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Antimicrobial and anti-inflammatory peptides of *Bacillus* strains isolated from Korean foods

조 선 대 학 교 대 학 원

약 학 과

최 윤 희

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한국전통 식품에서 분리한 바실러스 균주가 생산하는
항균, 항염 펩타이드에 관한 연구

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국문초록

한국전통 식품에서 분리한 바실러스 균주가 생산하는 항균, 항염 펩타이드에 관한 연구

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바실러스 속 CS61

한국 전통 식품에서 분리한 바실러스 속 균주 CS61 은 다약제 내성균주에 항균 효과를 보이는 펩타이드를 생산하는 것을 확인하였다. CS61 항균 펩타이드는 컬럼 크로마토 크래피를 통하여 분리정제 하여 분자량이 1100Da 정도임을 확인하였으며, 이 펩타이드는 pH2.0-10.0 사이, 온도는 80℃까지 활성이 유지되었다. 또한 단백질 분해효소인 lipase, proteinase K, α-chymotrypsin 에 안정하였다. 다양한 chemical 에 대한 안정성을 확인한 결과 EDTA, TCA, 계면활성제를 제외한 유기용매에 의해 활성이 감소하였다. CS61 항균펩타이드의 다약제 내성균인 메티실린 저항성 황색 포도상구균(MRSA), 반코마이신 저항성 황색 포도상 구균(VRSA), 반코마이신 저항성 장알균(VRE)에 대한 최소 저해 농도값은 0.625-20μg/ml 이었다. N-말단 아미노산 염기서열 분석 결과 A-I-N-X-D-A-A-Y-L 의 염기서열로 구성되어 있었으며, 네번째 자리에 읽히지 않은 아미노산을 20 가지 다양한 아미노산으로 치환하여 치환체의 항균활성을

비교하였을 때 시스테인으로 치환된 치환체에서 항균 활성을 보임을 알 수 있었다.

CS61 항균 펩타이드의 항산화 활성은 DPPH 라디칼 소거능과 환원력을 통해 분석하였다. 이 펩타이드의 DPPH 소거능은 1000 μg/ml 의 농도에서 77.789±1.8 의 소거능을 보였으며 대조군인 아스코르브산과 비교하였을 때 거의 비슷한 수준의 활성을 나타내었다. 환원력은 대조군인 아스코르브산 보다는 낮지만 농도 의존적으로 증가하였다.

Raw264.7 대식세포는 LPS 에 의해 일산화 탄소의 생성이 증가하는데 CS61 항균 펩타이드를 처리하였을 때 이의 생산을 억제하는 효과를 보였다. 또한 LPS 에 의해 유도되는 염증 단백질인 iNOS, COX-2 단백질의 발현을 농도 의존적으로 감소 시켰으며 RT-PCR 를 통해 유전자 수준에서의 발현 또한 감소함을 확인하였다. 염증반응에 관여하는 싸이토카인인 TNF-α, IL-6, IL-1β의 발현을 ELISA 를 통해 확인한 결과 농도의존적으로 감소하였고 이 싸이토카인의 유전자 수준에서의 발현 또한 감소함을 확인하였다.

바실러스 속 CS32

한국 전통식품에서 분리한 바실러스 CS32 균주는 항균펩타이드를 생산함을 확인하였다. 항생제 저항성 균주인 MRSA, VRE, VRSA 에 탁월한 효과를 보였으며, 이런 병원성 균주에 대한 최소저해농도는 0.156-80 μg/ml 이었다. 항균 펩타이드는 Sepharose CL-6B, Sephadex G50 컬럼을 통해 분리하였고 Tricine SDS-PAGE 와 MALDI-TOF 를 통해 5697.9Da 정도 크기를 보이는 펩타이드임을 확인하였다. N-말단 염기서열 분석을 통해 12 개의 아미노산 서열을 분석(APLEIXXIFHDN)하였고 이 서열은 기존에 보고 되었던 것과 다른 신규한 펩타이드임을 확인하였다. 이 펩타이드는 단백질 분해효소인 lipase, proteinase K, α-chymotrypsin, Trypsin 에 대해

안정하였으며, 다양한 chemical 에 대해서도 안정성을 보였다. 또한 pH5.0-10.0 사이에서 활성이 유지되었고 온도에도 안정하였다.

CS32 항균 펩타이드의 항산화 활성을 DPPH 라디칼 소거능과 환원력을 통해 시험하였다. DPPH 라디칼 소거능 시험 결과 CS32 펩타이드는 1000 μg/ml 의 농도에서 56.9±0.79 의 소거능을 보였으며 대조군인 아스코르브산과 비교하였을 때 같은 농도에서 절반정도의 활성을 나타내었다. 환원력은 대조군인 아스코르브산 보다는 낮지만 농도 의존적으로 증가하였다.

Raw264.7 대식세포는 LPS 에 의해 일산화 탄소의 생성이 증가하는데 CS32 항균 펩타이드를 처리하였을 때 이의 생산을 억제하는 효과를 보였다. 또한 LPS 에 의해 유도되는 염증 단백질인 iNOS, COX-2 단백질의 발현을 농도 의존적으로 감소 시켰으며 RT-PCR 를 통해 유전자 수준에서의 발현 또한 감소함을 확인하였다. 염증반응에 관여하는 싸이토카인인 TNF-α, IL-6, IL-1β의 발현을 ELISA 를 통해 확인한 결과 농도의존적으로 감소하였고 이 싸이토카인의 유전자 수준에서의 발현 또한 감소함을 확인하였다.

ABSTRACT

Antimicrobial and anti-inflammatory peptides of *Bacillus* strains isolated from Korean foods

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***Bacillus* sp. CS61**

In an attempt to isolate effective antimicrobial peptides (AMPs) from a microbial source for the treatment of multidrug-resistant (MDR) bacteria, peptide was purified from *Bacillus* sp. CS61 newly isolated from the traditional Korean fermented foods. CS61 AMP (ca. 1100Da) was purified to homogeneity by using sequential chromatographic steps. It was found to be stable at pH 2.0–10.0 and up to 80 ℃. The AMP activity was resistant to the proteolytic action of lipase, proteinase K and α-chymotrypsin. The effect of several chemicals on the AMP was evaluated. With an

exception to EDTA, TCA and detergent, the AMP was completely sensitive to organic solvents. CS61 AMP performed antimicrobial activity against MDR bacteria such as meticillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA) and vancomycin-resistant *enterococci* (VRE). Minimal inhibitory concentrations of CS61 AMP against MRSA, VRSA and VRE were in the range of 0.625–20μg/ml. The N-terminal amino acid sequence of CS61 AMP was A-I-N-X-D-A-A-Y-L, which differed from reported AMPs. The fourth unidentified amino acid was replaced and several peptides were synthesized. Among them, only cysteine replacement displayed antimicrobial activity.

The antioxidative activities of the AMP were determined by using 2,2-diphenyl-a-picrylhydrazyl (DPPH) and reducing antioxidant power methods. The CS61 AMP has good antiradical potency against the DPPH. Scavenging effect of DPPH radicals was 77.789±1.8 at 1000μg/ml. The reducing power of CS61 AMP was increased in a concentration dependent manner. However, the DPPH scavenging effect and reducing power of ascorbic acid (control) were more effective than CS61 AMP.

Bacillus sp. CS61 AMP was shown in this study to have potent inhibitory effects on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 macrophages. Consistent with these findings, CS61 AMP was further shown to suppress the expression of LPS-induced inducible nitric oxide synthase (iNOS) and cyclooxigenase-2 (COX-2) in a dose-dependent manner at both the protein and

mRNA gene expression levels. In addition, the release of tumor necrosis factor- α (TNF-α) and interleukin-6 (IL-6), IL-1β the mRNA expression levels of these cytokines were reduced by CS61 AMP in a dose-dependent manner. These results suggest that the anti-inflammatory properties of CS61 AMP are caused by iNOS, COX-2, TNF-α, IL-6 and IL-1β in RAW 264.7.

***Bacillus* sp. CS32**

In this study, an antibacterial peptide produced by an isolated *Bacillus licheniformis* CS32 from traditional Korean fermented foods, was identified as antimicrobial peptide. CS32 AMP showed antimicrobial activity against MDR bacteria such as meticillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA) and vancomycin-resistant *enterococci* (VRE). Minimal inhibitory concentrations of CS32 AMP against MRSA, VRSA and VRE were in the range 0.156–80μg/ml. The corresponding AMP was purified to homogeneity by ammonium sulfate precipitation, Sepharose CL-6B and Sephadex G50 column chromatography. Tricine Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis revealed that the active peptide had an apparent molecular weight about 5697.9 Da. Complete amino acid sequence of the peptide yielded twelve amino acids from the N-terminal end (APLEIXXIFHDN), which differed from reported AMPs. The antimicrobial peptide activity was resistant to the proteolytic action of lipase,

proteinase K, α-chymotrypsin and trypsin. The effect of several chemicals on the antimicrobial activity was evaluated. The antimicrobial activity was slightly decreased after its treatment with Triton X-100. Antimicrobial activity was not affected by treatment with organic solvents and EDTA. The antimicrobial activity was relatively heat resistant and also active over a wide range of pH 5-10. Such characteristics indicate that these AMPs may be potential candidate for alternative agents to control important clinical pathogens in animal diseases.

The antioxidative activities of the AMP were determined by using the 2,2-diphenyl-a-picrylhydrazyl (DPPH) and reducing antioxidant power methods. The CS32 AMP has good antiradical potency against the DPPH. Scavenging effect of DPPH radicals was 56.9±0.79 at 1000μg/ml. The reducing power of CS32 AMP was increased in a concentration dependent manner. However, the DPPH scavenging effect and reducing power of ascorbic acid (control) were more effective than CS32 AMP.

Bacillus sp. CS32 AMP was shown in this study to have potent inhibitory effects on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 macrophages. The level of nitric oxide (NO) production in the CS32 AMP and LPS-treated RAW 264.7 cells were determined by western blotting, RT-PCR and ELISA. The cytotoxicity of the CS32 AMP and LPS was measured by an MTT assay. CS32 AMP was shown to suppress the expression of LPS-induced inducible nitric oxide

synthase (iNOS) and cyclooxigenase-2 (COX-2) in a dose-dependent manner at both the protein and mRNA gene expression levels. In addition, the release of tumor necrosis factor- α (TNF-α), interleukin-6 (IL-6) and IL-1β the mRNA expression levels of these cytokines were reduced by CS32 AMP in a dose-dependent manner. These results suggest that the anti-inflammatory properties of CS32 AMP are caused by iNOS, COX-2, TNF-α, IL-6 and IL-1β in RAW 264.7 cells.

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List of Abbreviations

AMPS	Antimicrobial peptides
COX-2	Cyclooxygenase-2
DMEM	Dulbecco's Modified Eagle's Medium
DPPH	2,2-diphenyl-1-picrylhydrazyl.
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IL	Interleukin
LPS	Lipopolysaccharide
MDR	Multidrug-resistant
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTT	3-(4,5-dimethylthiazol-2-2,5-diphenyltetrazolium bromide)
PVDF	Polyvinylidene difluoride
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
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 kill Gram-negative and Gram-positive bacteria including important clinical pathogens, mycobacteria, protozoa, viruses, fungi, and cancer cell (Rana M *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 RE *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 M *et al.* 2003). The action mechanisms have been investigated for some antimicrobial peptides, including defensin (Tomas Ganz *et al*. 2003), maganin (Ludtke SJ *et al.* 1996), nisin (Rollema HS *et al.* 1995), prophenin (Harwig SS. *et al*. 1995), cecropin (Moore AJ *et al*. 1996), cathelicidin and histatin (De Smet K *et al*. 2005). Most AMPs do not target specific molecular receptors of pathogens but rather interact with each other and permeabilize microbial membrane (Park KH *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 generally consisted of between 12 and 50 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 i) α-helical, ii) β-

stranded due to the presence of 2 or more disulfide bonds, iii) β-hairpin or loop due to the presence of a single disulfide bond and/or cyclization of the peptide chain, and iv) extended (Figure 1) (Powers JS *et al*. 2003). 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 O. 2005).

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.

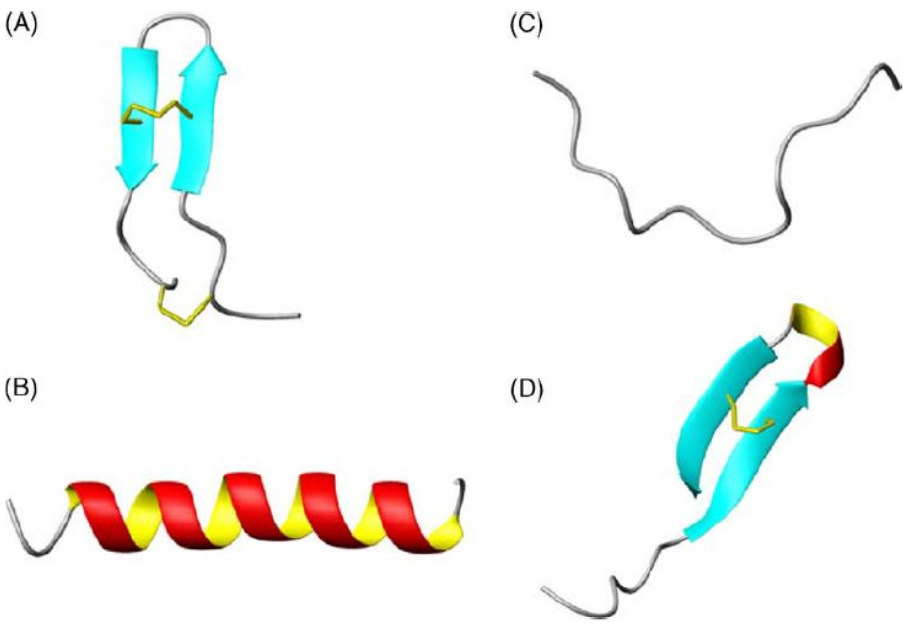

Figure 1. Structure classes of antimicrobial peptides: (A) β-sheet, tachyplesin I; (B) α-helical, magainin 2; (C) extended, indolicin; (D) loop, thanatin. Disulfied bons are indicated in yellow.

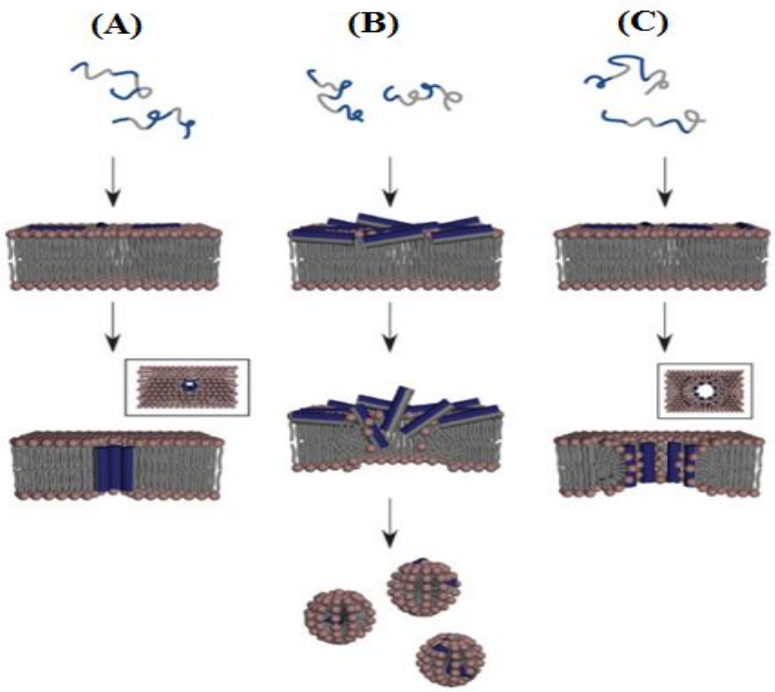

Figure 2. Antimicrobial peptide mechanisms of action.

