Journal of Food and Nutrition Research, 2016, Vol. 4, No. 10, 690-698
Available online at http://pubs.sciepub.com/jfnr/4/10/9

DOI:10.12691/jfnr-4-10-9

Inhibitory Effects and Molecular Mechanism of an Anti-inflammatory Peptide Isolated from Intestine of Abalone, *Haliotis Discus Hannai* on LPS-Induced Cytokine Production via the p-p38/p-JNK Pathways in RAW264.7 Macrophages

Zhong-Ji Qian[1], BoMi Ryu[2], Won Sun Park[3], IL-Whan Choi[4,*], Won-Kyo Jung[5,*]

[1]Key Laboratory of Advanced Processing of Aquatic Products of Guangdong Higher Education Institution, College of Food Science and Technology, Guangdong Ocean University, Zhanjiang 524088, China
[2]School of Pharmacy, the University of Queensland, Brisbane, Qld 4072, Australia
[3]Department of Physiology, School of Medicine, Kangwon National University, Chuncheon 24341, Republic of Korea
[4]Department of Microbiology, College of Medicine, Inje University, Busan 633-165, Republic of Korea
[5]Department of Biomedical Engineering, and Center for Marine-Intergrated Biomedical Technology (BK21 Plus) Pukyong National University, Busan 608-737, Republic of Korea
*Corresponding author: wkjung@pknu.ac.kr, cihima@inje.ac.kr

Abstract A marine mollusk, abalone (*Haliotis discus hannai*) is one of the important species in aquaculture industry, but nutraceutical and pharmaceutical benefits of *H. discus hannai* have been rarely identified and studied. To evaluate beneficial effects of *H. discus hannai*, an anti-inflammatory peptide (AAIP, abalone anti-inflammatory peptide) was purified from abalone intestines using consecutive HPLC purification system. In tandem MS analysis, the fragmentation results illustrate that the AAIP responsible for the nitric oxide (NO) inhibitory activity (IC_{50}=55.8μM) has amino acid sequence as Pro-Phe-Asn-Glu-Gly-Thr-Phe-Ala-Ser (1175.2 Da). To investigate anti-inflammatory effect of AAIP on lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages and elucidated the molecular mechanism. The results show that the AAIP peptide suppresses LPS-induced production of nitric oxide (NO) via inducible nitric oxide synthase (iNOS) expression in a dose-dependent manner. It also significantly reduced the gene transcription of proinflammatory cytokines, such as interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α). Furthermore, AAIP significantly suppresses phosphorylation of mitogen-activated protein kinases (MAPKs) such as p-p38 and p-JNK. These results indicated that AAIP inhibits LPS-induced inflammatory response via blocking of MAPK pathway in murine macrophages. Therefore, potent AAIP might suggest possibility for high valuable utilization and application as nutraceutical and therapeutic substances.

Keywords: *marine mollusk, inflammation, lipopolysaccharide (LPS), nitric oxide, nitric oxide synthase, mitogen-activated protein kinases (MAPK)*

Cite This Article: Zhong-Ji Qian, BoMi Ryu, Won Sun Park, IL-Whan Choi, and Won-Kyo Jung, "Inhibitory Effects and Molecular Mechanism of an Anti-inflammatory Peptide Isolated from Intestine of Abalone, *Haliotis Discus Hannai* on LPS-Induced Cytokine Production via the p-p38/p-JNK Pathways in RAW264.7 Macrophages." *Journal of Food and Nutrition Research*, vol. 4, no. 10 (2016): 690-698. doi: 10.12691/jfnr-4-10-9.

1. Introduction

Inflammation represents a highly coordinated set of events that allows tissues to respond to injury or infection, which requires the participation of various cell types expressing and reacting to diverse mediators in a sequential manner [1]. Macrophages are major immune cells in the innate immune system. The activation of macrophages plays a key role in inflammatory responses when infected with pathogens. Macrophages can kill pathogens directly by phagocytosis and indirectly through secretion of various pro-inflammatory mediators. Macrophages play an important role in inflammatory diseases relating to over production of pro-inflammatory cytokine including interleukin-1β (IL-1β), IL-6, and tumor necrosis factor (TNF-α), and inflammatory mediators including nitric oxide (NO), generated by activated inducible nitric oxide synthase (iNOS) [2,3]. NO is generated via the oxidation of terminal guanidine nitrogen of L-arginine by NOS, and is involved in inflammation and carcinogenesis [4]. Thus, the inhibition of NO overproduction by blocking iNOS expression may be a useful strategy for the treatment of various inflammatory disorders.

lipopolysaccharide (LPS), a major constituent of Gram-negative bacteria, initiates a number of major cellular

responses that play critical roles in the pathogenesis of inflammatory responses and has been employed to induce macrophage activation. Mitogen-activated protein kinases (MAPKs) also regulate key proinflammatory pathways following stimulation with LPS. The three major MAPKs proteins, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK are thought to play different roles in inflammatory diseases in different capacities. Therefore, participation of MAPKs has been frequently implicated in occurrence of diseases and expression of immune or inflammatory responses [5].

Bioactive peptides are specific protein fragments that have a positive impact on a body function or condition and ultimately may influence human health; which expected to be provided by a safe, reliable, and consistent oral delivery system. Gastrointestinal digestion by endogenous proteolytic enzymes such as pepsin, trypsin, α-chymotrypsin, and lipases could help in releasing these bioactive peptides. Furthermore, bioactive peptides derived from gastrointestinal digests have been reported for their beneficial effects on anti-inflammatory, antihypertensive, antioxidant, anticancer, antimicrobial, and anti-arthritis activities [6,7,8]. Especially, gastrointestinal digestion methods have offered as a rapid and reproducible protocol for the production of substantial amount of bioactive peptides [9].

Abalone is a marine gastropod, which is one of important fishery and food industrial resources, massively maricultured in Asia, Africa, Australia, and America. Pacific abalone, *H. discus hannai* has massively been maricultured from 1990' in southwestern coast of South Korea. To meet the increasing demand of the Asian market, abalone mariculture has been expanding in land- and sea-based systems in S. Korea and total yield is expected to produce 7,580 tons of abalone in 2009 (The Korea Marine Institute). In addition, manufacturing products of abalone (the dried, steamed, spiced abalone, and so on) have largely been increased [10].

In the present study, we report purification and characterization of a peptide from gastro digests of abalone intestine protein and examined the effects of peptide on its suppresses nitric oxide (NO) production via inducible nitric oxide synthase (iNOS) expression and reduced the gene transcription of pro-inflammatory cytokines in murine macrophages cells. These events are coordinated with the activation of mitogen-activated protein kinases (MAPKs). Inhibition of this signaling pathway by the peptide can modulate the LPS-induced inflammatory response.

