Carbohydrate Polymers 85 (2011) 80–85

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Anti-inflammatory activity of polysaccharide purified from AMG-assistant extract of *Ecklonia cava* in LPS-stimulated RAW 264.7 macrophages

Sung-Myung Kang[a], Kil-Nam Kim[b], Seung-Hong Lee[a], Ginnae Ahn[a], Seoun-Heui Cha[c], Areum-Daseul Kim[a], Xiu-Dong Yang[a], Min-Chul Kang[a], You-Jin Jeon[a,*]

[a] *Department of Marine Life Science, Jeju National University, Jeju 690-756, Republic of Korea*
[b] *Jeju Biodiversity Research Institute (JBRI), Jeju Hi-Tech Industry Development Institute (HiDI), Jeju 697-943, Republic of Korea*
[c] *Marine Bio Research Team, Korea Basic Science Institute (KBSI), Jeju 690-756, Republic of Korea*

ARTICLE INFO

Article history:
Received 6 December 2010
Received in revised form 26 January 2011
Accepted 27 January 2011
Available online 3 February 2011

Keywords:
Ecklonia cava
Polysaccharide
Anti-inflammatory
Brown seaweed

ABSTRACT

Algal fucoidan is a marine sulfated polysaccharide that evidences a variety of biological activities. We assessed the potential activity of the sulfated polysaccharide from *Ecklonia cava*, on anti-inflammatory activity in LPS-stimulated RAW 264.7 cells. In this study, *E. cava* was hydrolyzed by five carbohydrases and five proteases. Our findings demonstrated that the AMG extract has the highest yield and exerts the most profound inhibitory effects against NO production. To identify the active compounds, we conducted micro-filtration membrane, ethanol-added separation, and anion exchange and gel permeation chromatography; the purified polysaccharide (PPS) was subsequently obtained. PPS significantly inhibited NO production, prostaglandin-E_2 (PGE_2) production and suppressed inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression in LPS-stimulated RAW 264.7 cells. Thus, PPS inhibited NO and PGE_2 production via the inhibition of iNOS and COX-2. These results indicate that the anti-inflammatory activity of PPS may be attributable to the modulation of anti-inflammatory agents.

1. Introduction

Inflammation is the process by which the human body attempts to counteract potential injurious agents, including invading bacteria, viruses, and other pathogens (Henderson, Poole, & Wilson, 1996; Hersh, Weiss, & Zychlinsky, 1998; Ulevitch & Tobias, 1995). Although inflammation is a crucial process in living organisms, inflammation can also exert harmful effects on the host via multiple levels of biochemical, pharmacological, and molecular controls, involving a broad array of cell types and some soluble mediators, including cytokines (Boraschi et al., 1998; Dinarello, 2000; Nicod, 1993; Rouveix, 1997; Turcanu & Williams, 2001). When pro-inflammatory mediators and cytokines such as NO, PGE2, IL-1β, IL-6, and TNF-α are administered to humans, they can result in fever, inflammation, tissue destruction, and occasionally, shock and death (Dinarello, 2000).

In these mediators, NO is enzymatically generated by nitric oxide synthases (NOSs) and is formed by iNOS in macrophages and in other cells that play a role in the inflammatory response. Large quantities of NO can stimulate a variety of proteins and enzymes crucial to inflammatory reactions, including the NF-κB and MAPKs pathways (Nijkamp & Parnham, 2005). Many previous studies have reported that MAPKs mediate the activation of the transcriptional factor NF-κB (Aga et al., 2004; DeFranco, Hambleton, McMahon, & Weinstein, 1995) and, subsequently regulate COX-2 expression (Mestre et al., 2001) and iNOS–NO expression (Chan & Riches, 2001). Furthermore, iNOS expression and NO production, both of which are stimulated by LPS, have been demonstrated to contribute to septic shock (Jacobs & Ignarro, 2001). Prostaglandin E2, one of the prostaglandins, is produced by the cyclooxygenase pathway. Prostaglandins are known to regulate vascular permeability, platelet aggregation, and thrombus formation in the development of inflammation. The inhibition of COX-2 activity can ameliorate the deleterious consequences of sepsis (Knoferl et al., 2001). All of these cytokines may be valuable targets for inflammatory disease treatments, and a proper understanding of the inflammatory basis is indispensable to a thorough understanding of atherosclerosis, cancer, ischemic heart disease, and other maladies. NO has been identified as an molecule that is intimately involved in the regulation of biological activities in the vascular, neural, and immune systems (Moncada, Palmer, & Higgs, 1992). NO generated by activated macrophages has been shown to mediate host defense functions such as antimicrobial and anti-tumor activities, but excess NO production induces tissue damage associated with acute and chronic inflammation (MacMiking, Xie, & Nathan, 1997).

* Corresponding author. Tel.: +82 64 754 3475; fax: +82 64 756 3493.
E-mail address: youjinj@jejunu.ac.kr (Y.-J. Jeon).