(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.

Antimicrobial peptides (AMPs) are ubiquitously secreted by a wide range of microorganisms, including bacteria, to protect themselves from other microbes. 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 and other members of the *B.subtilis* group are considered as safe and have "generally recognized as safe" status. *Bacillus* species can be obligate aerobes of facultative anaerobes. *Bacillus* spp., particularly *B. subtilis*, are usually found in foods such as dry cured sausages, cheeses, traditional fermented milks, sourdough, etc.

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 S, *et.al.* 2005; Ahern M *et al*. 2003; Oscariz JC *et al*. 1999; Cherif A *et al*. 2001; Dischinger J *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 this 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 WJ *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 WJ *et al*. 2009).

This study describes the identification of an AMP-producing strain, *Bacillus* sp. CS61 and CS32, isolated from Korean traditional foods as well as purification and biochemical characterisation. And we examined the antioxidant activity and the anti-inflammatory effects of antimicrobial peptides from *Bacillus* sp. by measuring the production and mRNA expression of pro-inflammatory factors (TNF-α, IL-1ß, IL-6 and iNOS, COX-2) in murine macrophage RAW 264.7 cells.

II. *Bacillus* sp. CS61

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.

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin solution from Invitrogen (Grand Island, NY, USA). LPS (Lipopolysaccharide), dimethyl sulfoxide (DMSO), Griess reagent and MTT (3-(4,5-dimethylthiazol-2-2,5-diohenyltetrazolium bromide) were purchase from sigma. Rabbit anti-mouse iNOS polyclonal antibody was Santa Cruz Biotech Inc. (Santa Cruz, CA, USA) and anti-mouse COX-2 was Cayman Co. HRP-conugated donkey anti-rabbit Ig-G and anti-mouse IgG was purchased from Cell signaling. Alkaline phosphatase conjugated affinipure Donkey Anti mouse IgG was purchase from Jackson Immunoresearch laboratories INC. Mouse TNF-a, IL-6, IL-1β ELISA Kit were purchased from BD Biosciences (San Diego, CA, USA).

2. Isolation and production of biologically active antimicrobial peptide from *Bacillus* sp. CS61

a. *In vitro* screening and isolation

Traditional 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 CS61 was found to possess antimicrobial activity and was selected. *In vitro*, antimicrobial activity was primarily determined by the paper disk method using paper disk (8mm, Toyo) against MRSA 693E 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 18h 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 AMP production was determined using media supplemented with 1% yeast extract combined with 1% supplements such as maltose, mannitol, starch, lactose, fructose, sorbitol, sucrose, glucose. Fermentation was carried out in 250ml Erlenmeyer flasks containing 50ml media with constant shaking at 37℃, 180rpm. Afterwards, the influence of nitrogen source on peptide production was determined using medium containing 1% maltose (best carbon source) combined with 1% supplements such as beef extract, malt extract, tryptone, yeast extract, oat meal, peptone, soy. Furthermore, the effect of metal ions on peptide production was evaluated in media containing the best carbon source (1% maltose), the best nitrogen source (1% beef extract) and 0.01% supplements such as Na_2HPO_4, NaH_2PO_4, $MgSO_4$, $ZnSO_4$, $MgCl_2$, KH_2PO_4, $FeSO_4$, $CaCl_2$.

e. Purification of *Bacillus* sp. CS61 producing antimicrobial peptide

The culture broth of strain CS61 was centrifuged at 6000rpm for 1h at 4℃. In order to precipitate proteins, solid $(NH_4)_2SO_4$ was added to the supernatant to the final concentration of 20-40% saturation. Precipitate was collected by centrifugation at 6000rpm for 1h at 4℃, dissolved in 10mM Tris–HCl buffer (pH 8.0) and dialyzed against the same buffer for 24h. The dialysate was then loaded on Sepharose CL-6B column (2.2 cm×116 cm) equilibrated with 10mM Tris–HCl buffer (pH 8.0). All fractions were assayed for protein content and antimicrobial activity. Fractions showing antimicrobial activity were pooled, concentrated using 1kDa centricon (Amicon, USA), and then loaded on Sephadex G50 column (1.5cm×70cm) pre-equilibrated with 10mM Tris–HCl buffer (pH 8.0). Finally, fractions containing high antimicrobial activity were concentrated using 1kDa centricon. To assess antimicrobial activity conveniently, the disk diffusion method was employed with the indicator strain MRSA 693E.

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 50mM 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. CS61 producing antimicrobial peptide

The thermal stability of the CS61 AMP was assessed by exposing aliquots of the peptide solution to different temperatures (20, 40, 60, 80, 100 ℃ for 30min) 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 2-10 (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.

CS61 AMP was treated with different types of proteolytic enzymes at a final concentration of 1mg/ml; lipase (50mM Tris-HCl, pH7.5), protease K (50mM Tris-HCl, pH7.5), α-chymotrypsin (50mM Tris-HCl, pH7.5), trypsin (50mM Tris-HCl,

pH8.0). Chemicals (working concentrations in Table 8.) were added to the AMP and the samples were incubated for 1h at room temperature before being tested for residual antimicrobial activity. After the treatment, the samples were tested for residual antimicrobial activity against test organism.

h. N-terminal amino acid sequence and peptide synthesis

The N-terminal amino acid sequence of the purified preparation was determined by Edman degradation using a Procise Model 492 Protein Sequencing System (Applied Biosystems, Foster city, CA).

Based on the native amino acid sequence of CS61 AMP, several peptides were synthesized altering the unidentified fourth amino acid of the N-terminus by the peptide manufacturing company AnyGen (Gwangju, South Korea) (http://www.anygen.com). The molecular mass of the peptides was evaluated by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI–TOF/MS). Moreover, antimicrobial activity of the synthesized peptides was determined against MRSA 693E as a 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), 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 12h.

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 100ul were added to 100μl of DPPH (0.3mM) in methanol solution in 96 well plate. The mixture was kept at room temperature for 30min and the absorbance at 517nm 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 (200μl) of sample solution was mixed with 200μl of 200mM sodium phosphate buffer and 200μl of 1% ferricyanide aqueous solution. The mixture was incubated at 50℃ for 20min, followed by addition of 200μl of 10% trichloroacetic acid solution. Then, the mixture was centrifuged at 12,000 rpm for 10min. Finally, 500μl supernatant was mixed with 500μl 0.1% ferric chloride aqueous, and absorbance was measured at 700nm 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.

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 24h 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 24h incubation with or without peptide (10-100μg/ml), a 0.5mg/ml of MTT solution was added to each well in a 1/10 volume of media. Cells were incubated at 37℃ for 3h, and dimethylsulfoxide (DMSO) was added to dissolved the formazan crystals. The obsorbance was measured using an assay reader at 590nm. 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 CS61 peptide for 30min prior to stimulation with 1μg/ml of LPS for 24h. 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.

(4) Enzyme immune assay of TNF-α, IL-1β and IL-6

The inhibitory effects of CS61 peptide on the production of pro-inflammatory cytokines; TNF-α, IL-1β and IL-6 were determined by an enzyme-linked immune sorbent assay (ELISA). Cells were treated with different concentration of CS61 AMP (10-100μg/ml) for 1h followed by LPS (1μg/ml) treatment for 24h. Amount of nitrite of the supernatant was calculated from a sodium nitrite standard curve. Supernatant was analyzed for TNF-α, IL-1β and IL-6 by enzyme linked immunosorbent assay (ELISA) using commercial kits (BD Biosciences) according to the manufacturer's instructions.

(5) Western blotting

For detection of iNOS, COX-2 protein expression, Raw 264.7 cells were washed with cold PBS (phosphate-buffered saline). Cells were lysed in lysis buffer (mixture of RIPA buffer and protease inhibitor) and kept on ice for 30min. Cell lysates were centrifuged at 12,000rpm at 4℃ for 30min. Protein concentration of sample was determined by the Bradford method. Samples were stored at -80°C or immediately used for Western blot analysis. Aliquots of the lysates were separated on SDS polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked with 5% non-fat dried milk in Tris buffered saline- Tween (TBST, 10mM Tris-HCl, 150mM NaCl, 0.05% Tween 20, pH 7.6) at room temperature for 2h. After being washed, the membranes were incubated with specific primary antibody solution (anti-iNOS, anti- COX-2 or β-actin antibodies) for 12h at 4℃.

Each membrane was washed with TBST and incubated with HRP (for iNOS, COX-2 antibody) or alkaline phosphatase (for anti-β-actin antibodies) conjugated secondary antibody solution for 1h at room temperature. For detection of iNOS, COX-2 protein expression, the blots were washed with TBST and incubated with ECL reagent for 2min and viewed by chemilumunescence. Equal protein loading was assessed by the expression level of actin protein. For β-actin the blots were washed with TBST and incubated with AP color development buffer containing color reagent (AP conjugate substrate kit, Bio-rad laboratories Inc.).

(6) RNA preparation and mRNA expression analysis by RT-PCR

Total RNA from the peptide treated cells was prepared using RNAiso Reagent (Takara) according to the manufacturer's protocol, and was stored at -80°C until use. For the detection of iNOS, COX-2, TNF-α, IL-Iβ, IL-6, total RNA was extracted after stimulation and treatment. The mRNA expression levels of iNOS, COX-2, TNF-α, IL-I β, IL-6 in the treated cells were compared to the expression levels in control cells. One μg of the RNA was reverse-transcribed into cDNA and used as a template for RT-PCR amplication. The primers and the amplification conditions are listed in Table 1. PCR was performed with a DNA gene cycler, and the amplification was followed by denaturation at 94℃ for 30s, annealation at 58℃ for 30s, and primer extention at 72℃ for 40s. PCR products were analyzed on 1% agarose gels, and bands were visualized with ethidium bromide staining.

Table 1. Primers for RT-PCR

Primers	Primer sequences	
	Forward	**Reverse**
iNOS	5`-CCCTTCCGAAGTTTCTGGCAGCAGC-3`	5`-GGCTGTCAGAGCCTCGTGGCTTTGG-3`
COX-2	5`-CACTACATCCTGACCCACTT-3`	5`-ATGCTCCTGCTTGAGTATGT-3`
TNF-α	5`-TCTCATCAGTTCTATGGCCC-3`	5`-GGGAGTAGACAAGGTACAAC-3`
IL-1β	5`-TGGACGGACCCCAAAAGATG-3`	5`-AGAAGGTGCTCATGTCCTCA-3`
IL-6	5`-GTTCTCTGGGAAATCGTGGA-3`	5`-TGTACTCCAGGTAGCTATGG-3`
GAPDH	5`-CACTCACGGCAAATTCAACGGCAC-3`	5`-GACTCCACGACATACTCAGCAC-3`

B. Results

1. Production, isolation and purification of antimicrobial peptide from *Bacillus* sp. CS61

a. Identification of the *Bacillus* strain

For molecular phylogeny, the 16S rRNA sequence of the local isolate was compared to sequence of 19 *Bacillus* species. In order to determine the relation of the local isolate to these *Bacillus* strains. Multiple sequence alignment was done between the sequence of the 16s rRNA gene of *B. subtilis, B. vallismortis, B. tequilensis* and local isolate. Computer assisted RNA searches against bacterial database similarly revealed that the 16S rRNA sequence was 99.596% identical with both *Bacillus subtilis* CS61 (Table 2, Figure 3). 16S rRNA sequence of CS61 strain was deposited in GenBank under accession no. JN675406.

Table 2. Similarity of 16S rRNA gene sequence (CS61)

Rank	Name/Title	Strain	Accession	Similarity	Diff/ Total nt
1	***Bacillus subtilis sub sp. subtilis***	NCIB 3610(T)	ABQL01000001	99.596	6/1484
2	***Bacillus vallismortis***	DSM 11031(T)	AB021198	99.528	7/1484
3	***Bacillus subtilis sub sp.spizizenii***	NRRL B-23049(T)	AF074970	99.432	8/1409
4	***Bacillus tequilensis***	NRRL B-41771(T)	EU138487	99.401	7/1168
5	***Bacillus subtilis sub sp.inaquosorum***	BGSC 3A28(T)	EU138467	99.401	7/1168
6	***Bacillus mojavensis***	IFO 15718(T)	AB021191	99.124	13/1484
7	***Bacillus atrophaeus***	JCM 9070(T)	AB021181	99.122	13/1480
8	***Brevibacterium halotolerans***	LMG 21660(T)	AJ620368	99.122	13/1480
9	***Bacillus licheniformis***	ATCC 14580(T)	CP000002	97.842	32/1483
10	***Bacillus aerius***	24K(T)	AJ831843	97.772	33/1481
11	***Bacillus sonorensis***	NRRL B-23154(T)	AF302118	97.729	32/1409
12	***Bacillus altitudinis***	41KF2b(T)	AJ831842	97.23	41/1480
13	***Bacillus aerophilus***	28K(T)	AJ831844	97.1	43/1483
14	***Bacillus stratosphericus***	41KF2a(T)	AJ831841	97.1	43/1483
15	***Bacillus safensis***	FO-036b(T)	AF234854	96.932	44/1434
16	***Bacillus pumilus***	ATCC 7061(T)	ABRX01000007	96.831	47/1483
17	***Bacillus vietnamensis***	15-1(T)	AB099708	95.936	56/1378
18	***Bacillus aquimaris***	TF-12(T)	AF483625	95.74	63/1479
19	***Bacillus acidicola***	105-2(T)	AF547209	95.547	66/1482

TCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTA
GCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAAT
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CGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAAC
ACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCCAGCCGCCGAAGGTGGGACAGATGATTGGGGTGAAGTCTCA

Figure 3. 16S rRNA sequence of *Bacillus* sp. CS61

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 CS61 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 and nitrogen sources, maltose and beef extract, respectively, caused the maximum production of peptide. Furthermore, none of the tested metal ions enhanced the production of peptides, therefore optimized medium was prepared without metal ions (Figure 4,5,6). Finally, production of peptide was carried out MB (1% maltose and 1% beef extract) medium where significant production was achieved from 12h and reached a maximum at 48h. In addition, maximum production was achieved when the final pH of the medium was 8.26 (Table 3). Production of antimicrobial peptide in *Bacillus brevis* isolated from *kimchi* reached a maximum when the medium pH was 6.8. Similarly, significant production as well as growth was achieved from 12h and reached a maximum at 48h of cultivation, which is different from antimicrobial peptide production in *B.brevis*.