2. Materials and Methods

2.1. Materials

Murine macrophage (RAW264.7) cell lines were obtained from American Type of Culture Collection (Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM), trypsin-EDTA, penicillin/streptomycin/amphotericin (10,000 U mL^{-1}, 10,000 μg mL^{-1}, and 2,500 μg mL^{-1}, respectively), phosphate buffered-saline (PBS) and fetal bovine serum (FBS) were obtained from Gibco BRL, Life Technologies (USA). 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Griess reagent, lipopolysaccharide (LPS) from *Escherichia coli* serotype 0111:B4 reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Specific antibodies against iNOS, ERK, JNK, and p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phosphorylated (p)-ERK, p-p38, and p-JNK were purchased from Cell Signaling Technology (Beverly, MA). Other chemicals and reagents used were of analytical grade commercially available.

2.2. Preparation of in Vitro Gastrointestinal Digestion and Fractionated by UF Membranes Bioreactor System

The digestion process used the method described by Kapsokefalou and Miller [9]. One hundred milliliters of abalone intestine solution 4% (w/v) was brought to pH 2.2 in gastric digestion (phase I) using 1 M HCl and 1 M NaOH under vigorous mixing. Pepsin was added at an enzyme to substrate ratio of 1/100 (w/w), then incubated at 37 °C on a shaker. After 2 h, the pH was set to 6.5 to obtain the conditions of intestinal digestion (phase II). Similarly, trypsin and α-chymotrypsin were both supplemented at an enzyme to substrate ratio of 1/100 (w/w). The solution was further incubated at 37 °C for 2.5 h. When samples were taken at the beginning and end of digestion, the pH was adjusted to 6.5. Samples were centrifuged at 10,000 × g for 15 min at 4 °C, and the supernatant was lyophilized to obtain AIGIDs dry powder. The resultant AIGID was fractionated through UF membranes bioreactor system with a range of molecular weight cutoffs (MWCO) of 10, 5 and 1 kDa, respectively. Fractionates were designed as follows: AIGID-I with M_W distribution of > 10 kDa, AIGID-II with MW distribution of 10-5 kDa and AIGID-III with M_W distribution of 5-1 kDa. All AIGIDs recovered were lyophilized in a freeze drier for 5 days.

2.3. Purification of Inhibition NO Production Peptide

2.3.1. Ion Exchange Chromatography

The lyophilized AIGID III (20 mg/mL) was dissolved in 20 mM sodium acetate buffer (pH 4.0), and using fast protein liquid chromatography (FPLC AKTA, Amersham Bioscience Co., Uppsala, Swedern) onto a Hiprep 16/10 DEAE FF anion exchange column (16 × 100 mm) equilibrated with 20 mM sodium acetate buffer (pH 4.0), and eluted with a linear gradient of NaCl (0-2.0 M) in the same buffer at a flow rate of 2.4 mL/min. Each fraction was monitored at 280 nm, collected at a volume of 4 mL and concentrated using a rotary evaporator; antioxidant activity was also investigated. A strong antioxidant fraction was lyophilized, and chromatography was used as the next step.

2.3.2. High-Performance Liquid Chromatography (HPLC)

The fraction exhibiting inhibition NO production activity was further purified using reversed-phase high-performance liquid chromatography (RP-HPLC) on a Primesphere 10 C18 (20 · 250 mm) column with a linear gradient of acetonitrile (0-35% in 30 min) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 1.0 mL/min.

Elution peaks were detected at 215 nm, and active peak was concentrated using a rotary evaporator. Potent peaks were collected, evaluated NO production, and then lyophilized. The active fraction from analytical column was further applied onto a Synchropak RPP-100 analytical column with a linear gradient of acetonitrile (20% v/v, in 15 min) containing 0.1% TFA at flow rate of 1 mL/min. The finally purified peptide was analyzed amino acid sequence.

2.3.3. Determination of Amino Acid Sequence

Accurate molecular mass and amino acid sequence of the purified peptide were determined with a Q-TOF mass spectrometer (Micromass, Altrincham, UK) coupled with an electrospray ionization (ESI) source. The purified peptide (AAIP) was separately infused into the electrospray source following dissolution in methanol/water (1:1, v/v), and molecular mass was determined by a doubly charged $(M+2H)^{+2}$ state in the mass spectrum. Following molecular mass determination, the peptide was automatically selected for fragmentation, and sequence information was obtained by tandem mass spectroscopy (MS) analysis.

2.4. Culture of Cells and Viability Determination

Murine macrophage cell lines, RAW264.7 was grown in DMEM media containing 10% fetal bovine serum, 2 mM glutamine, and 100 µg/mL penicillin-streptomycin at 5% CO_2 and 37°C humidified atmosphere.

Cell viability was determined by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay as described by Hansen et al. [11]. In brief, cells were pre-incubated overnight in 96-well plates at a density of 1×10^4 cells per well, and were then washed with phosphate-buffered saline (PBS). Cells were treated with various concentrations (10-500 µM) of purified peptide for 24 h, 100 µL MTT was added and incubated at 37°C for 4 h. After the culture supernatants were removed, the resulting dark blue crystals were dissolved with DMSO. Absorbance values were read at 540 nm on an enzyme-linked immunosorbant assay (ELISA) microplate reader (ThermoMax, CA, USA). Relative cell viability was calculated compared with the absorbance of the untreated control group.

2.5. Nitric Oxide Production Assay

Nitric oxide (NO) levels in the culture supernatants were measured by the Griess reaction as described earlier by Lee et al. [12]. In brief, RAW264.7 cells were pre-incubated overnight in 96-well plates using DMEM without phenol red at a density of 1×10^4 cells per well, followed by the of treatment different concentrations of sample for 1 h. After, the NO production was stimulated by adding LPS (100 ng/mL final concentration) and incubated for 48 h. Then 50 µL of culture supernatants from each sample was mixed with the same volume of the Griess reagent following incubation for 15 min. Absorbance values were read at 540nm on an ELISA microplate reader (ThermoMax, CA, USA). The values obtained were compared with those of standard concentrations of sodium nitrite dissolved in DMEM, and the concentration of nitrite in conditioned media of sample treated cells were calculated.