0144-8617/$ – see front matter
doi:10.1016/j.carbpol.2011.01.052

Ecklonia cava species have been broadly employed as a source of fucoidan, which has been identified as an antitumor, hypoglycemic, and anti-angiogenic polysaccharide (Koyanagi, Tanigawa, Nakagawa, Soeda, & Shimeno, 2003; Synytsya et al., 2010). However, no investigations of anti-inflammatory activity have, thus far, been conducted with the polysaccharides of *E. cava*.

The principal objective of this study was to extract and purify the active polysaccharides from *E. cava*, and then to evaluate the anti-inflammatory effects of these polysaccharides on LPS-stimulated RAW 264.7 macrophages.

2. Reagents and materials

RAW 264.7 macrophage cell line was purchased from the Korea Cell Line Bank (KCLB, Seoul, Korea). DMEM medium and fetal bovine serum (FBS) were obtained from GIBCO-BRL (Grand Island, New York, USA). Dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and standard monosaccharides (D-glucose, D-xylose, D-galactose, L-rhamnose, D-mannose, and D-arabinose) were purchased from E. Merck (Darmstadt, Germany) and Sigma Co. (St. Louis, USA). DEAE-cellulose, Sepharose 4B and dextrans of different molecular weights were from Pharmacia Co. (Uppsala, Sweden). Five carbohydrate enzyme including Viscozyme L, Celluclast 1.5L FG, AMG 300L, Termamyl 120L, Ultraflo L, and the five protease including Protamex, Kojizyme 500 MG, Neutrase 0.8L, Flavourzyme 500 MG, Alcalase 2.4L FG were obtained from Novo Co. (Novozyme Nordisk, Bagsvaerd, Denmark). All other chemicals and reagents were analytical grade.

2.1. Plant material and extraction

E. cava was collected along Jeju Island coast of Korea during February and May 2008. Salt, sand and epiphytes were removed using tap water. Then, seaweed samples were rinsed carefully with fresh water and freeze-dried. Dried alga sample was ground and sifted through a 50-mesh standard testing sieve. The preparation of enzyme-assistant extract was followed by previously reported method (Heo, Jeon, Lee, Kim, & Lee, 2003; Athukorala, Jung, Vasanthan, & Jeon, 2006). Fifty gram of alga sample was homogenized with water (2 L), and mixed with 500 μL of enzymes. Each reactant was adjusted to be within the optimum pH and temperature range of the respective enzyme and enzymatic reactions were performed for 24 h. Following extraction, the extract was boiled for 10 min at 100 °C to inactivate the enzymes. Then, samples were clarified by centrifugation (3000 rpm, for 20 min at 4 °C) to remove the residue. This extracts were adjusted to pH 7.0 hereafter and designated to as enzymatic extract. The sample was kept in −20 °C for further experiments

2.2. Purification of polysaccharide

2.2.1. Molecular weight fractionation of AMG-assistant extract

AMG extract was passed through micro-filtration membrane (0.1 μm and 50 kDa) using Lab scale TFF system (PHILOS) to obtain different molecular weight fractions. Then, all the fractions (<0.1 μm, >50 kDa, <50 kDa) were separately evaluated for anti-inflammatory activity.

2.2.2. Crude polysaccharide separation

The >50 kDa fraction was (750 mL) mixed well with 1.5 L of 99.5% ethanol. Then, the mixture was allowed to stand for 30 min at room temperature and the crude polysaccharide fractions were collected by centrifugation at 10,000 × *g* for 20 min at 4 °C (Kuda, Taniguchi, Nishizawa, & Araki, 2002; Matubara, Matsuura, Hori, & Miyazawa, 2000).

2.2.3. Anion-exchange chromatography

The crude polysaccharide from *E. cava* (5 g) obtained by using the procedure described above was applied to a DEAE-cellulose column (17 cm × 2.5 cm) equilibrated in 50 mM sodium acetate (pH 5.0) and washed with the same buffer containing 0.2 M NaCl. Elution was carried out at a flow rate 15 mL/h with a linear gradient of 0.2–1.2 M NaCl containing 50 mM sodium acetate (pH 5.0). Fractions of 5 mL were collected and measured for polysaccharide by phenol–H_2SO_4 assay. Fractions showing anti-inflammatory activities against NO production were collected, dialyzed against distilled water, and fractions of 5 mL concentrated by rotary evaporator under reduced pressure below 40. The active fractions were pooled, dialyzed and freeze-dried for gel filtration chromatography.

2.2.4. Gel filtration chromatography

Purified sample (10 mg/mL in water) was applied to a Sepharose 4B column (72 cm × 2 cm) equilibrated and eluted with water at room temperature at a flow rate of 1 mL/min. Fractions (2 mL) were collected and assayed for phenol–H_2SO_4 at 490 nm and for total polysaccharide contents.

