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RESEARCH PAPER

Characterization and Immunostimulating Activity of a Water-soluble Polysaccharide Isolated from *Haematococcus lacustris*

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Abstract A water-soluble polysaccharide was isolated and purified from the culture filtrate of the photosynthetic green microalgae *Haematococcus lacustris* by 75% ethanol precipitation and Sepharose CL-6B column chromatography. The molecular mass of the purified polysaccharide (named HCP) was estimated to be approximately 135 kDa by size-exclusion HPLC and its monosaccharide composition was galactose, glucose and mannose at a relative molar ratio of 2.0, 1.0, and 4.1, respectively, suggesting that HCP is a galactomannan. Fourier-transform infrared and elemental analysis revealed that the purified HCP contains sulfate esters by 1.08% (in mass) and no detectable level of protein. The HCP significantly stimulated murine macrophage RAW264.7 cells to secrete the pro-inflammatory cytokine, TNF-α, in a dose-dependent manner and also enhanced the expression of COX-2 and iNOS genes at a concentration of lower than 10 μg/mL HCP. These results indicated that the sulfated HCP of *H. lacustris* has potent early innate immune stimulating activities.

Keywords: *haematococcus lacustris*, microalgae, polysaccharide, immuno-stimulating activity

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1. Introduction

Algal polysaccharides have emerged in recent years as abundant and important sources of new bioactive materials due to the current tendency of global markets to switch from synthetic to natural products and a growing understanding of their novel structures and diverse biological activities [1-6]. A group of the most extensively studied algal polysaccharides contain sulfate esters in their sugar residues, so called sulfated polysaccharides (SPS), and most of them are commonly found in a wide range of macroalgae (seaweeds) [1,3]. Numerous studies have shown that these seaweed polysaccharides, mostly sulfated ones including fucoidans of brown algae, exhibit a wide range of pharmacological activities, such as anti-inflammatory [7], antiviral [8], anticoagulant [3,9], antitumor [10], antioxidant [11], immunomodulating [12], and anti-angiogenesis activities [13]. Therefore, seaweeds including red and brown macroalgae have attracted great interests for many years as sources of biologically active materials, in particular as potent natural therapeutic agents and functional food ingredients.

In contrast to macroalgae, microalgae, photosynthetic unicellular organisms, can be cultivated in a photobioreactor system under relatively controlled conditions [14] and they have been used as functional materials for food and cosmetics, and as nutrient supplements for human consumption due to their high chlorophyll, protein and vitamin content [15,16]. Microalgae also synthesize diverse polysaccharides as part of their cell wall constituents [17,18]. Similar to fucoidan from macroalgae, microalgal polysaccharides are also known to exhibit various bioactivities

with pharmacological significance, although there are fewer reports of these compared to macroalgal polysaccharides [4,6]. Cell wall-derived sulfated polysaccharides from a red microalga *Porphyridium* sp. have shown antitumor [19], antiviral [20,21], anti-inflammatory [22], and antioxidant [23] activities. For these reasons, in addition to the importance for the human consumption as foods, microalgae can also be attractive sources of new bioactive polysaccharides.

Haematococcus lacustris, a photosynthetic unicellular green microalgae species, is being widely explored for the production and bioengineering of astaxanthin, a potent antioxidant [24,25]. It forms thick cell walls that are comprised of sulfated carbohydrates, some of which secrete into the culture medium [17,18]. However, little is known regarding the structures and potential therapeutic effects of active polysaccharides of *H. lacustris*. Accordingly, in an attempt to search for biologically active compounds from microalgal species, the present study reports the isolation and purification of a water-soluble sulfated polysaccharide (HCP) from the culture filtrate of *H. lacustris*, including its chemical characterization and immune stimulating effects on murine macrophage RAW264.7 cells.

2. Materials and Methods

2.1. Cell growth

H. lacustris, a unicellular green algae, purchased from the Culture Collection of Algae (CCA) at the University of Texas, Austin, USA, was cultivated photoautotrophically in MBBM culture medium (pH 6.6) [26], using a 0.4-L bubble-column photobioreactor (3.5 × 62 cm) made of Pyrex glass tubes. For supplementation of air and gas, filtered air containing 5% CO_2 was injected into the conical bottom part of the bioreactor at a constant flow rate of 0.2 $_{VVM}$, according to established conditions as described in a previous report [26]. *H. lacustris* was cultivated for 10 days under normal light intensity (40 μE/m^2/sec). Various parameters such as total cell concentration, average cell size, cell size distribution, and fresh cell weight (wet-based) were measured by using a Coulter Counter (Coulter Electronics, Inc., FL, USA). The temperature and pH were kept at 25ºC and 6.5 ± 0.5 during cultivation period.

