capacity of a peptide may serve as a significant indicator of its potential antioxidant activity. The ability of peptide to reducing iron (III) was determined according to the method developed by Oyaizu. Each sample was dissolved in distilled water at different concentrations. An aliquot (200 μl) of sample solution was mixed with 200 μl of 200 mM sodium phosphate buffer and 200 μl of 1 % ferricyanide aqueous solution. The mixture was incubated at 50 ℃ for 20 min, followed by addition of 200 μl of 10 % trichloroacetic acid solution. Then, the mixture was centrifuged at 12,000 rpm for 10 min. Finally, 500 μl supernatant was mixed with 500 μl 0.1 % ferric chloride aqueous, and absorbance was measured at 700 nm against a blank. Reducing power was proportional to the absorbance of the reaction mixture. Ascorbic acid was used as the positive control, and an increased absorbance reading indicated increased reducing power.

(3) Total phenol contents

The total phenol content was determined according to the modified Folin-Ciocalteu method (Lee *et al*. 2009). The peptide was placed in a test tube with distilled water (200 μl) and Folin-Ciocalteu reagent (200 mg/l, 200 μl) saturated with sodium carbonate solution (10 %, 200 μl), and allowed to stand for 60 min. The reaction was monitored by measuring the absorbance at 700 nm. Gallic acid was used as the positive control, and an increased absorbance reading indicated total phenol contents.

(4) Nitrite scavenging ability

The nitrite scavenging ability in each sample was determined using the Gray and Dugan method (Gray *et al.* 1975). The reaction, composed of 100 μl of sample and 100 μl of 1 mM $NaNO_2$, was adjusted to pH 1.2 with 0.1 N HCl to produce a final volume of 300 μl and incubated at 37 ℃ in a water bath for 1 h. The reaction mixture (200 μl) was combined with 1 ml of 2 % acetic acid and 80 μl of Griess reagent (1 % sulfanilic acid and 1 % naphthylamine in 30 % acetic acid, mixed at a 1:1 ratio), and vortexed. The sample was then incubated at room temperature for 15 min. The remaining nitrite was then measured at 520 nm. The control group was incubated with 400 μl of distilled water instead of Griess reagent.

c. Anti-inflammatory activity

(1) Cell culture

The murine macrophage cell line Raw 264.7 was cultured at 37 ℃ under 5 % CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10 % heat-inactivated fetal bovine serum (FBS). The cells were subcultured every three days.

(2) Cell viability

Cells were seeded on 96 well plates, and peptide treatment began 24 h after seeding. The general viability of cultured cells was determined by the MTT assay, in which MTT is reduced to formazan in viable cells. MTT is a pale yellow substrate that produces a dark blue formazan product when incubated with living cells. Briefly, after 24 h incubation with or without peptide (10-200 μg/ml), a 0.5 mg/ml of MTT solution was added to each well in a 1/10 volume of media. Cells were incubated at 37 ℃ for 3 h, and dimethylsulfoxide (DMSO) was added to dissolved the formazan crystals. The obsorbance was measured using an assay reader at 590 nm. Relative cell viability was calculated compared to the untreated control group.

(3) Nitric oxide production assay

Nitric oxide (NO) levels in the culture supernatants were measured by the Griess

reaction. Raw 264.7 cells (10^6 cells/ml) were plated onto 6 well plates and pretreated with the indicated concentrations of MP40 peptide for 30 min prior to stimulation with 1 μg/ml of LPS for 24 h. Briefly, the sample supernatants were mixed with equal volume of Griess reagent (1 % sulfanilamide in 5 % phosphoric acid and 0.1 % naphthylethylenediamine dihydrochloride) and then incubated at room temperature for 10 min. The absorbance was measured at 550 nm on a microplate reader (Thermo co.). Nitrite concentration was determined using a dilution of sodium nitrite as a standard.

B. Results

1. Production, isolation and purification of antimicrobial peptide from *Bacillus* sp. MP40

a. Identification of the *Bacillus* strain

For molecular phylogeny, the 16S rRNA sequence of the local isolate was compared to sequence of 10 *Bacillus* species. Multiple sequence alignment was done between the sequence of the 16s rRNA gene of *B. subtilis, B. vallismortis* and local isolate. In addition to the phenotypic identification, the BLAST analysis of 16S rRNA sequence revealed significant (99.52 %) with *Bacillus amyliquefaciens* (Table 1, Figure 3).

Table 1. Similarity of 16S rRNA gene sequence (MP40)

Rank	Name/Title	Strain	Accession	Similarity	Diff/ Total nt
1	***Bacillus amyliquefaciens subsp. plantarum***	FZB42(T)	CP000560	99.52	7/1458
2	***Bacillus subtilis sub sp. subtilis***	NCIB 3610(T)	ABQL01000001	99.596	6/1484
3	***Bacillus vallismortis***	DSM 11031(T)	AB021198	99.528	7/1484
4	***Bacillus subtilis sub sp.spizizenii***	NRRL B-23049(T)	AF074970	99.432	8/1409
5	***Bacillus tequilensis***	NRRL B-41771(T)	EU138487	99.401	7/1168
6	***Bacillus subtilis sub sp.inaquosorum***	BGSC 3A28(T)	EU138467	99.401	7/1168
7	***Bacillus mojavensis***	IFO 15718(T)	AB021191	99.124	13/1484
8	***Bacillus atrophaeus***	JCM 9070(T)	AB021181	99.122	13/1480
9	***Brevibacterium halotolerans***	LMG 21660(T)	AJ620368	99.122	13/1480
10	***Bacillus licheniformis***	ATCC 14580(T)	CP000002	97.842	32/1483

TCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTA
GCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAAT
ACCGGATGGTTGTCTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCG
GCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGA
CACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTC
TGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGTT
CAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATAC
GTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAC
CCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGT
GTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCT
GAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATCGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTT
AGGGGGTTTCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGACGGTCGCAAGACTGAA
ACTCAAAGGAATTGACGGGGGCCCGCACAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTT
ACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCAT
GGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCA
GCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCAT
GCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCC
AATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCG
CGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAAC
ACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCCAGCCGCCGAAGGTGGGACAGATGATTGGGGTGAAGTCTCA

Figure 3. 16S rRNA sequence of *Bacillus* sp. MP40

The data provided represented the 16S rRNA sequence to identify the name of the *Bacillus* strain.

b. Optimization of fermentation process

AMP production of the MP40 strain was optimized by using several cultural parameters such as carbon, nitrogen sources and metal ions. The effect of carbon, nitrogen sources and metal ion was studied. Among the tested carbon, nitrogen and metal ion sources, sorbitol and peptone, tryptone, soytone, $CaCl_2$, respectively, caused the maximum production of peptide.

Finally, production of peptide was carried out SPTSC (1 % sorbitol and 1 % peptone, 0.5 % tryptone, 0.5 % soytone, 0.01 % $CaCl_2$) medium where significant production was achieved from 6h and reached a maximum at 12 h (Figures 4-7).

Production of antimicrobial peptide in *B. subtilis* SK.DU.4 isolated from rhizosphere soil reached a maximum when the cultivation time was 24 h (Baindara *et al*., 2013). Similarly, significant production as well as growth was achieved from 12 h and reached a maximum at 48 h of cultivation, which is different from antimicrobial peptide production in *B. subtilis* CS61 (Choi *et al*. 2012).

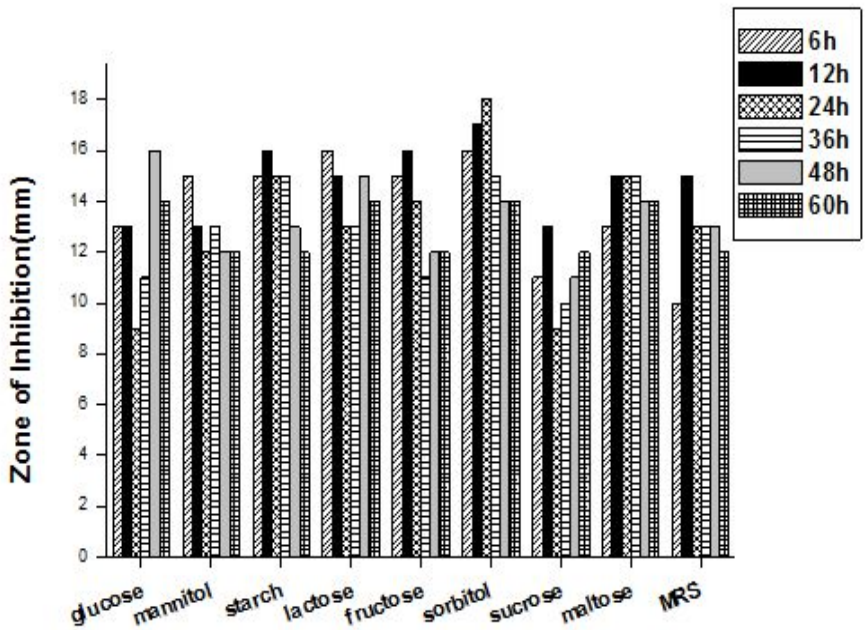


Figure 4. Effect of various carbon sources on the activity of antimicrobial peptide produced by *Bacillus* sp. MP40

Production medium was supplemented with various carbon sources such as glucose, mannitol, starch, lactose, fructose, sorbitol, sucrose, maltose each at a level of 1 % (w/v) by keeping the nitrogen sources constant.