2.3. Determination of the molecular mass of the purified polysaccharide

In order to determine molecular mass of the sample, the freeze-dried sample was introduced into PL-Aquaz OH 40 column and eluted with deionized water at 0.8 mL/min flow rate. Dextran standards (48.6, 148, 273, 410, 830, and 2000 kDa) were also introduced into the column under the same experimental condition for comparison purposes. The retention time was plotted against average molecular mass of the dextrans, and there by the molecular mass of the sample was calculated.

2.4. Neutral sugar analysis

E. cava polysaccharides were hydrolyzed in a sealed glass tube with 4 M of trifluoroacetic acid for 4 h at 100 °C to analyze neutral sugars. In order to analyze the mono-saccharide, the samples were digested using 6 N of HCl for 4 h. Then, *E. cava* fractions were separately applied to CarboPac PA1 (4.5 mm × 250 mm, Dionex, Sunnyvale, CA, USA) with CarboPac PA1 cartrige (4.5 mm × 50 mm) column to analyze neutral and amino sugar respectively. The column was eluted using 16 mM of NaOH at 1.0 mL/min flow rate. Each sugar of the sample was detected by using ED50 Dionex electrochemical detector and data were analyzed by Peack Net on-line software.

2.5. Sulfate content analysis

After acid hydrolysis of the purified polysaccharide, the sulfate content was measured by the $BaCl_2$/gelation method (Saito, Yamagata, & Suzuki, 1968).

2.6. Infrared spectral analysis of the polysaccharides

IR spectrum of the polysaccharide was determined using a Fourier transform infrared spectrometer (FTIR, Bruker, Germany) equipment. The purified polysaccharide was ground with KBr powder and then pressed into pellets for FTIR measurement in the frequency range of 4500–748 cm^{-1} (Wang et al., 2004).

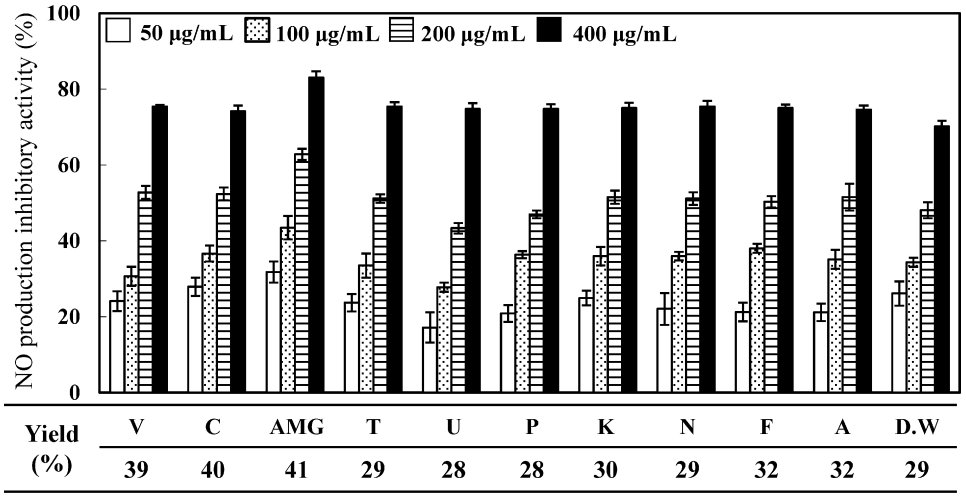

Fig. 1. Effects of enzymatic extract of *E. cava* on the inhibition activity of LPS-induced NO production in RAW 264.7 cells. □, 50 μg/mL; ▦ , 100 μg/mL; ▤ , 200 μg/mL; ■, 400 μg/mL. V, Viscozyme extract; C, Celluclast extract; AMG, AMG extract; T, Termamyl extract; U, Ultraflo extract; P, Protamex extract; K, Kojizyme extract; N, Neutrase extract; F, Flavourzyme extract; A, Alcalase extract; D.W., distilled water extract. Yield of enzymatic extracts of *E. cava* (%). Experiments were performed in triplicate and the data are expressed as mean ± SE.

2.7. Assay for cell culture experiments

Murine macrophage cell line RAW 264.7 was obtained from the KCLB (Korean Cell Line Bank). The cells were maintained at 37 °C in humidified atmosphere of 5% CO_2 in DMEM medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/mL and 100 μg/mL, respectively; GIBCO Inc, NY, USA). Exponential phase cells were used throughout the experiments.

2.7.1. Nitrite assay

The cells (1×10^5 cell/mL) were pretreated with various concentrations of purified compounds for 2 h and then incubated for 24 h with LPS (1 μg/mL). After incubation, the nitrite concentrations of supernatants (100 μL/well) were measured by adding 100 μL of Griess reagent (1% sulfanilamide in 2.5% phosphoric acid and 0.1% naphthylenediamine dihydrochloride in water) (Kim et al., 2007). The optical density at 540 nm was measured using an ELISA microplate reader (Amersham Pharmacia Biotech, UK, USA). The nitrite concentration was calculated by comparison with the absorbance at 540 nm of standard solutions of sodium nitrite prepared in culture medium.