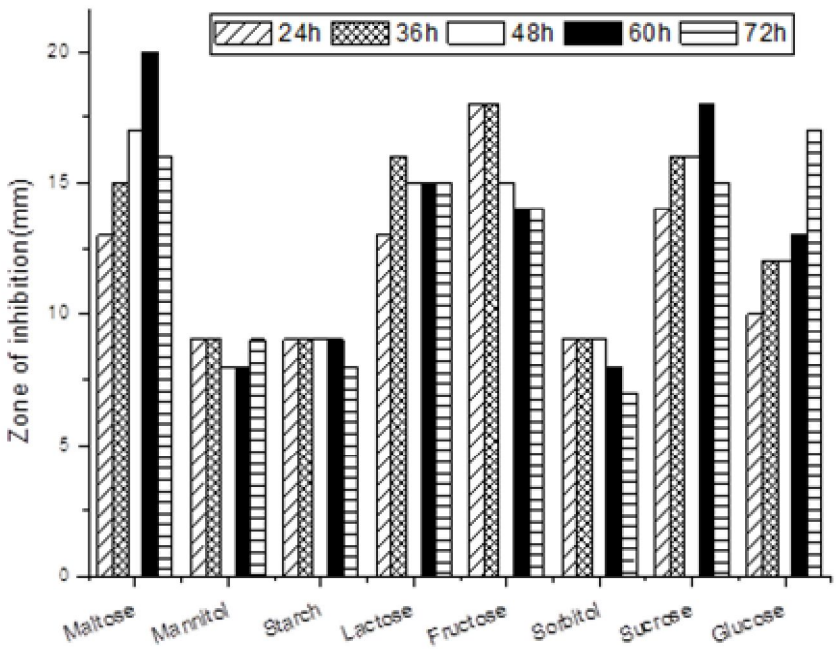


Figure 4. Effect of various carbon sources on the activity of antimicrobial peptide produced by *Bacillus* sp. CS61

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

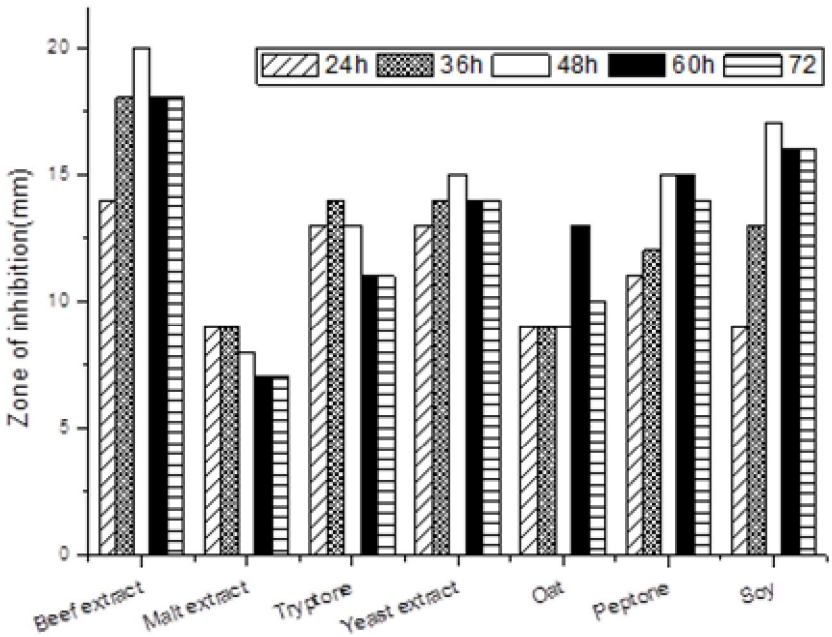


Figure 5. Effect of various nitrogen sources on the activity of antimicrobial peptide produced by *Bacillus* sp. CS61

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

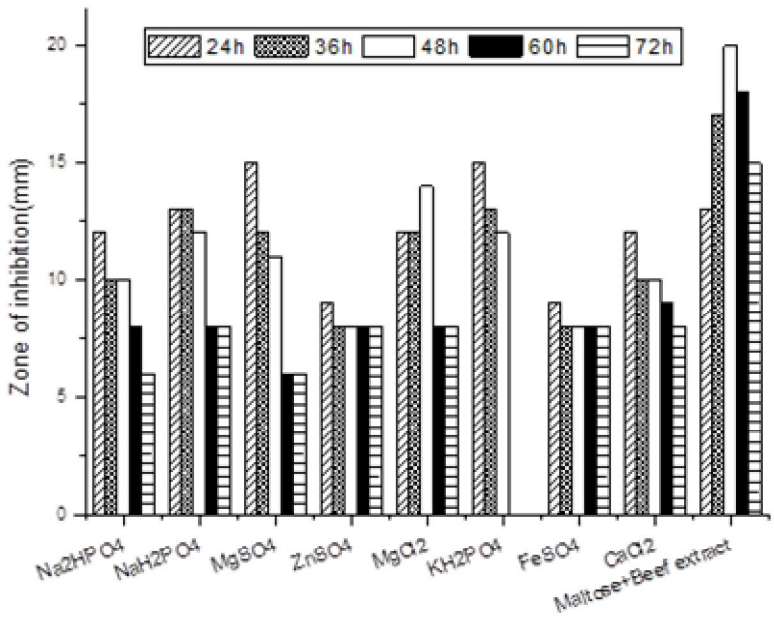


Figure 6. Effect of various metal ion sources on the activity of antimicrobial peptide produced by *Bacillus* sp. CS61

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

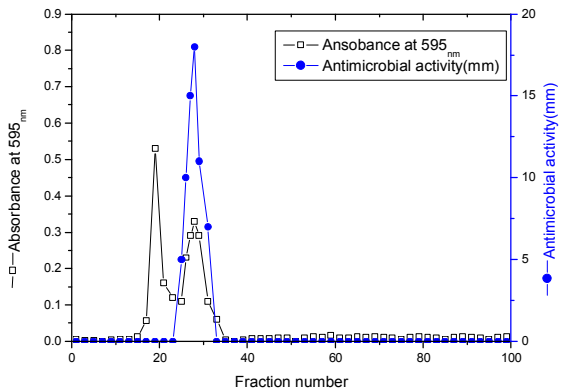
Table 3. Effect of physical parameters on bacterial growth and antimicrobial peptide production

Parameters	Final pH	Bacterial growth(OD660nm)	Inhibitory activities(mm)
Incubation period(hr)			
6	6.78	1.57	8
12	7.93	2.189	10
24	8.15	2.22	12
36	8.13	2.34	16
48	8.26	2.24	19
60	8.57	2.16	17
72	9.0	1.87	15
96	8.97	1.84	10

c. Fermentation and purification of antimicrobial peptide

Bacillus sp. CS61 was grown on rotatory shaker at 180rpm in MB medium for 48h at 37℃. Culture broth was centrifuged at 6,000rpm for 1h. AMP 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 7). Tricine SDS-PAGE and in situ detection inhibitory activity confirm the homogeneity and activity of the purified CS61 AMP. The peptide migrated as a single band and the molecular weight was estimated to be 1,100Da (Figure 8). The band was confirmed to be a peptide by in situ inhibitory activity of the gel.

(a)



(b)

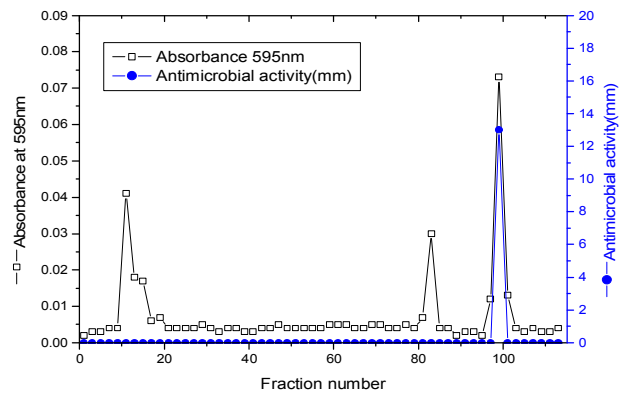


Figure 7. Elution profile of *Bacillus* sp. CS61 antimicrobial peptide

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

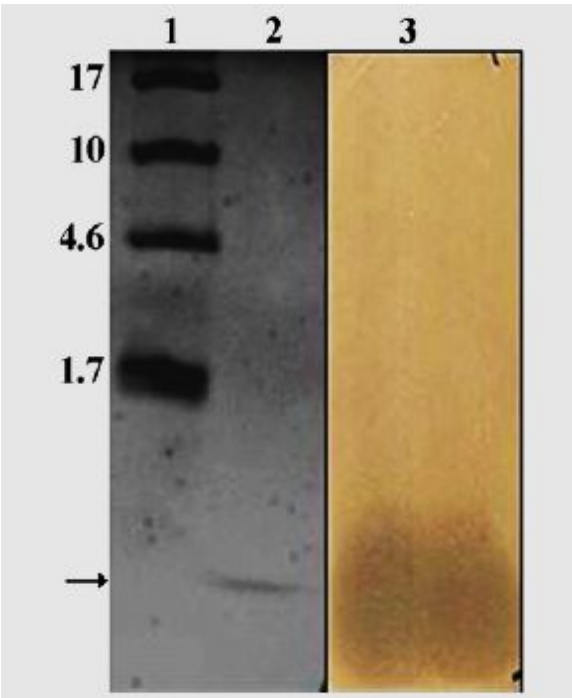


Figure 8. Determination of the molecular weight

Tricine SDS-PAGE and activity staining of CS61 peptide. Lane 1, protein size marker with the corresponding value in kDa on the left; Lane 2, purified CS61 peptide; Lane 3, activity staining.

d. Peptide synthesis and antimicrobial activity

The N-terminal amino acid sequence of CS61 AMP was A-I-N-X-D-A-A-Y-L. In a BLAST search, it showed homology with an unknown protein product (accession no. YP 004206141) from *Bacillus subtilis* BSn5. Although the antimicrobial function of this unknown product is unclear, >4.8% of the genome of *B. subtilis* BSn5 is supposed to be responsible for the synthesis of antimicrobial products (Deng Y *et al*. 2011). Although further study is required, the N-terminal sequence of CS61 peptide does not show homology with that of reported. AMPs, suggesting that it could be a novel type of AMP. Details of the synthesised peptides are given in Table 4. Of 20 peptides synthesised using hydrophilic amino acids for better water solubility, the peptide with a cysteine replacement in the unidentified fourth amino acid showed anti-MRSA 693E activity (final concentration 3mg/ml). The activity of cysteine replacement peptide was weaker than the native peptide. The others did not show such activity, suggesting that cysteine, a hydrophilic amino acid, is the key amino acid for the antimicrobial activity in CS61 antimicrobial peptide in association with the other unchanged amino acids. Taking this reference, further investigations such as complete antimicrobial spectra against MDR and non-MDR bacteria, mode of antimicrobial action, etc. are being carried out.

Table 4. Synthesised peptides and their antimicrobial activity against meticillin-resistant *Staphylococcus aureus* (MRSA) 693E

Peptide	Sequence	MW(Da)	Anti-MRSA activity (mm)
Original peptide of CS61	AIN**X**DAAYL	1100	20
Peptide-1(K)-lysine	AIN**K**DAAYL	1049.2	-
Peptide-2(R)-arginine	AIN**R**DAAYL	1077.2	-
Peptide-3(H)-histidine	AIN**H**DAAYL	1058.2	-
Peptide-4(E)-glutamate	AIN**E**DAAYL	1050.2	-
Peptide-5(D)-aspartate	AIN**D**DAAYL	1036.1	-
Peptide-6(C)-cysteine	AIN**C**DAAYL	1024.2	15
Peptide-7(Q)-glutamine	AIN**Q**DAAYL	1049.1	-
Peptide-8(N)-asparagine	AIN**N**DAAYL	1035.1	-
Peptide-9(S)-serine	AIN**S**DAAYL	1008.1	-
Peptide-10(T)-threonine	AIN**T**DAAYL	1022.1	-
Peptide-11(Y)-tyrosine	AIN**Y**DAAYL	1084.2	-
Peptide-12(A)-alanine	AIN**A**DAAYL	992.6	-
Peptide-13(V)-valnine	AIN**V**DAAYL	1020.5	-
Peptide-14(G)-glycine	AIN**G**DAAYL	978.0	-
Peptide-15(L)-leucine	AIN**L**DAAYL	1034.6	-
Peptide-16(I)-isoleucine	AIN**I**DAAYL	1034.1	-
Peptide-17(F)-phenylalanine	AIN**F**DAAYL	1068.8	-
Peptide-18(W)-tryptophan	AIN**W**DAAYL	1107.5	-
Peptide-19(P)-proline	AIN**P**DAAYL	1018.7	-
Peptide-20(M)-methionine	AIN**M**DAAYL	1052.8	-

MW, molecular weight

2. Biological activities of antimicrobial peptide

a. Antimicrobial activity of peptide

(1) Antimicrobial spectrum of antimicrobial peptide

As shown in Table 5, CS61 AMP showed antimicrobial activity against Gram-positive but not Gram-negative bacteria. Importantly, CS61 AMP displayed an antagonistic effect against multidrug-resistant (MDR) pathogens such as MRSA, VRSA and VRE, which was stronger than that shown by bacitracin and vancomycin. Although direct comparison in unavailable, the anti-MRSA activity of CS61 AMP appears much stronger than that of two peptide antibiotics antibiotics from *Bacillus licheniformis* DSM (Dischinger J *et al*. 2009) displaying a low range of inhibitory zone against most of CS61 peptide against MRSA and VRE strains is relatively weaker than that of epidermicin NI01 (Sandiford S *et al*. 2012)which showed a nanomolar range of MIC values against the strain.

Table 5. Minimum inhibitory concentration of *Bacillus* sp. CS61 peptide

Test organisms		MIC(ug/ml)		
		CS61	Bacitracin	Vancomycin
Alcaligenes faecalis ATCC 1004	G(-)	>80	>80	>80
Enterococcus faecalis ATCC 29212	G(+)	5	5	2.5
Bacillus subtilis ATCC 6633	G(+)	>80	40	0.3
Staphylococcus aureus KCTC 1928	G(+)	40	>80	5
Micrococcus luteus ATCC 9341	G(+)	>80	40	2.5
Mycobacterium smegmatis ATCC 9341	G(+)	40	>80	1.25
Salmonella typhimurium KCTC 1925	G(-)	>80	>80	>80
Escherichia coli KCTC 1923	G(-)	>80	>80	>80
Pseudomonas aeruginosa KCTC 1637	G(-)	>80	>80	>80
MRSA693E*	G(+)	10	1.25	1.25
MRSA 4-5*	G(+)	0.625	1.25	0.625
MRSA 5-3*	G(+)	0.625	1.25	0.625
VRE 82**	G(+)	20	40	>80
VRE 89**	G(+)	20	40	>80
VRE 98**	G(+)	20	>80	>80
VRSA***	G(+)	10	>80	>80

*MRSA, Methicillin resistant *Staphylococcus aureus*; **VRE, Vancomycin resistant *Enterococcus faecium*; ***VRSA, Vancomycin 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 6. Thermo stability of CS61 AMP was analyzed for various temperatures by measuring the residual activities. It was stable 80℃, but the activity gradually decreased with the increase in temperature. The residual activity was 66.7% after incubation at 100℃, and total loss of activity was observed after incubation at 121℃ at 15min. The stability of AMP was determined by measuring the residual activities after incubating the AMP for 1h 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%. Stability of CS61 against temperature and pH is comparable with epidermicin NI01 (Sandiford S *et al*. 2012), *Bacillus* sp. Strain 8A (D.Bizani *et al*. 2002) and *Bacillus thuringiensis* strain (Ahern M *et al*. 2003).