2.2. Isolation and purification of polysaccharide

Water soluble polysaccharides of *H. lacustris* were isolated and purified from the culture filtrate using previously described methods [9,12]. Briefly, cultures containing approximately 2.5 × 10^6 cells/mL grown for 10 days under the optimized conditions were subjected to centrifugation 2,100 × *g* for 30 min (Hanil Science, Korea) and the supernatants were then filtered through Whatman No. 4 filter paper to remove debris and insoluble materials. The resulting culture filtrates were treated with 75% ethanol (final concentration). The precipitated materials were then extensively dialyzed against dH_2O using a cellulose membrane dialysis tubing (MWCO 10,000, Sigma, USA) and then lyophilized. The freeze-dried polysaccharides were then dissolved in dH_2O (1%, w/v) containing 50 mM NaCl and subjected to Sepharose CL-6B column (1 × 90 cm, Sigma, USA) chromatography, and eluted with the same buffer. The main carbohydrate-positive fractions were pooled, dialyzed against dH_2O and lyophilized. The purified polysaccharide (named HCP in this study) was used for chemical analysis and assessment for immune-modulating activity.

2.3. Determination of molecular weight

The molecular weight of the HCP purified from the culture filtrates of *H. lacustris* was estimated by size-exclusion HPLC (Dionex, USA) equipped with an ELSD (Evaporative light scattering detector, Alltech, country?) [27], using a Shodex OHpak column (SB-806HQ, 8.0 × 300 mm, Showa Denko Co., Japan). A total of 10 μL of the sample was injected onto the column and eluted with water at a flow rate of 0.8 mL/min at 60ºC. The column was calibrated by dextrans as the relative molecular mass standards (Sigma, USA), as described in our previous study [12].

2.4. Monosaccharide composition and chemical analysis

High Performance Anion-Exchange Chromatography (HPAEC) was performed to determine the monosaccharide composition of the purified HCP, using a Bio-LC system (Dionex, USA) equipped with a pulsed amperometric detector (PAD). The purified HCP was dissolved in 1 mL dH_2O and mixed with an equal volume of 4.0 M trifluoroacetic acid (TFA). Samples were allowed to stand for 4 h at 100ºC and the acid-hydrolyzed sample was filtered through 0.45 μm syringe filter and vacuum dried using a Speed-Vac (Biotron, Korea). After removal of the residual acid by repeated vacuum drying, samples were analyzed on a CarboPac PA-1 column (Dionex, USA), following conditions for monosaccharide analysis by HPAEC-PAD as described previously [28]. Total carbohydrate was determined by a modified phenol-sulfuric acid method at 490 nm [29]. The amount of sulfate residues was determined by Loui's method [30], using Na_2SO_4 as a reference. Uronic acid content was determined by the carbazole assay [31], using D-glucuronic acid as a standard. Protein was quantified by Bradford method [32]. Phenolic compounds were quantified by Folin-Denis's method [33].

2.5. FT-IR and elemental analyses

Fourier-transform infrared (FT-IR) analysis [34] of the HCP sample was performed using the Nicolet 6700 (Thermo Scientific, USA) using KBr disks, after preparation of KBr pellets by mixing 100 μg KBr with 1.0 mL of the HCP solution (15 μg/mL) in dH_2O. Samples were stabilized under controlled humidity before acquiring the spectrum. IR spectra were recorded by accumulation of at least 64 scans, with a resolution of 4/cm. Weight percentages of the carbon, hydrogen, nitrogen and sulfur contents of the HCP were determined based on elemental analysis (EA) [35] using an EA1112 (CE Instrument, Italy) with a CHNS-Porapack PQS (CE Instrument, Italy) as described previously [12]. Approximately 1 mg of sample was injected, eluted with He, and detected with TCD (Thermal Conductivity Detector, CE Instruments, Italy). 2,5-Bis(5-tert-butyl-2-benzoxazol-2-yl) thiophene (BBOT) and aspartic acid were used as reference standards.