2.7.2. Western blot analysis of COX-2 and iNOS in RAW 264.7 cells

Murine macrophage cell line RAW 264.7 were pre-incubated for 16 h, and then stimulated by LPS (1 μg/mL) in the presence or absence of purified compounds for 24 h. The cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS), and the cell lysates were prepared with lysis buffer (50 mM/L Tris–HCl (pH 7.4), 150 mM/L NaCl, 1% Triton X-100, 0.1% SDS and 1 mM/L EDTA) for 20 min on ice. Cell lysates were centrifuged at 14,000 × *g* for 20 min at 4 °C. And then protein contents in the supernatant were measured using BCA™ protein assay kit. The lysate containing 20 μg of protein was subjected to electrophoresis on 7.5% sodium dodecyl sulfate-polyacrylamide gel, and the gel was transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were blocked with 5% non-fat dry milk in TBS buffer containing 0.2% Tween 20 (TBST) for 90 min at room temperature. Then the membrane was incubated with specific primary rabbit polyclonal anti-rabbit iNOS Ab (1:2000, Calbiochem, USA), or mouse monoclonal anti-mouse COX-2 Ab (1:5000, BD Biosciences, USA) at 4 for overnight. Membranes were washed with TTBS and incubated with goat anti-mouse or anti-rabbit IgG HRP conjugated secondary antibody (1:5000 dilution) in TBST that contained 2% non-fat dry milk for 90 min at room temperature. After three times washing with TBST for 10 min signals. Signals were developed using an ECL Western blotting detection kit and exposed to X-ray films.

2.7.3. PGE_2 enzyme-linked immunosorbent assay (ELISA)

PGE_2 levels were analyzed using the prostaglandin E_2 Biotrack ELISA system (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. RAW264.7 cells (1×10^5 cells/well) were pretreated with PPS for 2 h and then incubated with LPS (1 μg/mL) for 24 h. PGE_2 concentrations of supernatants were determined with a PGE_2 ELISA assay kit (Alexis, USA).

3. Results

3.1. Isolation and purification of active compounds from E. cava for anti-inflammation in RAW 264.7 cells

The enzyme-assistant extract of *E. cava* was obtained via hydrolysis using several carbohydrases (Viscozyme, Celluclast, AMG, Termamyl, and Ultraflo) and proteases (Kojizyme, Alcalase, Protamex, Flavourzyme, and Neutase) in order to evaluate their anti-inflammatory effects. All enzymatic extracts of *E. cava* evidenced stronger anti-inflammatory activities with higher extract yields than water extract (Fig. 1). Among the tested extracts, the AMG extract of *E. cava* evidenced a marked anti-inflammatory activity on NO assay and extract yield. AMG is able to digest 1,4 and 1,6-α linkages of the plant cell wall materials. The rate of hydrolysis depends on the type of linkage and on chain length (Athukorala et al., 2006). In order to identify the active compounds of the AMG extract, *a* >50 kDa fraction of the AMG extract was separated using a micro-filtration membrane system (PHILOS). The crude polysaccharide from the >50 kDa fraction was then separated via ethanol-added precipitation. The freeze-dried crude polysaccharide sample was subsequently introduced to a DEAE-cellulose column with a NaCl gradient to separate out the anti-inflammatory polysaccharide fraction (Fig. 2A). After evaporation under a rotary evaporator, the dialyzed sample was further purified via gel-filtration chromatography on Sepharose 4B (Fig. 2B).