To test the effect of proteolytic enzymes, CS61 AMP was treated with lipase, proteinase K, α-chymotrypsin, trypsin and residual activity was measured by agar diffusion assay against MRSA 693E. The antimicrobial peptide was sensitive to trypsin at the concentration of mg/ml (Table 7).

The effect of several chemicals on the antimicrobial activity was evaluated. With an exception to EDTA, TCA and detergent, the antimicrobial activity was completely sensitive to organic solvents (Table 8).

Table 6. Effect of different temperature and pH on inhibitory activity of peptide

Treatment	Residual activities (%)
Temperature(℃)	
None	100
20℃, 30min	100
40℃, 30min	100
60℃, 30min	100
80℃, 30min	100
100℃, 30min	66.7
121℃, 15min	0
pH	
2	100
4	100
6	100
8	100
10	100

Table 7. Effect of various proteolytic enzymes

Proteolytic enzymes	Residual activity (%)
None	100
Lipase	98
Proteinase K	99
a-chymotrypsin	98
trypsin	65

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

Table 8. Effect of various chemicals on antimicrobial activity

Treatment	Concentration	Residual activity(%)
None		100
Acetone	10% (v/v)	61
Chloroform	10% (v/v)	72
Ethanol	10% (v/v)	77.8
Methanol	10% (v/v)	66.7
Ethyl acetate	10% (v/v)	61
EDTA	10mM/ml	98
Trichloroacetic acid	100mg/ml	96
Triton X-100	1% (v/v)	98
Tween 20	10% (v/v)	99
Tween 80	10% (v/v)	99.3

* Antimicrobial peptide was pre-incubated for 1h 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 pH7.0 before testing for antimicrobial activity.

b. Antioxidant activities

(1) DPPH radical scavenging activity

To obtain information about the mechanisms of the antioxidative effects of the AMP, we examined their radical scavenging effects by measuring changes in absorbance of DPPH radical at 517nm. Both CS61 AMP and ascorbic acid showed a concentration dependent scavenging of DPPH radicals. CS61 AMP was found to be similar active radical scavenger to ascorbic acid. Figure 9, shows the results of the free radical (DPPH) scavenging activity in % inhibition. CS61 AMP exhibited a comparable antioxidant activity with that of standard ascorbic acid at varying concentration tested (1 to 1000μg /ml). There was a dose dependent increase in the percentage antioxidant activity for all concentrations tested (Figure 9). The AMP at a concentration of 1000 μg/ml showed a percentage inhibition of 77.789±1.8. 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 1 to 1000μg/ml. Ascorbic acid at a concentration of 1000μg/ml exhibited a percentage inhibition of 82.5±1.59. 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.

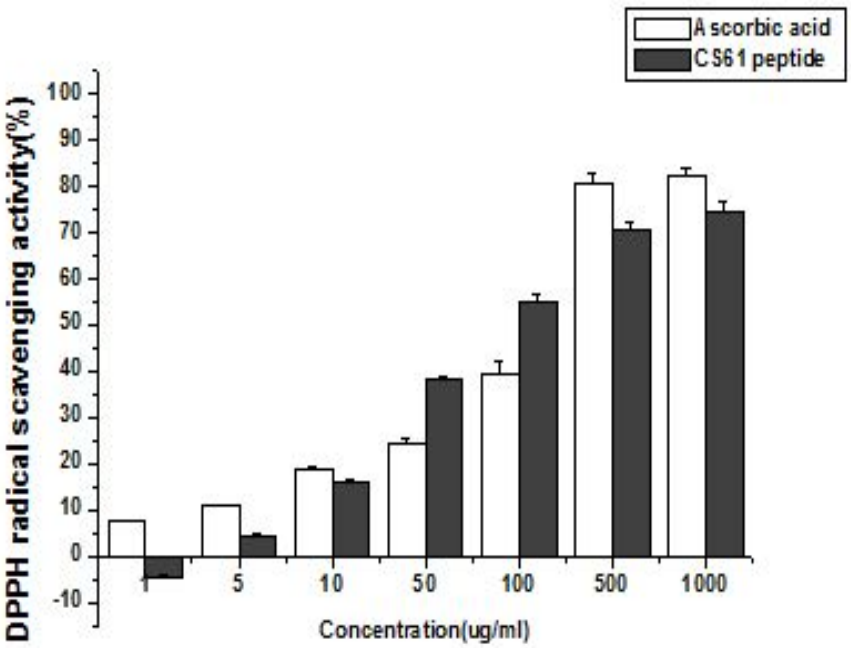


Figure 9. DPPH radical scavenging activity of CS61 antimicrobial peptide in different concentration.

(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 CS61 AMP was increased concentration dependent. However, the reducing power of ascorbic acid was much more effective than CS61 AMP (Figure 10).

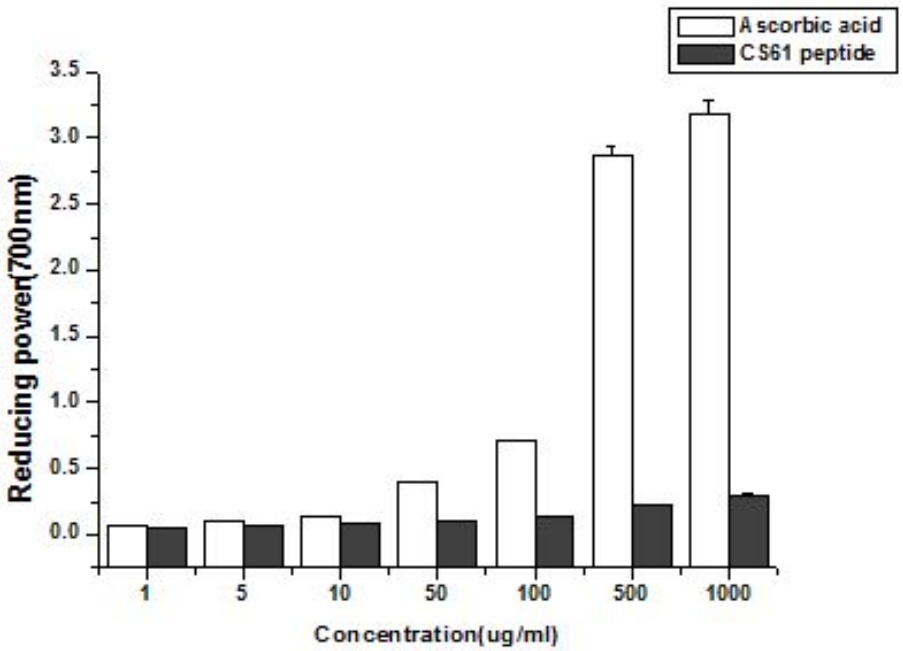


Figure 10. Reducing power of CS61 antimicrobial peptide

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

c. Anti-inflammatory activity of antimicrobial peptide

(1) Cytotoxicity

Raw 264.7 cells were treated with various concentrations of CS61 AMP for 24h and the viability was determined by MTT assay as described above. As shown in Figure 11, CS61 AMP did not exhibit cytotoxicity to Raw 264.7 cells at the range of 5-100μg/ml. Therefore peptide was used at 100μg/ml and below for further experiments.

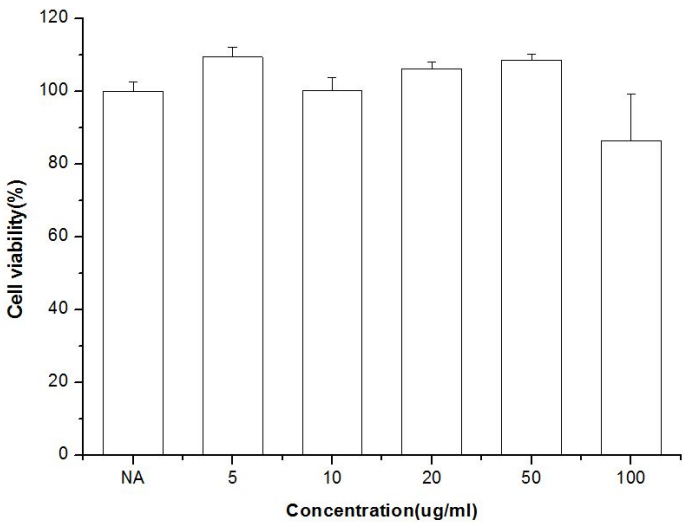


Figure 11. Effect of the CS61 antimicrobial peptide on cell viability

Cell viability was measured after 24h 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 5-100 μg/ml peptide for 24h. Each bar shows the mean ± S.D of three independent experiments performed in triplicate.

(2) Inhibition of nitric oxide production and iNOS, COX-2 protein expression in LPS-induced Raw 264.7 macrophage cells

Macrophages produce NO following stimulation with LPS. Excessive production of NO during inflammation causes tissue injury, nerve damage etc.. To assess the effect of CS61 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 Figure 12, LPS treatment significantly increased NO production compared to the untreated cells. Treatment of cells with CS61 AMP at 10, 50, 100μg/ml suppressed the LPS-induced production of NO to a statistically significant extent.

To confirm whether the inhibition of NO production is due to a decreased level of iNOS and COX-2 protein, the effect of CS61 AMP on the level of iNOS and COX-2 protein was determined by western blot analysis. The expression of iNOS and COX-2 protein was barely detectable in unstimulated Raw 264.7 cells, but was markedly increased after 24h of LPS (1μg/mL) treatment. However, CS61 AMP significantly attenuated iNOS and COX-2 protein expression in LPS-induced Raw 264.7 cells. These results indicate that LPS exposure increased the expression of iNOS and COX-2 protein, but treatment with CS61 AMP significantly suppressed the induction of LPS-induced mediators through transcriptional inhibition. (Figure 12).

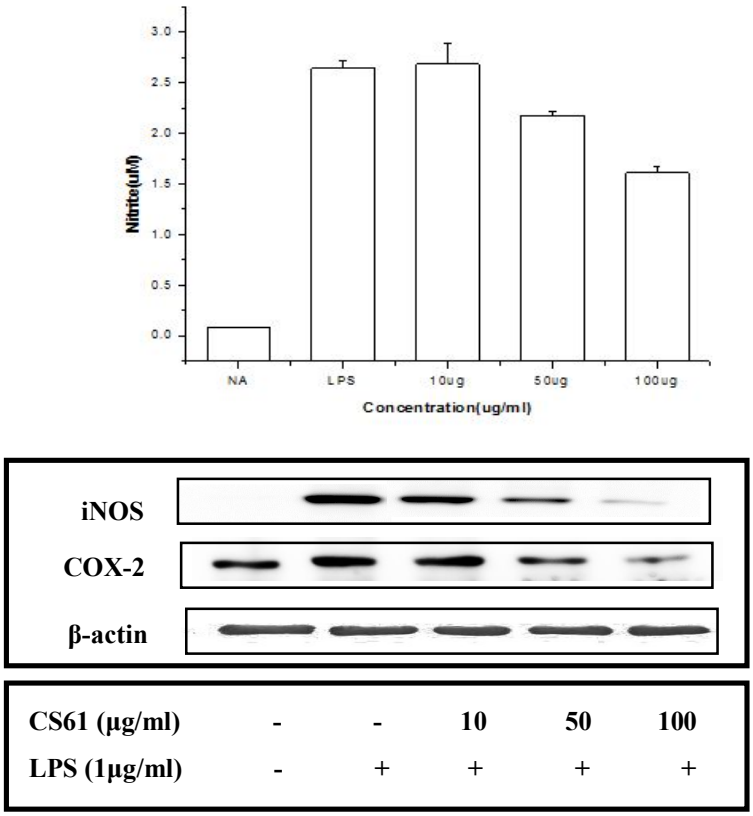


Figure 12. Inhibitory effect of CS61 antimicrobial peptide of NO production and iNOS, COX-2 protein expression in LPS-induced Raw 264.7 macrophage cells

Cells were incubated with the various concentration of peptide for 30 min, followed by treatment with 1μg/ml of LPS and incubated for 24h. The amounts of NO were determined using the Griess reagent in the culture medium (A). Equal amounts of cell lysate were resolved on SDS polyacrylamide gels, transferred to PVDF membranes, and probed with antibodies against iNOS and COX-2. β-actin was used as internal controls for western blot analysis (B).

(3) Inhibitory effects of CS61 peptide on the production of pro-inflammatory cytokines in LPS-induced Raw264.7 macrophage cells

Since TNF-α, IL-1β and IL-6 are early secreted pro-inflammatory cytokines, it determined the effects of CS61 AMP on secreted levels of TNF-α, IL-1β and IL-6 in treatments of LPS. To reach detectable ranges of secreted cytokines in cultured media, LPS stimulations were extended up to 24 h. The stimulation of RAW 264.7 cells with LPS increased levels of TNF-α, IL-1β and IL-6. Induced levels of pro-inflammatory cytokine were significantly decreased by CS61 AMP in a dose-dependent manner. This result indicated that CS61 AMP significantly inhibits TNF-α, IL-1β and IL-6 secretions in LPS stimulation, suggesting that CS61 AMP is a potential inhibitor for the initial phase of inflammatory cascades under LPS stimulations (Figure 13).

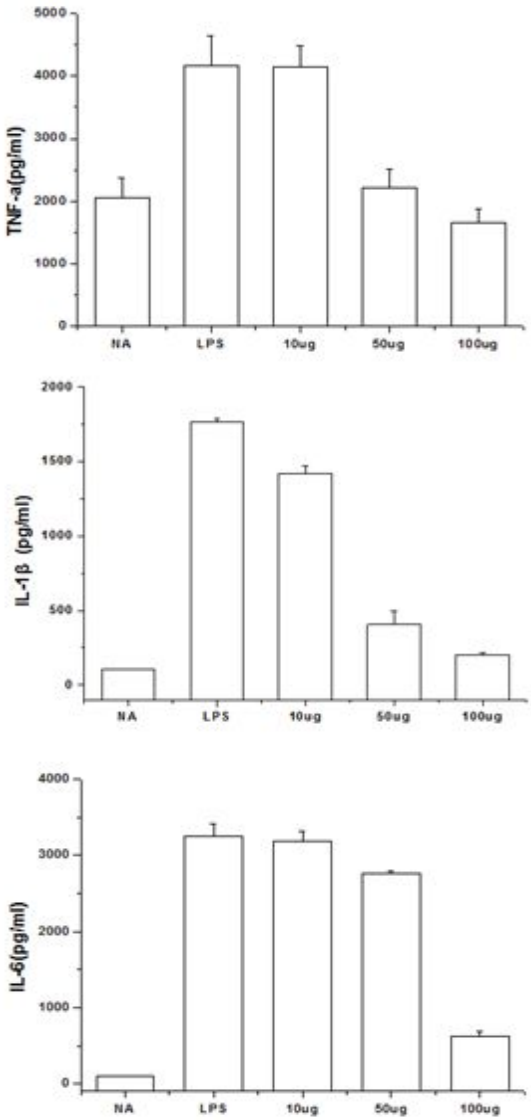


Figure 13. Effect of CS61 antimicrobial peptide on pro-inflammatory cytokines production in LPS- induced Raw 264.7 macrophage cells

Cells were incubated with the indicated concentrations of CS61 peptide for 30min before treatment with LPS (1μg/ml) for 24h. After incubation for 24h, the supernatant was collected, and the amount of proinflammatory cytokines were measured by ELISA assay.