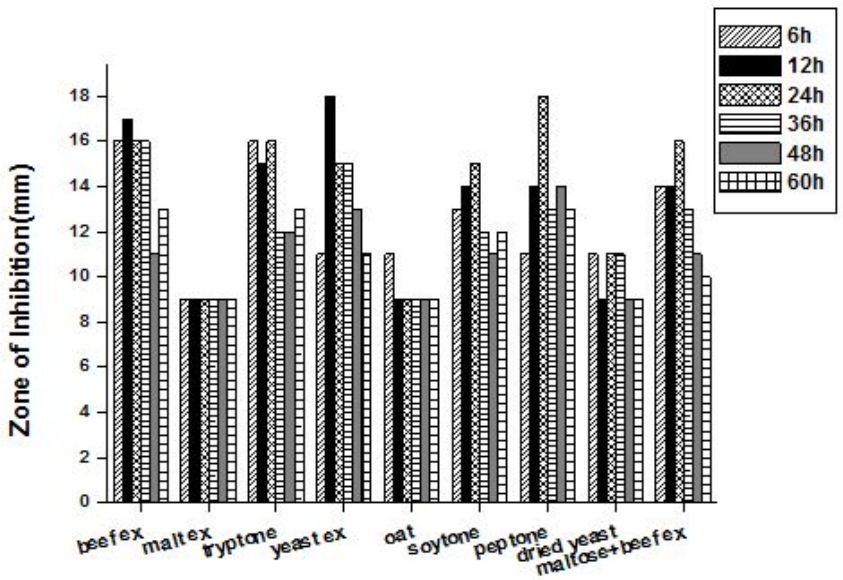


Figure 5. Effect of various nitrogen sources on the activity of antimicrobial peptide produced by *Bacillus* sp. MP40

Production medium was supplemented with various nitrogen sources such as beef extract, malt extract, tryptone, yeast extract, oat, soytone, peptone, dried yeast each at a level of 1 % (w/v) by keeping the metal ion sources constant.

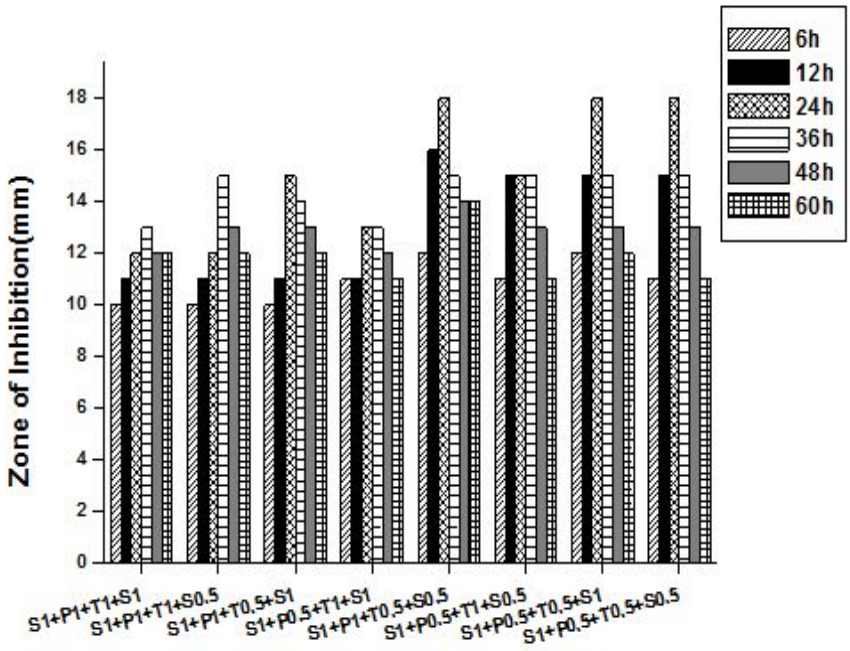


Figure 6. Effect of carbon and nitrogen sources on the activity of antimicrobial peptide produced by *Bacillus* sp. MP40

Production medium was supplemented with carbon, nitrogen sources such as Sorbitol 1 %+Peptone 1 %+Tryptone 1 %+Soytone 1 %, Sorbitol 1 %+Peptone 1 %+Tryptone 1 %+Soytone 0.5 %, Sorbitol 1 %+Peptone 1 %+Tryptone 0.5 %+Soytone 1 %, Sorbitol 1 %+Peptone 0.5 %+Tryptone 1 %+Soytone 1 %, Sorbitol 1 %+Peptone 1 %+Tryptone 0.5 %+Soytone 0.5 %, Sorbitol 1 %+Peptone 0.5 %+Tryptone 1 %+Soytone 0.5 %, Sorbitol 1 %+Peptone 0.5 %+Tryptone 0.5 %+Soytone 1 %, Sorbitol 1 %+Peptone 0.5 %+Tryptone 0.5 %+Soytone 0.5 %.

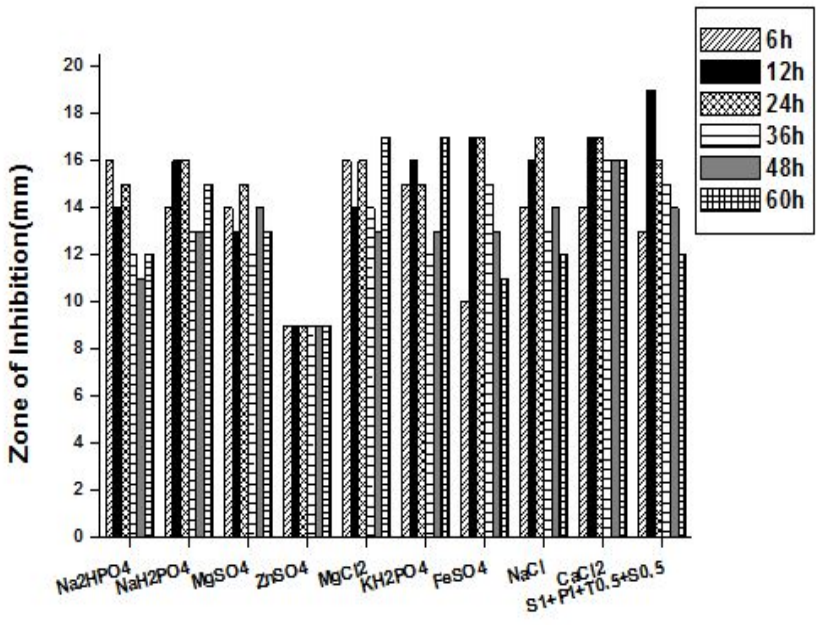


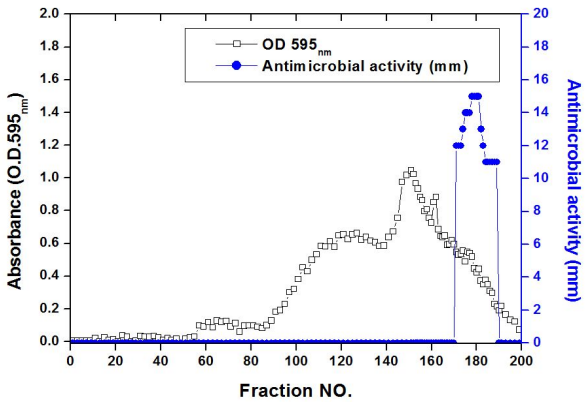
Figure 7. Effect of various metal ion sources on the activity of antimicrobial peptide produced by *Bacillus* sp. MP40

Production medium was supplemented with various nitrogen sources such as disodium phosphate, monosodium phosphate, magnesium sulfate, zinc sulfate, magnesium chloride, monopotassium phosphate, ferric sulfate, sodium chloride, calcium chloride each at a level of 0.01 % (w/v) to the production medium.

c. Fermentation and purification of antimicrobial peptide

Bacillus sp. MP40 was grown on rotatory shaker at 180 rpm in SPTSC medium for 12 h at 37 ℃. Culture broth was centrifuged at 6,000 rpm for 1 h. Peptide was purified by ammonium sulfate precipitation, sequential Sepharose CL-6B chromatography, and the final step of the Sephadex G50 purification procedure gave one active peptide peak (Figure 8). Tricine SDS-PAGE and in situ detection inhibitory activity confirm the homogeneity and activity of the purified MP40 AMP. The peptide migrated as a single band and the molecular weight was estimated to be 1,000 Da (Figure 9). The band was confirmed to be a peptide by in situ inhibitory activity of the gel.

