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Effects of γ-Aminobutyric Acid (GABA)-Enriched Sea Tangle *Laminaria japonica* Extract on Lipopolysaccharide-Induced Inflammation in Mouse Macrophage (RAW 264.7) Cells

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Abstract

γ-Aminobutyric acid-enriched sea tangle extract was obtained from the fermentation of *Lactobacillus brevis* BJ-20. The fermented sea tangle extract (FST) was separated into three fractions by molecular weight: FST I (greater than 10 kDa), FST II (1-10 kDa), and FST III (less than 1 kDa). The anti-inflammatory characteristics of the FST fractions were investigated by measuring the production of nitric oxide (NO) and the expression levels of inducible nitric oxide synthase (iNOS) in lipopolysaccharide (LPS)-induced mouse macrophage (RAW 264.7) cells. Both NO production and iNOS expression levels were significantly inhibited by FST treatments in a dose-dependent manner. FST III was the most effective inhibitor of processes. This demonstrates that the effect of FST on LPS-induced inflammation might be closely correlated with the inhibition of inflammatory cytokine expression.

Key words: Sea tangle, *Laminaria japonica*, Anti-inflammatory activity, γ-Aminobutyric acid, RAW 264.7 cells

Introduction

Inflammation results from a host response to pathogenic challenges or tissue injuries, and ultimately leads to the restoration of normal tissue structure and function. Normal inflammatory responses are self-limited by a process that involves the down-regulation of pro-inflammatory proteins and the up-regulation of anti-inflammatory proteins (Lawrence et al., 2002). Thus, the physiological benefits of acute inflammation are limited, particularly in response to infectious pathogens, and chronic inflammation can lead to several inflammatory diseases (Kaplanski et al., 2003). Prolonged inflammation contributes to the pathogenesis of inflammatory diseases such as bronchitis, gastritis (Fichtner-Feigl et al., 2005), inflammatory bowel disease, multiple sclerosis (Klotz et al., 2005), and rheumatoid arthritis (Ponchel et al., 2002). Macrophages play an important role in a variety of disease processes, including autoimmune diseases, infection, and inflammatory disorders (Pierce, 1990).

Lipopolysaccharide (LPS), an endotoxin derived from the outer membrane of Gram-negative bacteria, can directly activate macrophages to produce a variety of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukins (ILs), and other inflammatory mediators such as prostaglandins and nitric oxide (NO) (Yoon et al., 2011). NO is endogenously produced from L-arginine and molecular oxygen by NO synthases (NOSs). In mammals, there are three isoforms of NOS: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). nNOS and eNOS are constituvely expressed in neuronal and endothelial cells, respectively. In contrast, iNOS is inducible and its expression is increased in cells that are exposed to LPS or cytokines (Liu

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et al., 1998). Accordingly, high expression levels and activity of iNOS are observed in patients with chronic diseases such as inflammation and cancer (Liu et al., 1998; Maeda and Akaike, 1998).

Marine algae have been identified as rich sources of structurally diverse bioactive compounds with great pharmaceutical potential (Abad et al., 2008; Blunt et al., 2010). A variety of biological compounds including phlorotannins and fucoxanthin, have been isolated from Laminariacceae and characterized with regard to biological activity (Okuzumi et al., 1993; Kim et al., 2005; Woo et al., 2009). Sea tangle, *Laminaria japonica,* is a marine brown alga that is commonly used as a seasoning, condiment and health food in Korea, Japan, and China. A variety of *in vitro* and *in vivo* studies have focused on the antioxidative (Huang and Wang, 2004; Park et al., 2009) and chemopreventive (Zhang et al., 2008) activities of extracts from *L. japonica.* The present report is part of an ongoing investigation of the anti-inflammatory properties of *L. japonica*. There are no reports describing the anti-inflammatory activity of γ-Aminobutyric acid (GABA)-enriched sea tangle extract (FST). This prompted an investigation of the signaling mechanism between FST and inflammatory proteins in LPS-induced mouse macrophage (RAW 264.7) cells. This investigation included the anti-inflammatory activity of FST and its possible mechanisms in LPS-induced RAW 264.7 cells.

Materials and Methods

Preparation of FST

Sea tangle was added to water at a ratio of 1:15 (w/v), and 2% (w/w of dry sea tangle) rice flour was added to aid fermentation. After autoclaving at 121°C for 30 min, a *Lactobacillus brevis* BJ20 (accession no. KCTC 11377BP) culture was added to the sea tangle extract at a concentration of 2% (v/v). After thorough mixing, the sample was incubated at 37°C (Cha et al., 2011). The fermented product was obtained by filtration and was freeze-dried. GABA-enriched FST was separated into three fractions by molecular weight: FST I (more than 10 kDa), FST II (1-10 kDa), and FST III (less than 1kDa) by ultrafiltration.