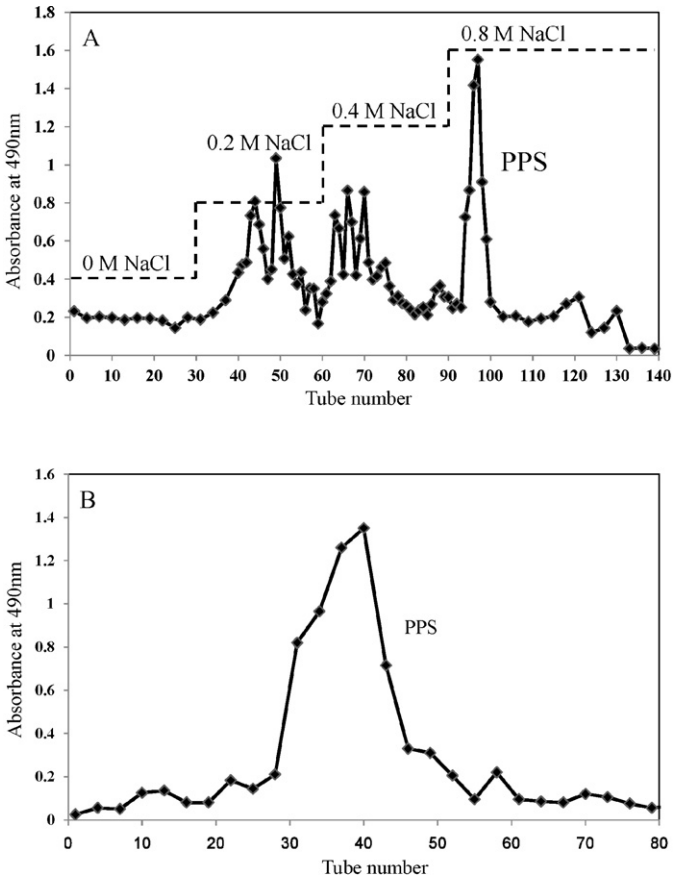

Fig. 2. DEAE-cellulose (A) and Sepharose 4B (B) chromatogram of the polysaccharides separated from *E. cava*.

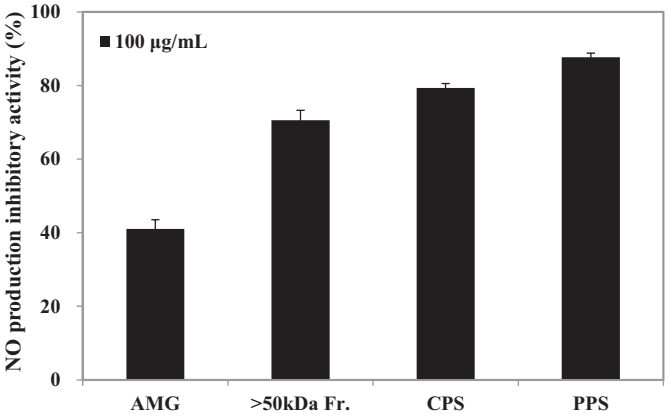

Fig. 3. Inhibitory effect of different fractions of AMG, >50 kDa Fr, CPS, and PPS on LPS-induced NO production in RAW 264.7 macrophage. Experiments were performed in triplicate and the data are expressed as mean ± SE.

Table 1
Monosaccharide composition of sulfated polysaccharide fraction isolated from AMG extract of *E. cava* by molecular weight membrane, ethanol precipitation, and column-chromatography.

Sugar (%)	AMG ext	>50 kDa Fr	[b]CPS Fr	[c]PPS
Fucose	48.17	65.13	69.55	82.11
Rhamnose	4.61	2.32	1.95	0.28
Galactose	15.28	17.32	14.95	12.21
Glucose	14.05	1.74	1.53	0.23
Mannose	8.53	4.01	2.69	2.07
Xylose	9.37	9.48	9.34	2.17
[a]Sulfate/total neutral sugar	0.41	0.52	0.71	0.92

[a] The mean degree of substitution of sulfate ester per total neutral sugar.
[b] Crude polysaccharide.
[c] Purified polysaccharide from AMG extract of *E. cava*.

3.2. Inhibition of NO production by separated polysaccharide fractions.

Stimulation of cells with LPS resulted in a significant enhancement of the nitrite concentration in conditioned medium relative to that of LPS non-stimulated blank cells (data not shown). This reflected increased NO production and the release of its stable product, NO_2^-, into the culture medium. However, cells pretreated with the separated polysaccharide evidenced a purity-dependent reduction in the production of NO after LPS stimulation. The NO production inhibition activities of the separated polysaccharide fractions were, in order from least to most profound inhibitory activity: AMG extract, >50 kDa fraction, CPS, and PPS (Fig. 3). At 100 µg/mL of PPS, the NO production level was similar to that observed in the non-stimulated cells.

3.3. Monosaccharide composition and sulfate contents analysis

The sugar composition of the active compound was evaluated by Bio-LC and compared with the absorption spectra of standard sugars. The monosaccharide composition of polysaccharide separated via molecular weight membrane, ethanol precipitation, and column-chromatography from the AMG extract of *E. cava* are provided in Table 1. According to our results, all of the fractions harbor substantial quantities of fucose (~48%, 65%, 69% and 82%) and lower quantities of galactose (~15%, 17%, 14% and 11%), respectively (Table 1). The fucose contents in the separated polysaccharide fractions, in order from lowest to highest, were: AMG extract, >50 kDa Fr, CPS Fr, and PPS. Moreover, all the fractions tested herein harbored minor quantities of other sugars. The total sulfate contents of the tested fractions were 0.41, 0.52, 0.71, and 0.92 (sulfate/total sugar), respectively.

3.4. Determination of the molecular mass of the purified polysaccharide

To determine the molecular weight, the PPS was subjected to HPLC gel filtration chromatography, and the retention time of the PPS was plotted using known dextran standards. The molecular weight of the PPS was approximately 1.381×10^6 Da (data not shown).