(a)



(b)

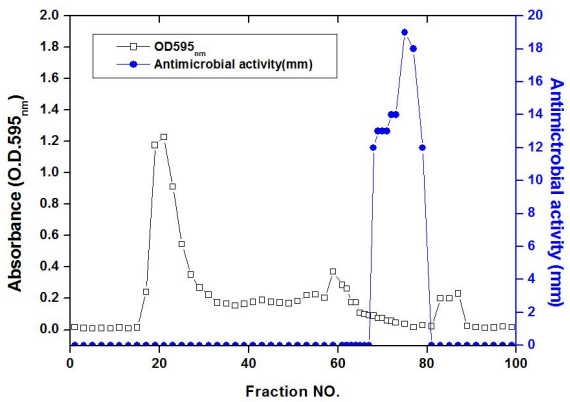


Figure 8. Elution profile of *Bacillus* sp. MP40 antimicrobial peptide

(a) Gel filtration chromatography with Sepharose CL-6B column (2.2 cm x 116 cm). The proteins were eluted at a flow rate of 5 ml/min. (b) Gel filtration chromatography with Sephadex G-50 column (1.5 cm x 70 cm). The proteins were eluted at a flow rate of 1 ml/min.

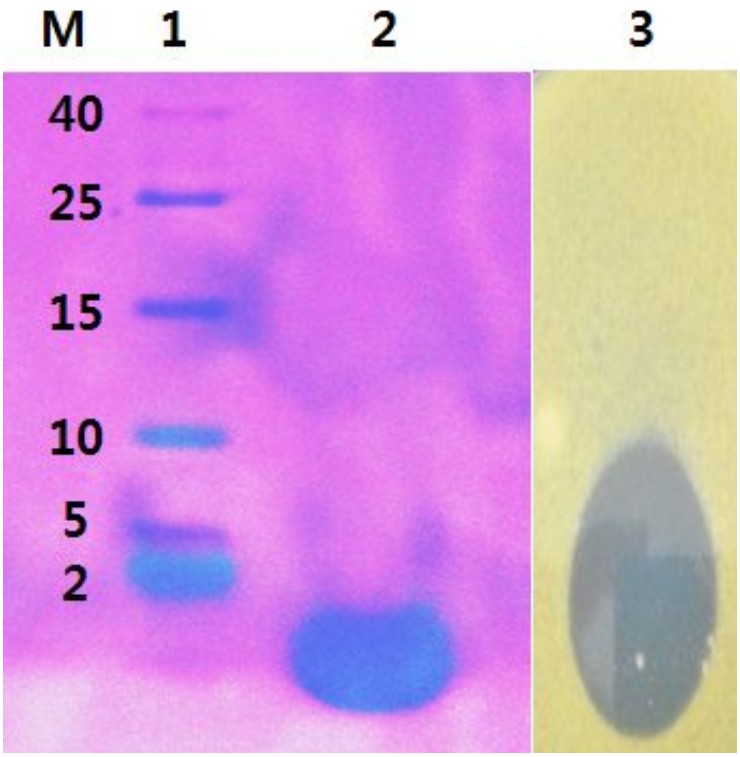


Figure 9. Determination of the molecular weight

Tricine SDS-PAGE and activity staining of MP40 peptide. Lane 1, protein size marker with the corresponding value in kDa on the left; Lane 2, purified MP40 peptide; Lane 3, activity staining.

2. Biological activities of antimicrobial peptide

a. Antimicrobial activity of peptide

(1) Antimicrobial spectrum of antimicrobial peptide

As shown in Table 2, MP40 AMP showed antimicrobial activity against Gram-positive but not Gram-negative bacteria. Importantly, MP40 AMP displayed an antagonistic effect against multidrug-resistant (MDR) pathogens such as VRE and Gram negative bacteria *E.coli* which was stronger than that shown by bacitracin and vancomycin. Although direct comparison in unavailable, the anti-ARE activity of MP40 AMP appears much stronger than that of another peptide antibiotics from *Bacillus licheniformis* DSM, *Bacillus subtillis* 168 and *Bacillus subtilis* CS61 (Dischinger *et al*. 2009; Tamehiro *et al*. 2002; Choi *et al*. 2012) displaying a low range of inhibitory zone against most of MP40 peptide against MRSA and VRE strains is relatively weaker than that of epidermicin NI01 (Sandiford *et al*. 2012).

Table 2. Minimum inhibitory concentration of *Bacillus* sp. MP40 peptide

Test organisms		MIC(ug/ml)		
		MP 40	Bacitracin	Vancomycin
Alcaligenes faecalis ATCC 1004	G(-)	>80	>80	>80
Salmonella typhimurium KCTC 1925	G(-)	>80	>80	>80
Escherichia coli KCTC 1923	G(-)	40	>80	>80
ESBL B1*	G(-)	>80	>80	>80
ESBL P3*	G(-)	>80	>80	>80
ESBL S1*	G(-)	>80	>80	>80
ESBL U4*	G(-)	>80	>80	>80
ESBL W1*	G(-)	>80	>80	>80
Pseudomonas aeruginosa KCTC 1637	G(-)	>80	>80	>80
IMP 123**	G(-)	>80	>80	>80
IMP129**	G(-)	>80	>80	>80
Bacillus subtilis ATCC 6633	G(+)	>80	40	0.3
Micrococcus luteus ATCC 9341	G(+)	>80	40	2.5
Mycobacterium smegmatis ATCC 9341	G(+)	5	>80	1.25
Enterococcus faecalis ATCC 29212	G(+)	40	5	2.5
VRE 2***	G(+)	20	5	>80
VRE 4***	G(+)	10	5	>80
VRE 6***	G(+)	20	>80	>80
VRE 82***	G(+)	10	40	>80
VRE89***	G(+)	10	40	>80
VRSA****	G(+)	>80	>80	>80

Test organisms		MIC(ug/ml)		
		MP 40	Bacitracin	Vancomycin
Staphylococcus aureus KCTC 1928	G(+)	20	>80	5
MRSA693E*****	G(+)	>80	1.25	1.25
MRSA 4-5*****	G(+)	40	1.25	0.625
MRSA 5-3*****	G(+)	10	1.25	0.625
MRSA S3*****	G(+)	40	10	0.625
MRSA U4*****	G(+)	>80	10	0.3125
MRSA P8*****	G(+)	0.6	1.25	0.15
MRSA B15*****	G(+)	10	5	0.625

*ESBL, Extended spectrum beta lactamase *Escherichia coli*; **IMP, Imipenem resistant *Pseudomonase aureus;* ***VRE, Vancomycin resistant *Enterococcus faecium*; ****VRSA, Vancomycin resistant *Staphylococcus aureus;* *****MRSA, Methicillin resistant *Staphylococcus aureus*.

(2) Temperature, pH, proteolytic enzymes and chemicals stability of antimicrobial peptide

The influence of temperature and pH in the antimicrobial stability is presented in Table 3. Thermo stability of MP40 AMP was analyzed for various temperatures by measuring the residual activities. It was stable 100 ℃, but the activity decreased with the increase in temperature. The residual activity was 81 % after incubation at 121 ℃ at 15 min. The stability of AMP was determined by measuring the residual activities after incubating the AMP for 1 h at room temperature in the different pH buffers (pH 2.0 ~ 10.0). The residual antimicrobial activities (without incubation in the pH buffer) were considered to be 100 %. Temperature and pH stability of MP40 is more stable against lichenin (Pattnaik *et al*. 2001), *Bacillus licheniformis* Strain P40 (Cladera-Olivera *et al*. 2004).

