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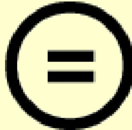

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2014 년 8 월
박사학위논문

Action mechanism of anti-inflammatory peptides from *Bacillus* strain

조 선 대 학 교 대 학 원

약 학 과

서 세 은

Action mechanism of anti-inflammatory peptides from *Bacillus* strain

Bacillus 속 세균이 생산하는

항염펩타이드의 작용기작 연구

2014 년 8 월 25 일

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Action mechanism of anti-inflammatory peptides from *Bacillus* strain

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국문초록

Bacillus 속 세균이 생산하는

항염펩타이드의 작용기작 연구

서 세 은

지도교수: 유 진 철

조선대학교 약학대학 약학과

한국 전통식품으로부터 다약제 내성균주에 항균 효과를 보이는 펩타이드 생산 미생물을 분리하였으며 이 균주를 각각 *Bacillus* sp. CS61과 *Bacillus* sp. CS32라 명명하였다. 이 미생물이 생산하는 펩타이드는 항균, 항산화, 항염 효과를 나타내었다.

Raw 264.7 대식세포는 LPS에 의해 일산화 탄소의 생성이 증가하는데 CS61과 CS32 균주가 생산하는 항균 펩타이드를 세포에 처리하였을 때 이의 생산을 억제하는 효과를 나타내었다. 또한 LPS에 의해 유도되는 염증

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단백질인 iNOS, COX-2 단백질의 발현을 농도 의존적으로 감소 시킴을 확인하였다.

이들 항균 펩타이드가 일산화 탄소의 생성을 농도 의존적으로 억제함을 확인하고, 이 펩타이드가 염증 관련 경로인 MAP kinase에 어떠한 영향을 미치는지 western blotting을 통하여 분석하였다. CS61 균주가 생산하는 펩타이드는 전염증성 사이토카인의 분비를 조절하는extracellular signal-regulated kinase (ERK1/2), c-Jun NH_2-terminal kinase (JNK), p38 mitogen-activated protein (MAP) kinase 를 포함한 mitogen-activated protein (MAP) kinase의 인산화를 억제하였다. 또한, NF-κB는 외부로부터 자극이 주어지면 핵 내로 이동이 활성화 되어 염증을 유발하게 되는데CS61 펩타이드 처리 시 LPS에 의해 유도되는 NF-κB의 핵 내로의 이동 및 IκBα의 분해를 억제하였다.

LPS에 의해 염증이 유도된 세포는 ERK, JNK, p38 MAPK의 인산화가 활성화 되는데 CS32 펩타이드는 이들의 인산화를 억제하였으며, NF-κB/IκBα 복합체의 안정화를 통해NF-κB의 활성과 IκBα의 분해를 억제하여 염증반응을 저해함을 하였다.

Raw 264.7 세포는 LPS에 의해 iNOS, COX-2 단백질이 mitogen-activated protein (MAP) kinase 경로인 ERK, JNK, p38의 인산화에 의해서 활성화 되는데 CS61과 CS32 균주가 생산하는 항균 펩타이드는 Raw 264.7 세포에서 LPS에 의해 유도되는 염증 반응을 효과적으로 억제하는 효과가 있음을 확

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인하였다.

CS32 균주가 생산하는 항균 펩타이드의 *in vivo*에서 항염증 효과가 있는지 확인하기 위하여 mouse ear edema 모델을 이용하였다. TPA로 마우스의 귀 부종을 유도하였으며 대조군으로 prednisolon을 사용하였다. TPA와 펩타이드를 함께 도포하여 항염증 효능을 확인한 결과 농도 의존적으로 부종이 억제 되었으며 귀조직의 iNOS 발현 또한 억제 됨을 확인하였다.

본 연구를 통하여 한국 전통 식품에서 분리한 바실러스가 생산하는 CS61, CS32 펩타이드는 항균 및 항염 효과를 나타내는 다 기능성 펩타이드임을 확인하였다.

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ABSTRACT

Action mechanism of anti-inflammatory

peptides from *Bacillus* strain

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To isolate effective antimicrobial peptides (AMPs) from a microbial source for the treatment of multidrug-resistant (MDR) bacteria, peptides were purified from *Bacillus* sp. CS61 and CS32 that were newly isolated from the traditional Korean fermented foods.

Bacillus sp. CS61 and CS32 AMPs have potent inhibitory effects on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in Raw 264.7 macrophage cells. Consistent with these findings, CS61 and CS32 AMPs suppress the expression of LPS-induced inducible nitric oxide synthase (iNOS) and cyclooxigenase-2 (COX-2) in a dose-dependent manner at the protein expression

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levels.

Dose-dependent effects of AMPs on NO production, especially, focusing on its effects on the MAP kinases were investigated. CS61 AMP inhibited the phosphorylation of mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK1/2), c-Jun NH_2-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38 MAPK), are involved in regulation of pro-inflammatory mediator secretion. Moreover, western blot analysis indicated that CS61 AMP inhibited completely LPS-triggered nuclear factor-κB (NF-κB) activation and IκBα degradation.

CS32 AMP did not reduced activation of JNK MAP kinase but strongly reduced activation of phosphorylated ERK kinase and p38 MAP kinase. In addition, CS32 AMP treatment stabilized NF-κB/ IκBα complex and resulted in inhibition NF-κB activation and IκBα degradation. CS61 and CS32 AMPs seem exert its anti-inflammatory and immune modulatory properties to the molecules such as NO and iNOS/COX-2 by suppressing the activation of ERK and p38 MAP kinase, and NF-κB/ IκBα signal transduction pathways

CS32 AMP was used to the TPA induced ear edema test, at the dose of 50 μg/ear evaluated the anti-inflammatory effect. CS32 AMP and prednisolone affect anti-inflammatory activity. CS32 AMP gives the highest contribution to the anti-inflammatory activity. Furthermore, I confirmed that the inhibition of CS32 AMP on iNOS expression in each ear by western blot analysis.

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Taken together, the results suggest that CS61 and CS32 AMPs are active anti-inflammatory constituents and that the anti-inflammatory property might be induced by the inhibition of iNOS and COX-2 expression from down-regulation of the ERK, JNK and p38 MAP kinase signal pathways and the inhibition of NF-κB activation.

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List of Abbreviations

AMPS	Antimicrobial peptides
COX-2	Cyclooxygenase-2
DMEM	Dulbecco's Modified Eagle's Medium
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JNK	c-Jun NH_2-terminal kinase
LPS	Lipopolysaccharide
MAP	Mitogen-activated protein kinase
MDR	Multidrug-resistant
MTT	3-(4,5-dimethylthiazol-2-2,5-diphenyltetrazolium bromide)
NF-κB	Nuclear factor of kappa B
NO	Nitric oxide
PVDF	Polyvinylidene difluoride
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TPA	12-O-tetradecanoylphorbol-13-acetate

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I. Introduction

Antimicrobial peptides (AMPs) are ubiquitously secreted by a wide range of microorganisms to protect themselves from various 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 and endospore-forming bacteria that spreads into the environment and other members of the *B.subtilis* group are considered as safe and have “generally recognized as safe” status. *B. subtilis* are usually found in foods such as dry cured sausages, cheeses, traditional fermented milks, sourdough, etc.