Cell culture and cell viability assay

RAW 264.7 cells were grown to confluence in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2. The cytotoxicity of the various components of the FST fractions was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, a method based on MTT (Weislow et al., 1989). The medium containing RAW 264.7 cells was cultured in a 96-well plate at a density of 10^5 cells per mL. The plate was incubated overnight and treated with 100 μL of DMEM medium containing different concentrations on FST. After 24 h of incubation, an MTT solution was added to each well and the plate was incubated for another 4 h at 37°C. The blue formazan salt was dissolved in dimethyl sulfoxide. Optical density was measured at 540 nm with a GENios microplate reader (Tecan, Austria GmbH, Grödig, Austria). The optical density of formazan formed by untreated cells was taken as the measure of 100% viability.

Measurement of NO

RAW 264.7 cells (1×10^6) were plated and incubated for 24 h with 0-100 μg per mL FST in the absence or presence of LPS (1 μg/mL). After treatment with LPS and FSTs, the nitrite concentration in the culture medium was measured as an indicator of NO production, according to the Griess reaction (Kim et al., 1995). For this measurement, 100 μL of culture supernatant was mixed with the same volume of Griess reagent (0.1% naphytylethylenediamine dihydrochloride and 1% sulfanilamide in 5% H_3PO_4), and the absorbance of the mixture was measured at 540nm with a microplate reader (Ultraspec 2100 Pro; Amersham Biosciences, Buckinghamshire, UK). The concentration of nitrite was calculated using sodium nitrite as a standard.

RNA extraction and reverse transcription-polymerase chain reaction

Total RNA was isolated using a Trizol reagent (Invitrogen Co., Carlsbad, CA, USA) following the manufacturer's recommendations. Total RNA was digested with RNase-free DNase (Roche, Indianapolis, IN, USA) for 15 min at 37°C and repurified using an RNeasy kit according to the manufacturer's protocol (Quiagen, Valencia, CA, USA). cDNA was synthesized in accordance with the manufaturer's instruction. Briefly, 2 μg of total RNA were incubated at 37°C for 1 h with AMV reverse transcriptase (Amersham Biosciences) and random hexanucleotides. The primers used to specifically amplify the genes of interest are given in Table 1. Amplification was performed in a master-cycler (Eppendorf, Hamburg, Germany) with denaturation at 95°C for 30 s, annealing at 60°C for 45 s, and extension at 72°C for 1 min. The amplified polymerase chain reaction (PCR) products were separated in 1.0% agarose gels and visualized by staining with ethidium bromide (EtBr).

Table 1. Gene-specific primers used for the reverse transcription PCR

Gene	Direction	Sequence
iNOS	Forward	5´-CAC CTT GGA GTT CAC CCA GT-3´
	Reverse	5´-ACC ACT CGT ACT TGG GAT GC-3´
β-actin	Forward	5´-CCA CAG CTG AGA GGG AAA TC-3´
	Reverse	5´-AAG GAA GGC TGG AAA AGA GC-3´

iNOS, inducible nitric oxide synthase.

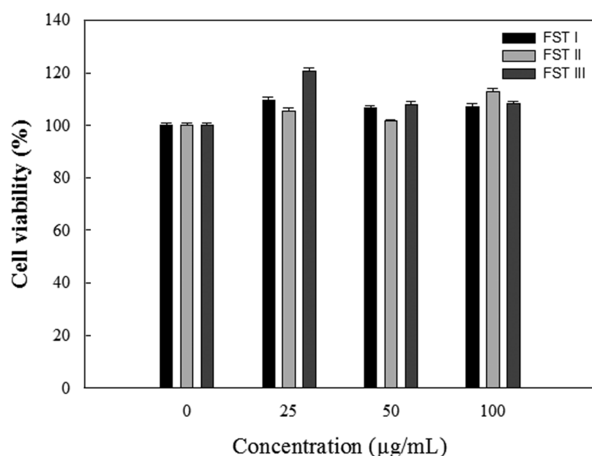

Fig. 1. Effect of fermented sea tangle extract (FST) on cell cytotoxicity in lipopolysaccharide-induced Raw 264.7 cells. FST I, >10 kDa; FST II, 1-10 kDa; FST III, <1 kDa.