2.6. Cytotoxicity assay

RAW264.7 cells were suspended in complete RPMI1640 medium, and the proliferation of the cells was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) micro-culture tetrazolium viability assay [36]. Briefly, RAW264.7 cells were seeded at a density of 5×10^4 cells/well and incubated in a 96-well microplate in 100 μL of the medium and allowed for cells to attach to the plate for 24 h. Different concentrations of the HCP were prepared with serial dilution ranging from 1.25 to 10 μg/mL, while the negative control was treated with the medium. A total of 20 μL of MTT solution (5 mg/mL) was added to each well 24 h later, and incubated further for 4 h at 37°C. The supernatant was aspirated out and 200 μL of dimethyl sulfoxide (DMSO) was added into each well. Absorbance was measured at 595 nm using a microplate reader. Experiments were performed at least in triplicates.

2.7. Assay for cytokine secretion

The concentration of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1 (IL-1) and prostaglandin E_2 (PGE_2) released from murine RAW264.7 cells treated with the purified HCP was determined using an ELISA assay (Quantikine Immunoassay Mouse TNF-α, Quantikine Immunoassay Mouse IL-6, R&D systems, USA, Prostaglandin E_2 EIA Kit, Cayman, USA), as previous study demonstrated [12]. RAW264.7 cells (5×10^4 cells/mL) seeded into a 96-well microplate in RPMI1640 medium were incubated for 24 h in the presence of various concentrations of the purified HCP or lipopolysaccharide (LPS, 0.1 μg/mL). LPS used in this study was *Escherichia coli* EH100 endotoxin (Sigma, USA), which causes a pro-inflammatory reaction. Cells were kept at 37°C for 72 h in a humidified 5% CO_2 incubator. Supernatants from each well were harvested and the concentration of TNF-α, IL-1, and IL-6 was measured by optical density following the manufacturer's instructions.

2.8. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RAW264.7 cells were cultured in complete RPMI 1640 media in 48-well plates with HCP present at various concentrations, and LPS (0.1 μg/mL) was used as positive control. The cells were then cultured at 37°C for 24 h in humidified 5% CO_2 incubator. The total RNA from either HCP-treated or untreated RAW264.7 cells was prepared by using a Total RNA extraction Kit (Intron Biotechnology, Korea), according to the manufacturer's protocol. Isolated total RNA was stored at −80°C for further study. Expression levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) genes, which are responsible for the production of nitric oxide (NO) and prostaglandin E2 (PGE2), respectively, were determined by RT-PCR using the ONE-STEP RT-PCR PreMix Kit (Intron Biotechnology, Korea). RT-PCR products were separated by electrophoresis using 2% agarose gel, stained with ethidium bromide, and the gels were viewed under UV transillumination. The primers used in this study were as described previously [12]: GAPDH forward, 5'-CATTTCTTCTCCTGCAGCC-3'; reverse, 5'-TCTCCATGGTGGTGAAGACA-3'; iNOS forward, 5'-CTGCAGCACTTGGATCAGGAACCTG-3'; reverse, 5'-GGGAGTAGCCTGTGTGCACCTGGAA-3' and COX-2 forward, 5'-CCCCCACAGTCAAAGACACT-3'; reverse, 5'-GAGTCCATGTTCCAGGAGGA-3'. Sequence information of these genes was collected from the NCBI GeneBank (http://www.ncbi.nlm.nih.gov/). All RT-PCR experiments were performed at least in triplicates.

2.9. Statistical analysis

Data were expressed as the mean ± SD of three different experiments, unless indicated as the mean of averages of at least tree times of each experiment. The statistical significance of the difference between mean values was determined by the student's *t*-test. *P*-values less than 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Isolation and purification of a water-soluble polysaccharide

Algal polysaccharides have become increasingly important due to their range of biological activities and have con-

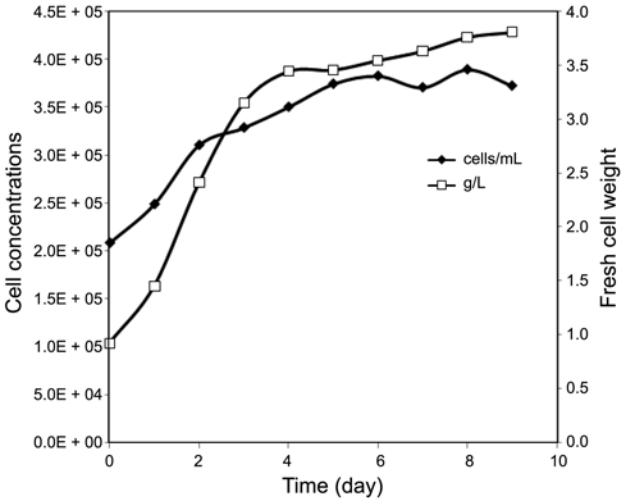

Fig. 1. Growth of *Haematococcus lacustris*, expressed as cell concentration (cell/mL) and fresh cell weight (g/L).