Furthermore, *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 center of attention for antimicrobial study (Wu et.al. 2005; Ahern et al. 2003; Oscariz et al. 1999; Cherif et al. 2001; Dischinger et al. 2009). Most of the peptides produced by *Bacillus* are antibacterial and a few are antifungal, antitumor, fibrinolysis-promoting, immunosuppressive, amylases, lipases and proteases.

These peptides are composed of 10-40 residues polypeptides and affect the active element of the innate immune response. Moreover, AMPs have been confirmed to kill gram-negative and gram-positive bacterias including important clinical pathogens,

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mycobacteria, protozoa, viruses, fungi, and cancer cell (Rana et al., 2006).

Inflammation is a 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 et al., 2012).

Macrophages are multi-potential cells that can be modulated to perform a variety of functions including production of pro-inflammatory cytokines and nitric oxide (NO), which are important mediators in host defense and inflammation (Denis et al., 1998). In these regards, lipopolysaccharide (LPS), a major component of the cell wall of gram-negative bacteria, is one of the most potent activator of the immune system and best characterized modulator of macrophage function (Cho *et al*., 2003). Stimulation of murine macrophages by LPS results in the expression of an inducible NO synthase (iNOS), which catalyzed the production of large amount of NO from L-arginine and molecular oxygen (Palmer et al., 1988).

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

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tumor cells. NO is a major product and its production is controlled by the nitric oxide synthases (iNOS), iNOS is highly expressed in macrophages; its activation leads to organ destruction in some inflammatory and autoimmune diseases. During inflammation, macrophages play a central role in managing many different immune-pathological phenomena, including the overproduction of pro-inflammatory cytokines and inflammatory mediators such as iNOS, COX-2 and TNF- α, IL-1ß, IL- 6 (Yoon et al., 2009). The induction of iNOS, COX-2 and cytokines are mainly triggered and regulated by a series of signaling pathways including NF-κB transcription factor and MAP kinases (Chakravortty et al., 2001) (Figure 1).

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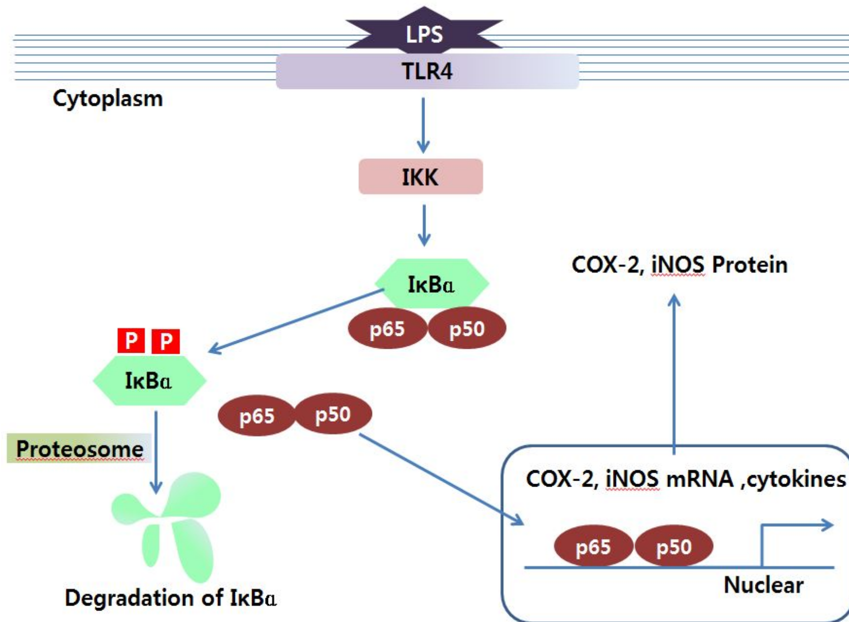

Figure 1. Pathway of iNOS, COX-2 inflammation action.

(Kang et al., 2011.)

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Nuclear factor-κB (NF-κB) is an important transcription factor complex that regulates the expression of many genes involved in immune and inflammatory responses (Ghosh et al,. 1998; Li and Verma 2002; Bonnizzi and Karin 2004). In unstimulated cells, NF-κB in not present in the nucleus but resides in a latent form in the cytoplasm. This inactive form is complex composed of a potentially DNA-binding dimer (NF-κB) and an inhibitory subunit (IκB).

Many stimuli, including lipopolysaccharides (LPS), cytokines, oxidants, and viruses, activate NF-κB via several signal transduction pathways that all lead to phosphorylation of IκB. Following activation, the NF-κB heterodimer is rapidly translocated to the nucleus, where it activates the transcription of target genes, including the genes encoding the pro-inflammatory cytokines, adhesion molecules, chemokines, and inducible enzymes such as COX-2 and iNOS (Finco et al., 1995)

The major pathway used to activate NF-κB involves the phosphorylation of IκBα at its regulatory N-terminus on serine 32 and 35 (Figure 2). This event leads to the subsequent conjugation with ubiquitin and the proteasome-mediated degradation of IκB (Traenckner et al., 1995). The degradation of IκB then unmasks nuclear localization sequences in the remaining NF-κB dimer which subsequently translocate to the nucleus and binds to DNA regulatory regions within NF-κB target genes. Some pathways obviously do not involve serine phosphorylation-induced degradation of IκB. In contrast to serine phosphorylation, this modification did not cause subsequent

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proteolytic degradation but was sufficient to release the inhibitor form NF-κB (Schulze-Osthoff et al., 1997). This process leads to the activation and translocation of NF-κB into the nucleus, where it up-regulated the expression of genes involved in inflammatory responses (Cho et al., 2003).

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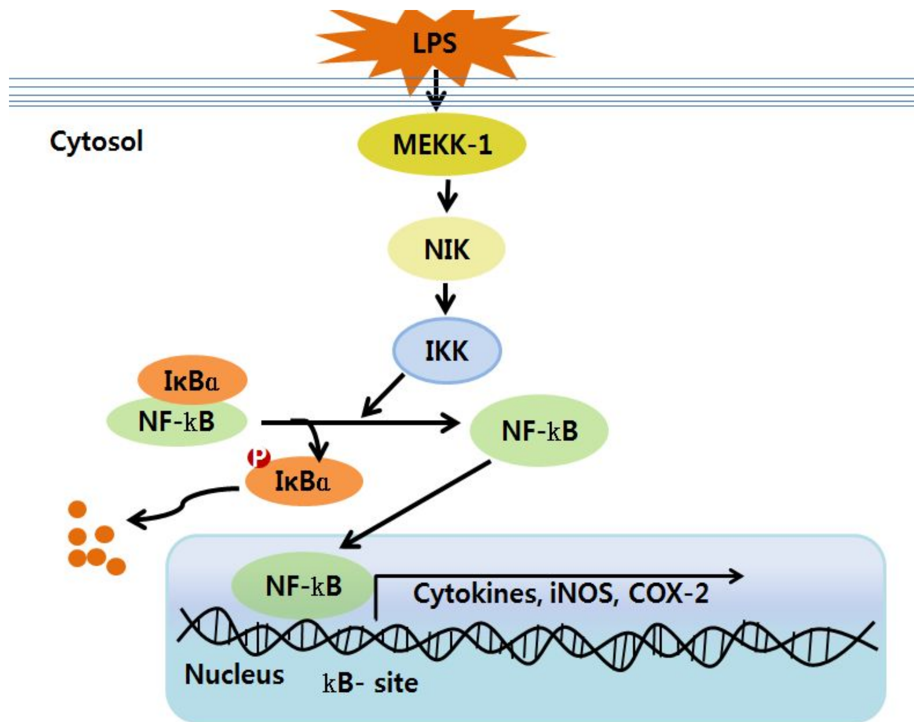

Figure 2. Mechanism of NF-κB action.