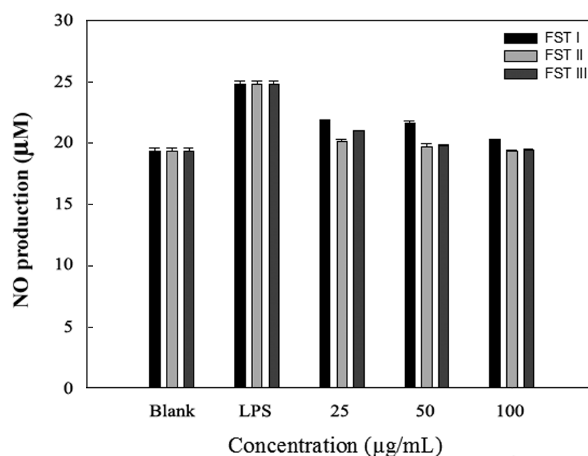

Fig. 2. Effect of fermented sea tangle extract (FST) on nitric oxide (NO) production in lipopolysaccharide (LPS)-induced Raw 264.7 cells. Blank, -LPS; LPS, +LPS (1 μg/mL); FST I, >10 kDa; FST II, 1-10 kDa; FST III, <1 kDa.

Western blot analysis

Western blotting was performed according to standard procedures. Briefly, cells were lysed at 4°C for 30 min in RIPA buffer containing 50 mM Tris-HCl (pH 8.0), 0.4% Nonidet P-40, 120 mM NaCl, 1.5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, leupeptin (80 μg/mL), 3 mM NaF and 1 mM dithiothreitol. Cell lysates (50 μg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech., Buckinghamshire, UK), blocked with 5% skim milk, and hybridized with primary antibodies (diluted 1:1000). After incubation with horseradish-peroxidase-conjugated secondary antibodies at room temperature, immunoreactive proteins were detected using a chemiluminescent ECL assay kit (Amersham Biosciences) according to the manufacturer's instructions. Western blot bands were visualized using a LAS3000 Luminescent image analyzer (Fujifilm Life Science, Tokyo, Japan).

Results and Discussion

Effects of FSTs on RAW 264.7 cell viability

Inflammation is the first response of the immune system to infection or irritation. During inflammation, macrophages provide a host defense against extracellular foreign agents and stimuli. LPS is one of the major inflammatory stimuli that can induce the production of a variety of pro-inflammatory cytokines, including TNF-α, IL-1, and IL-6 in macrophages. Thus, inhibitors of these cytokines have been considered as candidate for anti-inflammatory drugs (Yoon et al., 2011).

In East Asia, sea tangle is consumed in a variety of ways: as a side dish, in soups and salads, and as a sandwich ingredient. Traditionally, sea tangle has been used to lessen inflammation in oriental medicine. However, few studies have directly and systematically evaluated the effects of sea tangle extract on inflammation. This study examines the anti-inflammatory activity of FST using LPS-induced Raw 264.7 cells.

The cytotoxicity of FSTs on LPS-induced RAW 264.7 cells was determined using MTT assays. LPS-induced RAW 264.7 cells were treated with FSTs (0, 25, 50 or 100 μg/mL). As shown in Fig. 1, the FSTs did not show any significant cytotoxicity at concentrations less than 100 μg/mL. Thus, FSTs were deemed safe for *in-vitro* cell culture experiments at concentrations up to 100 μg/mL. Song et al. (2011) also reported a negligible cytotoxicity for *Hizikia fusiforme* extract fermented by *L. brevis* BJ20 at concentration of 100 μg per mL.

Effects of FSTs on NO production in RAW 264.7 cells

NO, an important cellular secondary messenger is a free radical that is produced at the cellular level from its precursor L-arginine by NOSs. Several beneficial biological activities have been attributed to NO, including antimicrobicidal, antiviral, immunomodulatory, and antitumoral effects. However, high levels of NO are involved in the physiology and pathophsiology of several human diseases, and its uncontrolled release can cause destruction of target tissues during the inflammation processes (Yoon et al., 2011).