(4) Inhibition of iNOS, COX-2, TNF-α, IL-1β, IL-6 mRNA gene expression in LPS-induced Raw 264.7 macrophage cells

Since iNOS or COX-2 protein is regulated at the transcription level, it investigated the effects of CS61 AMP on iNOS or COX-2 mRNA gene expression. Raw 264.7 cells were treated with or without LPS (1μg/ml) for 6h in the absence or presence of CS61 AMP and mRNA levels were determined by RT-PCR. As shown in Figure 14, LPS stimulation significantly increased iNOS and COX-2 mRNA levels and CS61 AMP treatments significantly inhibited inducible levels of iNOS and COX-2 mRNA expressions in a dose dependent manner. These results suggest that the CS61 AMP mediated inhibition of iNOS and COX-2 gene expression in LPS-treated Raw 264.7 cells was associated with transcriptional regulation of iNOS and COX-2.

We also examined the expression of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6 mRNA expression. LPS treatment increased TNF-α, IL-1β and IL-6 mRNA levels and treatment of cells with CS61 AMP dose-dependently inhibited the LPS-increased expression. Moreover, upon LPS treatment, increased expression of their mRNA was attenuated by CS61 AMP pretreatment in a dose-dependent manner. These results indicated inhibitory action of CS61 AMP on LPS-induced production of NO and cytokines take place at transcription levels (Figure 14).

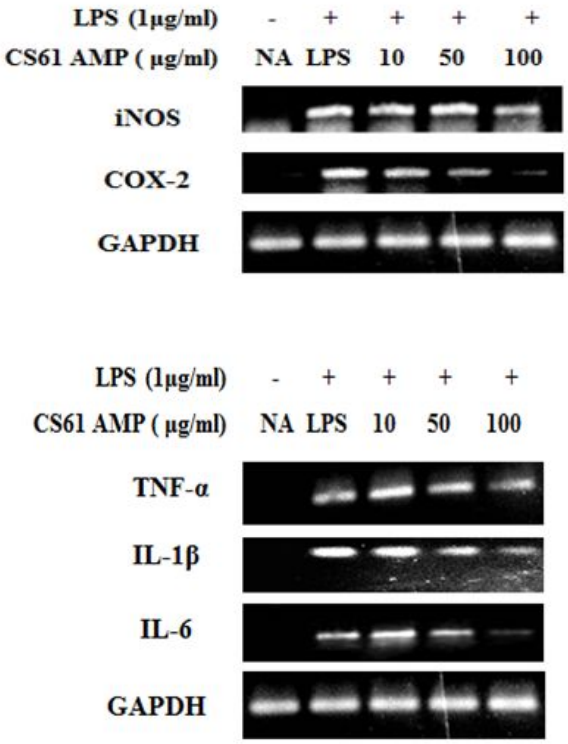


Figure 14. Effect of CS61 antimicrobial peptide on iNOS, COX-2, TNF-α, IL-1β, IL-6 mRNA expression in LPS-induced Raw 264.7 macrophage cells

Effects of CS61 peptide on LPS-induced iNOS, COX-2 and TNF-α, IL-1β, IL-6 mRNA expressions in RAW 264.7 cells. Total RNA was prepared for RT-PCR of gene expressions from RAW 264.7 macrophages stimulated with LPS (1 μg/ml) in the presence or absence of CS61 peptide (10, 50, 100μg/ml). These bands were detected by agarose gel electrophoresis, as described in methods. The experiment was repeated three times and similar results were obtained. GAPDH was used as internal control for RT-PCR assays. Values shown are means ± S.D. of three independent experiments.

III. *Bacillus* sp. CS32

A. Materials & Methods

1. Materials

Sepharose CL-6B and Sephadex G-50 were obtained from Pharmacia (Uppsala, Sweden). Trypsin, Proteinase K, α-chymotrypsin, lipase were from Sigma (St. Louis, USA). All other chemicals and reagent were of analytical grade.

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin solution from Invitrogen (Grand Island, NY, USA). LPS (Lipopolysaccharide), dimethyl sulfoxide (DMSO), Griess reagent and MTT (3-(4,5-dimethylthiazol-2-2,5-diohenyltetrazolium bromide) were purchase from sigma. Rabbit anti-mouse iNOS polyclonal antibody was Santa Cruz Biotech Inc. (Santa Cruz, CA, USA) and anti-mouse COX-2 was Cayman Co.. HRP-conguated donkey anti-rabbit Ig-G and anti-mouse IgG was purchased from Cell signaling. Alkaline phosphatase conjugated affinipure Donkey Anti mouse IgG was purchase from Jackson Immuno-research laboratories INC. Mouse TNF-a, IL-6, IL-1B ELISA Kit were purchased from BD Biosciences (San Diego, CA, USA).

2. Isolation and production of biologically active antimicrobial peptide from *Bacillus* sp. CS32

a. *In vitro* screening and isolation

Traditional 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, 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

In vitro, antimicrobial activity was primarily determined by the paper disk method using paper disk (8mm, Toyo) against *Micrococcus luteus* ATCC 9341 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 18h 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 minerals.

The influence of various carbon sources on antimicrobial 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 250ml Erlenmeyer flasks containing 50ml media with constant shaking at 180rpm. Afterwards, the influence of nitrogen source on peptide production was determined using medium containing 1% glucose (best carbon source) combined with 1% supplements such as beef extract, malt extract, tryptone, yeast extract, oat meal, soytone, peptone. Furthermore, Percent of carbon and nitrogen sources on peptide production was evaluated in media containing the best carbon source (1, 2% glucose), the best nitrogen sources (0.5, 1% beef extract and peptone).

e. Purification of *Bacillus* sp. CS32 producing antimicrobial peptide

Bacillus sp. CS32 was cultured for 60h in glucose-beef extract-peptone medium (1% glucose, 0.5% beef extract and 0.5% peptone). Cells were removed by centrifugation (6000rpm, 30min, 4℃) and peptides in the supernatant were precipitated at 4℃ overnight with ammonium sulfate of 60% saturation. The precipitation was collected by centrifugation (6,000rpm, 60min, 4℃), resuspended in 10mM Tris-HCl buffer (pH 8.0) and dialyzed using 1kDa cut-off membrane (Millipore). The crude extracts were applied to Sepharose CL-6B column (2.2x116cm) and Sephadex G50 column (1.5x70cm), eluted 10mM Tris-HCl (pH 8.0). Active fractions were pooled, concentrated using 1 YM amicon filter and stored at -20℃. To assess antimicrobial activity conveniently, the disk diffusion method was employed with the indicator strain *Micrococcus luteus* ATCC 9341.

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). For in situ detection of inhibitory activity, the gel was washed with 50mM 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. CS32 producing antimicrobial peptide

To analyze thermo stability, AMP was evaluated by measuring the residual activities at temperatures (30-121℃, 10-60min). After treatment, the samples were tested for residual antimicrobial activity against indicator strain *Micrococcus luteus* ATCC 9341. Effects of pH on antimicrobial activity were estimated by varying pH levels. Samples were diluted in the following buffers: Citrate and Na_2HPO_4 (pH 3.0-7.0), Tris (pH 7.0-9.0), and $NaHCO_3$ and NaOH (pH 10.0-11.0). After incubation for 1 h at room temperature, samples were adjusted to pH 7.0 and tested for antimicrobial activity against indicator strain. Antimicrobial peptide was treated with different types of proteolytic enzymes at a final concentration of 1mg/ml; lipase (50mM Tris-HCl, pH7.5), protease K (50mM Tris-HCl, pH7.5), α-chymotrypsin (50mM Tris-HCl, pH7.5), Trypsin (50mM Tris-HCl, pH8.0). After 1h incubation at room temperature, the enzymes were boiled for 2 min to inactivate the enzyme. Chemicals (working concentrations in Table 15) were added to the antimicrobial peptide and the samples were incubated for 1h at room temperature before being tested for residual antimicrobial activity. After the treatment, the samples were tested for antimicrobial activity against test organism.

h. Analysis of N-terminal amino acid sequence

For N-terminal amino acid sequencing, the purified peptide was confirmed single band and lyophilized the peptide. Sequencing was carried out in the procise 492 amino sequencer (Applied Biosystems, Foster City, CA) according to the automated Edman degradation method. The molecular mass of the peptide was evaluated by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-MASS).

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), 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 12h.

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. A 100 μl peptide solution was added to 100 μl of DPPH (0.3mM) in methanol solution in 96-well-plate. The plate was incubated for 30min at room temperature and the absorbance was determined at 517nm using a microplate. 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 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. Samples were dissolved in distilled

water at different concentrations. An aliquot (200μl) of sample solution was mixed with 200μl of 0.2M sodium phosphate buffer and 200μl of 1% ferricyanide aqueous solution. The mixture was incubated at 50℃ for 20min, followed by addition of 200μl of 10% trichloroacetic acid solution. Then, the mixture was centrifuged at 12,000 rpm for 10min. Finally, 500μl supernatant was mixed with 500μl 0.1% ferric chloride aqueous, and absorbance was measured at 700nm. 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.

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 24h 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 24h incubation with or without peptide (10-100μg/ml), a 0.5mg/ml of MTT solution was added to each well in a 1/10 volume of media. Cells were incubated at 37℃ for 3h, and dimethylsulfoxide (DMSO) was added to dissolved the formazan crystals. The absorbance was measured using an assay reader at 590nm. Relative cell viability was calculated compared to the untreated control group.

(3) Nitric oxide analysis and quantification of cytokine production

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 CS32 AMP for 30min prior to stimulation with 1 μg/ml of LPS for 24h. 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 10min. 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.

(4) Enzyme immune assay of TNF-α, IL-1β and IL-6

The inhibitory effects of CS32 AMP on the production of pro-inflammatory cytokines; TNF-α, IL-1β and IL-6 were determined by an enzyme-linked immune sorbent assay (ELISA). Cells were treated with different concentration of CS32 AMP (10-100μg/ml) for 1h followed by LPS (1μg/ml) treatment for 24h. Amount of nitrite of the supernatant was calculated from a sodium nitrite standard curve. Supernatant was analyzed for TNF-α, IL-1β and IL-6 by enzyme linked immunosorbent assay (ELISA) using commercial kits (BD Biosciences) according to the manufacturer's instructions

(5) Western blotting

For detection of iNOS, COX-2 expression, Raw 264.7 cells were washed with cold PBS (phosphate-buffered saline). Cells were lysed in lysis buffer (mixture of RIPA buffer and protease inhibitor) and kept on ice for 30min. Cell lysates were centrifuged at 12,000rpm at 4℃ for 30min. Protein concentration of sample was determined by the Bradford method. Samples were stored at -80°C or immediately used for Western blot analysis. Aliquots of the lysates were separated on SDS polyacrylamide gels and transferred PVDF membranes. The membranes were blocked with 5% non-fat dried milk in Tris buffered saline- Tween (TBST, 10mM Tris-HCl, 150mM NaCl, 0.05% Tween 20, pH 7.6) at room temperature for 2h. After being washed, the membranes

were incubated with specific primary antibody solution (anti-iNOS, anti- COX-2 or β-actin antibodies) for 12h at 4℃.

Each membrane was washed with TBST and incubated with HRP (for iNOS, COX-2 antibody) or alkaline phosphatase (for anti-β-actin antibodies) conjugated secondary antibody solution for 1h at room temperature. For detection of iNOS, COX-2 expression, the blots were washed with TBST and incubated with ECL reagent for 2min and viewed by chemilumunescence. Equal protein loading was assessed by the expression level of actin protein. For β-actin the blots were washed with TBST and incubated with AP color development buffer containing color reagent (AP conjugate substrate kit, Bio-rad laboratories Inc.).

(6) RNA preparation and mRNA expression analysis by RT-PCR

Total RNA from the peptide treated cells was prepared using RNAiso Reagent (Takara) according to the manufacturer's protocol, and was stored at -80°C until use. For the detection of iNOS, COX-2, TNF-α, IL-Iβ, IL-6, total RNA was extracted after stimulation and treatment. The mRNA expression levels of iNOS, COX-2, TNF-α, IL-Iβ, IL-6 in the treated cells were compared to the expression levels in control cells.

One μg of the RNA was reverse-transcribed into cDNA and used as a template for RT-PCR amplication. The primers and the amplification conditions are listed in Table 1.

PCR was performed with a DNA gene cycler, and the amplification was followed by

denaturation at 94℃ for 30s, annealation at 58℃ for 30s, and primer extention at 72℃ for 40s. PCR products were analyzed on 1% agarose gels, and bands were visualized with ethidium bromide staining.

B. Results

1. Production, isolation and purification of antimicrobial peptide from *Bacillus* sp. CS32

a. Identification of the *Bacillus* strain

For molecular phylogeny, the 16S rRNA sequence of the local isolate was compared to sequence of 15 *Bacillus* species. In order to determine the relation of the local isolate to these *Bacillus* strains. Multiple sequence alignment was done between the sequence of the 16s rRNA gene of *B. licheniformis, B. sonorensis, B.aerius* and local isolate. Computer assisted RNA searches against bacterial database similarly revealed that the 16S rRNA sequence was 99.392% identical with both *Bacillus licheniformis* CS32 (Table 9, Figure 15).