To test the effect of proteolytic enzymes, MP40 AMP was treated with lipase, proteinase K, α-chymotrypsin, trypsin and residual activity was measured by agar diffusion assay against *Mycobacterium smegmatis*. The antimicrobial peptide was non-sensitive all protease enzymes (Table 4).

The effect of several chemicals on the antimicrobial activity was evaluated. The antimicrobial activity was completely non-sensitive to organic solvents and detergents (Table 5).

Table 3. Effect of different temperature and pH on inhibitory activity of peptide

Treatment	Residual activities (%)
Temperature, Time	
None	100
20 ℃, 30 min	114.3±10
40 ℃, 30 min	119±5.77
60 ℃, 30 min	114.3±10
80 ℃, 30 min	116.7±11.54
100 ℃, 30 min	97.6±5.7
121 ℃, 15 min	81±5.77
pH	
2	105.7±5.7
4	102±5.0
6	100
8	112.5
10	100

Table 4. Effect of various proteolytic enzymes

Proteolytic enzymes	Residual activity (%)
None	100
Lipase	111.9±5.7
Proteinase K	126.2±6.0
α-chymotrypsin	111.9±5.8
trypsin	116.7±5.77

* Peptide was treated with 1 mg/ml of each enzyme and incubated at room temperature for 1 h and then boiled for 2 min at 100 ℃ for enzyme inactivation. Peptide solution without any proteolytic enzymes treatment was taken as none (100 %).

Table 5. Effect of various chemicals on antimicrobial activity

Treatment	Concentration	Residual activity(%)
None		100
Acetone	10 % (v/v)	97.4±5.77
Chloroform	10 % (v/v)	94.73±10.0
Ethanol	10 % (v/v)	94.73
Methanol	10 % (v/v)	94.73
Ethyl acetate	10 % (v/v)	94.73
EDTA	10 mM/ml	102.63
Trichloroacetic acid	100 mg/ml	100±5.7
Triton X-100	1 % (v/v)	118.42
Tween 20	10 % (v/v)	94.74
Tween 80	10 % (v/v)	84.21±5.7

* Antimicrobial peptide was pre-incubated for 1 h at room temperature with chemicals and then assayed for antimicrobial activity. Antimicrobial peptide activity measured in the absence of any chemicals was considered as none (100 %).

* After treatment with TCA samples were centrifuged 8,000 rpm for 5 min and the supernatant was neutralized to pH 7.0 before testing for antimicrobial activity.

b. Antioxidant activities

(1) DPPH radical scavenging activity

We examined radical scavenging effects by measuring changes in absorbance of DPPH radical at 517 nm. Both MP40 AMP and ascorbic acid showed a concentration dependent scavenging activity of DPPH radicals. Figure 10, shows the results of the free radical (DPPH) scavenging activity in % inhibition. MP40 AMP exhibited a comparable antioxidant activity with that of standard ascorbic acid at varying concentration tested (2 to 200 μg /ml). There was a dose dependent increase in the percentage antioxidant activity for all concentrations tested. The AMP at a concentration of 200 μg/ml showed a percentage inhibition of 124±0.85. Ascorbic acid was used as the positive control for the determination of the antioxidant activity by DPPH method. The concentration of ascorbic acid varied from 2 to 200 μg/ml. Ascorbic acid at a concentration of 200 μg/ml exhibited a percentage inhibition of 89.5±2.21. A graded increase in percentage of inhibition was observed for the increase in the concentration of ascorbic acid. All determinations were done in triplicate and the mean values were determined. Hence DPPH is usually used as a substance to evaluate the antioxidant activity.

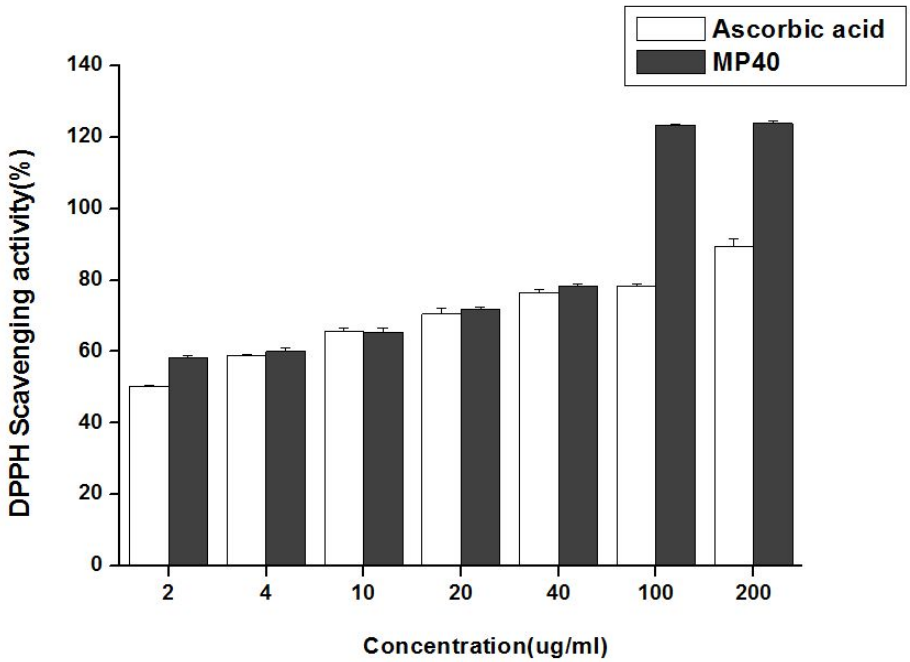


Figure 10. DPPH radical scavenging activity of MP40 antimicrobial peptide in different concentration.

(2) Reducing power

Reducing power assay method is based on the principle that AMP, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm. Ascorbic acid was used as the positive control for the determination of the antioxidant activity by reducing power method. The reducing power of MP40 AMP was increased concentration dependent. The reducing power of MP40 AMP was much more effective than ascorbic acid (Figure 11).

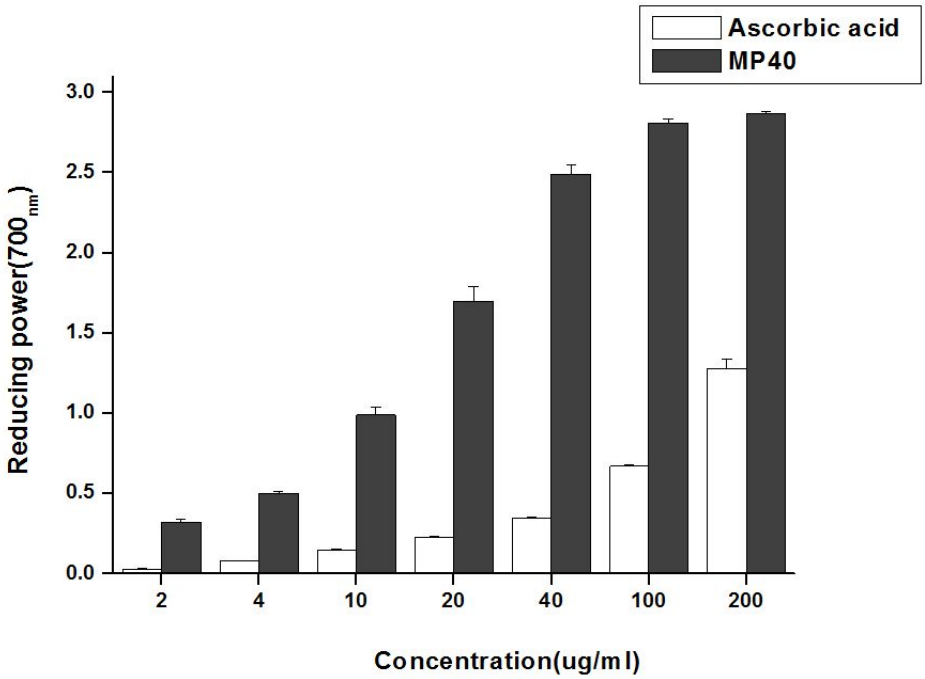


Figure 11. Reducing power of MP40 antimicrobial peptide

The absorbance (700 nm) was plotted against concentration of sample. All values are mean ± SD of triplicates.

