

Immune-enhancing effects of polysaccharides with different molecular weights obtained from *Gloiopeltis furcata*

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불등분가사리 다당류의 분자량에 따른 면역증강 효과

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Abstract

This study investigated the immune-enhancing effects of polysaccharides extracted from *Gloiopeltis furcata* (red seaweed) with different molecular weights. A crude polysaccharide mixture was hydrolyzed using acid treatment (0.1 N HCl) and three molecular weight fractions were generated and filtered using centrifugation: (≤ 10 kDa, 10 to 100 kDa, and ≥ 100 kDa). Nitric oxide (NO) production in RAW264.7 cells treated with 0.01-0.5 $\mu\text{g/mL}$ polysaccharides ≥ 100 kDa was 12.28-19.05 μM . Treatment with polysaccharides ≥ 100 kDa increased cytokine levels, including TNF- α and IL-6 levels, in a dose-dependent manner. Polymerase chain reaction analysis also revealed marked increases in iNOS and COX-2 mRNA expression levels. These findings lead us to conclude that macrophage activation induced by polysaccharides ≥ 100 kDa was greater than that induced by polysaccharides ≤ 10 kDa or between 10 and 100 kDa. The polysaccharides ≥ 100 kDa extracted from *Gloiopeltis furcata* investigated herein are potentially useful as natural immune-enhancing agents. These findings provided further insights into the potential use of ≥ 100 kDa as immunopotentiator or new function food.

Key words : *Gloiopeltis furcata*, polysaccharides, immune-enhancing, nitric oxide, cytokine production

Introduction

Marine algae, also known as seaweed or macroalgae, are photosynthetic eukaryotic organisms that can be found in coastal areas. There are three main seaweed taxa that are classified based on their pigmentation: red algae, green algae, and brown algae (1). Seaweed can survive in harsh environmental conditions because they are able to use numerous strategies to adapt to new challenging environments. To survive in such diverse and extreme environments, seaweed produces a variety of natural bioactive

compounds and metabolites, such as polysaccharides, polyunsaturated fatty acids, and phlorotannins (2,3).

Polysaccharides are a complex group of macromolecules that have a wide ranges of bioactivities. These polymers are ubiquitous in nature, occurring in a great number of different organisms, such as mammals and invertebrates. Marine seaweeds are the most important source of non-animal polysaccharides. The molecular structure of these polysaccharides vary depending on the species of seaweed. Based on this, polysaccharides from marine seaweed could be compounds with unique structures and novel biological activities. These polysaccharides have been extensively studied because of their various biological activities including, immunomodulatory, anti-cancer, and antioxidant activities (4-6).

Gloiopeltis furcata (*G. furcata*) is a red alga belonging to the genus *Gloiopeltis* of the *Endocladiaaceae* family, that

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been widely used in traditional herbal medicine, and as a food thickener (7). Polysaccharides with different physiological activities (antitumor, antiviral, anti-inflammatory) have been found in water and ethanol extracts prepared from *G. furcata* (8,9). However, the physiological activities of *G. furcata* polysaccharides originating from south Korea, have not been studied in detail.

The biological activity of polysaccharides depends on their structural features such as molecular weight, degree of sulfation, type of sugar, and glycosidic branching. Therefore, when polysaccharides of different molecular weights are involved, changes in biological activity are assured. Moreover, the molecular interactions may be affected by the molecular weights of polysaccharides, there by resulting in different biological properties (10-12).

In this study, the immune-enhancing effect of polysaccharides with different molecular weights from *G. furcata* were investigated.

Materials and Methods

Materials

Gloiopeltis furcata (*G. furcata*) was purchased from a local supermarket (Daegu, Korea). *G. furcata* was thoroughly washed with water and air-dried at 50°C. The dried samples were milled using a Wiley-type cutting mill (J-NCM; Jisico Co., Seoul, Korea), sieved (<0.5 mm), and stored at -20°C.