3.5. FT-IR analysis of purified polysaccharide

The FT-IR spectra of PPS revealed bands characteristic of sulfates and uronic acids. PPS showed bands at 3336.85, 2924.08, 2364.72, 1600.91, 1257.58 and 825.53 cm^{-1} (Fig. 4). A band between the 1240 and 1260 cm^{-1} regions is related to the S=O stretching vibration of the sulfate group (Percival & Wold, 1963). Additionally, the signal nearest to 1600 cm^{-1} was attributed to the asymmetric and symmetric stretch vibration of the –COO– of uronic acid. In our study we detected absorption bands at 3336.85, 2924.08, 2364.72 cm^{-1}, which were reflective of the O–H, C–H, O–C–O, and C–OH bonds. The sulfate absorption band at 825.53 cm^{-1} indicates that most sulfate groups are located at positions 2 and 3. Based on the FT-IR spectrum, it could be anticipated that the PPS of *E. cava* may be predominantly acidic in nature.

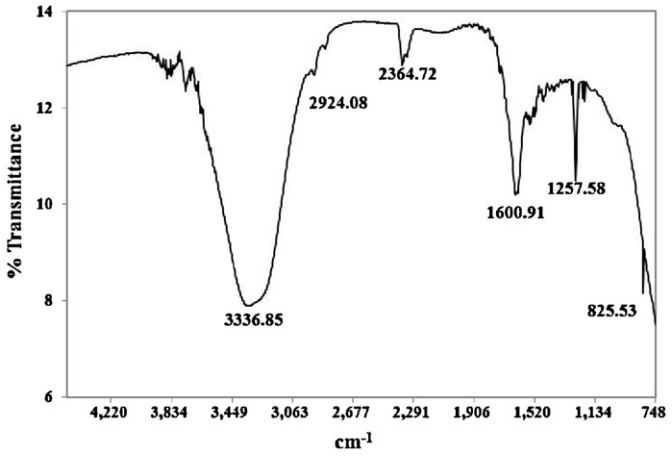

Fig. 4. FT-IR spectra of PPS in the frequency range 4500–748 cm^{-1}.

3.6. Inhibition of iNOS expression and NO production by PPS

Inducible NO synthase (iNOS) is one of the major inflammatory mediators contributing to the pathogenesis of cancer and inflammation. In response to LPS, the iNOS of macrophages is induced and consequently leads to NO overproduction, which may perform a crucial role in the pathogenesis of a variety of inflammatory diseases. Our results demonstrated that PPS significantly inhibited NO production and iNOS expression in LPS-stimulated RAW 264.7 macrophages, which may partly explain its anti-inflammatory effects. PPS significantly inhibited this increase in NO production in a concentration-dependent manner, with 100 μg/mL of PPS affecting a complete blockage of LPS-inducible NO production. It remains unclear as to whether the PPS-mediated inhibition of NO formation is the consequence of the inhibition of iNOS gene expression. Therefore, the inhibitory effects of different PPS concentrations on LPS-induced (1 μg/mL) iNOS protein expression were assessed. As shown in Fig. 5A, PPS inhibited iNOS protein expression at 25–200 μg/mL, in a concentration-dependent manner. These results demonstrated that PPS suppresses the *de novo* synthesis of iNOS in LPS-stimulated macrophages.

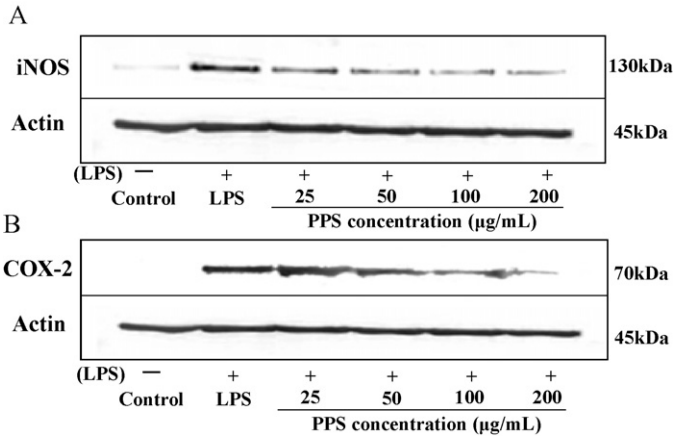

Fig. 5. Inhibition of iNOSand COX-2 protein expression by isolated PPS. RAW 264.7 cells were pretreated with different concentrations of sulfated PPS for 1 h and stimulated with LPS (1 μg/mL) for another 24 h.

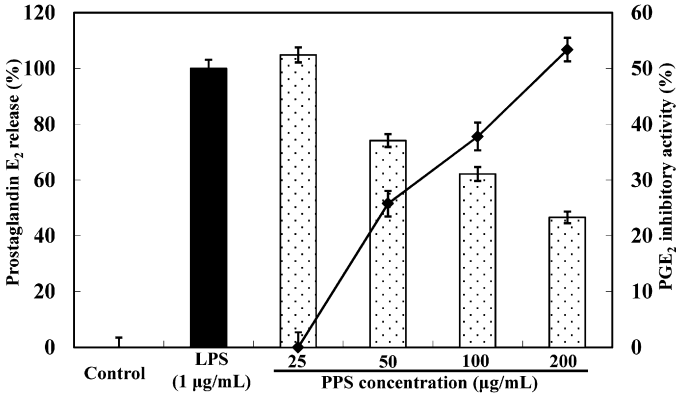

Fig. 6. Inhibition of isolated PPS on PGE_2 production in RAW 264.7 macrophage cells. PGE_2 production in supernatant fluid was measured using an ELISA kit. Experiments were performed in triplicate and the data are expressed as mean ± SE.