(3) Total phenol contents

Since phenolic compounds generally have antioxidant activity, we initially examined the total phenol contents in MP40 AMP. Total phenol content (TPC) was determined spectrophotometrically according to the Folin-Ciocalteu colorimetric method using gallic acid as the standard. Maximum wavelength for blue colored complex was at 700 nm. The TPC of MP40 AMP was increased concentration dependent. Also, TPC of peptide was significantly higher than gallic acid in same concentration (Figure 12).

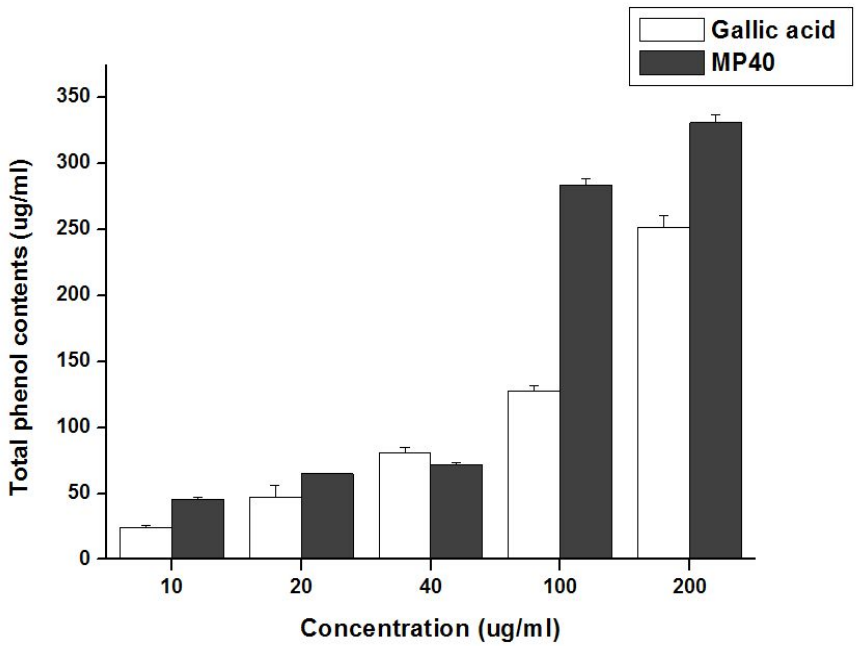


Figure 12. Total phenol contents of MP40 antimicrobial peptide

Total phenolic content was measured using the Folin-Ciocalteu method. Absorbance values represent triplicates of different samples analysed.

(4) Nitrite scavenging activity

We examined the nitrite scavenging effect of MP40 AMP by analyzing nitrite (NO^{2-}). The nitrite scavenging activity in the 10-200 μg/ml MP40 AMP significantly increased compared to ascorbic acid, which showed relatively high scavenging activity. However, in low concentrations, there was no significant difference in nitrite scavenging activity from 10-20 μg/ml (Figure 13).

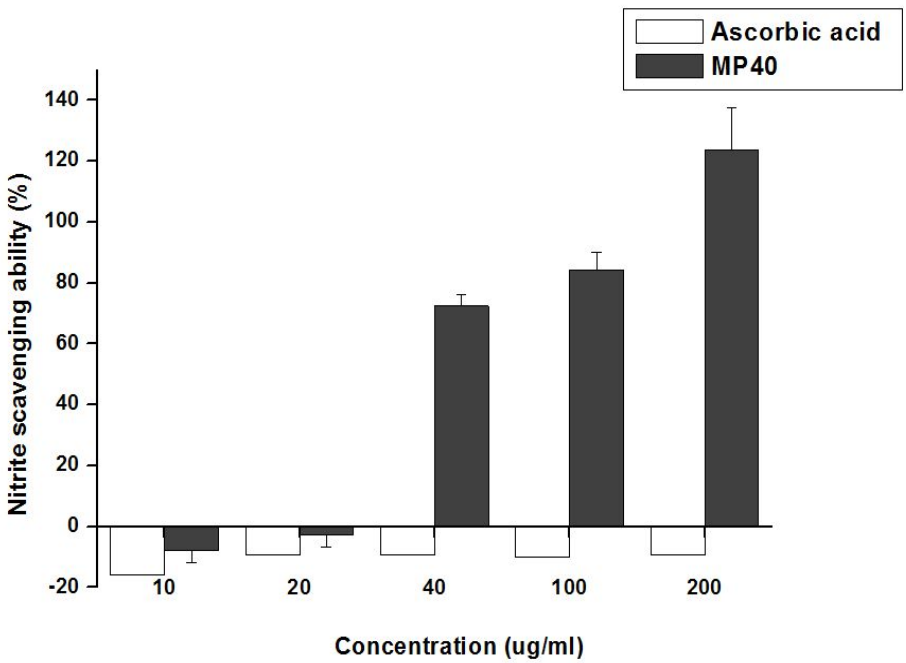


Figure 13. Nitrite scavenging activity of MP40 antimicrobial peptide

The nitrite concentrations in the sample-treated reactions were determined at 520 nm. Nitrite scavenging activity was calculated using the previous formula. All experiments were performed in triplicate and measured as the standard deviation of three replicates.

a. Anti-inflammatory activity of antimicrobial peptide

(1) Cytotoxicity and NO production

Raw 264.7 cells were treated with various concentrations of MP40 AMP for 24 h and the viability was determined by MTT assay as described above. The cell viability of untreated group was designated as 100 %, indicating no cytotoxicity. As shown in Figure 14 (a), MP40 AMP did not exhibit cytotoxicity to Raw 264.7 cells at the range of 10-40 μg/ml. However, MP40 AMP showed the decreasing cell viability according to its increasing doses, indicating the higher cytotoxicity.

Macrophages produce No following stimulation with LPS. To assess the effect of MP40 AMP on NO production by LPS-induced Raw 264.7 cells, we measured the nitric concentration in the culture medium using the Griess reagent method. As shown in Figure 14 (b), LPS treatment significantly increased NO production compared to the untreated cells. Treatment of cells with MP40 AMP at 2-200 μg/ml suppressed the LPS-stimulated production of NO to a statistically significant extent.

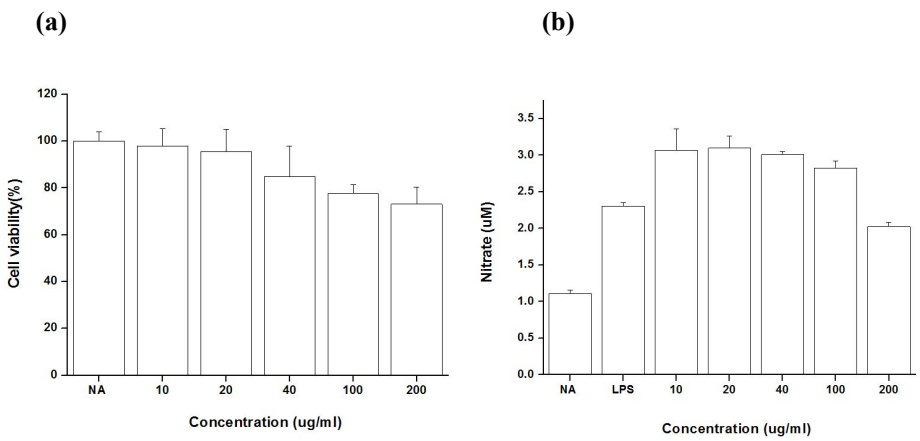


Figure 14. Effect of the MP40 antimicrobial peptide on cell viability and NO production

(a) Cell viability was measured after 24 h incubation. Survival rates were tested with MTT assay in Raw 264.7 cells. Raw 264.7 cells were incubated in the presence or absence of 10-200 μg/ml peptide for 24 h. (b) Cells were incubated with the various concentration of peptide for 30 min, followed by treatment with 1 μg/ml of LPS and incubated for 24 h. The amounts of NO were determined using the Griess reagent in the culture medium . Each bar shows the mean ± S.D of three independent experiments performed in triplicate.

III. Discussion

Microbial strains capable of producing AMPs were recently isolated from Korean foods. Strain MP40 was selected among the numerous screened strains since it showed highest antimicrobial activity. 16S rRNA gene sequences of the strains were analyzed. This strain was identified as *Bacillus* sp. MP40 [(*Bacillus amyliquefaciens* (99.52 %)]. This study was aimed to isolate and identify antimicrobial, antioxidant and anti-inflammation properties of peptide from *Bacillus* sp. strains. Sorbitol and peptone, tryptone, soytone, $CaCl_2$ were found the most suitable nutrient sources for the maximum production of MP40 antimicrobial peptide (Figures 4-7).