While in an inactivated state, NF-κB is located in the cytosol complexed with the inhibitory protein IκBα. Through the intermediacy of integral membrane receptors, a variety of extracellular signals can activate the enzyme IκB kinase (IKK). IKK, in turn, phosphorylates the IκBα protein, which results in ubiquitination, dissociation of IκBα from NF-κB, and eventual degradation of IκBα by the proteosome. The activated NF-κB is then translocated into the nucleus where it binds to specific sequences of DNA.

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One of the LPS-induced pathways involves the mitogen-activated protein kinases (MAPKs), including ERK1/2, c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPKs), and p38 MAPK. MAPKs regulate inflammatory and immune responses and the MAPK signaling pathways are known to be involved in LPS-induced COX-2 and iNOS expression in macrophages.

The MAPKs comprise an important group of serine and threonine signaling kinases that transduce a variety of extra-cellular stimuli through a cascade of protein phosphorylations that lead to the activation of transcription factors. There are three principal MAPK family members: (1) p42 and p44 extracellular signal-regulated kinase (ERK: extracellular signal-regulated kinase), (2) p46 and p54 c-Jun N-terminal kinase (JNK: c-Jun NH_2-terminal kinase), or stress-activated protein kinase, with multiple subisoforms, (3) p38 MAPK, with α, β, γ, δ isoforms (Bhattacharyya et al., 2002). Phosphorylation of MAPKs is known to be a critical component of production of NO and pro-inflammatory cytokines in activated macrophages (Zhou et al., 2008).

ERK1 and ERK2 are widely expressed and are involved in the regulation of meiosis, mitosis, and post-mitotic functions in differentiated cells. Many different stimuli, including growth factors, cytokines, virus infection, ligands for heterotrimeric guanine nucleotide–binding protein (G protein)–coupled receptors, transforming agents, and carcinogens, activate the ERK1 and ERK2 pathways.

The c-Jun NH_2-terminal kinase (JNK) represents one subgroup of MAP kinases

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that is primarily activated by cytokines and exposure to environmental stress. The JNKs were isolated and characterized as stress-activated protein kinases on the basis of their activation in response to inhibition of protein synthesis (Johnson et al., 2002).

The p38 kinases were first defined in a screen for drugs inhibiting tumor necrosis factor α mediated inflammatory responses. The p38 MAP kinase pathway shares many similarities with the other MAP kinase cascades, being associated with inflammation, cell growth, cell differentiation, and cell death. p38 MAPK positively regulates expression of many genes involved in inflammation, such as those coding for TNF-a, IL-1h, IL-6, IL-8, cyclooxygenase- 2. p38 is activated in immune cells by inflammatory cytokines and has an important role in activation of the immune response (Johnson et al., 2002) (Figure 3).

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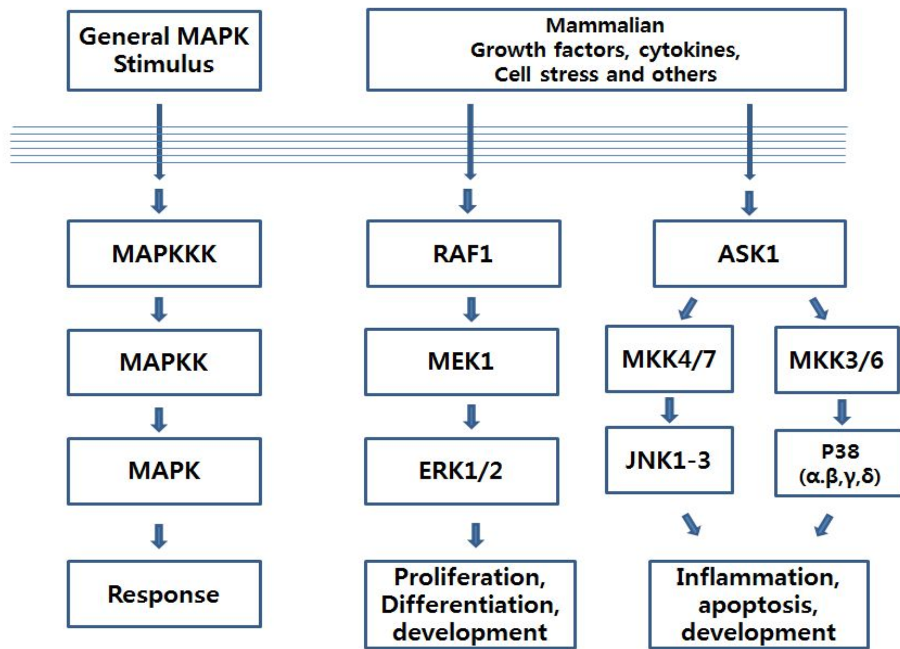

Figure 3. A simplified overview of MAPK pathways in mammals.

(Turjanski et al., 2007)

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The objective of the present study was to assess the effect of AMPs on the inflammatory reaction. I investigated whether the antimicrobial peptides would inhibit the production of NO as well as iNOS and COX-2 expression in macrophages following stimulation with LPS. In addition, I demonstrate that the AMPs inhibit NF-κB activity and MAPK phosphorylation in LPS-stimulated RAW 264.7 macrophage cells.

In the present study, I clearly demonstrated that AMPs have anti-inflammatory effect on in vitro in Raw 264.7 macrophage cells.

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II. Materials & Methods

1. Materials

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin solution from Invitrogen (Grand Island, NY, USA). LPS (*Escherichia coli* O11:B4), dimethyl sulfoxide (DMSO), Griess reagent and MTT (3-(4,5-dimethylthiazol-2-2,5-diohenyltetrazolium bromide) were purchase from Sigma (St. Louis, USA). The antibodies for immunoblotting that JNK, p-JNK, ERK, p-ERK, p38, p-p38, mouse were obtained from Cell signaling Technology, Danvers, MA. NF-κB, IκBα and p- IκBα polyclonal antibody was Santa Cruz Biotech Inc. (Santa Cruz, CA, USA). 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.

2. Purification of antimicrobial peptides

Microbial strains capable of producing AMPs were 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*

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subtilis (99.596 %)] and *Bacillus* sp. CS32 [(*Bacillus licheniformis* (99.392 %)]. Maltose and beef extract were found the most suitable nutrient sources for the maximum production of CS61 antimicrobial peptide. While, the most suitable nutrient sources of CS32 were 1 % glucose, 0.5 % peptone and 0.5 % beef extract.

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.

3. Macrophage 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), 100 unit/ml penicillin, and 100 μg/ml streptomycin, amphotericin B 25 μg/ml. The cells were subcultured every three days. Cells were washed twice with fresh medium and stimulated with 1 μg/ml LPS.