LPS can induce the formation of iNOS and NO in macrophage cells. Therefore, we investigated the inhibition of NO production by FSTs in LPS-induced RAW 264.7 cells. As an indicator of NO production, nitrite (NO_2^-) accumulation was measured in the culture media using the Griess method (Yoon et al., 2011). Fig. 2 shows that the FSTs inhibit LPS-induced NO production in macrophages. The inhibition may be due to

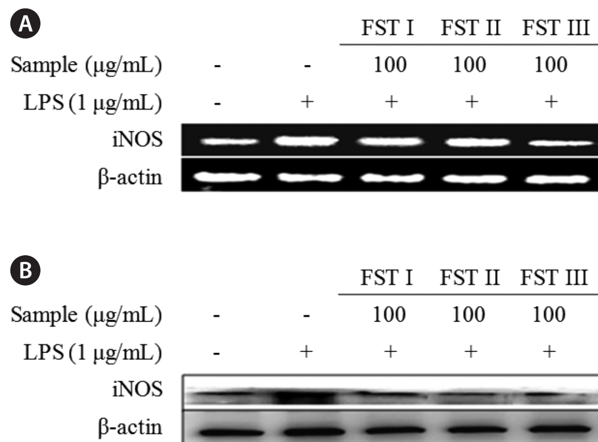

Fig. 3. Effect of fermented sea tangle extract (FST) on inducible nitric oxide synthase (iNOS) expression of mRNA (A) and protein (B) level in lipopolysaccharide (LPS)-induced Raw 264.7 cells.

the suppression of LPS-induced iNOS transcription. Song et al. (2011) previously reported that *H. fusiforme* extract fermented by *L. brevis* BJ20 inhibits NO production in LPS-stimulated HepG2 cells. Eom et al. (2010) reported the inhibition of NO production by sea tangle extract fermented with yeast in LPS-induced Raw 264.7 cells. To the best of our knowledge, the current report is the first to describe the inhibitory effects of FSTs on NO production in RAW 264.7 cells.

Effects of FSTs on the regulation of inflammatory response genes and proteins

FST treatment of LPS-activated RAW 264.7 macrophages resulted in a decrease in NO production. Nuclear factor-κB is critical to the regulation of cell survival genes and the coordination of pro-inflammatory mediators such as iNOS and NO (Kang et al., 2011). Therefore, the modulation of iNOS expression by sea tangle extract will impact transcription complex activity. Several studies have shown an elevation of iNOS levels in LPS-induced acute inflammatory responses. Since the modulation of iNOS-mediated NO production is one of the major contributing factors in the inflammatory process, the selective inhibition of iNOS may be beneficial for the treatment of inflammatory disease (Song et al., 2011).

The effects of FSTs on cytokine mRNA levels and protein expression in LPS-induced RAW 264.7 cells were examined using reverse transcription PCR and Western blot analyses, respectively (Fig. 3). Levels of both mRNA and iNOS were reduced by FST treatment (at 100 μg/mL), which is consistent with the results obtained above showing an FST-related inhibition of NO production. This indicates that FSTs are an effective inhibitor of LPS-stimulated iNOS expression and NO secretion in RAW 264.7 macrophages. These results are consistent with those obtained with *H. fusiforme* extract fermented by *L. brevis* BJ-20, which also significantly decreased iNOS levels in LPS-stimulated HepG2 cells.

In conclusion, the present study demonstrates that the anti-inflammatory effects of FSTs are the result of reductions in the levels of pro-inflammatory cytokines and the inhibition of iNOS expression and NO release in LPS-stimulated RAW 264.7 macrophages. Thus, FSTs may be used as a therapeutic agent for inflammatory diseases without side effects.

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References

Abad MJ, Bedoya LM and Bermejo P. 2008. Natural marine anti-inflammatory products. Mini Rev Med Chem 8, 740-754.

Blunt JW, Copp BR, Munro MHG, Northcote PT and Prinsep MR. 2010. Marine natural products. Nat Prod Rep 27, 165-237.

Cha JY, Lee BJ, Je JY, Kang YM, Kim YM and Cho YS. 2011. GABA-enriched fermented *Laminaria japonica* protects against alcoholic hepatotoxicity in Sprague-Dawley rats. Fish Aquat Sci 14, 79-88.

Eom SH, Lee BJ and Kim YM. 2010. Effect of yeast fermentation on the antioxidant and anti-inflammatory activity of sea tangle water extract. Kor J Fish Aquat Sci 43, 117-124.

Fichtner-Feigl S, Fuss IJ, Preiss JC, Strober W and Kitani A. 2005. Treatment of murine Th1- and Th2-mediated inflammatory bowel disease with NF-κB decoy oligonucleotides. J Clin Invest 115, 3057-3071.

Huang HL and Wang BG. 2004. Antioxidant capacity and lipophilic content of seaweeds collected from the Qingdao coastline. J Agric Food Chem 52, 4993-4997.

Kang YM, Qian ZJ, Lee BJ and Kim YM. 2011. Protective effect of GABA-enriched fermented sea tangle against ethanol-induced cytoxicity in HepG2 cell. Biotechnol Bioprocess Eng 16, 966-970.