The *Bacillus* isolate, MP40 exhibited a wide spectrum antimicrobial activity. The antimicrobial peptide produced in the optimal medium by *Bacillus* sp. MP40 was purified to homogeneity by gel permeation chromatography using Sepharose CL-6B and Sephadex G-50 column chromatography.

Effect of peptide MP40 purified form *Bacillus* sp. was evaluated against various pathogenic gram-positive and gram-negative bacteria. The production of AMP by some others sp. of *Bacillus* such as *B. subtilis, B. licheniformis* and other *Bacillus* species has been reported. Although several strains are active against a narrow spectrum of bacteria, some strains produce peptides with a broad range of activity

against clinical important pathogens. The AMP produced by *Bacillus* sp. MP40 exhibited antimicrobial effect towards broad spectrum of Gram-positive organisms. Such a broad spectrum of activity is comparable with the effect of antimicrobial peptides of *Bacillus cereus* Bc7 (Oscariz *et al*. 1999) and *Bacillus subtilis* SK.DU.4 (Baindara *et al*. 2013). Antimicrobial effects, in terms of MIC values, of all the purified peptide and two well-known reference antibiotic named bacitracin and vancomycin are illustrated in Table 2. AMP displayed activities against gram-positive bacteria. It is remarkable that the effect shown by MP40 AMP against VRE and *E. coli* was comparable with bacitracin and vancomycin. Minimal inhibitory concentrations of MP40 AMP for VRE were in the range 10–20 μg/ml.

The MP40 peptide showed stability between pH 2.0 and pH 10.0 and up to 100 ℃ (Table 3). Sensibility test of MP40 towards various proteolytic enzymes were tested and residual activity was measured by agar diffusion assay against *Mycobacterium smegmatis*. The peptide was not sensible to all proteolytic enzymes (Table 4). The effect of several chemicals on the antimicrobial activity was evaluated. AMP activity was not affected by treatment with detergents and various organic solvents (Table 5).

The antioxidant activities of the AMP were tested using free radical DPPH, reducing power, total phenol contents, nitric scavenging activity. A freshly prepared DPPH solution exhibits a deep purple color generally fades / disappears when they

are present in medium. Thus antioxidant molecule can quench DPPH free radicals (by providing by hydrogen atom or by electron transfer, conceivably via a free radical attack on the DPPH molecule). Radical scavengers may protect tissues from free radical attack on the DPPH molecule and convert them to a colourless product (2,2-diphenyl-1-hydrazine, or a substituted analogous hydrazine) analogous of hydrazine resulting in a decreasing absorbance at a 517 nm. Hence more rapidly the absorbance decreases implies more potent antioxidant activity of AMP in terms of hydrogen atom donating capacity / electron transfer capability (Figure 10).

The reducing power of the peptide was detected using potassium ferricyanide reduction method. It has been reported that the reducing power was associated with the antioxidant activity. The presence of peptide causes the reduction of Fe^{3+}/ Ferric cyanide complex to ferrous form. Therefore Fe^{2+} complex can be monitored by measuring the formation. The absorbance value and concentration of peptide show linear relationship. Therefore the increasing OD value indicates increasing trend of reducing power. The reducing power of peptide was concentration dependent. There was significantly increase on the reducing power with increase in concentration of peptide solution. When compared with the standard (ascorbic acid), peptide showed higher reducing power activity (Figure 11).

Phenolic compounds have been found to have therapeutic applications against different diseases caused by oxidative stress. Phenolic compounds are very important

antioxidant agents, by chelating redox-active metal ions, inactivating lipid free radical chains and preventing hydroperoxide conversion into reactive oxyradicals (Oliveira *et al*. 2009.). Total phenol content (TPC) was determined according to the Folin-Ciocalteu colorimetric method. This method allows the estimation of all the flavonoids, anthocyanins and nonflavonoid phenolic compounds that is, of all the phenolics present in the samples (Benvenuti *et al.,* 2004). The TPC of MP40 AMP was increased concentration dependent. Also, TPC of peptide was significantly higher than gallic acid in same concentration (Figure 12). Nitric Oxide (NO) scavenging assay is based on the scavenging ability of the peptide as well as ascorbic acid, which is used as standard. The NO scavenging of peptide was found to increase in dose dependent manner. Maximum inhibition of NO was observed in the peptide of highest concentration (20 μg/ml)(Figure 13).

The MP40 peptide was evaluated for their inhibitory effect on NO production in LPS-induced Raw 264.7 cells. Lipopolysaccharide (LPS), an outer membrane component of Gram negative bacteria, is a potent activator of monocytes and macrophages. LPS triggers the abundant secretion of nitric oxide and many cytokines from macrophages (Meng *et al*., 1997). When the peptide was placed in the NO production system, the nitrite accumulation was inhibited in a dose-dependent manner (Figure 14 (b)). The cytotoxicity of peptide was not detected in the range of the test concentrations indicating that the inhibition was due to the effect of the compounds

(Figure 14 (a)). This study revealed that a *Bacillus* sp. MP40 AMP inhibited NO production by LPS-induced Raw 264.7 cells.

In conclusion, MP40 AMP with various activities was purified from *Bacillus* strain isolated from Korean foods. The results from this study suggest a possible use of MP40 peptide as a source of natural antimicrobial and antioxidant agents.

IV. References

Ahern M, Verschueren S, van Sinderen D. 2003. Isolation and characterization of a novel bacteriocin produced by *Bacillus thuringiensis* strain B439. Microbiology Letters. 220, 127-131.

Ahmed AS, Elgorashi EE, Moodley N, McGAW LJ, Naidoo V, Eloff JN. 2012. The antimicrobial, antioxidative, anti-inflammatory activity and cytotoxicity of different fractions of four South African *Bauhinia* species used traditionally to treat diarrhea. Journal of Ethnopharmacology. 143(3), 826-839.

Ahren M, Verschueren S, van Sinderen D. 2003. Isolation and characterization of a novel bacteriocin produced by *Bacillus thuringiensis* strain B439. FEMS Microbiol Lett. 220, 127-131.

Bahar AA, Ren D. 2013. Antimicrobial peptides. Pharmaceuticals. 6(12), 1543-1575.

Baindara P, Mandal SM, Chawla N, Singh PK, Pinnaka AK, Korpole S. 2013. Characterization of two antimicrobial peptides produced by a halotolerant *Bacillus subtilis* strain SK. DU. 4 isolated from a rhizosphere soil sample. AMB Express. 3(2)

Benitez LB, Velho RV, Lisboa MP, Mendina LF, Brandelli A. 2010. Isolation and characterization of antifungal peptides produced by *Bacillus amyloliquefaciens* LBM5006. The Journal of Microbiology. 48(6), 791-797.

Benvenuti S., Pellati F., Melegari M., Bertelli D. 2004. Polyphenols, anthocyanins, ascorbic acid, and radical scavenging activity of *Rubus*, *Ribes,* and *Aronia*. Food Chemistry and Toxicology. 69(2), 164-169.

Bizani D, Brandelli A. 2002. Characterization of a bacteriocin produced by a newly isolated *Bacillus* sp. Strain 8A. Journal of Applied Microbiology. 93, 512-519.

Caldeira AT, Feio SS, Arteiro JM, Coelho AV, Roseiro JC. 2008. Enviromental dynamic of *Bacillus amyloliquefaciens* CCMI 1051 Antifungal Activity under Different Nitrogen Patterns. Journal of Applied Microbiology. 104(3), 808-816.

Chen H, Wang L, Su C.X., Gong G.H., Wang P, Yu Z.L.. 2008. Isolation and characterization of lipopeptide antibiotics produced by Bacillus subtilis. Letters in Applied Microbiology. 47(3), 180-186.

Chen N, Yang H, Sun Y, Niu J, Liu S. 2012. Purification and identification of antioxidant peptides from walnut (*Juglans regia* L.) protein hydrolysates. Peptides. 38(2), 344-349.

Cherif A, Chehimi S, Limem F. 2003. Detection and characterization of the novel bacteriocin entomoxin 9, and safety evaluation of its producer, *Bacillus thuringiensis* ssp. Entomocidus HD9. Journal of Applied Microbiology. 95(5), 990-1000.

Cherif A, Ouzari H, Daffonchio D, et.al. 2001. Thuricin 7: A novel bacteriocin produced by *Bacillus thuringiensis* BMG1.7, a new strain isolated from soil.