2.6. RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted from RAW 264.7 cells after treatment with the AAPI at different concentrations (10-250 µM) [13]. For that, cells were lysed with Trizol® and centrifuged at 12,000 rpm for 15 min at 25°C following the addition of chloroform. Isopropanol was dded to the supernatant at a 1:1 ratio and the RNA pellet was obtained following centrifugation. After washing with ethanol, extracted RNA was solubilized in diethyl pyrocarbonate-treated RNase-free water and quantified by measuring the absorbance at 260 nm using the GENios® microplate reader (Tecan Austria GmbH). Equal amounts of RNA (1 µg) were reverse transcribed in a mastermix containing 1× reverse transcriptase (RT) buffer, 1mM dNTPs, 500 ng of oligo (dT)15 primers, 140U of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase and 40U of RNase inhibitor, for 45 min at 42°C. Polymerase chain reaction was carried out in an automatic Whatman thermocycler (Biometra, Kent, UK) to amplify IL-6, iNOS, and IL-1β mRNA. RNA was stored at -70°C until used.

The PCR was performed using selective primers for the IL-6 (5'-ATGAGCACAGAAAGCATGATC-3' and 5'-TACAGGCTTGTCACTCGAATT-3'), iNOS (5'-ATGTCCGAAGCAAACATCAC-3' and 5'-TAATGTCCAGGAAGTAGGTG-3'), IL-1β (5'-ATGGCAACTGTTCCTGAACTCAACT-3' and 5'-TTTCCTTTCTTAGATATGGACAGGAC-3'), TNF-α (5'-ATGAGCACAGAAAGCATGATC-3' and 5'-TACAGGCTTGTCACTCGAATT-3') and GAPDH (5'-TTTGTGATGGGTGTGAACCACGAG-3' and 5'-GGAGACAACCTGGTCCTCAGTGTA-3'). The PCR products were electrophoresed in 12 % agarose gels and stained with ethidium bromide.

2.7. Western Blot Analysis

The cells were seeded in 6-well culture plates at a density of 1×10^6 cells/well and grown in 2 ml of growth media for 24 h. Typically the cells were pretreated with various test materials for 1 h and then incubated for 24 hr and protein extracts were separated using 10 % SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose membranes. The membranes were pre-incubated with blocking solution (5 % skim milk in Tris buffered saline containing Tween-20) at room temperature for 2 h and then incubated with iNOS, ERK, p-ERK, JNK, p-JNK, P-38 and p-P-38 (1:1000) for 2 h at room temperature. After washing, the blots were incubated with horseradish peroxidase conjugated goat anti-mouse IgG secondary antibody (1:5000; Amersham Pharmacia Biotech, Little Chalfont, UK) for 30 min. The bands were visualized on X-ray film using ECL detection reagent (Amersham Biosciences, Piscataway, NJ, USA).

2.8. Statistical Analyses

Statistical analysis was performed by Student's *t*-test. A value of $p < 0.05$ based on at least three or more independent experiments was considered to be statistically significant.

3. Results

3.1. Purification Profiles of Inhibition NO Production Peptides from Abalone Intestine Digests

In prior studies, the formation of abalone intestine gastrointestinal digests (AIGIDs), two infant formulas, gastric digests (phase 1) and intestine digests (phase 2), with different behavior were subjected to a hydrolysis process, which simulates physiological digestion. Gastric digests (phase 1) corresponded to a pepsin-hydrolysed abalone protein-based formula and intestine digests (phase 2) to hydrolysed pepsin-hydrolysed abalone protein by two enzyme (trypsin and α-chymotrypsin). Further separated abalone intestine digests (phase 2) by four MW groups using UF membranes (MWCO=10, 5 and 1 kDa) into AIGID-I (M_W > 10 kDa), AIGID-II (M_W = 10-5 kDa), AIGID-III (M_W = 5-1 kDa) and AIGID-IV (M_W = 5 <1 kDa). The four groups were investigated by NO production activity. The results showed that AIGID-III with an MW=1-3 kDa exhibited inhibition NO production effects higher than those of other groups (data not shown). Therefore, AIGID III was selected for further purification.

The lyophilized AIGID III was dissolved in 20 mM sodium acetate buffer (pH 4.0), and using fast protein liquid chromatography (FPLC) onto a Hiprep 16/10 DEAE FF anion exchange column with a linear gradient of 2.0 M NaCl (0-100 %). Elution peaks were monitored at 280 nm, and each fraction was collected as 4 ml and fractionated into two non-adsorptive portion and two adsorptive portions (Figure 1A). Each fraction was pooled, lyophilized, and its inhibition NO production activity measured. Fraction IV exhibited the highest inhibition NO production activity (Figure 1B, 78.6% at 500 μg mL^{-1} in RAW264.7 cell). The lyophilized active fraction IV was further separated by RP-HPLC on a Primesphere $10C_{18}$ (20 mm×250 mm) column with a 15 % acetonitrile containing 0.1% trifluoroacetic acid (TFA). The fraction was divided into three clear fractions FrIV-1, FrIV-2, and FrIV-3 (target peptide) (Figure 2A). Each fraction was pooled, lyophilized, and its inhibition NO production activity measured. The FrIV-3 (target peptide) exhibited the highest inhibition NO production activity (Figure 2B, 82.5% at 200 μg/mL in RAW264.7 cells lines) then other fractions. FrIV-3 was pooled and further purified on a Synchropak RPP-100 analytical column (10 mm × 250 mm) using 10 % acetonitrile containing 0.1% TFA (Figure 2C). The amino acid sequence of the purified peptide was determined to be Pro-Phe-Asn-Glu-Gly-Thr-Phe-Ala-Ser (target peptide, M_W AAIP; 1175.2 Da, Figure 2D). The mass determined by ESI/MS spectroscopy was in excellent agreement with the theoretical mass according to the sequence.