4. Cell viability and nitric oxide production assay

Cells were seeded on 96 well plates, and peptide treatment began 24 h after seeding. The general viability of cultured cells was determined by the MTT assay, in which

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MTT is reduced to formazan in viable cells. MTT is pale yellow substrate that produces a dark blue formazan product when incubated with living cells. Briefly, after 24 h incubation with or without peptide (10-100 μg/ml), a 0.5 mg/ml of MTT solution was added to each well in a 1/10 volume of media. Cells were incubated at 37 ℃ for 3 h, and dimethylsulfoxide (DMSO) was added to dissolved the formazan crystals. The obsorbance was measured using an assay reader at 590 nm. Relative cell viability was calculated compared to the untreated control group.

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, CS32 peptides for 30min prior to stimulation with 1 μg/ml of LPS for 24 h. Briefly, the sample supernatants were mixed with equal volume of Griess reagent (1 % sulfanilamide in 5 % phosphoric acid and 0.1 % naphthylethylenediamine dihydrochloride) and then incubated at room temperature for 10 min. The absorbance was measured at 550 nm on a microplate reader (Thermo co.). Nitrite concentration was determined using a dilution of sodium nitrite as a standard.

5. Preparation of nuclear extracts

Nuclear extracts were prepared by a modified method of Wadsworth and Koop

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(Wadsworth and Koop, 1999). Treated cells were washed, then scraped into 1.5 ml of ice-cold Tris buffered saline (pH 7.9), and centrifuged at 12,000 rpm for 30 s. The pellet was suspended in 10 mM HEPES [(*N*-[2-hydroxyethyl] piperazine-*N'*-[2-ethanesulfonic acid])], pH 7.9, with 10mM KCl, 0.1mM EDTA (ethylenediamine tetraacetic acid), 0.1 mM EGTA [ethyleneglycol-bis-(β-amini ethyl ether *N*,*N*,*N'*,*N'*-tetraacetic acid)], 1 mM DTT (dl-Dithiothreitol), 0.5 mM PMSF (phenylmethyl-sulfonyl fluoride), 5 μg/ml of leupeptin, aprotinin, and pepstatin, incubated on ice for 15 min, and then vortexed for 10 s with 0.6 % Nonidet P-40. Nuclei were separated from cytosol by centrifugation at 12,000 rpm for 60 s. The supernatant was removed, and the pellet was suspended in 50–100 μl of 20 mM HEPES, pH 7.9, with 25 % glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml of leupeptin, aprotinin, and pepstatin. The samples were incubated with rocking at 4 ℃ for 15 min, and centrifuged for 5 min at 10,000 rpm. Protein concentration of the supernatant was determined by Bradford assay.

6. Western blot analysis

Macrophages were incubated with or without LPS in the presence or absence of the antimicrobial peptides. Cells were harvested, washed twice with ice cold Tris–HCl buffered saline (TBS), and resuspended in lysis buffer 100 mM Tris, 5 mM EDTA, 50

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mM NaCl, 50 mM β- glycerophosphate, 50 mM NaF (sodium fluoride), 0.5 % NP-40, 1 % sodium deoxycholate, 0.1 mM sodium orthovanadate and 1% PMSF. The cytosolic fraction was obtained from the supernatant after 12,000 rpm centrifugation at 4 ℃ for 20 min. Samples (20 μg of protein) were separated on 10 % SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and transferred to PDVF membranes. The membranes were blocked with 5 % non-fat milk in TBS–Tween 20 (0.1 %) for 2 h and then incubated with specific primary antibodies for ERK, phosphor ERK, JNK, phospho JNK, p38 MAPK, phospho p38 MAPK (all them from Cell signaling Technology, Danvers, MA) and NF-κB, IκB, phospho IκB (1:1000; Santa Cruz Biotechnology). To prove equal loading, the blots were analysed for β-actin expression using an anti-β-actin antibody (Santa Cruz Biotechnology, CA, California, USA.). After washing three times with TBS–Tween 20, the membrane was hybridized with secondary antibody conjugated with horseradish peroxidase for 1 h. The membranes were washed three times for 10 min and developed with ECL Western blotting detection system. The immune reactive proteins were detected by LAS-3000 Luminescent image analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan).

7. Assay of TPA induced ear edema in mice

TPA induced ear edema was prepared by a modified method of Young et al.

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(Young et al., 1984). Samples (50, 100 μg/ear) were applied on the left ear for 10 min. And TPA (0.25 mg/ml) and dissolved in acetone were applied to the inner and outer surfaces in the ear of mice (10 μg/ear). After, the ear thickness was measured with a calibrated digital thickness gauge before and 5 h after. TPA challenge, and the difference in thickness was calculated. Prednisolone at 50 and 100 μg/ear was used as positive control.

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Table 1. Antibodies used in this study

Antibodies	Species	Clonal	Manufactures	Dilute
iNOS	Rabbit	Polyclonal	Santa Cruz Biotechnology, CA, California, USA	1:1000
COX-2	Mouse	Polyclonal	Cell signaling technology, Berverly, MA	1:2000
NF-κB	Rabbit	Polyclonal	Santa Cruz Biotechnology, CA, California, USA	1:1000
IκBα	Rabbit	Polyclonal	Santa Cruz Biotechnology, CA, California, USA	1:1000
phospho-IKBα	Mouse	Polyclonal	Cell signaling technology, Berverly, MA	1:2000
ERK	Rabbit	Polyclonal	Cell signaling technology, Berverly, MA	1:1000
phospho-ERK	Rabbit	Polyclonal	Cell signaling technology, Berverly, MA	1:1000
JNK	Rabbit	Polyclonal	Cell signaling technology, Berverly, MA	1:1000
phospho-JNK	Mouse	Polyclonal	Cell signaling technology, Berverly, MA	1:2000
P38	Mouse	Polyclonal	Cell signaling technology, Berverly, MA	1:2000
phpospho-p38	Rabbit	Polyclonal	Cell signaling technology, Berverly, MA	1:1000

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Table 2. Comparison of antimicrobial peptides produced *Bacillus* strains.

		CS 61	CS 32
Strain		*Bacillus subtilis* (99.596 %)	*Bacillus licheniformis* (99.392 %)
Molecular weight (Da)		1,100 Da	5,800 Da
N-terminal amino acid secuences		AINXDAAYL	APLEIXXIFHDN
Optimum cultivation conditions		1 % Maltose, 1 % beef extract, 48 h, 37 ℃, 180 rpm	1 % glucose, 0.5 % peptone, 0.5 % beef extract, 60 h, 37 ℃, 180 rpm
Stability	Temperature (℃)	80 ℃	90 ℃
	pH	2.0-10.0	5.0-10.0

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III. Results

1. Effects of antimicrobial peptides on cytotoxicity and NO production

Raw 264.7 cells were treated with various concentrations of CS61 and CS32 AMPs for 24 h and the viability was determined by MTT assay as des cribed above. As shown in Figure 4, all AMPs did not exhibit cytotoxicity to Raw 264.7 cells at the range of 5-100 μg/ml. Therefore peptide was used at 100 μg/ml and below for further experiments.