Kaplanski G, Marin V, Montero-Julian F, Mantovani A and Farnarier C. 2003. IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation. Trends Immunol 24, 25-29.

Kim H, Lee HS, Chang KT, Ko TH, Baek KJ and Kwon NS. 1995. Chloromethyl ketones block induction of nitric oxide synthase in murine macrophages by preventing activation of nuclear factor-kappa B. J Immunol 154, 4741-4748.

Kim YC, An RB, Yoon NY, Nam TJ and Choi JS. 2005. Hepatoprotective constituents of the edible brown alga *Ecklonia stolonifera* on tacrine-induced cytotoxicity in Hep G2 cells. Arch Pharm Res 28, 1376-80.

Klotz L, Schmidt M, Giese T, Sastre M, Knolle P, Klockgether T and Heneka MT. 2005. Proinflammatory stimulation and pioglitazone treatment regulate peroxisome proliferator-activated receptor gam-

ma levels in peripheral blood mononuclear cells from healthy controls and multiple sclerosis patients. J Immunol 175, 4948-4955.

Lawrence T, Willoughby DA and Gilroy DW. 2002. Anti-inflammatory lipid mediators and insights into resolution of inflammation. Nat Rev Immunol 2, 787-795.

Liu CY, Wang CH, Chen TC, Lin HC, Yu CT and Kuo HP. 1998. Increased level of exhaled nitric oxide and up-regulation of inducible nitric oxide synthase inpatients with primary lung cancer. Br J Cancer 78, 534-541.

Maeda H and Akaike T. 1998. Nitric oxide and oxygen radicals in infection, inflammation, and cancer. Biochemistry Mosc 63, 854-865.

Okuzumi J, Takahashi T, Yamane T, Kitao Y, Inagake M, Ohya K, Nishino H and Tanaka Y. 1993. Inhibitory effects of fucoxanthin, a natural carotenoid, on N-ethyl-N'-nitro-N-nitrosoguanidine-induced mouse duodenal carcinogenesis. Cancer Lett 68, 159-168.

Park PJ, Kim EK, Lee SJ, Park SY, Kang DS, Jung BM, Kim KS, Je JY and Ahn CB. 2009. Protective effects against H_2O_2-induced damage by enzymatic hydrolysates of an edible brown seaweed, sea tangle (*Laminaria japonica*). J Med Food 12, 159-166.

Pierce GF. 1990. Macrophages: important physiologic and pathologic sources of polypeptide growth factors. Am J Respir Cell Mol Biol 2, 233-234.

Ponchel F, Morgan AW, Bingham SJ, Quinn M, Buch M, Verburg RJ, Henwood J, Douglas SH, Masurel A, Conaghan P, Gesinde M, Taylor J, Markham AF, Emery P, van Laar JM and Isaacs JD. 2002. Dysregulated lymphocyte proliferation and differentiation in patients with rheumatoid arthritis. Blood 100, 4550-4556.

Song HS, Eom SH, Kang YM, Choi JD and Kim YM. 2011. Enhancement of the antioxidant and anti-inflammatory activity of *Hizikia fusiforme* water extract by lactic acid bacteria fermentation. Kor J Fish Aquat Sci 44, 111-117.

Weislow OS, Kiser R, Fine DL, Barder J, Shoemaker RH and Boyd MR. 1989. New soluble-formazan assay for HIV-1 cytopathic effects: application to high-flux screening of synthetic and natural products for AIDS-antiviral activity. J Natl Cancer Inst 81, 577-586.

Woo MN, Jeon SM, Shin YC, Lee MK, Kang MA and Choi MS. 2009. Anti-obese property of fucoxanthin is partly mediated by altering lipid-regulating enzymes and uncoupling proteins of visceral adipose tissue in mice. Mol Nutr Food Res 53, 1603-1611.

Yoon HD, Jeong EJ, Choi JW, Lee MS, Park MA, Yoon NY, Kim YK, Cho DM, Kim JI and Kim HR. 2011. Anti-inflammatory effects of ethanolic extracts from *Codium fragile* on LPS-stimulated RAW 264.7 macrophages via nuclear factor kappaB inactivation. Fish Aquat Sci 14, 267-274.

Zhang Z, Zhang P, Hamada M, Takahashi S, Xing G, Liu J and Sugiura N. 2008. Potential chemoprevention effect of dietary fucoxanthin on urinary bladder cancer EJ-1 cell line. Oncol Rep 20, 1099-1103.