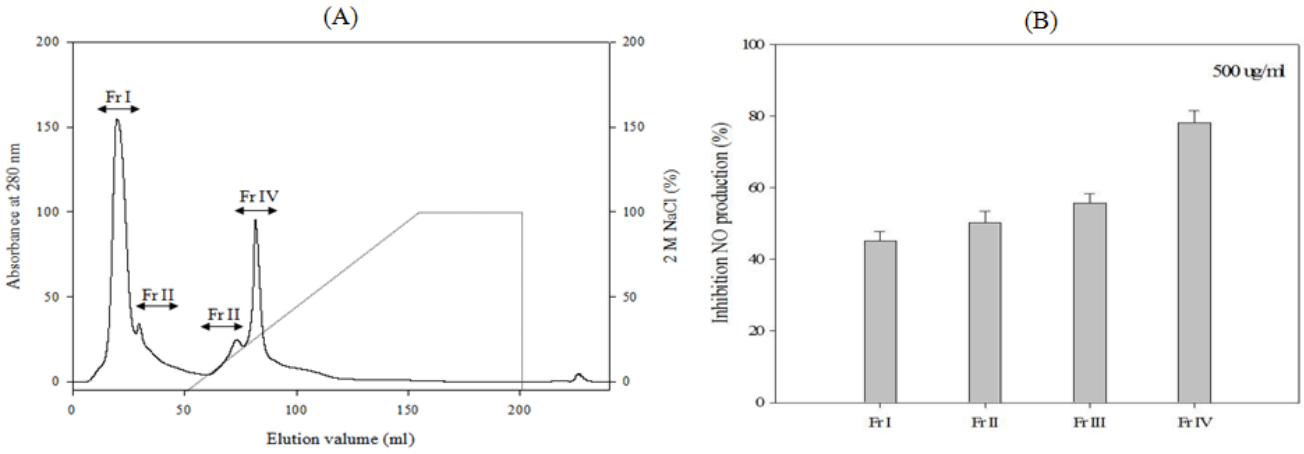

Figure 1. Purification of inhibition NO production peptides from AIGID III. (**A**) Fast protein liquid chromatography (FPLC) of AIGID III by Hiprep 16/10 DEAE FF anion exchange chromatography and elution was performed at 2.4 mL/min of flow rate with al linear gradient of 2.0 M NaCl (0-100%) in 20 mM sodium acetate buffer, pH 4.0. (**B**). Inhibition NO production activities of fractions on Hiprep 16/10 DEAE FF column using FPLC. Error bars represent mean and S.D from triplicate experiments after deduction of background values from raw values (Qian et al. (2016))

3.2. Cell Viability and Inhibition of NO Production by AAIP

The cytocompatible effects of the AAIP on viability of RAW 264.7 cells. The results suggest that AAIP displayed no cytotoxic effects at various concentration of 10-500 μM on these three tested cultured cell lines as shown in Figure 3A. Stimulation of RAW264.7 cells with LPS (100 ng/mL) showed a significant increase in cellular nitrite (NO) levels compared to the non-stimulated group. Different concentrations of AAIP were tested for the ability to inhibit this LPS stimulated NO production. NO is converted stable product of released NO by LPS stimulated cells. As shown in Fig. 3B, AAIP showed the highest potential to inhibit NO production in LPS stimulated RAW264.7 cells and the inhibitions were dose dependent (50-500 μM).

3.3. Inhibition of NO Production by AAIP through Suppressing the iNOS Expression and Inhibition of Pro-inflammatory Cytokines

To examine the action mechanism responsible for the inhibition of LPS stimulated NO production, mRNA and protein expression levels of iNOS were investigated. As shown in Figure 3A and Figure 3B, were clear that LPS treatment sufficiently increase iNOS mRNA and protein expression compared to the non-treated cells. This stimulated iNOS mRNA and protein expression levels

were evidently decreased with the AAIP treatment (10, 50, 100 and 250 μM) for 24 h. Collective analysis of this data shows that the inhibition of NO production is a resultant of down regulation of iNOS mRNA and protein expression by AID treatment in LPS stimulated RAW264.7 cells. The resulted dose dependent inhibition of NO aroused our interest on studying their respective gene and protein expressions. The RAW264.7 cells were stimulated with LPS for 24 h to analyze the effects of AAIP on iNOS transcription and translation, because in previous studies it has been reported that maximum iNOS mRNA expression occurs around this time period. As anticipated, both transcriptional and translational levels of iNOS were dose dependently inhibited by AAIP treatment.