Macrophages produce NO following stimulation with LPS. Excessive producti on of NO during inflammation causes tissue injury, nerve damage etc.. To asse ss the effect of CS61 and CS32 AMPs on NO production by LPS-induced Raw 264.7 cells, the nitric concentration in the culture medium were measured using the Griess reagent method. As shown in Figure 5, LPS treatment significantly i ncreased NO production compared to the untreated cells. Treatment of cells wit h CS61 and CS32 AMPs at 10, 50, 100 μg/ml suppressed the LPS-induced prod uction of NO to a statistically significant extent.

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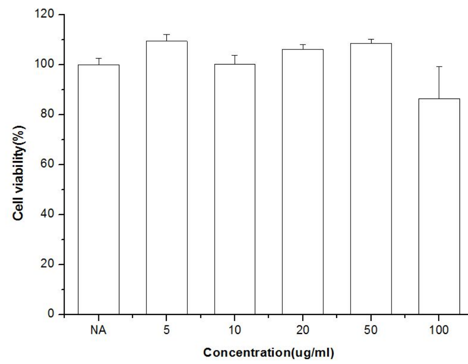
(a) CS61



(b) CS32

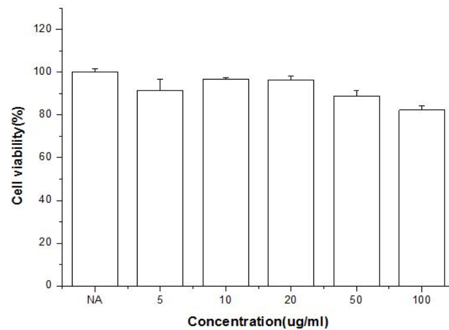


Figure 4. Effect of the antimicrobial peptides on cell viability.

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 peptides for 24 h. Cells were incubated with the various concentration of peptides for 30 min, followed by treatment with 1 μg/ml of LPS and incubated for 24 h. Each bar shows the mean ± S.D of three independent experiments performed in triplicate. (a) CS61 AMP, (b) CS32 AMP.

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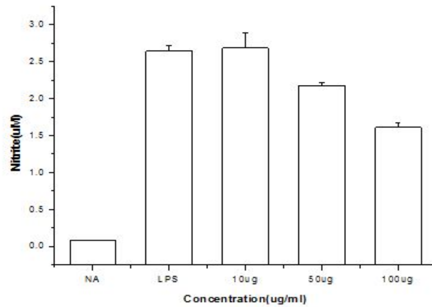
(a) CS61



(b) CS32

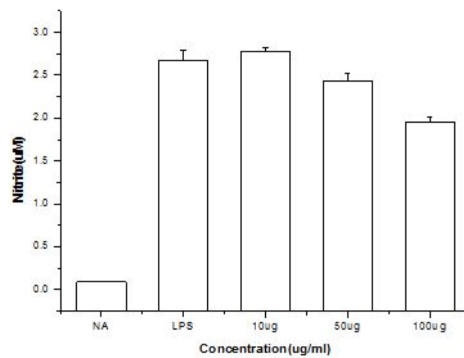


Figure 5. Effect of the antimicrobial peptides on NO production.

Cells were incubated with the various concentration of peptides (5-100 μg/ml) for 30 min, fo llowed by treatment with 1 μg/ml of LPS and incubated for 24 h. The amounts of NO were determined using the Griess reagent in the culture medium. Each bar shows the mean ± S. D of three independent experiments performed in triplicate. (a) CS61 AMP, (b) CS32 AMP.

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2. Effects of iNOS, COX-2 protein expression in LPS-induced Raw 264.7 macrophage cells

To confirm whether the inhibition of NO production is due to a decreased level of iNOS and COX-2 proteins, the effects of CS61 and CS32 AMPs on the level of iNOS and COX-2 proteins were determined by western blot analysis. The expression of iNOS and COX-2 proteins were barely detectable in unstimu lated Raw 264.7 cells, but was markedly increased after 24 h of LPS (1 μg/ml) treatment. However, CS61 and CS32 AMPs significantly inhibited iNOS and COX-2 protein expression in LPS-induced Raw 264.7 cells. These results indic ate that LPS exposure increased the expression of iNOS and COX-2 proteins, but treatment with AMPs significantly suppressed the induction of LPS-induced mediators through transcriptional inhibition. (Figure 6).

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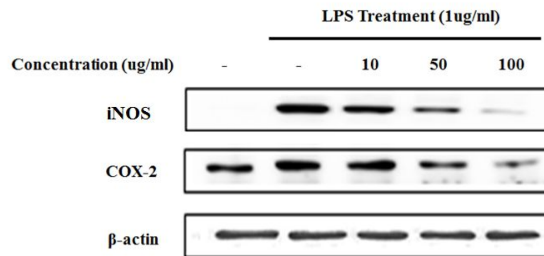
(a) CS61



(b) CS32

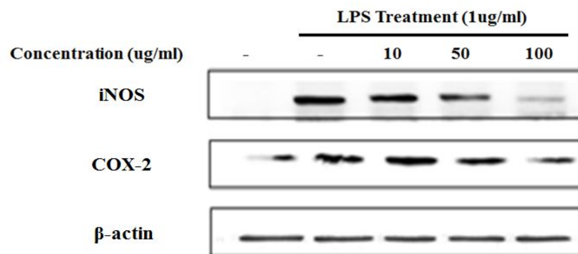


Figure 6. Inhibitory effect of antimicrobial peptides of NO production and iNOS, COX-2 protein expression in LPS-induced Raw 264.7 macrophage cells.

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. (a) CS61 AMP, (b) CS32 AMP.

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3. Effects of antimicrobial peptides on LPS-induced NF-κB transcriptional activity in Raw 264.7 macrophage cells

NF-κB transcription factor has been evidenced to play an important role in LPS-induced expression of pro-inflammatory proteins. In unstimulated cells, NF-κB is constitutively localized in the cytosol as a heterodimer by physical association with an inhibitory protein called inhibitor κB (IκB) (Baeuerle et al., 1988; Baldwin, 1996).

Therefore, Inhibition of the phosphorylation and degradation of IκBα by AMPs was evaluated. Accordingly, Raw 264.7 cells were pretreated for 1 h with AMPs, and IκBα protein levels were determined after 20 min of further LPS exposure (1 μg/ml). AMPs were shown to significantly suppress the LPS-induced phosphorylation of IκBα (Figure 7). These suggest show that AMPs inhibit LPS induced NF-κB activation by preventing the degradation of IκBα phosphorylation. In order to confirm the result, the nuclear translocation of p65 and p50, and a component of the p65/p50 heterodimer of NF-κB were evaluated. LPS treatment was emplyed to induce the translocation of p65 and p50 from the cytosol to the nucleus, and AMPs markedly suppressed the nuclear translocation of p65 and p50 (Figure 8).