Table 9. Similarity of 16S rRNA gene sequence (CS32)

Rank	Name/Title	Strain	Accession	Similarity	Diff/ Total nt
1	***Bacillus licheniformis***	ATCC 14580(T)	CP000002	99.392	9/1480
2	***Bacillus sonorensis***	NRRL B-23154(T)	AF302118	99.362	9/1410
3	***Bacillus aerius***	24K(T)	AJ831843	99.323	10/1478
4	***Bacillus subtilis subsp. spizizenii***	NRRL B-23049(T)	AF074970	98.652	19/1409
5	***Bacillus mojavensis***	IFO 15718(T)	AB021191	98.648	20/1479
6	***Brevibacterium halotolerans***	LMG 21660(T)	AJ620368	98.642	20/1473
7	***Bacillus atrophaeus***	JCM 9070(T)	AB021181	98.642	20/1473
8	***Bacillus subtilis subsp. subtilis***	NCIB 3610(T)	ABQL0100 0001	98.58	21/1479
9	***Bacillus vallismortis***	DSM 11031(T)	AB021198	98.513	22/1479
10	***Bacillus amyloliquefaciens subsp. amyloliquefaciens***	DSM 7(T)	FN597644	98.377	24/1479
11	***Bacillus amyloliquefaciens subsp. plantarum***	FZB42(T)	CP000560	98.242	26/1479
12	***Bacillus siamensis***	PD-A10(T)	GQ281299	98.174	27/1479
13	***Bacillus methylotrophicus***	CBMB205(T)	EU194897	98.125	27/1440
14	***Bacillus altitudinis***	41KF2b(T)	AJ831842	96.685	49/1478
15	***Bacillus stratosphericus***	41KF2a(T)	AJ831841	96.685	49/1478

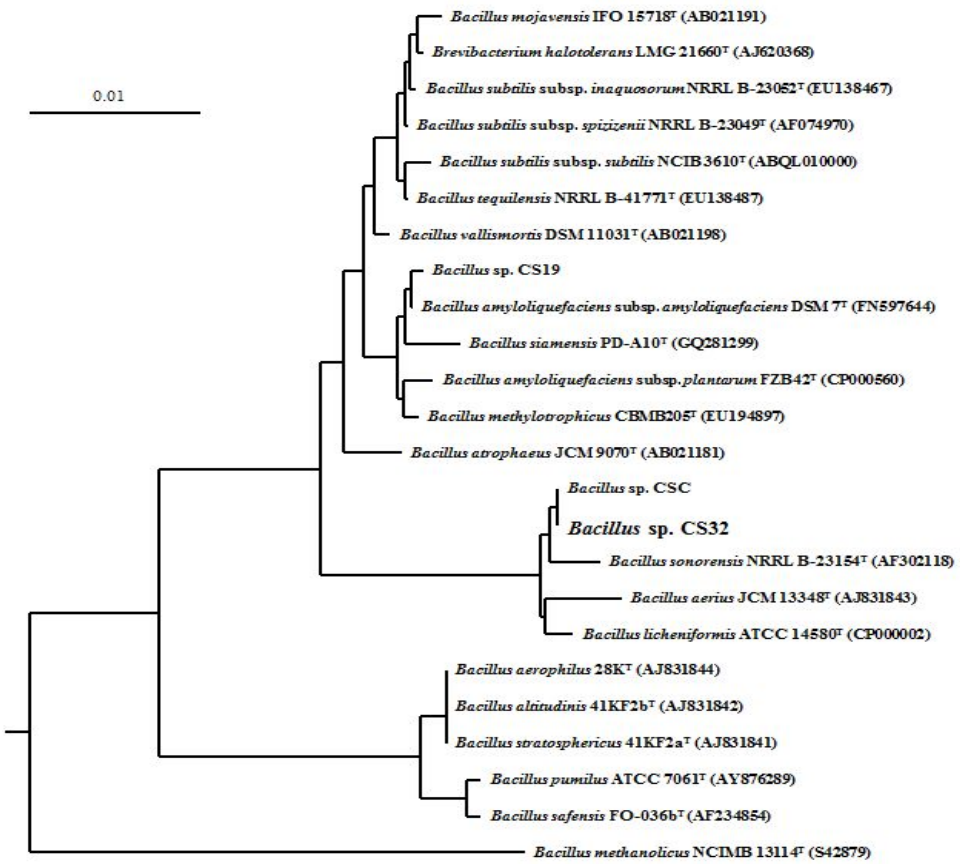


Figure 15. Phylogenic tree of the *Bacillus* species

Neighbor-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between strain CS32 and related members of the genus *Bacilllus* sp. The percentage numbers at the nodes are the levels of bootstrap support based on neighbor-joining analyses of 1000 resampled data sets. The sequence of *Bacillus lucheniformis* ATCC 14580(T) was used as an outgroup. Bar : 0.01 nucleotide substitution per position.

b. Optimization of fermentation process

Antimicrobial peptide production of the CS32 strain was optimized by using several cultural parameters such as carbon, nitrogen sources. The effect of carbon, nitrogen sources was studied. Among the tested carbon and nitrogen sources, glucose, peptone and beef extract, respectively, caused the maximum production of antimicrobial peptide. Finally, production of peptide was carried out GPB (1% glucose, 0.5% peptone and 0.5% beef extract) medium where significant production was achieved from 12h and reached a maximum at 60h (Figure 16,17,18).

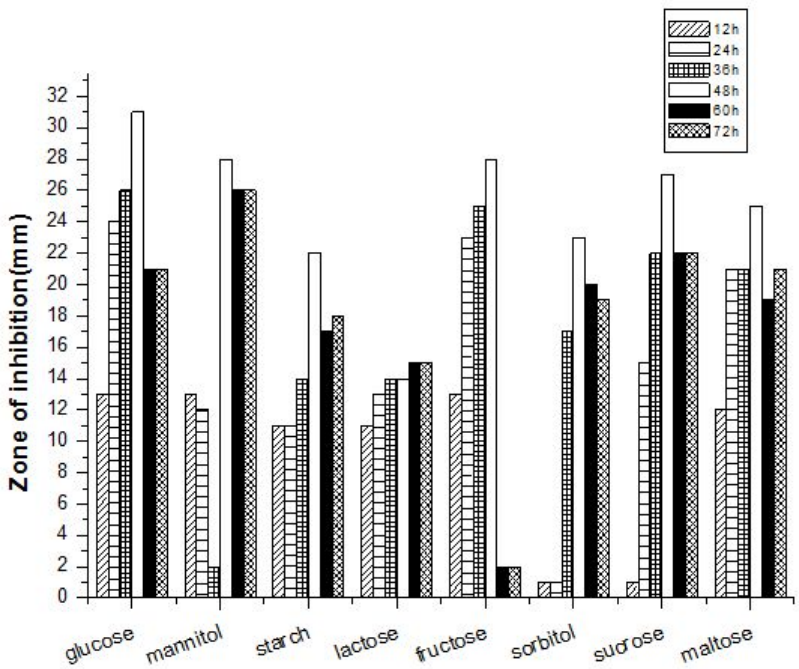


Figure 16. Effect of various carbon sources on the activity of antimicrobial peptide produced by *Bacillus* sp. CS32

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

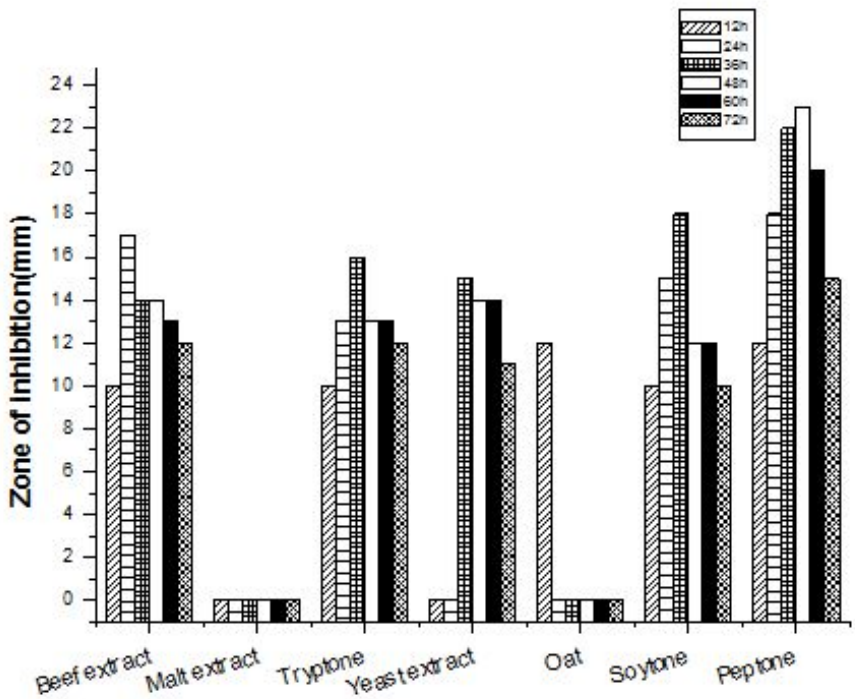


Figure 17. Effect of various nitrogen sources on the activity of antimicrobial peptide produced by *Bacillus* sp. CS32

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

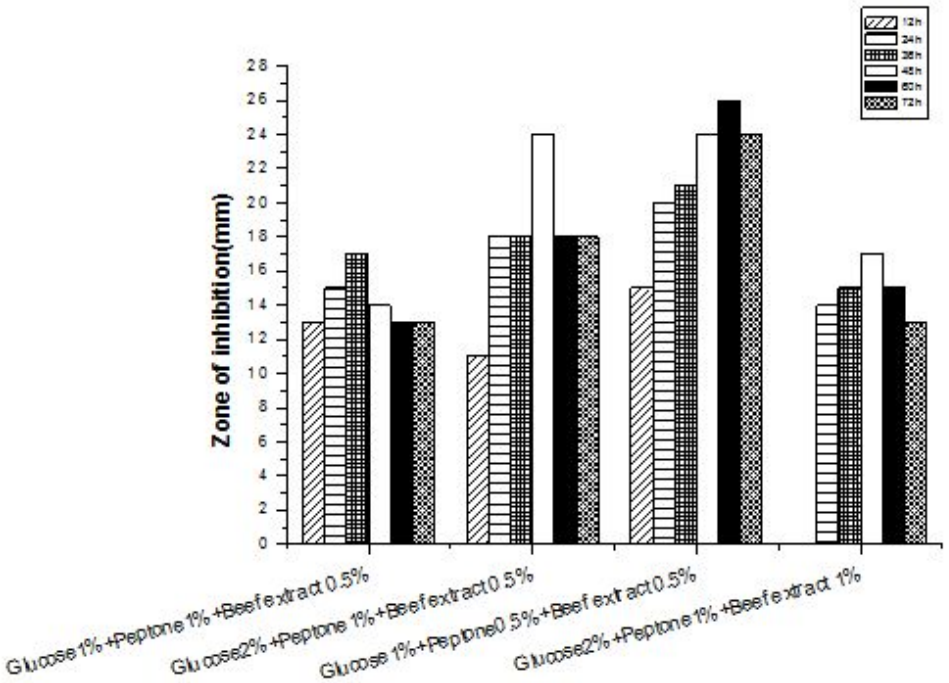


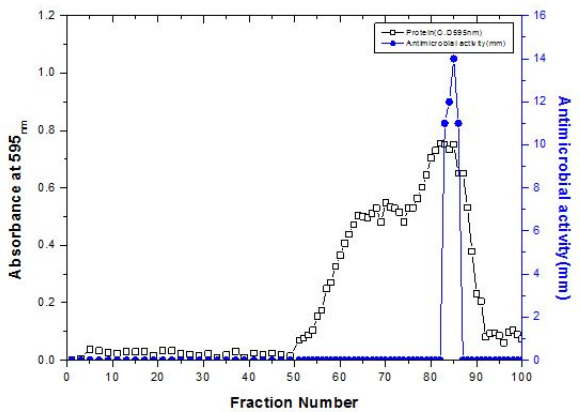
Figure 18. Effect of carbon and nitrogen sources on the activity of antimicrobial peptide produced by *Bacillus* sp. CS32

Production medium was supplemented with carbon, nitrogen sources such as glucose 1%+peptone 1%+beef extract 0.5%, glucose 2%+peptone 1%+beef extract 0.5%, glucose 1%+peptone 0.5%+beef extract 0.5%, glucose 2%+peptone 1%+beef extract 1%.

c. Fermentation and purification of antimicrobial peptide

Bacillus sp. CS32 was grown on rotatory shaker at 180rpm in GPB medium for 60h at 37℃. Culture broth was centrifuged at 6,000rpm for 30min. Antimicrobial 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 19). Tricine SDS-PAGE and in situ detection inhibitory activity confirm the homogeneity and activity of the purified CS32 AMP. The peptide migrated as a single and the molecular weight was estimated to be 5,697.9Da (Figure 20). The band was confirmed to be a peptide by in situ inhibitory activity of the gel.

(a)



(b)

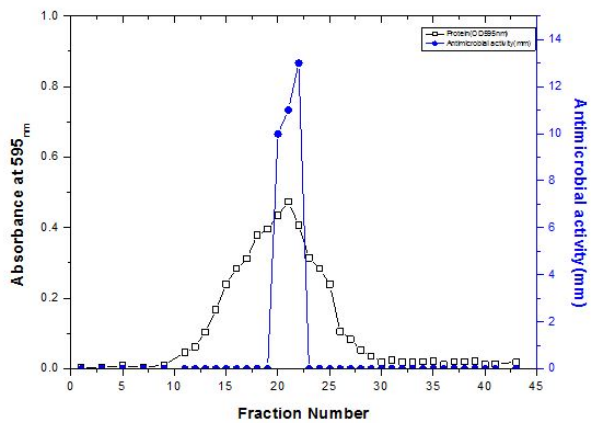
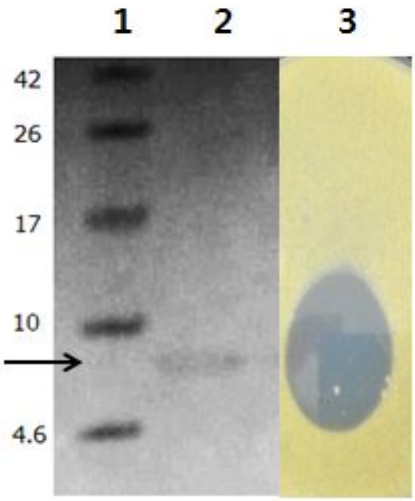


Figure 19. Elution profile of *Bacillus* sp. CS32 peptide

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

(a)



(b)

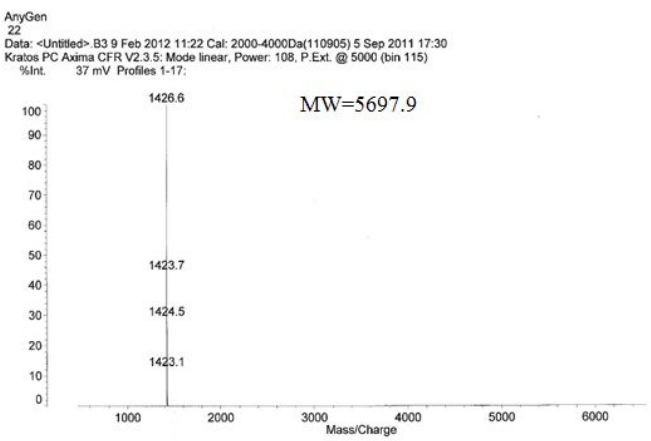


Figure 20. Determination of the molecular weight

Tricine SDS-PAGE and activity staining of CS32 peptide. (a) Tricine SDS-PAGE : Lane 1, protein size marker with the corresponding value in kDa on the left; Lane 2, purified CS32 peptide; Lane 3, activity staining (b) MALDI-TOF-MASS.

d. N-terminal sequence analysis

After Tricine SDS–PAGE and the N-terminal amino acid sequence of the purified antimicrobial peptide was analyzed via the automated Edman method. First twelve amino acid residues of the N-terminal were determined to be APLEIXXIFHDN. The sequences were compared with related antimicrobial peptides and presented in Table 10. In NCBI Blast search, the sequences of CS32 AMP exhibited highest homology with bacteriocin-like compound (Lichenin) from *Bacillus licheniformis* (accession no. P8290701) (P.Pattnaik *et al*. 2001). Besides, the sequences also showed some identity with AMPs from *Bacillus subtilis* JM4 (Shimei Wu *et al*. 2005) with shared sequences XXXEIXXIFHDN.

Table 10. Analysis of N terminal amino acid sequence

Antimicrobial peptides	N-terminal amino acid sequence	Identity (%)
***Bacillus* sp. CS32**	**A P L E I X X I F H D N**	100
***B. licheniformis* 26L-10/3RA**	I S **L E I** C **X I F H D N**	75
***B. subtilis* Subpeptin JM4-A**	X X K **E I** X W **I F H D N**	58.33
***B.subtilis* Subpeptin JM4-B**	X X K **E I** X H **I F H D N**	58.33

2. Biological activities of antimicrobial peptide

a. Antimicrobial activity of peptide

(1) Antimicrobial spectrum of antimicrobial peptide

As shown in Table 11, CS32 AMP showed antimicrobial activity against Gram-positive but not Gram-negative bacteria. Importantly, CS32 AMP displayed an antagonistic effect against multidrug-resistant (MDR) pathogens such as *Micrococcus luteus*, MRSA, VRSA and VRE, which was similar or stronger than that shown by bacitracin and vancomycin.