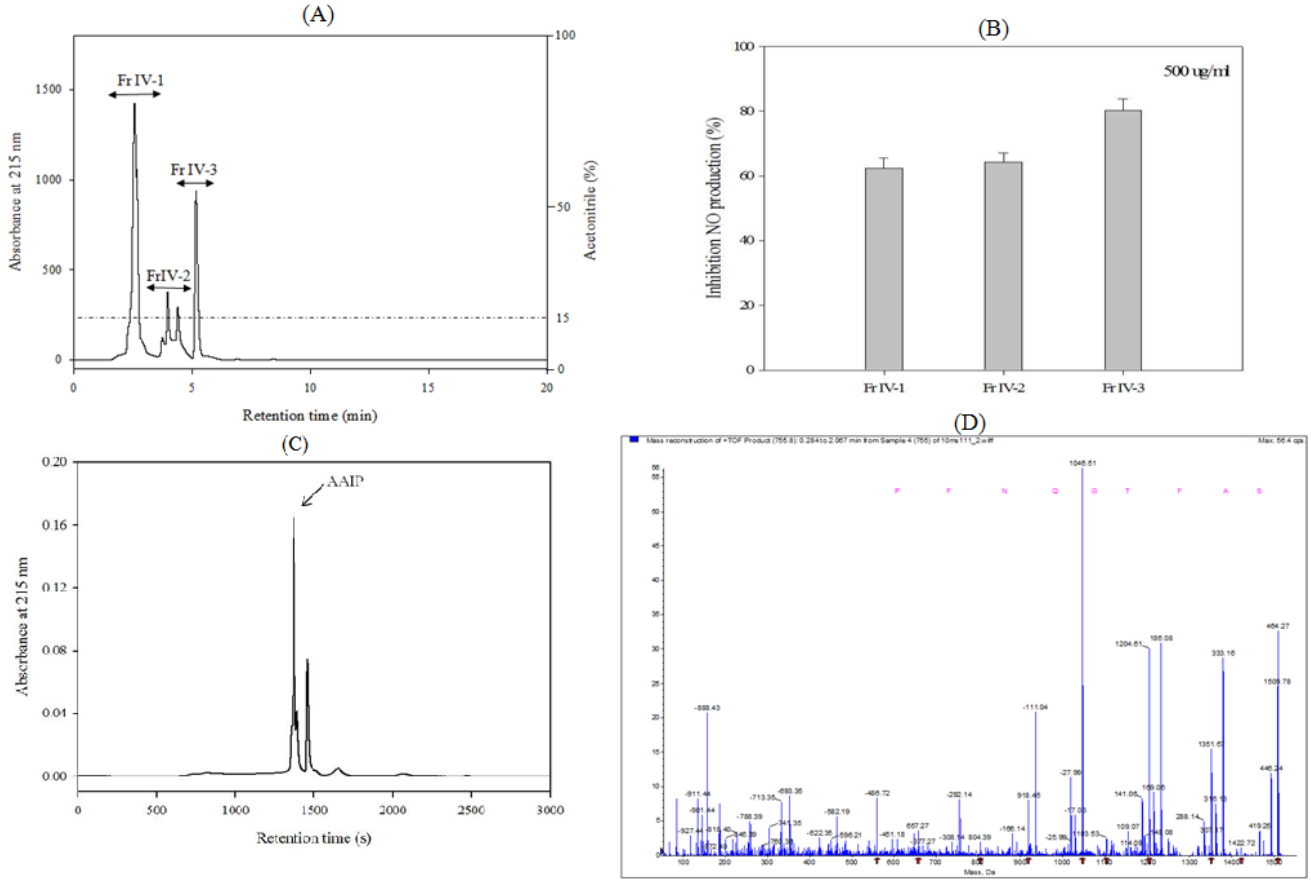

Figure 2. (**A**) Reversed-phase HPLC pattern on a Primesphere 10 C_{18} column of active fraction from Fr IV and HPLC operation was carried out with 15% acetonitrile as mobile phase at 1 mL/min of a flow rate using UV detector at 215 nm. (**B**). Inhibition NO production activities of fractions of Reversed-phase HPLC pattern on a Primesphere 10 C18 column chromatography. Error bars represent mean and S.D from triplicate experiments after deduction of background values from raw values. (**C**) Further separation of active fraction peak responsible for the highest production activity was finally purified on a Synchropak RPP-100 analytical column. HPLC operation was carried out with 10% acetonitrile as mobile phase at 1 mL/min of a flow rate using UV detector at 215 nm. (**D**). Identification of molecular mass and amino acid sequence of AAIP. MS/MS experiments were performed on a Q-TOF tandem mass spectrometer (Micromass Co., Manchester, UK) equipped with a nano-ESI source. Sequencing of active peptide was acquired over the m/z range 50-2500 and sequenced by using the PepSeq de nove sequencing algorithm (Qian et al. (2016))

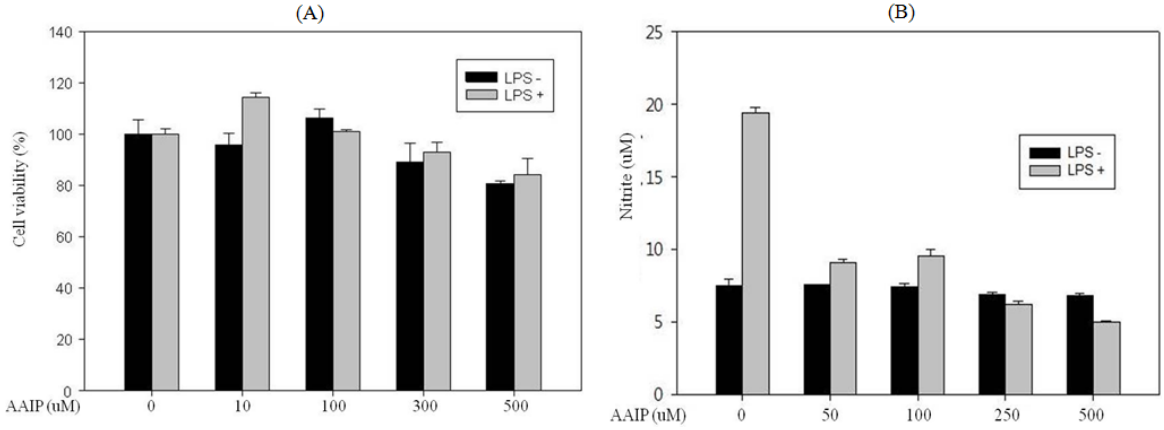

Figure 3. (**A**). The cytocompatible effects of the AAIP on viability of RAW 264.7 cells. Cells were treated with AAIP at the indicated concentrations (0-500 μM). After 24 h to treat AAIP, cell viability was assessed by MTT assay as described in the text. Results are means ± standard error of three independent experiments. (**B**). Effects of AAIP on LPS-induced NO production in RAW264.7 cells. Cells cultured in serum-free media were pretreated with different concentrations (10-500 μM) of AAIP for 1 h, and then stimulated with LPS (100 ng/mL). Conditioned medium was collected after 48 h and NO concentrations were measured using the Griess reaction (Qian et al. (2016))

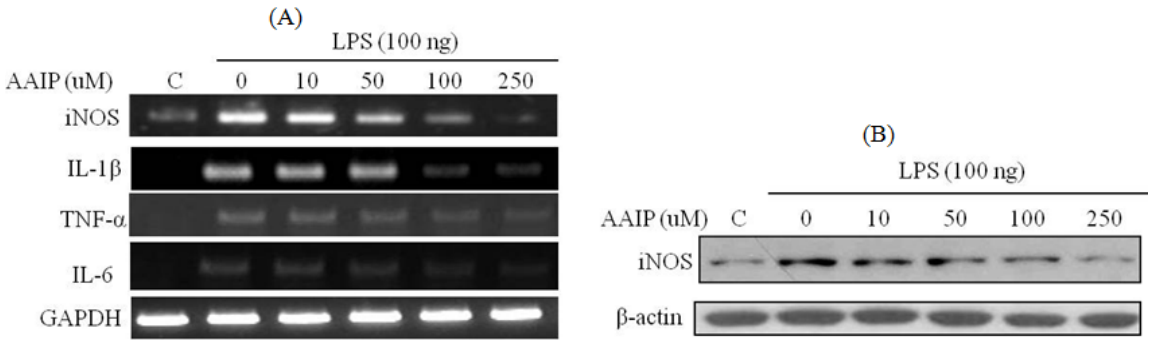

Figure 4. Effects of AAIP on LPS-induced mRNA and iNOS protein expression in RAW264.7 cells. Cell cultured in serum-free media were pretreated with different concentrations (10-250 μM) of AAIP for 1 h, and then stimulated with LPS (100 ng/mL). Cell lysates were extracted, and gene and protein levels of iNOS were analyzed by RT-PCR and Western blotting respectively. GAPDH and β-actin expressions were used as an internal control for gene and protein in RT-PCR and Western blotting respectively. (**A**). Cell lysates were extracted, and gene (IL-1β, TNF-α, and IL-6) were analyzed by RT-PCR. (**B**). Cell lysates were extracted, protein levels of iNOS were analyzed by Western blotting respectively (Qian et al. (2016))

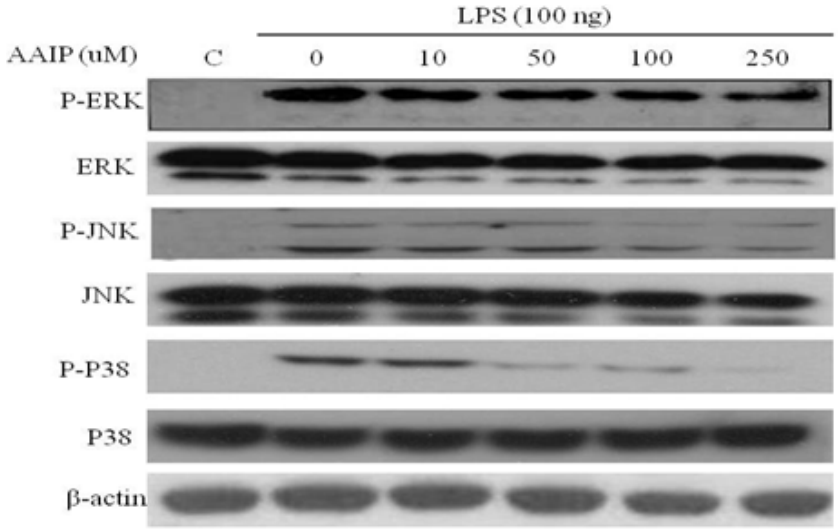

Figure 5. Effects of AAIP on LPS-induced phosphorylation of ERK 1/2, JNK 1/2 and p38 MAP kinase in RAW264.7 cells. Cell were treated with vehicle or the indicated concentrations of AAIP (10-250 μM) for 1 h before incubation with LPS (100 ng/mL). Cell lysates were then prepared and subjected to Western blotting with antibodies specific for phosphorylated forms of ERK 1/2, JNK 1/2 and p38. Results represent three independent experiments (Qian et al. (2016))