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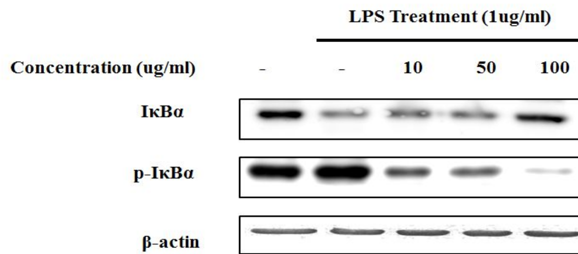
(a) CS61



(b) CS32

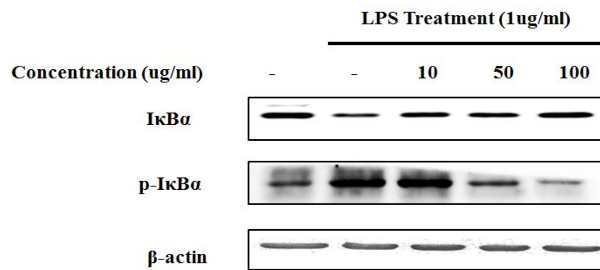


Figure 7. AMPs inhibited LPS-induced IκBα phosphorylation and in Raw 264.7 cells.

(a) Cells were pretreated with AMPs (10, 50, 100 μg/ml) for 1 h and then with LPS (1 μg/ml) for 15 min. Total cellular proteins were prepared and western blotted for p-IκBα, IκBα and using specific p-IκBα, IκBα antibodies. β-actin was used as an internal control. Experiments were repeated three times and similar results were obtained. (a) CS61 AMP, (b) CS32 AMP

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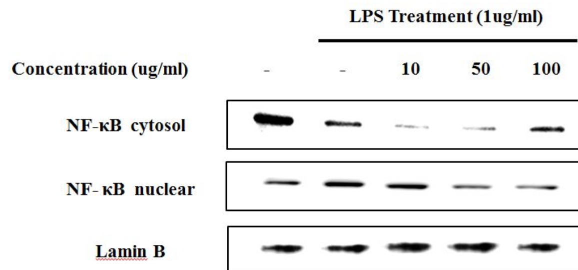
(a) CS61



(b) CS32

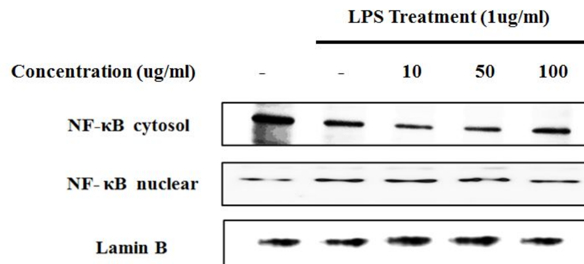


Figure 8. AMPs inhibited LPS-induced NF-κB activation in Raw 264.7 macrophage cells.

Raw 264.7 macrophage cells were pretreated with AMPs (10, 50, 100 μg/ml) for 1 h and then with LPS (1 μg/ml) for 20 min. Nuclear extracts were prepared for the western blot analysis of p65 and p50 of NF-κB protein using a specific anti-p65 and p50 NF-κB monoclonal antibody. Lamin-B was used as an internal control. Experiments were repeated three times and similar results were obtained. (a) CS61 AMP, (b) CS32 AMP.

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4. Effects of antimicrobial peptides on MAP kinases activation in LPS-induced Raw 264.7 macrophage cells

The three MAPKs - ERK, JNK and p38 MAPK, are known to be activated by LPS, and various stimuli have demonstrated the pathways of MAPKs in the LPS-induced macrophages (Chen et al., 1999; Kim et al., 2004). To determine whether the inhibition of inflammatory responses by AMPs are mediated via an MAPK pathway or not, the effect of different concentrations of AMPs (10, 50, 100 μg/ml) on the LPS-induced phosphorylation of ERK1/2, JNK and p38 MAPK was evaluated by western blot analysis.

The result suggested that CS61 AMP inhibited LPS-induced phosphorylation of ERK and p38 MAPK in a concentration-dependent manner. The activation of JNK MAP kinase by CS61 AMP was not inhibited whereas phosphorylation of JNK was not affected (Figure 9).

As shown in Figure 10, LPS (1 μg/ml) significantly promoted the phosphorylation of ERK1/2, JNK, and p38 MAPKs in Raw 264.7 cells. CS32 AMP (10, 50, 100 μg/ml) treatment dramatically reduced the phosphorylations of ERK1/2, p38 and JNK MAPKs in a dose-dependent manner. However, the expressions of nonphosphorylated ERK1/2, p38, and JNK MAPKs were unaffected by LPS or LPS in combination with CS32 AMP. These results suggest that p38 MAPK and JNK

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conjunction with NF-κB pathway may contribute to the inhibitory effect of AMPs on NO production in LPS-stimulated Raw 264.7 cells.

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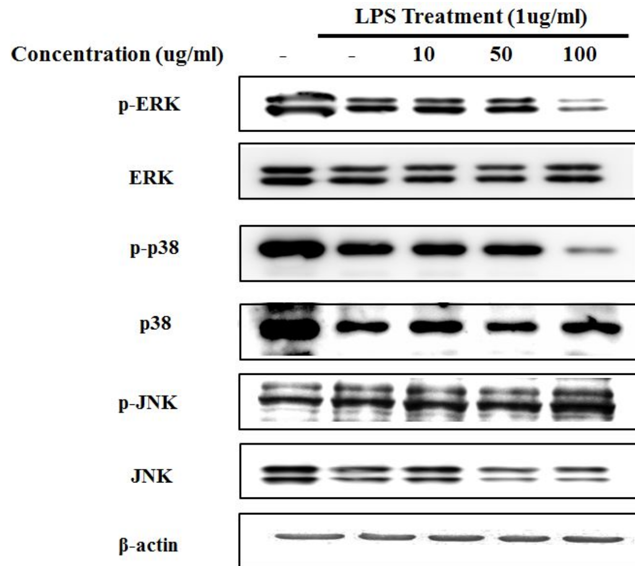

Figure 9. Effect of CS61 AMP on LPS-induced phosphorylation of MAPKs in Raw 264.7 macrophage cells.

Raw 264.7 macrophage cells were treated with 10, 50, 100 μg/ml CS61 AMP for 2 h before the addition of 1 μg /ml LPS for 30 min. Phosphorylation levels of ERK, JNK, and p38 MAPKs were determined by Western blot analysis. The total MAPK levels were used as internal controls.

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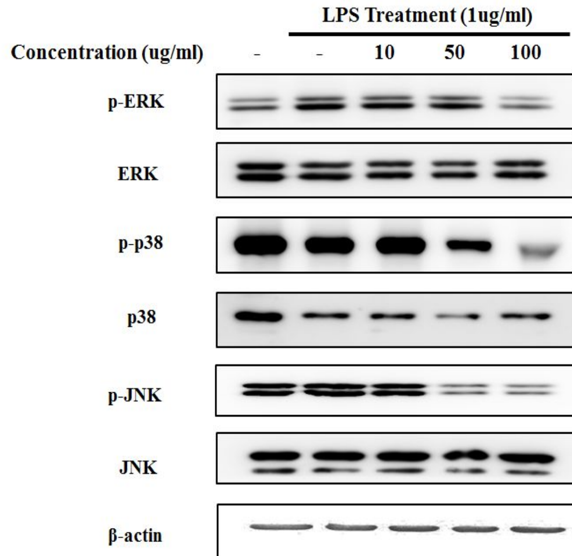

Figure 10. Effect of CS32 AMP on LPS-induced phosphorylation of MAPKs in Raw 264.7 macrophage cells.