Extraction of crude polysaccharides

G. furcata (100 g) was suspended in distilled water (10 L), and 3% Viscozyme L (enzyme to substrate ratio) was added. The hydrolytic reaction was performed for 14 h at 60°C. After centrifugation at 3,000 ×g for 30 min (Supra 30K; Hanil Science Industrial Co., Seoul, Korea) to remove debris, the filtrate was concentrated in a rotary evaporator and then precipitated with 5 volumes of 94% ethanol for 24 h at 4°C. The precipitates were acquired after centrifugation and dissolved in distilled water.

Preparation of polysaccharides with different molecular weights

Polysaccharides with different molecular weights were identical to the sample used in our previous study (13). Briefly, crude polysaccharide preparation (10 g) was dissolved in 0.1 N HCl (1 L) and maintained at 50°C for different periods of time. The pH of the solution was then adjusted to 7.0

by the addition of ice-cold 0.1 N NaOH. The Amicon ultra-15 centrifugal filter unit (Merck Millipore, Tullagreen Carrigtwohill, Ireland) used for the separation was made of polysulfone material, which is composed of the pore size with molecular weight cut off (MWCO) 10 kDa and 100 kDa Dissolved crude polysaccharides (15 mL) were placed in a centrifugal filter unit (15 mL capacity) and centrifuged at 3,000 ×g for 30 min (1236MG, Gyrozen Co., Daejeon, Korea). after which they were lyophilized at -70°C under reduced pressure (Free Zone 2.5, Labconco Co., Kansas, MO, USA).

Cell culture

The RAW264.7 cells (Korean Cell Line Bank, Seoul, Korea) were cultured in DMEM supplemented with 10% fetal bovine serum (WELGENE Inc., Daegu, Korea) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin; GIBCO, Rockville, MD, USA) at 37°C in a humidified atmosphere with 5% CO₂ (MCO-18AIC, SANYO Co., Sakata, Japan).

Cell proliferation

Proliferation of the RAW264.7 cell lines treated with polysaccharides with different molecular weights was evaluated using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. Briefly, a total of 5×10⁴ cells/well were seeded in to 96-well plates for 24 h, and the cells were treated with various concentrations of the isolated compounds for 24 h. After treatment with each sample, MTT solution, diluted in phosphate buffered saline (PBS, 1 mg/mL final concentration, Sigma-Aldrich Co.), was then added to each well at 1/10 volume of media. The cells were incubated for 4 h at 37°C, and dimethyl sulfoxide was added to dissolve the formazan crystals. The absorbance was measured at 570 nm using a microplate reader (UVM-340, ASYS, Biochrom, Eugendorf, Austria). Proliferation was calculated as follows: Activity (%)=(absorbance sample/absorbance control)×100.

Nitric oxide production

Nitric oxide (NO) secretion from macrophages and the nitrite concentration were determined by the Griess reaction. RAW264.7 cells were placed in a 96-well plate (5×10⁴ cells/well in a volume of 100 µL) and incubated for 24 h at 37°C. Cultured cells were treated with 100 µL of each sample or LPS (final concentration 0.1 µg/mL), which was used as a positive control. After incubation for 24 h at 37°C, 100 µL of the cultured cell supernatant was mixed with an

equal volume of griess reagent (1% sulfanilamide, and 0.1% N-[1-naphthyl] ethylenediamine hydrochloride in distilled water) and left at 25 °C for 10 min. Absorbance at 540 nm was measured using a microplate reader (UVM-340, ASYS Co.). NO secretion from RAW264.7 cells was calculated with reference to a standard curve obtained using NaNO₂.

Cytokines production

For cytokine determination, RAW264.7 cells were cultured in the presence of each sample or LPS (final concentration 0.1 µg/mL). Cells were incubated for 24 h, and the supernatant was collected and used to determine cytokine production following stimulation by each sample. The production of TNF-α and IL-6 was measured using commercially available ELISA kits (Pepro Tech Inc., Rocky Hill, NJ, USA).