3.7. Inhibition of PPS on COX-2 protein expression in RAW 264.7 cells

In order to confirm that the dose-dependent NO decrease is due to its influence on iNOS, the inhibitory effect of PPS on iNOS was measured by Western blot analysis. In addition, the inhibitory activity of PPS on COX-2 was also confirmed by means of Western blot analysis. The COX-2 protein was induced upon LPS stimulation for 24 h. As is shown in Fig. 5B, reductions of COX-2 were clearly regulated in a dose-dependent manner by PPS in LPS-stimulated RAW 264.7 cells.

3.8. Inhibition of PPS on PGE_2 production in RAW 264.7 cells

In an effort to determine whether or not the stimulatory effect of PPS on COX-2 protein expression were related to the modulation of PGE_2 release, the levels of COX-2 production were evaluated via PGE_2 immunoassays. RAW 264.7 macrophages were treated with PPS (25, 50 100, and 200 μg/mL) for 24 h, and the concentration of PGE_2 was estimated in cultured supernatants. The results showed that upon treatment with PPS, the macrophages generated significantly lower levels of PGE_2 in a dose-dependent manner relative to the values observed with the LPS-treated cultures, and 200 μg/mL of PPS profoundly blocked the LPS-inducible PGE_2 production. The addition of LPS resulted in a clearly defined increase in COX-2 expression, which was markedly attenuated in a dose-dependent manner upon treatment with PPS (Fig. 6), thus verifying that PPS induced a reduction in COX-2, which translated into a dramatic reduction in PGE_2. We noted no difference in PGE_2 production between the cells cultured in the presence of PPS and control cells.

4. Conclusion

In the previous studies, it was determined that the polysaccharides contained in plants not only function as energy resources, but also perform key biological roles in many life processes. The structure and pharmaceutical effect mechanisms of bioactive polysaccharides on diseases have been studied extensively, and more natural polysaccharides with different curative effects have been tested, and even applied in actual therapies (Wang & Fang, 2004). The results of this study confirmed the presence of sulfate and uronic acid linkages in the purified polysaccharide of *E. cava*. Additionally, the expression levels of iNOS and COX-2 genes, as well as the production levels of NO and PGE_2 by LPS-stimulated RAW 264.7 macrophages were evaluated in this study. PPS was shown to dose-dependently inhibit LPS-induced iNOS and COX-2 gene expression, as well as the subsequent production of NO and

PGE_2 by LPS in RAW 264.7 macrophages. Therefore, we propose that PPS may prove to be an effective immunomodulatory mediator with a variety of beneficial effects.

Acknowledgement

This research was supported by a grant from Marine Bioprocess Research Center of the Marine Biotechnology Program funded by the Ministry of Land, Transport and Maritime, Republic of Korea.

References

Aga, M., Watters, J. J., Pfeiffer, Z. A., Wiepz, G. J., Sommer, J. A., & Bertics, P. J. (2004). Evidence for nucleotide receptor modulation of cross talk between MAP kinase and NF-kappa B signaling pathways in murine RAW 264.7 macrophages. *American Journal of Physiology: Cell Physiology*, *286*, 923–930.

Athukorala, Y., Jung, W. K., Vasanthan, T., & Jeon, Y. J. (2006). An anticoagulative polysaccharide from an enzymatic hydrolysate of Ecklonia cava. *Carbohydrate Polymers*, *66*, 184–191.

Boraschi, D., Cifone, M. G., Falk, W., Flad, H. D., Tagliabue, A., & Martin, M. U. (1998). Cytokine in inflammation. *European Cytokine Network*, *9*(2), 205–212.

Chan, E. D., & Riches, D. W. (2001). IFN-gamma + LPS induction of iNOS is modulated by ERK, JNK/SAPK, and p38(mapk) in a mouse macrophage cell line. *American Journal of Physiology: Cell Physiology*, *280*, 441–450.

DeFranco, A. L., Hambleton, J., McMahon, M., & Weinstein, S. L. (1995). Examination of the role of MAP kinase in the response of macrophages to lipopolysaccharide. *Progress in Clinical and Biological Research*, *392*, 407–420.

Dinarello, C. A. (2000). Proinflammatory cytokines. *Chest*, *188*, 503–508.

Henderson, B., Poole, S., & Wilson, M. (1996). Bacterial modulins: A novel class of virulence factors which cause host tissue pathology by including cytokine synthesis. *Microbiological Review*, *60*, 316–341.