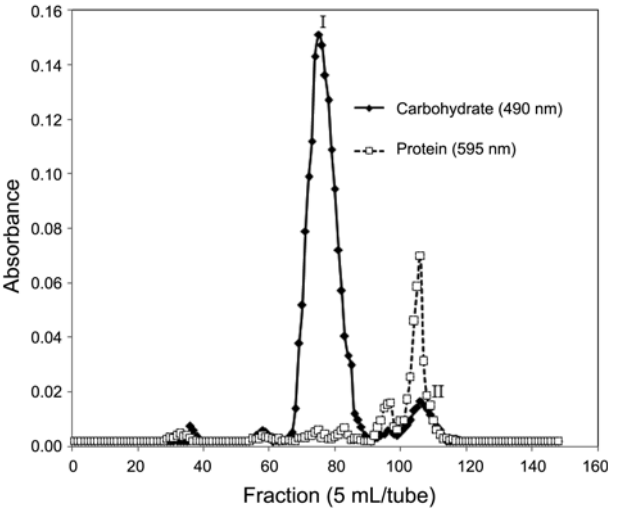

Fig. 2. Fractionation of polysaccharide by Sepharose CL-6B column chromatography. Each fraction was assayed for carbohydrates by a modified phenol-sulfuric acid method and assayed for protein by the Bradford method.

sequently been widely studied for their potential as natural therapeutic agents. In contrast to polysaccharides from macroalgae (seaweeds), such as fucoidans and carrageenan, microalgal polysaccharides have not been well studied and thus searching for new bioactive polysaccharides from microalgal species poses an exciting challenge [3,6]. Microalgae are known to produce a variety of consumer products such as omega 3 oil, chlorophyll, biodiesel and bioethanol [25]. Owing to recent progress in metabolic engineering technologies and large scale cultivation in a controlled photobioreactor system, microalgal species represent one of the most promising sources for new biomaterials and their industrial applications [6,25,26]. In this context, we isolated and characterized a water-soluble polysaccharide from cultures of *H. lacustris* and examined its immune modulating activity.

As shown in Fig. 1, cells reached a maximum cell density of 3.7×10^5 cell/mL and a fresh cell weight of 3.44 g/L on day 10 of cultivation. This crude preparation was then applied to the size-exclusion column chromatography (SEC) after dissolving in dH_2O (1%, w/v) containing 50 mM NaCl. The sample was eluted as two major carbohydrate-positive peaks (Peak I and II) and the fractions of 66 ~ 84 (Peak I) were combined, dialyzed against dH_2O, freeze-dried and designated as the HCP fraction, which yielded 0.12 g (dry mass) from 1 L culture (Fig. 2).

3.2. Chemical composition of the purified polysaccharide (HCP)

Chemical composition of the purified polysaccharide (HCP) was determined as shown in Table 1. The HCP was shown to be composed of neutral sugars (40.5% in mass), uronic acids (2.2%), phenolic compounds (0.6%) and sulfate groups (1.08%) as determined by the biochemical methods described in the Methods section. The protein content was, if any, negligible as determined by Bradford method [32]. In addition, four elements of the purified HCP were determined to be: carbon (5.31% w/w), hydrogen (3.50%), nitrogen (0.47%), and sulfur (0.97%), as summarized in

Table 1. Chemical composition of HCP and its molecular weight

	Neutral sugar[a] (mass %)	Uronic acid[b] (mass %)	Sulfate esters[c] (mass %)	Protein[d] (mass %)	Phenolic compounds[e] (mass %)	Proportion of monosaccharides[f] (mole %) Galactose	Glucose	Mannose	Molecular weight[g] (kDa)
HCP	40	2.2	1.08	N[h]	0.6	2.0	1.0	4.1	135

[a]Neutral sugars were determined by a modified phenol-sulfuric acid method at 490 nm.
[b]Uronic acid content was determined by the carbazole reaction, using D-glucuronic acid as a standard.
[c]Sulfate ester content was quantified by Loui's method, using Na_2SO_4 as a reference.
[d]Protein was quantified by Bradford method.
[e]Phenolic compounds of HCP were examined by Folin-Denis's method.
[f]Values were obtained by setting the sum of each mole number at 100%.
[g]The molecular weight of HCP was estimated by size-exclusion HPLC using dextrans as standard size markers (Sigma).
[h]Negligible.