Raw 264.7 macrophage cells were treated with 10, 50, 100 μg/ml CS32 AMP for 2 h before the addition of 1 μg/ml LPS for 30 min. Phosphorylation levels of ERK, JNK, and p38 MAPKs were determined by Western blot analysis. The total MAPK levels were used as internal controls.

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5. Effects of AMP on TPA-induced ear edema of mice

CS32 AMP was used to the TPA induced ear edema test, at the dose of 50 μg/ear evaluated the anti-inflammatory effect. These AMPs were investigated in comparision to that of the positive control prednisolone. Positive controls suppressed ear edema at a dose 50 and 100 μg/ear in edema models.

As reported in Table 3, CS32 AMP and prednisolone affect anti-inflammatory activity. CS32 AMP gives the highest contribution to the anti-inflammatory activity. Furthermore, I investigated the effect of CS32 AMP on iNOS expression in each ear by western blot analysis.

I confirmed that iNOS expression was evaluated in mouse edema with TPA for 5 h. Pretreatment with CS32 AMP 1 h prior to the application of TPA resulted in decrease in the level of iNOS in mouse ear with TPA-induced acute inflammation (Figure 11).

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Table 3. Anti-inflammatory activity of AMP on TPA induced mouse ear edema

Group	Dose (μg/ear)	left ear (μg/ear)	right ear (μg/ear)	Weight of left ear (mg)	Weight of right ear (mg)
Control	0	none	TPA 2.5	3.8	16.2
Prednisolon	50	TPA 2.5	TPA+pred 50	8.4	6.6
	100	TPA 2.5	TPA+pred 100	6.9	5.3
CS32 AMP	50	TPA 2.5	TPA+AMP 50	12.2	10.2
	100	TPA 2.5	TPA+AMP100	9.6	7

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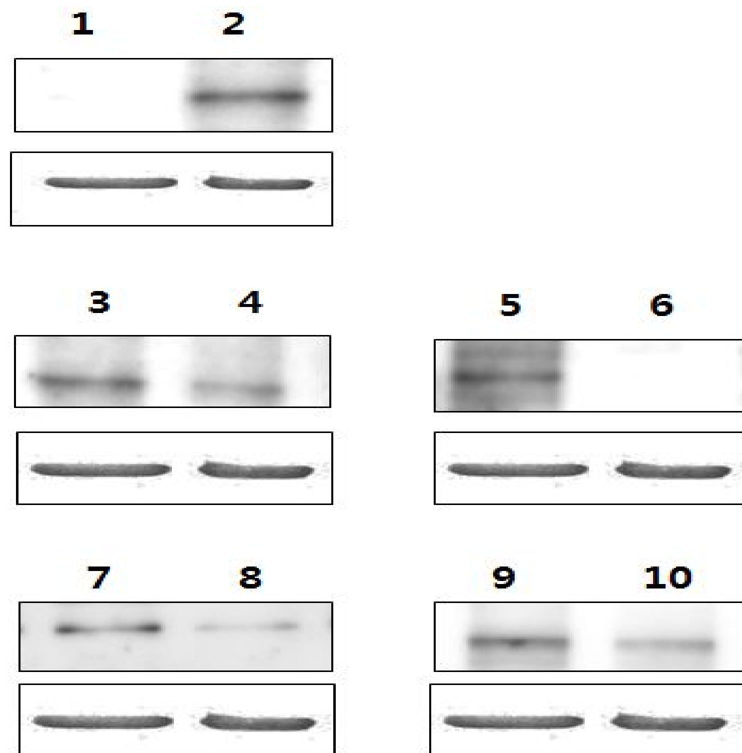

Figure 11. Effects of CS32 AMP iNOS expression on TPA induced ear edema in mice

iNOS expression in ICR mouse ear edema was determined by Western blot analysis. The β-actin was used as internal controls. (1: none,; 2: TPA 2.5 μg/ear,; 3: TPA 2.5 μg/ear,; 4: TPA+pred 50 μg/ear,; 5:TPA 2.5 μg/ear,; 6: TPA+pred 100 μg/ear,; 7: TPA 2.5 μg/ear,; 8: TPA+AMP 50 μg/ear,; 9: TPA 2.5 μg/ear,; 10: TPA+AMP 100 μg/ear)

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IV. Discussion

The results suggest that the antimicrobial peptides (CS61 and CS32 AMP) isolated from Korean traditional foods exhibit anti-inflammatory properties; these properties appear to be attributable to the down regulation of MAPK signal pathways and the inhibition of NF-κB activation in LPS induced Raw 264.7 macrophage cells.

Inflammation is the first response of the immune system against infection or irritation. During the inflammatory process, sizeable quantities of the pro-inflammatory mediators NO and PGE_2 were generated by the inducible isoforms of iNOS and COX-2 (Posadas et al,. 2000). Therfore, AMPs that inhibit the production of these inflammatory mediators have been previously considered as potential candidates for anti-inflammatory drugs.

Western blotting was performed to assess expression of iNOS and COX-2 in LPS-induced Raw 264.7 macrophage cells. The production of NO in culture supernatants was evaluated. Furthermore, the cytotoxicity of AMPs on the Raw 264.7 macrophage cells was evaluated by MTT assay. Up to 100 μg /ml of AMPs had no significant effect on cell viability in untreated cells or cells stimulated with 1 μg/ml LPS (Figure 4, 5). 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 expressions were detectable in Raw 264.7 macrophage cells.

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iNOS and COX-2 protein levels were significantly increase after LPS-treatment. This increase in iNOS and COX-2 protein expression in Raw 264.7 macrophage cells after LPS activation was significantly reduced by CS61 and CS32 AMPs treatment at concentrations of 10 to 100 μg/ml (Figure 6). The results of this study demonstrated that AMPs inhibited NO production by the suppression of iNOS and COX-2 expression.

LPS exerts its inflammatory effects by the activation of both the MAPK signaling pathway and the classical NF-κB pathway. Therefore, the inhibition of these signaling pathways may explain the potent activity of AMPs as suppressor of inflammatory mediators and cytokines. NF-κB commonly exists as a homodimer or heterodimer, and one heterodimer of p50 and one of the Rel family p65s is known to mediate the expression of genes associated with innate immune responses (Baeuerle and Baltimore, 1996). In unstimulated cells, NF-κB dimers are bound to inhibitory κBα (IκBα), and are consequently retained within the cytoplasm. However, when the cells are stimulated with pro-inflammatory stimuli, IκBα are rapidly phosphorylated and degraded by the action of the IκB kinase (IKK) complex, and the free NF-κB is translocated into the nucleus (Li and Verma, 2002) (Figure 2).

The result demonstrated that the relationship between AMPs and its inhibition effect on LPS-mediated NF-κB transcription was concentration dependence (Figure 8). Additionally, determination of IκBα protein by western blot analysis showed that

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10 to 100 μg/ml of AMPs treatment the induced degradation and resynthesis of IκBα protein. Meanwhile, AMPs treatment moderately inhibited the phosphorylation of IκBα (Figure 7). Therefore, these results suggest that AMPs inhibit the expression of iNOS and COX-2, and NO production through inactivation of NF-κB by reducing IκBα degradation and phosphorylation (Figure 12).