To investigate more about the potential anti-inflammatory activities of AAIP, its effect on production and transcription of pro-inflammatory cytokines; the TNF-α, IL-1β, and IL-6 were analyzed (Figure 4A). We performed RT-PCR to determine whether AAIP inhibits the expression of these cytokines at a transcriptional level. As shown in Figure 4A, due to treatment of AAIP (10, 50, 100 and 250 μM), LPS-induced production of IL-6, IL-1β, and TNF-α mRNA transcriptional levels showed a significant decrease and it was concentration dependent. These results strongly suggest that AAIP treatment is effective in suppressing the production of pro-inflammatory cytokines at their transcriptional level in LPS-induced RAW264.7 cells.

3.4. AAIP down Regulate the MAPK Signaling

Inflammation could be induced by different signaling pathways. MAPK pathway is identified as one the major signaling pathway mediating inflammatory responses. To find out the pathways that may probably involve in above explained inhibitions of iNOS and pro-inflammatory cytokines by AAIP, non-phosphorylation and phosphorylation of three different MAPK molecules; p38, Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), were analyzed. LPS induce rapid phosphorylation of ERK, JNK and p38, and it has been reported that this induction of phosphorylation is done within 10-30 min of LPS treatment. Therefore, cells for Western blots were collected after 1 h of AAIP treatment followed by 30 min of LPS (100 ng/mL) stimulation. As shown in Fig. 5, the inhibitory action was dose dependent on JNK, ERK and p38 phosporylation by AAIP (10, 50, 100 and 250 μM). Therefore, these results indicated that inhibition of iNOS mediated via inhibition of signal transduction through MAP kinases mediated pathways in LPS stimulated RAW264.7 cells.

4. Discussion

Abalone is a marine gastropod and an important fishery and food industrial resource that is massively fishery aquaculture in Asia, Africa, Australia, and America. To meet the increasing demand of the Asian market, abalone mariculture has been expanding in land- and sea-based systems in S. Korea and the total yield was estimated at 7,580 tons of abalone in 2009 (The Korea Marine Institute). In addition, manufacturing products of abalone (dried, steamed, spiced abalone, and so on) have also been

significantly increased [10]. It is currently agreed that that marine organisms possess various bioactive natural components with many nutraceutical and pharmaceutical activities-related physiological functions such as antioxidant, anti-inflammatory, antitumor, antimicrobial, antihypertension, anticoagulation, anti-cardiovascular disease, and etc [14]. However, the health beneficial effects of abalone have rarely been reported, although a number of studies have been performed on the biologicaland physiological properties of abalones, considering pathology, genetics, and aquaculture technology [15,16,17,18].

Recently, bioactive peptides from protein hydrolysates have received much attention due to unraveling the structural, compositional, and sequential properties and their biological activities. They can be used as versatile raw materials for producing nutraceuticals and pharmaceuticals for humans. The bioactive peptides have been obtained from various raw materials that contain high protein concentration [19,20]. Thus, abalone (*Haliotis discus hannai*) is considered a great source to produce bioactive peptides such as anti-inflammatory peptides. Its was hydrolysed with gastro intestinal endopeptidases including pepsin, trypsin, and a-chymotrypsin under optimal conditions. It is believed that prior hydrolysis with the endopeptidases may increase the bioavailability of the active peptide and avoid the further digestion in the gastrointestinal tract. The previous studies have shown that gastrointestinal enzyme digestion results in more potent peptides compared with single enzyme digestions [19]. Notably, the bioactive peptides with low molecular weight are possible to cross the intestinal barrier. A number of studies have confirmed that low molecular weight peptides show potent anti-inflammatory bioactivities [19,21,22,23]. Lee et al. [21] has prepared egg white peptides with M_W < 1.3 kDa, and these peptides could attenuate the symptoms of inflammatory bowel disease. Both anti-inflammatory peptides, LDAVNR (M_W = 683 Da) and MMLDF (M_W = 655 Da) have been isolated from microalgae *spirulina maxima* [19]. Our study also revealed that the AAIP (Pro-Phe-Asn-Glu-Gly-Thr-Phe-Ala-Ser, 1175.2 Da) isolated from abalone intestines se exhibited potent anti-inflammatory effect (Figure 2).

Macrophages are the major cells involved in inflammation and they are responsible for the major functions of inflammation, particularly immune-modulation through the production of various cytokines and growth factors [24]. Inflammation is a complex biological process in which macrophages play a critical role in the initiation, maintenance, and resolution, including the overproduction of pro-inflammatory cytokines and inflammatory mediators such as TNF-a, IL-1b, IL-6, NO and prostaglandin(PGE2) [25,26,27]. TNF-a and IL-1b are proinflammatory cytokines which mediate both acute and chronic inflammation by triggering a cascade of inflammatory mediators, like platelet-activating factor and leukotrienes, prostaglandins, NO, IL-6 and IL-8 [28,29]. Suppressing production of TNF-a and IL-1b in vitro and in vivo has been widely applied to screen anti-inflammatory agents [27,30]. IL-6 is an interleukin that acts as both a pro-inflammatory and anti-inflammatory cytokine [31,32]. Although IL-6 can down-regulate the synthesis of IL-1 and TNF-a [32], overproduction of IL-6 has been shown to underlie a number of autoimmune and inflammatory diseases, and blocking of IL-6 signaling is considered to be therapeutic in diseases characterized by pathological IL-6 overproduction [33]. The agents which can reduce production of IL-6 are thought to be anti-inflammatory [34,35]. In cell experiment, AAIP significantly reduced release of proinflammatory factors including TNF-a, IL-1b, IL-6 and NO in LPS activated RAW264.1 cells (Figure 4).