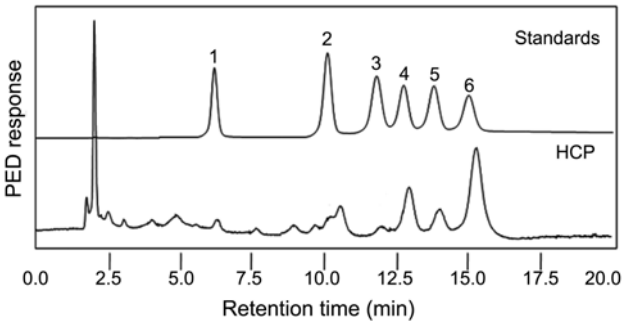

Fig. 3. HPAEC-PAD analysis for monosaccharide composition of the purified HCP. Standard monosaccharides used as references: 1. fucose; 2. galactosamine; 3. glucosamine; 4. glactose; 5. glucose; and 6. mannose.

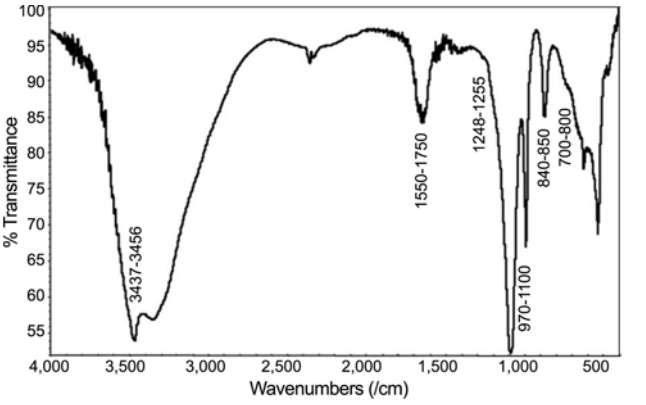

Fig. 4. The FT-IR spectrum of the purified HCP.

Table 1. The results clearly demonstrated the presence of sulfate groups in this polysaccharide and this was further evidenced by FT-IR analysis (Fig. 4). Interestingly, the high ratio of carbon versus proton from the EA analysis suggested for the presence of aromatic compounds (Table 2) and this was further evidenced by the FT-IR analysis (Fig. 4) as well as Folin-Denis's method [33].

3.3. Monosaccharide composition of the purified HCP

The monosaccharide composition of the purified HCP was shown to be fucose, galactosamine, glucosamine, galactose, glucose and mannose (Fig. 3). Among these, galactose, glucose and mannose were the major monosaccharides at a relative molar ratio of 2.0, 1.0, and 4.1, respectively (Fig. 3 and Table 1). A few minor peaks on the HPAEC-PAD HPLC chromatogram in Fig. 3 remained unidentified, indicating that this polysaccharide may contain other sugars as minor components. Taken together, these results suggest that the HCP is a sulfated heteropolysaccharide, a galactomannan mainly consisting of galactose and mannose. The apparent molecular mass of the purified HCP was estimated to be approximately 135 kDa by size-exclusion HPLC. The major monosaccharide composition of the purified HCP, a galactomannan, was similar to the water-soluble polysaccharide (SPS-CF) which was isolated and purified from Korean *Capsosiphon fulvescens*, as described in our previous paper. However, HCP has much lower sulfur content and other minor components compared to the SPS-CF. This difference in component sugars may have arisen mainly from the differences of species and may also give different biological activities.