Letters in Applied Microbiology. 32(4), 243-247.

Cho SJ, Oh SH, Pridmore RD, Juillerat MA, Lee CH. 2003. Purification and characterization of proteases from *Bacillus amyloliquefaciens* isolated from traditional soybean fermentation starter. Journal of Agricultural and Food Chemustry. 51(26), 7664-7670.

Choi YH, Cho SS, Simkhada JR, Yoo JC. 2012. A novel thermotolerant and acidotolerant peptide produced by a Bacillus strain newly isolated from a fermented food (kimchi) shows activity against multidrug-resistant bacteria. International Journal of Antimicrobial Agents. 40(1), 80-83.

Cook Rj, Thomashow LS, Weller DM, Fujimoto D, Mazzola M, Bangera G, Kim DS. 1995. Molecular mechanisms of defense by Rizobacteria against Root disease. Proceedings of the National Academy of Sciences of the United States of America. 92(10), 4197-4201.

Cladera-Olivera F, Caron G.R., Brandelli A. 2004. Bacteriocin-like substance production by Bacillus licheniformis strain P40. Letters in Applied Microbiology. 38(4), 251-256.

De Smet K, Contreras R. 2005. Human antimicrobial peptides :defensins, cathelicidins and histatins. Biotechnology Letters. 27, 1337-1347.

Dowling DN, O'Gara F. 1994. Metabolites of *Pseudomonas* involved in the biocontrol of plant disease. Trends in Biotechnology. 12, 133-141.

Dischinger J, Josten M, Szekat C, Sahl HG, Bierbaum G. 2009. Production of the novel two-peptide lantibiotic lichenicidin by *Bacillus licheniformis* DSM 13. PLOS One. 4(8), 1-11.

Elias RJ, Kellerby SS, Decker EA. 2008. Antioxidant activity of proteins and peptides. Critical Reviews in Food Science and Nutrition. 48(5), 430-441.

Friedrich CL, Moyles D, Beveridge TJ, Hancock RE. 2000. Antibacterial action of structurally diverse cationic peptides on gram-positive bacteria. Antimicrobial Agents and Chmotheraphy. 44(8), 2086-2092.

Ganie SA, Jan A, Muzaffar S, Zargar BA, Hamid R, Zargar MA. 2012. Radical scavenging and antibacterial activity of Arnebia benthamii methanol extract. Asian Pacific Journal of Tropical Medicine. 5(10), 766-772.

Gray JI, Dugan JLR. 1975. Inhibition of N-nitrosamine formation in a model food system. Journal of Food Science. 40(5), 981-985.

Hagelin G, Oulie I, Raknes A, Undheim K, Clausen OG. 2004. Preparative high-performance liquid chromatographic separation and analysis of the Maltacine Complex-A family of cyclic peptide antibiotics from *Bacillus Subtilis*. Journal of Chromatography B. 811, 243-251.

Hancock RE, Chapple DS. 1999. Peptide antibiotics. Antimicrobial Agents and Chemotherapy. 43(6), 1317-1323.

Harwig SS, Kokryakov VN, Swiderek KM, Aleshina GM, Zhao C, Lehrer RI. 1995.

Prophenin-1, an exceptionally proline-rich antimicrobial peptide from porcine leukocytes. FEBS Letters 362, 65-69.

Hiradate S, Yoshida S, Sugie H, Yada H, Fujii Y. 2002. Mulberry anthracnose antagosists (iturins) produced by *Bacillus amyloliquefaciens* RC-2. Phytochemistry. 6, 693-698.

Ingolf FN, Yoon SS, Azung BD. 2007. Ribosomally synthesiszed antimicrobial peptides (Bacteriocins) in lactic acid bacteria : A review. Food Science and Biotechnology. 16(5), 675-690.

Jeon HJ, Choi HS, Lee OH, Jeon YJ, Lee BY. 2012. Inhibition of Reactive Oxygen Species (ROS) and Nitric Oxide (NO) by *Gelidium elegans* Using Alternative Drying and Extraction Conditions in 3T3-L1 and RAW 264.7 Cells. Prevention Nutrition and Food Science. 17(2), 122-128.

Joo LH, Park CS, Joo JY, Kim HS, Yoon JH, Park YH, Hwang IK, Ahn JS, Mheen TI. 1999. Identification and characterization of Bacteriocin-producing lactic acid bacteria isolated from *Kimchi*. Journal of Microbiology Biotechnology. 9(3), 282-291.

Katz E, Demain A. 1977. The peptide antibiotics of *Bacillus* : Chemistry, Biogenesis and Possible Functions. Bacteriological Reviews. 41(2), 449-474.

Kayalvizhi N, Gunasekaran P. 2008. Production and Characterization of a low-molecular-weight bacteriocin from *Bacillus licheniformis* MKU3. Letters in

Applied Microbiology. 47, 600-607.

Khalil R, Elbahloul Y, Djadouni F, Omar S. 2009. Isolation and partial characterization of a bacteriocin produced by a newly isolated *Bacillus megateium* 19 strain. Pakistan Journal of Nutrition . 8(3), 242-250.

Klich MA, Arthur KS, Lax AR, Bland JM. 1994. Iturin A :A potential new fungicide for stored grains. Mycophathologia. 127(2), 123-127.

Koumoutsi A, Chen XH, Henne A, Liesegang H, Hitzeroth G, Franke P, Vater J, Borriss R. 2004. Structural and functional characterization of gene clusters directing non-ribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. Journal of Bacteiology. 186(4), 1084-1096.

Kim ID, Ha BJ. 2009. Paeoniflorin protects RAW 264.7 macrophages from LPS-induced cytotoxicity and genotoxicity. Toxicology in Vitro. 23(6), 1014-1019.

Kumar A, Saini P, Shrivastava JN. 2009. Production of peptide antifungal antibiotic and biocontrol activity of *Bacillus subtilis*. Indian Journal of Experimental Biology. 47, 57-62.

Lee H, Lee YJ, Choi H, Ko EH, Kim JW. 2009. Reactive oxygen species facilitate adipocyte differentiation by accelerating mitotic clonal expansion. Journal of Biological Chemistry. 284(16), 10601-10609.

Lin HY, Rao YK, Wu WS, Tzeng YM. 2007. Ferrous ion enhanced lipopeptide antibiotic Iturin A production from *Bacillus amyloliquefaciens* B128. International

Journal of Applied Science and Engineering. 5(2), 123-132.

Ludtke SJ, He K, Heller WT, Harroun TA, Yang L, Huang HW. 1996. Membrane pores induced by Magainin. Biochemistry. 35, 13723-13728.

Marx R, Stein T, Entian KD, Glaser SJ. 2001. Structure of the *Bacillus subtilis* peptide antibiotic Subtilosin A determined BY 1H-NMR and Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. Journal of Protein Chemistry. 20(6), 501-506.

Mendo S, Faustino NA, Sarmento AC, Amado F, Moir AJ. 2004. Purification and characterization of a new peptide antibiotic produced by a thermotolerant *Bacillus licheniformis* strain. Biotechnology Letters. 26(2), 115-9.

Meng F., Lowell CA. 1997. Lipopolysaccharide (LPS)-induced macrophage activation and signal transduction in the absence of Src-family kinases Hck, Fgr, and Lyn. Journal of Experimental Medicine. 185(9), 1661-1670.

Mikkola R, Andersson MA, Grigoriev P, Telpova VV, Saris NE, Rainey FA, Salonen MS. 2004. *Bacillus amyloliquefaciens* strains isolated from moisture-damaged buildings produced Surfactin and a substance toxic to mammalian cells. Archives of Microbiology. 181(4), 314-323.

Moore AJ, Beazley WD, Bibby MC, Devine DA. 1996. Antimicrobial activity of cecropins. Journal of Antimicrobial Chemotherapy. 37, 1077-1089.

Oliveira AC de, Valentim IB, Silva CA, Bechara EJH, Baros MP de, Mano CM,

Goulart MOF. 2009. Total phenolic content and free radical scavenging activities of methanolic extract powders of tropical fruit residues. Food chemistry. 115(2), 469-475.

Oscariz JC, Lasa I, Pisabarro AG. 1999. Detection and characterization of Cerein 7, a new bacteriocin produced by *Bacillus cereus* with a broad spectrum of activity. FEMS Microbial Letters. 178(2), 337-341.

Oyaizu M. 1986. Studies on products of browning reactions: antioxidative activities of browning reaction prepared from glucosamine. Japan Journal of Nutrition. 44, 307-315.