Studies on signaling pathways that regulate anti-inflammatory activity play a vital role in drug discovery [36]. Inflammation could be induced by different signaling pathways. We determined which signaling pathways were involved in the AAIP-mediated regulation of iNOS and pro-inflammatory cytokine production induced by LPS. MAPK pathway is identified as one the major signaling pathway mediating inflammatory responses [37,38]. MAPKs (ERK 1/2, p38, and JNK) are a group of serine/threonine kinases that are activated in response to a diverse array of extracellular stimuli and that mediate signal transduction from the cell surface to the nucleus [39]. Furthermore, MAPKs have previously been implicated in the signaling pathways relevant to LPS-induced inflammation. The phosphorylation and activation of three major MAPKs have been shown to initiate inflammatory gene expression in LPS-induced macrophages [40]. LPS treatment results in regulating the expression of iNOS via MAPK signaling pathway by phosphorylating p38, ERK and JNK, which are the main three distinct groups of MAPK subfamily [41]. Therefore, inhibition of the phosphorylation of these molecules will be therapeutically valued. In our investigations of LPS-induced phosphorylation of these molecules, p38, JNK and ERK showed a dose dependant inhibition of its phosphorylation, whereas the observed inhibition of ERK phosphorylation was not dose dependent. Also we investigated the inhibition of total forms of MAPK molecules by AAIP treatment and only p38 and JNK molecules were inhibited dose dependently and ERK was not inhibited. Collectively, these results suggest that p38 and JNK pathways would be the molecular mechanisms mediating the inhibitory activities of iNOS expressions following AAIP. In addition, in previous studies it has been reported that JNK and p38 are important modulators of iNOS and proinflammatory cytokine production, and their down regulation may result in inhibition of those inflammatory mediators [42].

5. Conclusions

Considering all obtained results it could be concluded that AAIP attenuated the LPS-induced gene and protein expression of iNOS and pro-inflammatory cytokines probably via MAPK, ERK and p38MAPK pathway. Thus it could be suggested that AAIP possess potential in anti-inflammation therapy and could be used as an effective functional food. Finally the authors like to declare that this is the first report of the anti-inflammatory effects of peptide from abalone (*Haliotis discus hannai*) intestine gastrointestinal digests on LPS-induced murine macrophages, up to the best of our knowledge.

Acknowledgements

This research was supported by Marine Biotechnology Program (20150220) and a part of the titled 'Development of biomedical materials for regenerating bone tissue using bioactive substances from marine algae' funded by Ministry of Oceans and Fisheries, Republic of Korea. And this research also supported from the project sponsored by the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry (The Project-sponsored by SRF for ROCS, SEM).And also this research was supported by Yangfan Scarce Top Talent Project of Guangdong Province [to Zhong-Ji Qian].

References

[1] Sebban, H., Courtois, G. NF-kB and inflammation in genetic disease. *Biochemical Pharmacology,* 7. 1153-1160. 2006.

[2] Lee, H.J., Hyun, E.A., Yoon, W.J., Kim, B.H., Rhee, M.H., Kang, H.K., Cho, J.Y., Yoo, E.S. In vitro anti-inflammatory and anti-oxidative effects of Cinnamomum camphora extracts. *Journal of Ethnopharmacology,* 103. 208-216. 2006.

[3] Walsh, N.C., Crotti, T.N., Goldring, S.R., Gravallese, E.M. Rheumatic diseases: the effects of inflammation on bone. *Immunological Reviews,* 208. 228-251. 2005.

[4] Wong, J.M., Billiar, T.R. Regulation and function of inducible nitric oxide synthase during sepsis and acute inflammation. *Advances in Pharmacology,* 34. 155-170. 1995.

[5] Inoue, H., Kubo, M. SOCS proteins in T helper cell differentiation: implications for allergic disorders?. *Expet Reviews in Molecular Medicine,* 6. 1-11. 2004.

[6] Jung, W.K., Rajapakse, N., Kim SK. Antioxidative activity of low molecular peptide derived from the sauce of fermented blue mussel, Mytilus edulis. *European Food Research Technology,* 220. 535-539. 2005.

[7] Rajapakse, N., Mendis, E., Jung, W.K., Je, J.Y., Kim, S.K. Purification of radical scavenging peptide from fermented mussel sauce and its antioxidant properties. *Food Research International,* 38. 175-182. 2005.

[8] Ryu, B.M., Qian, Z.J., Kim, S.K. SHP-1, a novel peptide isolated from seahorse inhibits collagen release through the suppression of collagenases 1 and 3, nitric oxide products regulated by NF-kB/p38 kinase. *Peptides,* 31. 79-87. 2010.

[9] Kapsokefalou, M., Miller, D.D. Effects of meat and selected food components on the valence of nonheme iron during *in vitro* digestion. *Journal of Food Science,* 56. 352-355. 1991.

[10] Qian, Z.J., Kim, S.A., Lee, J.S., Kim, H.J., Choi, I.W., Jung, W.K. The Antioxidant and Anti-inflammatory Effects of Abalone Intestine Digest, Haliotis discus hannai in RAW 264.7 Macrophages. *Biotechnology and Bioprocess Engineering,* 17. 475-484. 2012.

[11] Hansen, M.B., Nielson, S.E., Berg, K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *Journal of Immunological Methods,* 119. 203-210. 1989.

[12] Lee, M.H., Lee, J.M., Jun, S.H., Lee, S.H., Kim, N.W., Lee, J.H., Ko, N.Y., Kun, S.H., Kim, B.K., Lim, B.O., Choi, D.K., Choi, W.S. The anti-inflammatory effects of Pyrolae herba extract through the inhibition of the expression of inducible nitric oxide synthase (iNOS) and NO production. *Journal of Ethnopharmacology,* 112. 49-54. 2007.

[13] Ren, G.L., Bai, X.F., Zhang, Y., Chen, H.M., Huang, C.X., Wang, P.Z, et al. Stable inhibition of hepatitis B virus expression and replication by expressed siRNA. *Biochemical Biophysical Research Communications,* 335. 1051-1059. 2005.

[14] Mayer, A.M.D., Rodríguez, A.D., Berlinck, R., Fusetani, N. Marine pharmacology in 2007-2008: Marine compounds with antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiprotozoal, antituberculosis, and antiviral activities; affecting the immune and nervous system, and other miscellaneous mechanisms of action. *Comparative Biochemistry and Physiology C,* 153. 191-222. 2011.

[15] Sun, L., Zhu, B., Li, D., Wang, L., Dong, X., Murata, Y., Xing, R., Dong, Y. Purification and bioactivity of a sulphated polysaccharide conjugate from viscera of abalone *Haliotis discus* hannai Ino. *Food and Agriculture Immunology,* 21. 15-26. 2010.