PCR analysis of mRNA expression

To evaluate the mRNA expression levels of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), total RNAs from each samples or LPS (final concentration 0.1 µg/mL) treated RAW264.7 cells were prepared using a total RNA (1 µg) Extraction Kit (Enzynomics Co., Daejeon, Korea). RT-PCR (Mastercycler® Nexus; Eppendorf AG, Hamburg, Germany) was performed using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). Reverse transcriptase amplification was performed by initial denaturation (94 °C for 30 sec), annealing (55 °C for 30 sec), and extension (72 °C for 1 min), followed by a final extension step at 72 °C for 10 min. The products obtained by RT-PCR were separated by electrophoresis (Mupid-ex, Advance, Tokyo, Japan) using a 1.2% agarose gel and stained with Eco View (Daemyung Science Co., Ltd., Seoul, Korea). The gels were then viewed under UV transillumination. The primer sequences (Bioneer, Daejeon, Korea) used in this experiment were as Table 1.

Table 1. Sequence of the primers used for RT-PCR

| Gene | Primer sequence | Size (bp) |
|---------|---|-----------|
| iNOS | F: 5'CCCTTCCGAAGTTTCTGGCAGCAGC-3' R: 5'GGCTGTGACAGCCCTCGTGGCTTTGG-3' | 496 |
| COX-2 | F: 5'CACTACATCCTGACCCACTT-3' R: 5'ATGCTCTGCTTGAGTATGT-3' | 696 |
| β-Actin | F: 5'GTGGGCCGCCCTAGGCACCAG-3' R: 5'GGAGGAAGAGGATGCGGCAGT-3' | 603 |

Statistical analysis

All experiments were performed in triplicate, and means±SD or average values are reported. Duncan's

multiple-range test was used to adjust for multiple comparisons, and null hypotheses were rejected at the 0.05 level. All data were analyzed using SPSS/Windows software (Version 19.0; SPSS Inc., Chicago, IL, USA).

Results and Discussion

Cell proliferation

In our previous study, we preparation of polysaccharides with different molecular weights from *G. furcata*, further investigated the chemical compositions (13). The major constituents in molecular weight fractions were total sugar (81.37-85.82%), uronic acid (27.89-32.85 g/100 g), sulfate (33.38-39.04%), and protein (0.35-3.16%), respectively.

Macrophages play critical roles in host defense, including the phagocytosis of pathogens and apoptotic cells, cytokine production, and the proteolytic processing and presentation of foreign antigens (14). The effect of a crude polysaccharide preparation from *G. furcata*, and sub-fractions of the same polysaccharide preparation (0.01-0.5 µg/mL) having different molecular weights, on RAW264.7 cell proliferation are shown in Fig. 1. When RAW264.7 cells were cultured with the different sub-fractions of the polysaccharides with different molecular weights, their proliferation increased only slightly, suggesting that these polysaccharides are not cytotoxic to RAW264.7 cells over the range of concentrations tested.

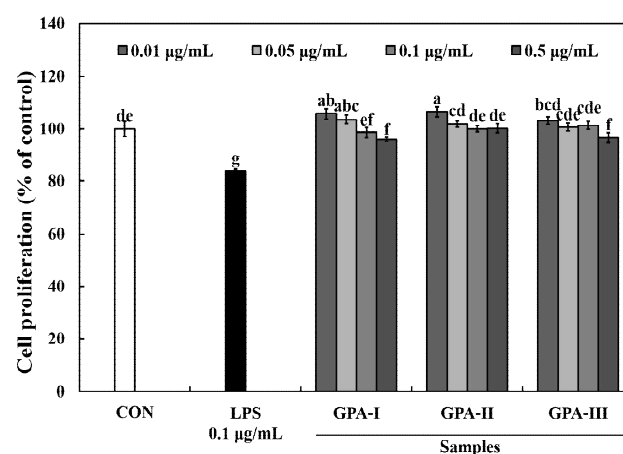


Fig. 1. Effect of polysaccharides with different molecular weights from *G. furcata* on the cell proliferation of RAW264.7 cell.

GPA- I , polysaccharides with molecular weight ≤10 kDa; GPA- II, polysaccharides with molecular weight 10-100 kDa; GPA-III, polysaccharides with molecular weight ≥100 kDa.