Table 11. Minimum inhibitory concentration of *Bacillus* sp. CS32 peptide

Test organisms	MIC		
	CS32	Bacitracin	Vancomycin
Alcaligenes faecalis **ATCC 1004**	>80	>80	>80
Salmonella typhimurium **KCTC 1925**	>80	>80	>80
Escherichia coli **KCTC 1923**	>80	>80	>80
Pseudomonas aeruginosa **KCTC 1637**	>80	>80	>80
Micrococcus luteus **ATCC 9341**	0.156	>80	0.156
Mycobacterium smegmatis **ATCC 9341 9341**	>80	>80	0.3125
Enterococcus faecalis **ATCC 29212**	5	5	2.5
Bacillus subtilis **ATCC 6633**	>80	40	>80
Listeria monocytogenes **KCTC3569**	10	0.3125	0.156
Staphylococcus aureus **KCTC 1928**	5	40	0.3125
MRSA 639E	2.5	40	0.3125
MRSA 4-5	5	1.25	0.3125
MRSA 5-3	2.5	1.25	0.3125
MRSA(methicillin resistance *Staphylococcus*) U4	40	1.25	0.3125
MRSA S3	10	1.25	0.3125
MRSA P3	40	1.25	0.625
MSRA S1	40	1.25	0.625
VRSA	40	>80	>80
VRE(Vancomycine resistance *Enterococcus*) 2	10	0.3125	>80
VRE3	.>80	20	>80
VRE4	10	5	>80
VRE5	10	1.25	>80
VRE6	>80	>80	>80
VRE82	80	40	>80
VRE89	>80	40	>80

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

The effects of temperature and pH on antimicrobial activity and stability are shown in Table 12 and Table 13. *Bacillus* sp. CS32 AMP completely stables at pH 5.0-12.0 and up to 100℃. Its activity decreased sharply at or above 121℃. Stability of peptide at various pH and temperature was comparable with Lichenin and AMPs produced by *Bacillus subtilis* strain JM4.

To test the effect of proteolytic enzymes, CS32 AMP was test of residual activity to lipase, proteinas K, α-chymotyrpsin and trypsin was measured by agar diffusion assay against *Micrococcus luteus*. The peptide was not sensible to proteolytic enzymes (Table 14).

The effect of several chemicals on the antimicrobial activity was evaluated. The antimicrobial activity slightly decreases its activity after treatment with Triton X-100. Antimicrobial activity was not affected by treatment with organic solvents and EDTA (Table 15).

Table 12. Thermo stability of CS32 antimicrobial peptide on different conditions

Temperature	Time	Residual activity(%)
None		100
	10 min	100
30℃	30min	100
	60min	100
	10min	100
50℃	30min	98
	60min	98
	10 min	98
70℃	30min	98
	60 min	98
	10 min	98
90℃	30 min	95.9
	60 min	95.9
121℃	105kPa/15 min	65.3

Table 13. Effect of various pH on the CS32 antimicrobial peptide activity

pH	Residual activity (%)
None	100
pH 2	61.5
pH 3	63
pH 4	89.8
pH 5	95.9
pH 6	100
pH 7	100
pH 8	100
pH 9	100
pH 10	98
pH 11	91.8
pH 12	91.8

Table 14. Effect of various proteolytic enzymes

Proteolytic enzymes	Residual activity (%)
None	100
Lipase	98.3
Proteinase K	95.7
α-chymotrypsin	94.3
trypsin	100

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

Table 15. Effect of various chemicals on CS32 antimicrobial peptide activity

Treatment	Concentration	Residual activity (%)
None		100
Acetone	10% (v/v)	103.6
Chloroform	10% (v/v)	103.8
Ethanol	10% (v/v)	105.9
Methanol	10% (v/v)	101.56
Ethyl acetate	10% (v/v)	101.8
EDTA	10mM/ml	101.8
Trichloroacetic acid	100mg/ml	101.3
Triton X-100	1% (v/v)	91.6
Tween 20	10% (v/v)	109.6
Tween 80	10% (v/v)	108.3

* Antimicrobial peptide was pre-treated for 1h 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 8,000rpm for 5min and the supernatant was neutralized to pH7.0 before testing for antimicrobial activity.

b. Antioxidant activities

(1) DPPH radical scavenging activity

To obtain information about the mechanisms of the antioxidative effects of the peptide, we examined their radical scavenging effects by measuring changes in absorbance of DPPH radical at 517nm. Both CS32 AMP and ascorbic acid showed a concentration dependent scavenging of DPPH radicals. CS32 AMP was found to be low active radical scavenger to ascorbic acid.

Figure 21, show the results of the free radical (DPPH) scavenging activity in % inhibition. 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 1 to 1000 μg/ml. The results revealed that the CS32 AMP exhibited the radical scavenging activity with (56.9±0.79) at the concentration of 1000μg/ml. In comparison to ascorbic acid the known antioxidant which shows (82.5±1.59) at the same concentration. 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.

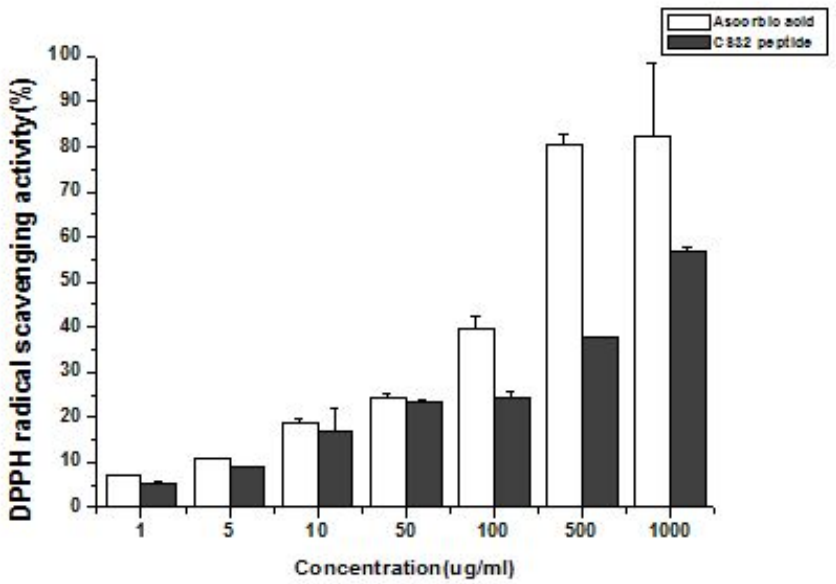


Figure 21. DPPH radical scavenging activity of CS32 antimicrobial peptide in different concentration.

(2) Reducing power

Reducing power assay method is based on the principle that peptide, 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 700nm. The reducing power assay is often used to evaluate the ability of an antioxidant to donate electrons, through which the free radicals can be stabilized. The reducing power of CS32 AMP was not affect concentration dependent. The reducing power of ascorbic acid was much more effective than CS32 AMP (Figure 22).

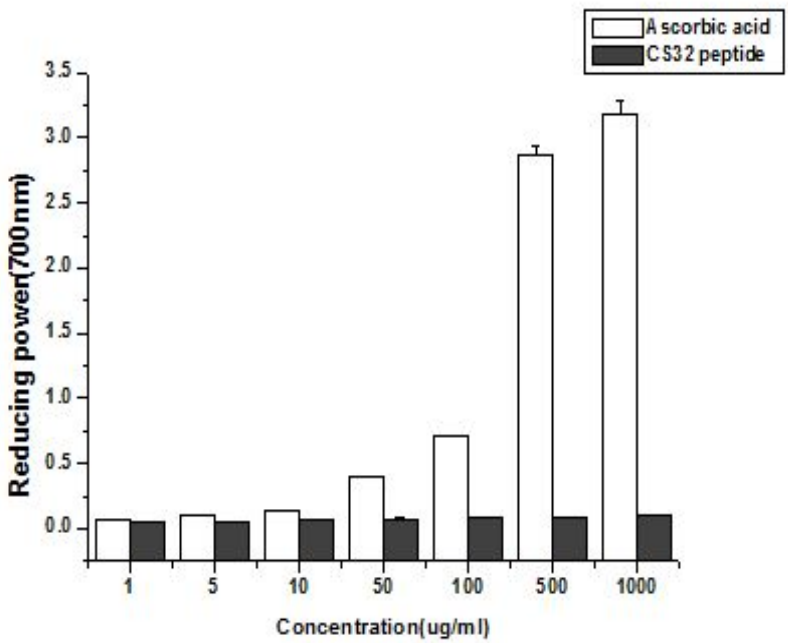


Figure 22. Reducing power of CS32 antimicrobial peptide

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

c. Anti-inflammatory activity of antimicrobial peptide

(1) Cytotoxicity

Raw 264.7 cells were treated with various concentrations of CS32 AMP for 24h and cell viability was tested by MTT assay as described above. As shown in Figure 23, peptide did not exhibit cytotoxicity at the range of 5-100μg/ml against Raw 264.7 cells. Therefore peptide was used at 100μg/ml and below for further experiments.

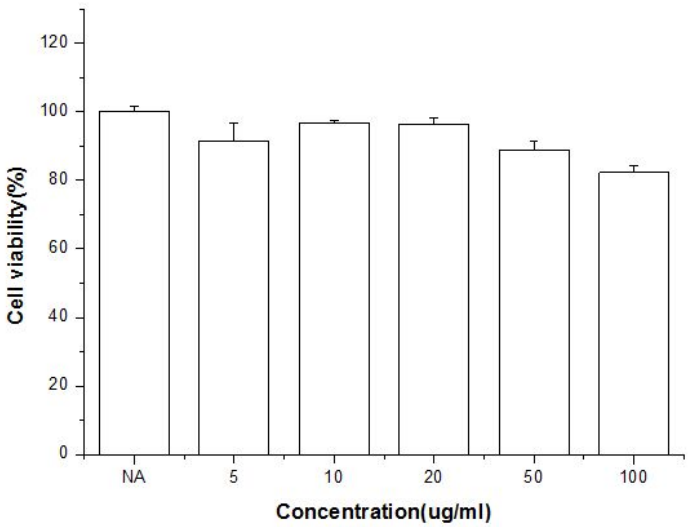


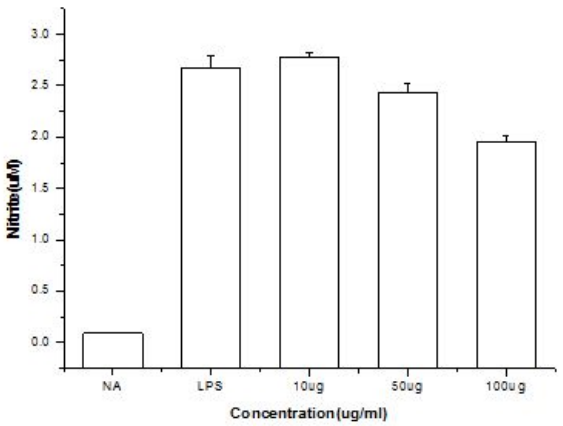
Figure 23. Effect of the CS32 antimicrobial peptide on cell viability

Cell viability was measured after 24h 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 5-100μg/ml peptide for 24h. Each bar shows the mean ± S.D of three independent experiments performed in triplicate.

(2) Inhibition of nitric oxide production and iNOS, COX-2 protein expression in LPS-induced Raw 264.7 macrophage cells

Macrophages produce No following stimulation with LPS. To assess the effect of CS32 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 24, LPS treatment significantly increased NO production compared to the untreated cells. Treatment of cells with CS32 AMP at 10, 50, 100μg/ml suppressed the LPS-stimulated production of NO to a statistically significant extent.

In an attempt to assess whether the inhibition of NO production by CS32 AMP was related to the down regulation of iNOS, COX-2, we next examined the protein expression levels by western blotting. As shown in Figure 24, both iNOS, COX-2 protein levels increased in LPS-stimulated cells when compared to the controls. However, this induction was significantly inhibited in a dose-dependent manner by treatment with CS32 AMP (Figure 24).



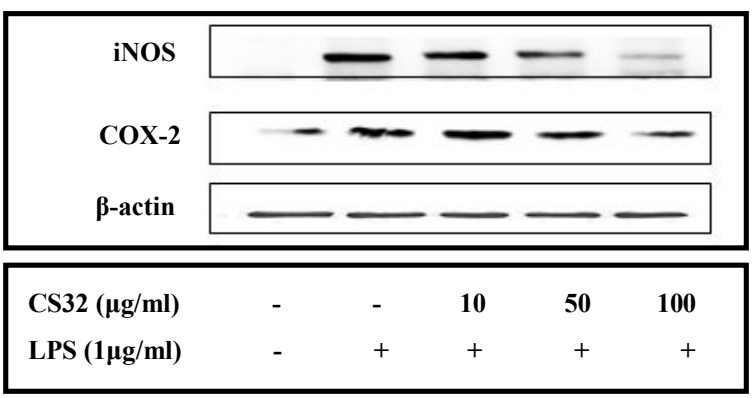


Figure 24. Effect of CS32 antimicrobial peptide of NO production and iNOS, COX-2 protein expression in LPS-induced Raw 264.7 macrophage cells. Cells were incubated with the various concentration of peptide for 30 min, followed by treatment with 1μg/ml of LPS and incubated for 24h. The amounts of NO were determined using the Griess reagent in the culture medium (A). Equal amounts of cell lysate were resolved on SDS polyacrylamide gels, transferred to PVDF membranes, and probed with antibodies against iNOS and COX-2. β-actin was used as internal controls for western blot analysis (B).

(3) Inhibitory effects of CS32 antimicrobial peptide on the production of pro-inflammatory cytokines in LPS-induced Raw 264.7 macrophage cells

LPS-induced macrophages promote inflammation by secreting pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6. To determine if peptide affects cytokine production by LPS-induced Raw 264.7 cells, we measured TNF-α, IL-1β and IL-6 concentrations in culture supernatants by ELISA assay. LPS treatment significantly increased TNF-α, IL-1β and IL-6 productions compared to the untreated cells. As shown in Figure 25, LPS-induced cytokines release were significantly blocked by CS32 AMP in a dose-dependent manner. These results demonstrated that CS32 AMP blocked LPS-induced pro-inflammatory mediators such as TNF-α, IL-1β and IL-6 in macrophage, which might be responsible for its anti-inflammatory application.