[16] Ekanayake, P.M., De, Zoysa M., Kang, H.S., Wan, Q., Jee, Y., Lee, Y.H., Kim, S.J., Lee, J. Cloning, characterization and tissue expression of disk abalone (*Haliotis discus discus*) catalase. *Fish and Shellfish Immunology,* 24. 267-278. 2008.

[17] Zoysa, M.D., Whang, I., Lee, Y.D., Lee, S.K., Lee, J.S., Lee, J. Transcriptional analysis of antioxidant and immune defense genes in disk abalone (*Haliotis discus discus*) during thermal, low-salinity and hypoxic stress/comparative biochemistry and physiology. *Comparative Biochemistry and Physiology B,* 154. 387-395. 2009.

[18] Wan, Q., Whang, I., Lee, J. Molecular characterization of mu class glutathione-Stransferase from disk abalone (*Haliotis discus discus*), a potential biomarker of endocrine-disrupting chemicals. *Comparative Biochemistry and Physiology B,* 150. 187-199. 2005.

[19] Vo, T.S., Ryu, B.M., Kim, S.K. Purification of novel anti-inflammatory peptides from enzymatic hydrolysate of the edible microalgal Spirulina maxima. *Journal of Functional Foods,* 5. 1336-1246. 2013.

[20] Harnedy, P.A., FitzGerald, R.J. Bioactive peptides from marine processing waste and shellfish: A review. *Journal of Functional Foods,* 4. 6-24. 2012.

[21] Lee, M., Kovacs-Nolan, J., Archbold, T., Fan, M.Z., Juneja, L.R., Okubo, T., Mine, Y. Therapeutic potential of hen egg white peptides for the treatment of intestinal inflammation. *Journal of Functional Foods,* 1(2). 161-169. 2009.

[22] Hwang, J.W., Lee, S.J., Kim, Y.S., Kim, E.K., Ahn, C.B., Jeon, Y.J., Moon, S.H., Jeon, B.T., Park, P.J. Purification and characterization of a novel peptide with inhibitory effects on colitis induced mice by dextran sulfate sodium from enzymatic hydrolysates of *Crassostrea gigas*. *Fish and Shellfish Immunology,* 33(4). 993-999. 2012.

[23] Lee, S.J., Kim, E.K., Kim, Y.S., Hwang, J.W., Lee, K.H., Choi, D.K., Kang, H., Moon, S.H., Jeon, B.T., Park, P.J. Purification and characterization of a nitric oxide inhibitory peptide from *Ruditapes philippinarum*. *Food Chemistry and Toxicology,* 50(5). 1660-1666. 2012.

[24] Fujiwara, N., Kobayashi, K. Macrophages in inflammation. *Current Drug Targets,* 4. 281-286. 2004.

[25] Mosser, D.M., Edwards, J.P. Exploring the full spectrum of macrophage activation. *Nature Reviews Immunology,* 8. 958-969. 2008.

[26] Kim, E.Y., Moudgil, K.D. Regulation of autoimmune inflammation by proinflammatory cytokines. *Immunology Letters,* 120. 1-5. 2008.

[27] Yoon, W.J., Ham, Y.M., Kim, S.S., Yoo, B.S., Moon, J.Y., Baik, J.S., Lee, N.H., Hyun, C.G. Suppression of pro-inflammatory cytokines, iNOS, and COX-2 expression by brown algae Sargassum micracanthum in RAW 264.7 macrophages. *European and Asian Journal of BioScience,* 3. 130-143. 2009.

[28] Dinarello, C.A. Proinflammatory cytokines. *Chest,* 118. 503-508. 2000.

[29] Feghali, C.A., Wright, T.M. Cytokines in acute and chronic inflammation. *Frontieres in Bioscience,* 2. 12-26. 1997.

[30] Dinarello, C.A. Anti-inflammatory agents: present and future. *Cell,* 140. 935-950. 2010.

[31] Petersen, A.M., Pedersen, B.K. The role of IL-6 in mediating the anti-inflammatory effects of exercise. *Journal of physiology and pharmacology,* 57. 43-51. 2006.

[32] Opal, S.M., DePalo, V.A. Anti-inflammatory cytokines. *Chest,* 117. 1162-1172. 2000.

[33] Nishimoto, N. Interleukin-6 as a therapeutic target in candidate inflammatory diseases. *Clinical Pharmacology and Therapeutics,* 87. 483-487. 2010.

[34] Park, J.S., Lee, E.J., Lee, J.C., Kim, W.K., Kim, H.S. Anti-inflammatory effects of short chain fatty acids in IFN-[gamma]-stimulated RAW 264.7 murine macrophage cells: involvement of NF-[kappa] B and ERK signaling pathways. *International immunopharmacology,* 7, 70-77. 2007.

[35] Tedelind, S., Westberg, F., Kjerrulf, M., Vidal, A. Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: a study with relevance to inflammatory bowel disease. *World Journal of Gastroenterology,* 13. 2826-2832. 2007.

[36] Lee, J.H., Jung, H.S., Giang, P.M., Jin, X., Lee, S., Son, P.T et al. Blockade of nuclear factorkappaBsignaling pathway and anti-

inflammatory activity of cardamomin, a chalcone analog from Alpinia conchigera. *Journal of Pharmacology and Experimental Therapeutics*, 316. 271-278. 2006.

[37] Jung, H.W., Yoon, C.H., Park, K.M., Han, H.S., Park, Y.K. Hexane fraction of Zingiberis Rhizoma crudus extract inhibits the production of nitric oxide and pro-inflammatory cytokines in LPS stimulated BV2 microgilial cells via the NF-kB pathway. *Food Chemistry and Toxicology*, 47. 1190-1197. 2009b.

[38] Himaya, S.W.A., Ryu, B.M., Qian, Z.J., Kim, S.K. Sea cucumber, Stichopus japonicus ethyl acetate fraction modulates the lipopolysaccharide induced iNOS and COX-2 via MAPK signaling pathway in murine macrophages. *Environmental Toxicolocy and Pharmacology*, 30. 68-75. 2010.

[39] Cobb, M.H., Goldsmith, E.J. How MAP kinases are regulated. *Journal of Biological Chemistry*, 270. 14843-14846. 1995.

[40] Hommes, D.W., Peppelenbosch, M.P., van Deventer, S.J. Mitogen activated protein (MAP) kinase signal transduction pathways and novel anti-inflammatory targets. *Gut*, 52, 144-151. 2004.

[41] Cario, E., Rosenberg, I.M., Brandwein, S.L., Beck, P.L., Reinecker, H.C., Podolsky, D.K. Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. *Journal of Immunology*, 164, 966-972. 2000.

[42] Jongeneel, C.V. Transcriptional regulation of the tumor necrosis factor gene. *Immunobiology*, 193. 210-216. 1995.