3.4. FT-IR analysis

The primary structural information on the HCP was obtained by FT-IR analysis (Fig. 4). Band assignment was made according to the literature [12,38-41]. Broad IR bands seen at 3,437 ~ 3,456/cm correspond to OH and H_2O stretching vibrations. The envelope of strong bands at 970 ~ 1,100/cm is caused mainly by CC and CO stretching in pyranoid ring structures, and COC stretching of glycosidic bonds. These structure sensitive bands are a common characteristic of all polysaccharides. The bands and shoulders within bands at the 970 ~ 1,200/cm region varied in their position and intensities reflecting some differences in composition and substitution of monomeric units. Weak IR band at 1,248 ~ 1,255/cm was attributed to O=S=O stretching of sulfates, which is a good marker of sulfation. An additional sulfate absorption band of C-O-S at 840 ~ 850/cm was identified [42], where this region represents the vibrations of secondary sulfates at axial (C-4) positions of fucose or galactose units. The position of C-O-S bending band, however, may depend on other substitutions at the pyranoid ring, such as glycosylation, *O*-acetylation and/or additional sulfation [43]. On the other hand, the peak in the region of 1,550 ~ 1,750/cm indicated the presence of aromatics, probably polyphenols (C=C and C=O stretching vibrations) (Fig. 4). The minor broad shoulder that appeared in the range of 700 ~ 800/cm was assigned to out-of-plane CH bending vibrations in aromatics, which was in good agreement with the high carbon to proton ratio from the EA analysis (Table 2) [12]. The content of phenolic compounds was quantified by biochemical method following the Folin-Denis's method [33]. The FT-IR and elemental analyses of the purified HCP clearly demonstrated that HCP contains sulfate ester groups, indicating that it is a sulfated polysaccharide (SPS) (Fig. 4 and Table 2). The sulfate content in HCP was shown to be 1.08% (in mass), which is somewhat lower than those of fucoidan (7.4%) isolated from the sporophyll of Korean *Undaria pinnatifida* [9] and the polysaccharide SPS-CF (5.7%) of *Capsosiphon fulvescence* [12]. Like those from most macroalgal species, SPS are now also commonly found in microalgae and their sulfate contents are also variable ranging from the highest in *Porphyridium* sp. (9%) to the lowest in *P. aerrugineum* with

Table 2. Elemental analysis of HCP isolated from *H. lacustris*

Elements	Theoretical composition (%, *w/w*)		Experimental composition (%, *w/w*)
	BBOT*	Aspartic acid	HCP**
% N	6.50	10.49	0.476
% C	72.44	36.12	5.312
% H	6.11	5.27	3.503
% S	7.24	–	0.968

*2,5-Bis (5' tert-butyl-2-benzoxazol-2-yl) thio-phene (BBOT) and aspartic acid were used as references.
**Approximately 1 mg of the purified HCP was used for elemental analysis.

less than 1% [6]. Generally, it is known that the biological activity of algal polysaccharides depends on their structure [44] and degree of sulfation [6,22,45]. Proteins or peptides are found as covalently attached chemical components in some algal SPS [3,46]. However, the FT-IR, elemental analysis and Bradford protein assay clearly demonstrated no or negligible level of nitrogenous compounds in HCP, suggesting that it does not contain protein (Fig. 4 and Tables 1 and 2). Interestingly, this polysaccharide was shown to contain a small fraction of phenolic compounds (0.6%) (Fig. 4 and Table 1). The presence of phenolics in the purified HCP may suggest possible antioxidant activity of this polysaccharide [47], although this was not examined in the present study. Collectively, the FT-IR analysis suggested that HCP is a sulfated polysaccharide and may exist as a complex with a small fraction of phenolic compounds.

3.5. Immunostimulating activity of HCP

A number of immunomodulating polysaccharides modulate macrophage cytokine and/or chemokine production. Thus, we examined the effects of HCP on murine macrophage TNF-α production. *In vitro* activation of macrophages was examined with the different concentrations (0 ~ 10 μg/mL) of the purified HCP by measuring production of TNF-α and the expression levels of iNOS and COX-2 genes. Macrophage RAW264.7 cells are known to produce pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, and others in response to LPS, and/or pathogens. Among many cytokines, TNF-α has been implicated as a key cytokine playing an important role in the immune response as well as anti-tumor activity. The HCP did not show any detectable level of cytotoxicity on the RAW264.7 cells at the concentrations (up to 10 μg/mL) tested (data not shown). Macrophages are the first line of defense among innate immune responses against microbial infection. The defense mechanism of macrophages against pathogens includes pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, and others [48]. Macrophage RAW264.7 cells are known to produce cytokines in response to the addition of LPS