The activation of NF-κB is regulated by cellular kinases, including MAPK. MAPKs are a highly conserved family of protein serine/threonine kinases and include the ERK1/2, JNK and p38 subgroups (Garrington and Johnson, 1999).

To investigate the mechanisms of NF-κB inactivation and NO inhibition by AMPs, the effects of AMPs on the LPS induced phosphorylation of ERK1/2, JNK and p38 MAPK were examined. The result demonstrated that CS61 AMP significantly inhibited the phosphorylation of ERK 1/2, and p38 MAPK, but not JNK in LPS-induced Raw 264.7 macrophage cells (Figure 9). However, CS32 AMP significantly inhibited the phosphorylation of ERK 1/2, JNK and p38 MAPK in LPS-induced Raw 264.7 macrophage cells (Figure 10).

To relationship between AMPs and its inhibition effect on TPA induced ear edema was concentration dependence.CS32 AMP showed slight inhibition of edema formation. Moreover, CS32 AMP inhibited TPA-induced expression of iNOS protein.

This study describes the identification of the AMP-producing strain, *Bacillus* sp. CS61 and CS32, isolated from Korean traditional foods. The results demonstrated that

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the AMPs significantly inhibits LPS-induced NO production as well as the expressions of iNOS and COX-2 by down-regulation of the ERK, JNK and p38 MAP kinase signal pathways and inhibition of NF-κB activation. The importance of MAPKs in controlling cellular responses to the environment and in regulating gene expression, cell growth, and apoptosis has made them a priority for research related to many diseases. In this study, phosphorylation of ERK1/2, JNK, and p38 MAPK followed by LPS stimulation in Raw 264.7 macrophage cells were inhibited by the AMPs in a dose-dependent manner, implying that the AMPs may inhibit MAPK signaling cascade. The results of this study suggest that the AMPs have potentiality as a candidate of anti-inflammatory agents.

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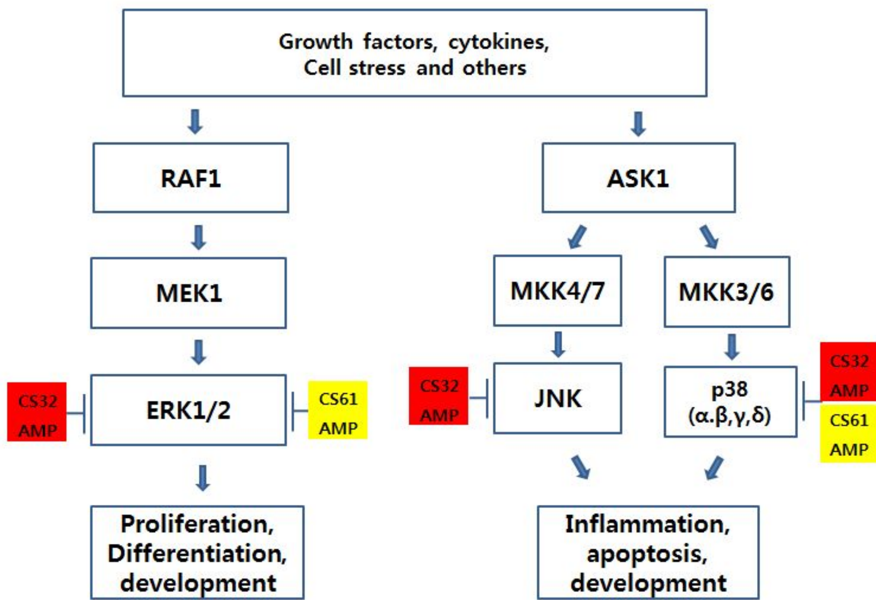

Figure 12. Schematic diagram illustrating MAPK signaling cascades involved in antimicrobial peptides inhibition of LPS-induced inflammation in Raw 264.7 macrophage cells.

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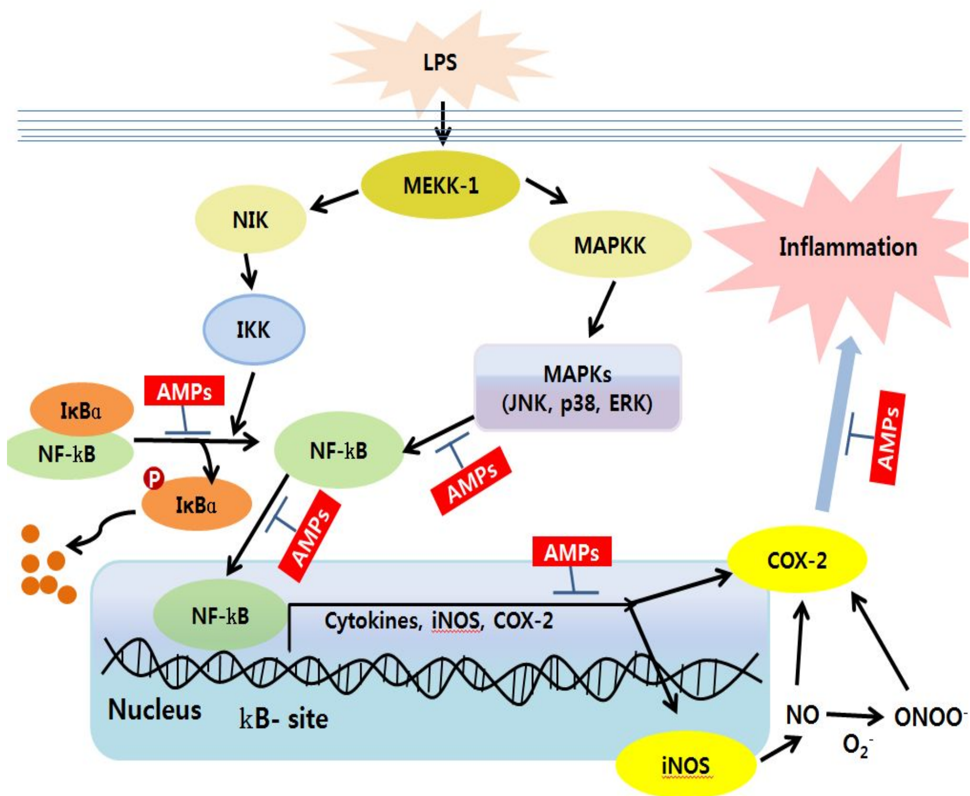

Figure 13. Proposed mechanism of action by antimicrobial peptides inhibited inflammation in Raw 264.7 macrophage cells.

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먼저 박사 과정 동안 수많은 조언과 함께 아낌없이 지도 해주신 유진철 교수님께 진심으로 감사 드립니다. 그리고 저의 논문을 열과 성으로 심사해 주신 김태성 교수님, 홍준희 교수님, 지준필 교수님, 조승식 교수님께도 깊은 감사를 드립니다. 교수님들께서는 단순히 박사로서의 소양뿐만 아니라 인생을 마주하는 자세에 대한 가르침을 주셨습니다. 너무도 훌륭한 교수님들의 따뜻한 조언 한마디 한마디 모두 마음에 새겨두겠습니다.

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