Paik SH, Chakicherla A, Hansen JN. 1998. Identification and characterization of Tochicin, a bacteriocin produced by *Bacillus thuringiensis* sub Tochigiensis. Journal of Industrial Microbiology and Biotechnology. 19, 294-298.

Papagianni M. 2003. Ribosomally synthesized peptides with antimicrobial properties : biosynthesis, structure, function, and applications. Biotechnol Advanceds. 21(6), 465-499.

Park KH, Nan YH, Park YK, Kim JI, Park IS. 2009. Cell specificity, anti-inflammatory activity, and plausible bactericidal mechanism of designed Tryp-rich model antimicrobial peptides. Biochemica et Biophysica Acta. 1788, 1193-1203.

Pattnaik P, Kaushik JK, Grover S, Batish VK. 2001. Purification and characterization of a bacteriocin-like compound (Lichenin) produced anaerobically by *Bacillus*

licheniformis isolated from water buffalo. Journal of Applied Microbiology. 91, 636-645.

Peters BM, Shirtliff ME, Jabra-Rizk MA. 2010. Antimicrobial Peptides: Primeval Molecules or Future Drugs?. PLOS Pathogens. 6(10)

Powers JS, Hancock RE. 2003. The relationship between peptide structure and antibacterial activity. Pepties. 24, 1681-1691.

Ramos HJ, Souza EM, Soares-Ramos JR, Pedros FO. 2006. Antibiosis by *Bacillus amyloliquefaciens* ribonuclease barnase expressed in *Escherichia coli* and against symbiotic and endophytic nitrogne-fixing bacteria. Journal of Biotechnology. 126(3), 291-294.

Rana M, Chatterjee S, Kochhar S, Pereira BMJ. 2006. Antimicrobial peptides : A new dawn for regulating fertility and reproductive tract infections. Journal of Endocrinol Reprod 10. 2, 88-95.

Riley MA, Wertz JE. 2002. Bacteriocins: Evolution, ecology, and application. Annual Review of Microbiology. 56, 117-137.

Rollema HS, Kuipers OP, Both P, de Vos WM, Siezen RJ. 1995. Improvement of solubility and stability of the antimicrobial peptide Nisin by protein engineering. Applied and Environmental Microbiology. 61(8), 2873-2878.

Sandiford S, Upton M. 2012. Identification, characterization, and recombinant expression of epidermicin NI01, a novel unmodified bacteriocin produced by

Staphylococcus epidermis that displays potent activity against *Staphylococci*. Antimicrobial Agents and Chemotherapy. 56(3), 1539-1547.

Sen S, De B, Devanna N, Chakraborty R. 2013. Total phenolic, total flavonoid content, and antioxidant capacity of the leaves of Meyna spinosa Roxb., an Indian medicinal plant. Chinese journal of natural medicines. 11(2), 149-157.

Sheng ZW, Ma WH, Gao JH, Bi Y, Zhang WM, Dou HT, Jin ZQ. 2011. Antioxidant properties of banana flower of two cultivars in China using 2,2-diphenyl-1-picrylhydrazyl (DPPH), reducing power, 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate (ABTS) and inhibition of lipid peroxidation assays. African Journal of Biotechnology. 10(21), 4470-4477.

Sitaram N, Sai KP, Singh S, Sankaran K, Nagaraj R. 2002. Structure-function relationship studies on the frog skin antimicrobial peptide Tigerinin 1 : Design of analogs with improved activity and their action on clinical bacterial isolates. Antimicrobial agents and Chemotherapy. 46(7), 2279-2283.

Stachelhaus T, Schneider A, Marahiel M. 1995. Rational design of peptide antibiotics by targeted replacement of bacterial and fungal domains. Science. 269(5220), 69-72.

Sutyak KE, Wirawan RE, Aroucheva AA, Chikindas ML. 2008. Isolation of *Bacillus sibtilis* antimicrobial peptide Subtilosin from the dairy product-derived *Bacillus amyloliquefaciens*. Journal of Applied Microbiology. 104(4), 1067-1074.

Tamehiro N, Okamoto-Hosoya Y, Okamoto S, Ubukata M, Hamada M, Naganawa H, Ochi K. 2002. Bacilysocin, a novel phospholipid antibiotic produced by *Bacillus subtilis* 168. Antimicrobial agents and chemotherapy. 46(2), 315-320.

Toka O. 2005. Antimicrobial peptides : New candidates in the fight against bacterial infections. Biopolymers. 80, 717-73.

Tomas Ganz. 2003. Defensins : Antimicrobial peptides of innate immunity. Nature. 3, 710-720.

Toure Y, Ongena M, Jacques P, Guiro A, Thonart P. 2004. Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reductionof grey moulld disease caused by *Botrytis cinerea* on apple. Journal of Applied Microbiology. 96(5), 1151-1160.

Yaltirak T, Aslim B, Ozturk S, Alli H. 2009. Antimicrobial and antioxidant activities of *Russula delica* Fr. Food and chemical toxicology. 47, 2052-2056.

Ye Y, Guo Y, Luo YT, Wang YF. 2012. Isolation and free radical scavenging activities of a novel biflavonoid from the shells of *Camellia oleifera* Abel. Fitoterapia. 83(8), 1585-1589.

Yilmaz M, Soran H, Beyatli Y. 2006. Antimicrobial activities of some *Bacillus* spp. strains isolated form the soil. Microbiological research. 161(2), 127-131.

Yoon WJ, Heo SJ, Han SC, Lee HJ, Kang HK, Hyun JW, Koh YS, Yoo ES. 2012. Anti-inflammatory effect of sargachromanol G isolated from Sargassum siliquastrum in Raw 264.7 cells. Archives of Pharmacal Research. 35(8), 1421-

1430.

Yoon WJ, Kim SS, Oh TH, Lee NH, Hyun CG. 2009. Cryptomeria japonica essential oil inhibits the growth of drug-resistant skin pathogens and LPS-induced nitric oxide and pro-inflammatory cytokine production. Polish Journal of Microbiology. 58(1), 61-68.

Yu GY, Sinclair JB, Hartman GL, Bertagnolli BL. 2002. Production of iturin A by *Bacillus amyloliquefaciens* suppressing *Rhizoctomia solani*. Solil Biology and Biochemistry. 34, 955-963.

Wakayama S, Ishikawa F, Oishi K. 1984. Mycocerein, a novel antifungal peptide antibiotic produced by *Bacillus cereus*. Antimicrobial Agent and Chemotheraphy. 26(6), 939-940.

Wu S, Jia S, Sun D, Chen M, Chen X, Zhong J, Huan L. 2005. Purification and characterization of two novel antimicrobial peptides Subpeptin JM4-A and Subpeptin JM4-B produced by *Bacillus subtilis* JM4. Current Microbiology. 50, 292-296.

Zainol M. K., Abd-Hamid A., Yusof S., Muse R.. 2003. Antioxidative activity and total phenolic compounds of leaf, root and petiole of four accessions of Centella asiatica (L.) Urban. Food chemistry. 81(4), 575-581.

감사의 글

2010 년 박사학위를 시작하면서 어느덧 저의 작은 연구결과가 결실을 맞게 되었습니다. 박사학위 과정과 논문을 완성하기까지 많은 관심과 도움을 주신 모든 분들께 감사의 말씀을 드립니다.

먼저 하나님께 모든 영광을 드립니다. 그리고, 항상 부족한 저를 아낌없는 충고와 가르침으로 지도해주신 유진철 지도교수님께 마음을 담아 깊이 감사 드립니다. 교수님의 많은 가르침 항상 잊지 않고 기억하겠습니다. 또한 바쁘신 가운데 열정적으로 제 논문을 심사해주신 고려대학교 김승욱 교수님, 선문대학교 송재경 교수님, 광주대학교 이효정 교수님, 조선대학교 김은애 교수님께도 진심으로 감사를 드립니다.

대학원 과정 동안 저에게 많은 도움을 주신 조승식 교수님, 최윤희 박사님, 최윤석 그리고 항상 옆에서 힘이 되어준 약품미생물 실험실 식구들과 선배님들께 감사를 드립니다.

무엇보다도 저에게 항상 응원과 격려, 지원을 아끼지 않으셨던 어머니와 하늘나라에서 보고계신 아버지께 진심으로 감사를 드리며, 제 곁에서 묵묵히 지켜주던 아내 김은순과 기훈와 수진이에게 고마움을 표합니다.