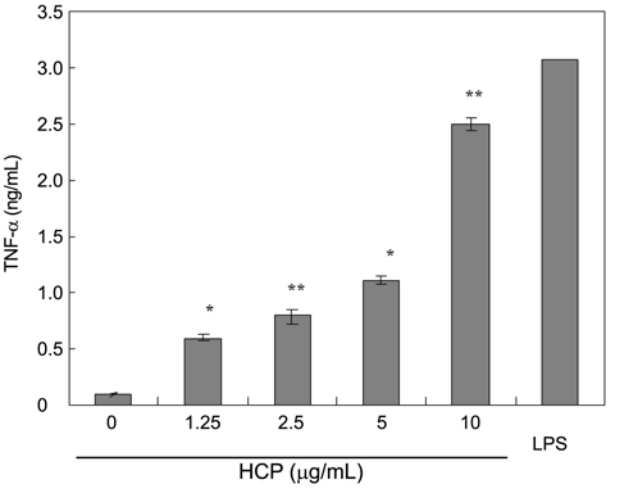

Fig. 5. Effect of HCP on TNF-α production. Murine macrophage RAW264.7 cells were stimulated by different concentration (0 ~ 10 μg/mL) of the purified HCP for 24 h. LPS (0.1 mg/mL) was used as positive control. Production of TNF-α was determined by ELISA. Values represent the mean ± S.D. The statistical significance of the difference between mean values was determined by the student's *t*-test. $*P < 0.05$ and $**P < 0.01$ was considered significant. Experiments were performed at least in triplicates.

(lipopolysaccharide), and this system is used to detect the modulating activities of compounds on cytokine production [49]. Thus LPS was used as a positive control to compare the degree of immunomodulating activity of our HCP in this study. As shown in Fig. 5, untreated murine RAW264.7 cells produced negligible amounts of TNF-α; whereas LPS (0.1 μg/mL), as a positive control, greatly enhanced the TNF-α production as expected.

The ELISA assay demonstrated that the sulfated HCP of *H. lacustris* significantly stimulates the release of the pro-inflammatory cytokine, TNF-α, in a dose-dependent manner in RAW264.7 macrophage cells to a level 27-times greater than the normal (untreated). At the highest concentration (10 μg/mL) of HCP used in this study, the amount of TNF-α produced by macrophages reached approximately 80% of that seen in LPS-treated control (Fig. 5). Searching for new biomaterials which can potentiate the immune function has become an important issue in immunopharmacology and oncotheraphy. Photosynthetic microalgae are also being recognized as rich sources of such compounds owing to their enormous species biodiversity and because the bioactivities and structures of microalgal polysaccharides have been relatively less extensively studied than those of macroalgal species, thus providing a better chance to find novel compounds [4,6,25].

3.6. Immune modulating activity of HCP

Expression of iNOS and COX-2 in macrophages is regulated by transcriptional activation during immune responses

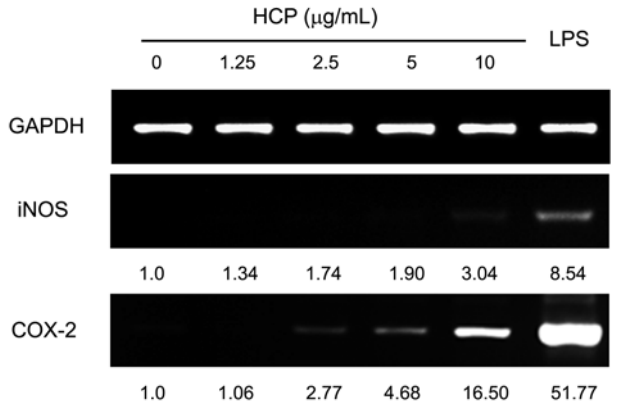

Fig. 6. Effects of the purified HCP on the expression of iNOS and COX-2 in RAW264.7 cells. Cells (5×10^4 cells/mL) were incubated with different concentration (0 ~ 10 μg/mL) of the purified HCP for 24 h. LPS (0.1 μg/mL) was used as positive control. The mRNA levels of iNOS and COX-2 were determined by RT-PCR and the numerical values under each blot represent the amount of iNOS and COX-2 mRNA, expressed as the ratio of densitometric measurement to the corresponding internal standard (GAPDH). Experiments were performed at least in triplicates.