Heo, S. J., Jeon, Y. J., Lee, J., Kim, H. T., & Lee, K. W. (2003). Antioxidant effect of enzymatic hydrolyzates from marine algae. *Food Industry and Nutrition*, *10*(1), 31–41.

Hersh, D., Weiss, J., & Zychlinsky, A. (1998). How bacteria initiate inflammation: Aspects of the emerging story. *Current Opinion in Microbiology*, *1*, 43–48.

Jacobs, A. T., & Ignarro, L. J. (2001). Lipopolysaccharide-induced expression of interferon-beta mediates the timing of inducible nitric-oxide synthase induction in RAW 264.7 macrophages. *Journal of Biological Chemistry*, *276*, 47950–47957.

Kim, J. B., Han, A. R., Park, E. Y., Kim, J. Y., Cho, W., Lee, J., et al. (2007). Inhibition of LPS-induced iNOS, COX-2 and cytokines expression by poncirin through the NF-kappaB inactivation in RAW 264.7 macrophage cells. *Biological and Pharmaceutical Bulletin*, *30*(12), 2345–2351.

Knoferl, M. W., Diodato, M. G., Schwacha, W. G., Cioffi, K. I., Bland, & Chaudry, I. H. (2001). Cyclooxygenase-2-mediated regulation of kupffer cell interleukin-6 production following trauma-hemorrhage and subsequent sepsis. *Shock*, *16*, 479–483.

Koyanagi, S., Tanigawa, N., Nakagawa, H., Soeda, S., & Shimeno, H. (2003). Oversulfation of fucoidan enhances its anti-angiogenic and antitumor activities. *Biochemical Pharmacology*, *65*, 173–179.

Kuda, T., Taniguchi, E., Nishizawa, M., & Araki, Y. (2002). Fate of Water-soluble polysaccharides in dried *Chorda filum* a brown alga during water washing. *Journal of Food Composition and Analysis*, *15*, 3–9.

MacMiking, J., Xie, Q. W., & Nathan, C. (1997). Nitric oxide and macrophage function. *Annual Review Immunology*, *15*, 323–350.

Matubara, K., Matsuura, Y., Hori, K., & Miyazawa, K. (2000). Anticoagulant proteoglycan from marine green alga, *Codium pugnifomis*. *Journal of Applied Phycology*, *12*, 9–14.

Mestre, J. R., Mackrell, P. J., Rivadeneira, D. E., Stapleton, P. P., Tanabe, T., & Daly, J. M. (2001). Redundancy in the signaling pathways and promoter elements regulating cyclooxygenase-2 gene expression in endotoxin-treated macrophage/monocytic cells. *Journal of Biological Chemistry*, *276*, 3977–3982.

Moncada, S., Palmer, R. M., & Higgs, E. A. (1992). Nitric oxide: Physiology, pathophysiology. *Pharmacological Reviews*, *43*, 109–142.

Nicod, L. P. (1993). Cytokines: Overview. *Thorax*, *48*, 660–667.

Nijkamp, F. P., & Parnham, M. J. (2005). *Principles of immunopharacology* (2nd ed.). Basel, Switzerland: Birkhauser., pp. 188.

Percival, E., & Wold, J. K. (1963). The acid polysaccharide from the green seaweed *Ulva lactuca*. Part II. The site of the ester sulphate. *Journal of Chemical Society*, *1040*, 5456–5468.

Rouveix, B. (1997). Clinical pharmacology of cytokines. *European Cytokine Network*, *8*, 291–293.

Saito, H., Yamagata, T., & Suzuki, S. (1968). Enzymatic methods for the determination of small quantities of isomeric chondroitin sulfates. *Journal of the Biological Chemistry*, *243*, 1536–1542.

Synytsya, A., Kim, W. J., Kim, S. M., Pohl, R., Syntsya, A., Kvasnicka, F., et al., & Park, Y. I. (2010). Structure and antitumor activity of fucoidan isolated from sporophyll of Korean brown seaweed *Undaria pinnatifida*. *Carbohydrate Polymers*, *81*(1), 41–48.

Turcanu, V., & Williams, N. A. (2001). Cell identification and isolation on the basis of cytokine secretion: A novel tool for investigating immune responses. *Nature Medicine*, *7*, 373–376.

Ulevitch, R. J., & Tobias, P. S. (1995). Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Annual Review of Immunology*, *13*, 437–457.

Wang, Q. J., & Fang, Y. Z. (2004). Analysis of sugars in traditional Chinese drugs. *Journal of Chromatography B*, *812*, 309–324.

Wang, Y., Zhang, M., Ruan, D., Shashkov, A. S., Kilcoyne, M., Savage, A. V., et al. (2004). Chemical components and molecular mass of six polysaccharides isolated from the sclerotium of *Poria cocos*. *Carbohydrate Research*, *339*, 327–334.