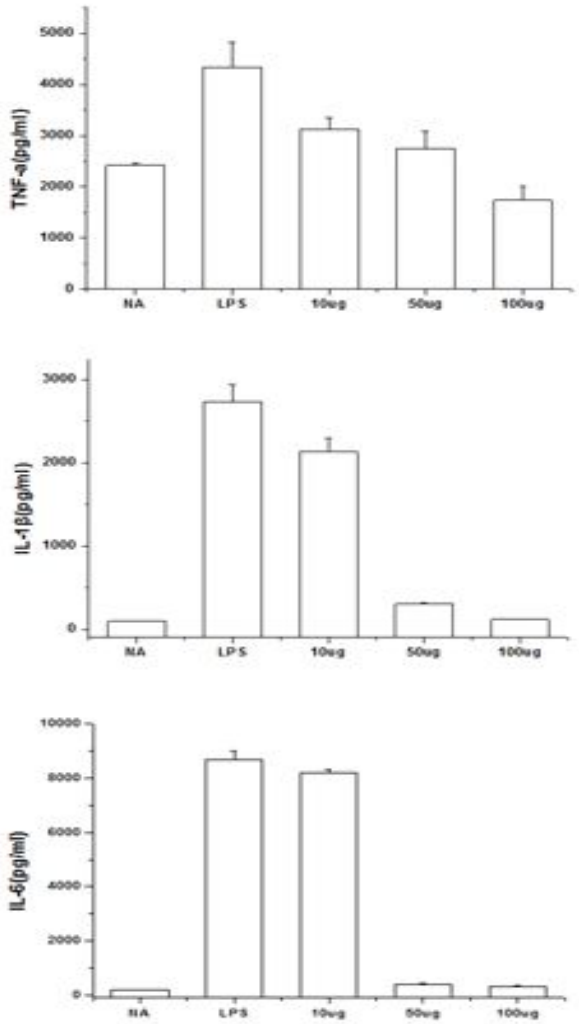


Figure 25. Effect of CS32 antimicrobial peptide in the suppression of TNF-α, IL-1β, IL-6 production in LPS-induced Raw 264.7 macrophage cells

Cells were incubated with the indicated concentrations of CS32 peptide for 30min before treatment with LPS (1μg/ml) for 24h. After incubation for 24h, the supernatant was collected, and the amount of proinflammatory cytokines were measured by ELISA assay.

(4) Inhibition of iNOS, COX-2, TNF-α, IL-1β, IL-6 mRNA gene expression in LPS-induced Raw 264.7 macrophage cells

To determine if the above effect on NO production was related to the changes in the level of iNOS and COX-2, the expression of iNOS and COX-2 mRNA was measured with RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize gene expression. The cells treated with LPS along with the peptide significantly inhibited iNOS, COX-2 mRNA expression in a dose-dependent manner. We also examined the expression of several pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 mRNA. LPS treatment increased TNF-α, IL-1β, IL-6 mRNA levels and treatment of cells with CS32 AMP dose-dependently inhibited the LPS-increased expression (Figure 26).

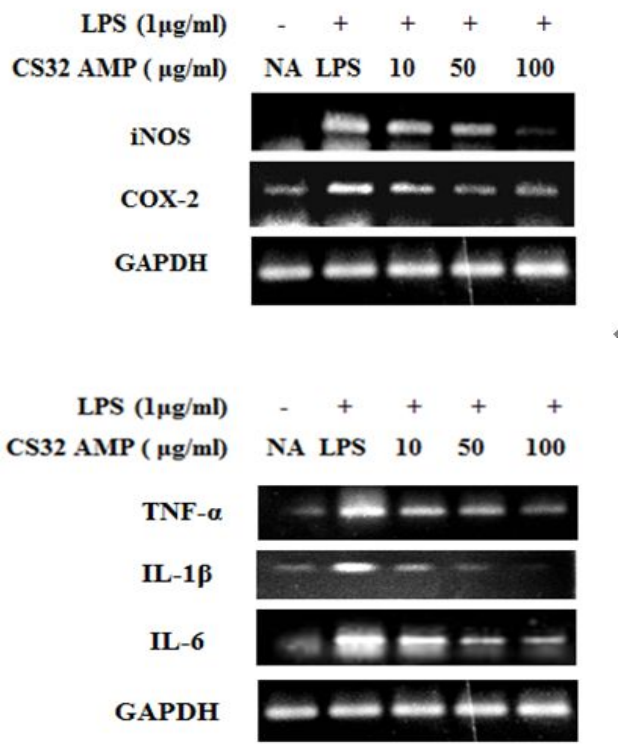


Figure 26. Effect of CS32 antimicrobial peptide on LPS-induced iNOS, COX-2, TNF-α, IL-1β, IL-6 mRNA gene expression

After LPS treatment 2-12h, the levels of iNOS, COX-2, TNF-α, IL-1β, IL-6 mRNA were determined by RT-PCR. GAPDH was used as internal control for RT-PCR assays.

III. Discussion

Microbial strains capable of producing AMPs were recently isolated from Korean traditional foods. Strain CS61 and CS32 were selected among the numerous screened strains since it showed highest antimicrobial activity. 16S rRNA gene sequences of the strains were analyzed. These strains were identified as *Bacillus* sp. CS61 [(*Bacillus subtilis* (99.596%)] and *Bacillus* sp. CS32 [(*Bacillus licheniformis* (99.392%)]. This study was aimed to isolate and identify antimicrobial, antioxidant and anti-inflammation properties of peptide from *Bacillus* sp. strains. Maltose and beef extract were found the most suitable nutrient sources for the maximum production of CS61 antimicrobial peptide (Figure 4, 5, 6). While, the most suitable nutrient sources of CS32 were 1% glucose, 0.5% peptone and 0.5% beef extract (Figure 16, 17, 18).

The *Bacillus* isolate, CS61 and CS32 exhibited a wide spectrum antimicrobial activity. Two antimicrobial peptides (AMPs) individually produced in the optimal medium by *Bacillus* sp. CS61 and CS32 were purified to homogeneity by gel permeation chromatography using Sepharose CL-6B and Sephadex G-50 column chromatography.

Effect of peptides CS61 and CS32 purified form *Bacillus* sp. were evaluated against various pathogenic gram-positive and gram-negative bacteria. The production of

AMPs 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 AMPs produced by *Bacillus* sp. CS61 and CS32 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* sp. JM4 (Wu S *et al*. 2005) and *Bacillus licheniformis* A89 (Mendo S *et al*. 2004). 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 5 and 11. Two AMPs displayed activities against gram-positive bacteria. It is remarkable that the effect shown by CS61 and CS32 AMPs against MRSA, VRSA and VRE was comparable with bacitracin and vancomycin. Minimal inhibitory concentrations of CS61 and CS32 AMPs for MRSA, VRSA and VRE were in the range 0.625–20μg/ml and 0.156–80μg/ml. In contrast, it did not show antimicrobial activity against other gram negative bacteria.

The antimicrobial peptide of CS61 and CS32 was obtained by a two steps purification procedure. CS61 sequences were consisted of 9 amino acids. The N-terminal amino acid sequence was A-I-N-X-D-A-A-Y-L. The N-terminal amino acid sequence of CS61 showed no significant homology with reported AMPs. Previous

works showed that some antimicrobial peptides produced by *Bacillus* genus often contained unusual residues, such as amino acids of formylated, acylated, and covalent linked to another function group. It is well known that such unusual residues interfere with peptide sequencing. Cysteine replacement in the unidentified fourth amino acid of CS61 showed antimicrobial activity against MRSA 693E (Table 4). CS61 with unique N-terminal amino acid sequences and activity against MDR bacteria could be a novel AMP with potential therapeutic application. Molecular mass of CS32 AMP was estimated 5kDa by Tricine SDS-PAGE (Figure 20). First 12 amino acid residues of the CS32 AMP were APLEIXXIFHDN. The molecular size integrated with different N-terminal amino acid sequences makes CS32 AMP a novel type of antimicrobial peptide (Table 10).

The CS61 peptide showed stability between pH 2.0 and pH 10.0 and up to 80℃ (Table 6). Sensibility test of CS61 towards various proteolytic enzymes were tested and residual activity was measured by agar diffusion assay against MRSA 693E. The CS61 AMP was sensitive to trypsin, but was not sensible to other enzymes (Table 7). The effect of several chemicals on the antimicrobial activity was evaluated. The lost its activity after treatment with organic solvents and EDTA. However, AMP activity was not affected by treatment with detergents (Table 8).

The CS32 antimicrobial peptide showed thermal resistance up to 100℃ (Table 12). In addition, the antimicrobial peptide was inactive at acidic pH, showing

maximum activity at neutral pH values (Table 13). Purified CS32 AMP was not sensitive to all proteases tested, suggesting that a peptide moiety is associated with its activity (Table 14). Although high proteolytic enzyme concentrations were necessary to inactivate the antimicrobial activity, some antimicrobials produced by *Bacillus* spp. are cyclic peptides containing unusual amino acids, which are more resistant to proteolytic enzymes. The resistance to temperature and proteolytic enzymes resembled that of an antimicrobial peptide produced by *Bacillus licheniformis*.

The antioxidant activities of the AMPs were tested using free radical DPPH and reducing power. 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 517nm. Hence more rapidly the absorbance decreases implies more potent antioxidant activity of AMPs in terms of hydrogen atom donating capacity / electron transfer capability (Figure 9 and 21).

The reducing power of the AMPs was detected using potassium ferricyanide reduction method. It has been reported that the reducing power was associated with

the antioxidant activity. The yellow color of the test solution changes to various shades of green and blue depending upon the reducing power of each AMP. The presence of AMPs 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 AMPs show linear relationship. Therefore the increasing OD value indicates increasing trend of reducing power. The reducing power of AMPs was concentration dependent. There was slight increase on the reducing power with increase in concentration of peptide solution. However when compared with the standard (ascorbic acid), all AMPs showed lower reducing power activity (Figure 10 and 22).

This study revealed that a *Bacillus* sp. AMPs, CS61 and CS32, significantly inhibited the LPS-induced production of proinflammatory mediators NO, iNOS and COX-2 in Raw 264.7 cells. The inducible enzymes iNOS and COX-2 as well as the second messenger. NO have been shown to be the major elements in mediating inflammatory processes.

This study performed western blotting and RT-PCR to assess expression of iNOS and COX-2 in LPS-induced Raw 264.7 cells. Also we evaluated the production of NO in culture supernatants. First, we performed the MTT assay to assess the cytotoxicity of AMPs on the Raw 264.7 cells. Various concentrations of AMPs up to 100ug/ml had no significant effect on cell viability in untreated cells or cells stimulated with 1ug/ml

LPS. The levels of iNOS and COX-2 protein were measured quantitatively by immunoblotting and normalized to the protein expression of control β-actin. iNOS and COX-2 protein expression was detectable in Raw 264.7 cells. iNOS and COX-2 protein levels were significantly induced after LPS-treatment. This increase in iNOS and COX-2 protein expression in Raw 264.7 cells after LPS activation was significantly reduced by CS61 and CS32 AMPs treatment at concentrations of 10 to 100ug/ml (Figure 12, 24).

To further determine whether the inhibition of LPS stimulated NO production by AMPs were mediated by the regulation of iNOS and COX-2 mRNA expression, RT-PCR analyses were performed. As shown in Figure. 14 and 26, the expressions of iNOS and COX-2 mRNA were significantly elevated in macrophages treated with LPS (1μg/mL) compared to unstimulated cells (control). RT-PCR analyses indicated that AMPs reduced iNOS and COX-2 mRNA without affecting the mRNA of GAPDH, a housekeeping protein.

In general, these results indicate that the inhibitory effects of AMPs on LPS-induced NO productions are caused by iNOS and COX-2 suppression. Furthermore, the RT-PCR analysis indicates that mRNA levels of iNOS and COX-2 correlated with their protein levels. Therefore, the inhibitory effect of AMPs on iNOS and COX-2 gene expression appears to be one of the mechanisms responsible for the anti-inflammatory action of AMPs. In conclusion, CS61 and CS32 AMPs suppress the

expression of genes implicated in inflammation.

We evaluated proinflammatory cytokines production in LPS-stimulated cells, specifically TNF-α, IL-1β and IL-6. These cytokines are produced mainly by activated macrophages. Suppression of cytokine production is an important mechanism to counter the inflammatory process. Since AMPs were found to most potently inhibit the pro-inflammatory mediators, we further investigated its effects on LPS-induced cytokines release by enzyme immunoassay (ELISA) and RT-PCR. mRNA gene expression of pro-inflammatory cytokines were determined from the pre-culture stimulated with LPS (1μg/mL) in the presence of CS61 and CS32 AMPs. There was no basal change cytokine expression following incubation with only the AMPs without LPS. After 24h incubation with both LPS (1μg/mL) and CS61 and CS32 AMPs, there was remarkable inhibition of cytokine production (Figure 13, 25) and mRNA expression (Figure 14, 26) in Raw 264.7 cells. These results suggest that AMPs are potent inhibitor of LPS-induced iNOS and COX-2 gene and protein expression, cytokines production in the Raw 264.7 cells.

In conclusion, CS61 and CS32 AMPs with various activities were purified from *Bacillus* strain isolated form Korean traditional foods. The results of this study that that AMPs may be candidates for development as antimicrobial, antioxidative and anti-inflammatory 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(2003), 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(2003), 127-131.

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.

Bizani D, Brandelli A. 2002. Characterization of a bacteriocin produced by a newly isolated *Bacillus* sp. Strain 8A. J Appl Microbiol. 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. J. Appl. Microbiol. 104(3), 808-816.

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. J.Appl Microbiol. 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. Lett Appl Microbiol. 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.

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.

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(1994), 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.

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.

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(2004), 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.

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*. J. Microbiol. Biotechnol. 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(2008), 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 JY, Park SJ, Yun KJ, Cho YW, Park HJ, Lee KT. 2008. Isoliquiritigenin isolated from the roots of *Glycyrrhiza uralensis* inhibits LPS-induced iNOS and COX-2 expression *via* the attenuation of NF-kB in Raw 264.7 macrophages. European Journal of Pharmacology. 584(1), 175-184.

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

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. *Biotechnol Lett*. 26(2), 115-9.

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.

Moon GS, Kang CH, Pyun YR, Kim WJ. 2004. Isolation, identification of a Bacteriocin-producing *Enterococcus* sp. from *Kimchi* and its application to *Kimchi* fermentation. 14(5), 924-931.

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

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 Lett. 178(2), 337-341.

Oyaizu M. 1986. Studies on products of browning reactions: antioxidative activities of browning reaction prepared from glucosamine. Jpn. J. Nutr. 44, 307-315.

Paik SH, Chakicherla A, Hansen JN. 1998. Identification and characterization of Tochicin, a bacteriocin produced by *Bacillus thuringiensis* sub Tochigiensis. J Ind Microbiol Biotechnol. 19, 294-298.

Papagianni M. 2003. Ribosomally synthesized peptides with antimicrobial properties : biosynthesis, structure, function, and applications. Biotechnol Adv.

21(6), 465-499.

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

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.

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. J Endocrinol Reprod 10. 2, 88-95.

Riley MA, Wertz JE. 2002. Bacteriocins: Evolution, ecology, and application. Annu Rev. Microbiol. 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. Antimicrob Agents Chemother. 56(3), 1539-1547.

Sarkar D, Sha P, Gamre S, Bhattacharjee S, Hariharan C, Ganguly S, Sen R, Mandal G, Chattopadhyay S, Majumdar S, Chatterjee M. 2008. Anti-inflammatory effect of allylpyrocatechol in LPS-induced macrophages is mediated by suppression of iNOS and COX-2 via the NF-kB pathway. International Immunopharmacology. 8(9), 1264-1271.

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*. J.Appl. Microbiol. 104(4), 1067-1074.

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.

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.

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. Arch Pharm Res. 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. Pol J Microbiol. 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&Biochemistry. 34(2002), 955-963.

Yu HY, Kim KS, Lee YC, Moon HI, Lee JH. 2012. Oleifolioside A, a new active compound, attenuates LPS-stimulated iNOS and COX-2 expression through the downregulation of NF-kB and MAPK activities in Raw 264.7 macrophages. Evidence-based complementary and alternative medicine. 2012(2012), 1-8.

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(2005), 292-296.