and inflammation [12,50,51]. Therefore, RT-PCR analysis was performed to evaluate the effects of purified HCP on macrophage activation by measuring the expression levels of iNOS and COX-2 mRNA from RAW264.7 cells. The expression levels of mRNAs of iNOS and COX-2 were gradually increased in a dose-dependent manner of HCP (0 ~ 10 μg/mL) (Fig. 6). As estimated by densitometric quantification, 10 mg/mL of the HCP stimulated the expression of iNOS and COX-2 from macrophages approximately 2.7 and 16 times higher, respectively, than the normal samples (untreated), although their levels were shown to be much lower than those obtained with 0.1 μg/mL LPS treatment. It was notable that stimulation of COX-2 gene expression was more significant than that of iNOS over the concentrations tested in this study. Collectively, the results clearly demonstrated that the sulfated HCP from *H. lacustris* has potent immune modulating activity by stimulating the release of the pro-inflammatory cytokine, TNF-α, and inducing the expression of iNOS and COX-2 genes in RAW264.7 murine macrophages. However, the effects of HCP on the secretion of IL-6 and IL-1 were very low or negligible (data not shown). Macrophages are known to regulate innate immunity as well as adaptive immune responses by production of cytokines such as interleukin-1β (IL-1β), IL-6, and TNF-α [52], which are signaling molecules that are involved in cellular defense processes such as immune responses and inflammatory reactions. Many macroalgal polysaccharides are known to show immune modulating activities by activating macrophage functions as examplified by the polysaccharides from *Porphyra yezoensis* [53] and *Gracilaria verrucosa* [54], SPS-CF from a green alga *C. fulvescens* [12] and Ulvan from *Ulva rigida* [55]. Compared to these macroalgal polysaccharides, reports on the immune modulating activities of microalgal polysaccharides are relatively rare. The polysaccharides isolated from microalgal species, *Chlorella stigmatophora* and *Phaeodactylum tricornutum*, have shown to exhibit anti-inflammatory and immunomodulatory [56] activities. Cell wall-derived sulfated polysaccharides from *Porphyridium* sp. have been reported for their antitumor [19] and anti-inflammatory activities [22]. Like many other macroalgal polysaccharides ever reported (Table 3), the purified sulfated polysaccharide, HCP, of the microalga *H. lacustris*, also showed significant immune stimulating activity in the macrophage RAW264.7 cells by dose-dependently stimulating the expression of iNOS and COX-2 genes (Fig. 6).

NO plays important roles in a variety of physiological processes and is a key participant in host defense by acting as an intracellular messenger and regulating cellular functions, such as inflammation, as well as eliminating pathogens and tumor cells [57]. Prostaglandins (PGs) are important mediators of the inflammatory response, and are involved in diverse regulatory functions, including immune responses. Therefore, the fact that the sulfated HCP, isolated from the microalga *H. lacustris*, is able to activate macrophages to produce pro-inflammatory mediators, TNF-α, NO, and PGE2, clearly indicates that HCP has potent immune modulating activity by activating macrophages

Table 3. Biological activities of polysaccharides derived from algal species

Algal species	Biological activity	Major component	References
Porphyridium cruentum	Antiviral activity	Sulfated polysaccharides	20
Chlorella stigmatophora & *Phaeodactylum tricornutum*	Anti-inflammatory & Immunomodulatory activities	Glucose, glucuronic acid, xylose, and ribose/fucose and/or mannose	56
Laminaria japonica	Antioxidant activity	Galactose, fucose	11
Capsosiphon fulvescens	Immunostimulating activity	Glucuronogalactomannan	12
Ecklonia kurome	Anticoagulant activity	Fucan sulfate	47
Sargassum thunbergii	Antitumor activity	Fucoidan or L-fucan	10
Haematococcus lacustris	Immunostimulating activity	**Galactomannan**	**In this study**

during early stages of the immune response.

4. Conclusion

A water-soluble polysaccharide was isolated and purified from the culture filtrate of a photosynthetic green microalga *H. lacustris*. It was shown to be an acidic polysaccharide, a sulfated galactomannan, with no proteinaceous materials and named as HCP in this study. The observed immune modulating activity of this polysaccharide on RAW264.7 murine macrophages, without any detectable level of cytotoxicity, suggests that it can activate early innate immune responses. Since *H. lacustris* can be cultivated in a photobioreactor system under controlled conditions and is being widely explored for the production and bioengineering of astaxanthin, a potent antioxidant, the production of the sulfated polysaccharide, HCP, having immunomodulating activity augments the potential of *H. lacustris* for its industrial applications as a functional material for healthy foods.

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