Mean±SD (n=3) followed by the same letter are not significantly different (p<0.05).

Nitric oxide production

Activated macrophages (RAW264.7) release immunomodulatory

factors, such as NO, as well other factors, to remove pathogens and inhibit the growth of invading cells. In particular, the nitric oxide released from macrophages is a highly reactive free radical that plays an important role in the regulation of various immune responses. Therefore, RAW264.7 cells are often used to determine the immunomodulatory activities of various compounds by examining nitric oxide production, as well as their cytokine production profile (15). The polysaccharides with different molecular weights (0.5 $\mu\text{g/mL}$) were able to significantly stimulate macrophage cells and produce a considerable amount of NO (range of 14.10-19.05 μM), in a dose-dependent manner (Fig. 2). The amount of NO released was higher for polysaccharides of GPA-III (≥ 100 kDa) compared to polysaccharides with GPA- I (≤ 10 kDa) or GPA- II (10-100 kDa) which resulted in increased levels of NO (range from 12.28-19.05 μM). Generally, NO plays an important role as a vasodilator, neurotransmitter, and in the immunological system as a defense against tumor cells, parasites, and bacteria (16). These results suggest that the sulfate content and the molecular weight of sulfated polysaccharides may contribute to NO production. Moreover, Leiro et al. (17), and Tabarsa (18) have reported that polysaccharides from *Ulvarigida* and *Ulva intestinalis* induce high NO production, proportionate to their sulfate contents and MW. These findings suggest that the MW and high sulfate content of polysaccharides are pivotal for its high bioactivity.

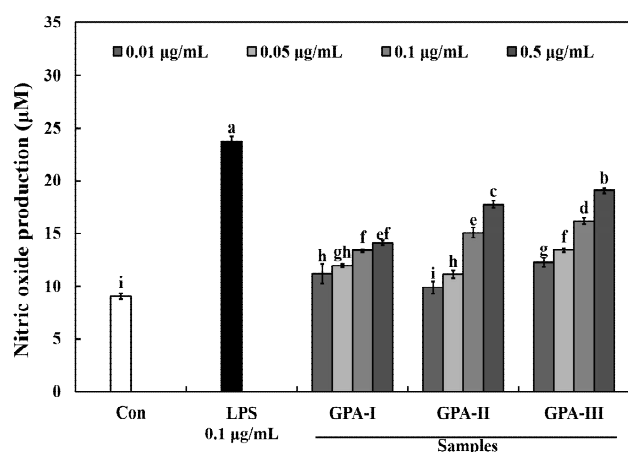


Fig. 2. Effect of polysaccharides with different molecular weights from *G. furcata* on the nitric oxide production of RAW264.7 cell.

GPA- I, polysaccharides with molecular weight ≤ 10 kDa; GPA- II, polysaccharides with molecular weight 10-100 kDa; GPA-III, polysaccharides with molecular weight ≥ 100 kDa.

Mean \pm SD (n=3) followed by the same letter are not significantly different (p<0.05).

Cytokine production

Part of the defense mechanism used by macrophages against pathogens includes the release of pro-inflammatory

cytokines such as TNF- α , and IL-6. Polysaccharides with a GPA-III (≥ 100 kDa) stimulated the production of TNF- α (35.77-62.37% of positive control), and IL-6 (48.42-67.87% of positive control) from RAW264.7 macrophages to levels that were 2-4 times greater than the negative control (13.02%, and 15.85% of positive control). These increases in both TNF- α , and IL-6 secretion from RAW264.7 cells occurred in a dose-dependent manner (Fig. 3). These data suggest that polysaccharides with different molecular weights have a beneficial effect on immune stimulation. Cytokines are signaling molecules that control homeostasis of the organism by regulating cell differentiation, proliferation, and apoptosis, as well as defense functions such as immune responses and inflammatory reactions. Cytokines are produced by cells that possess regulatory properties and are found very close to, or in direct contact with, target cells. By binding to specific

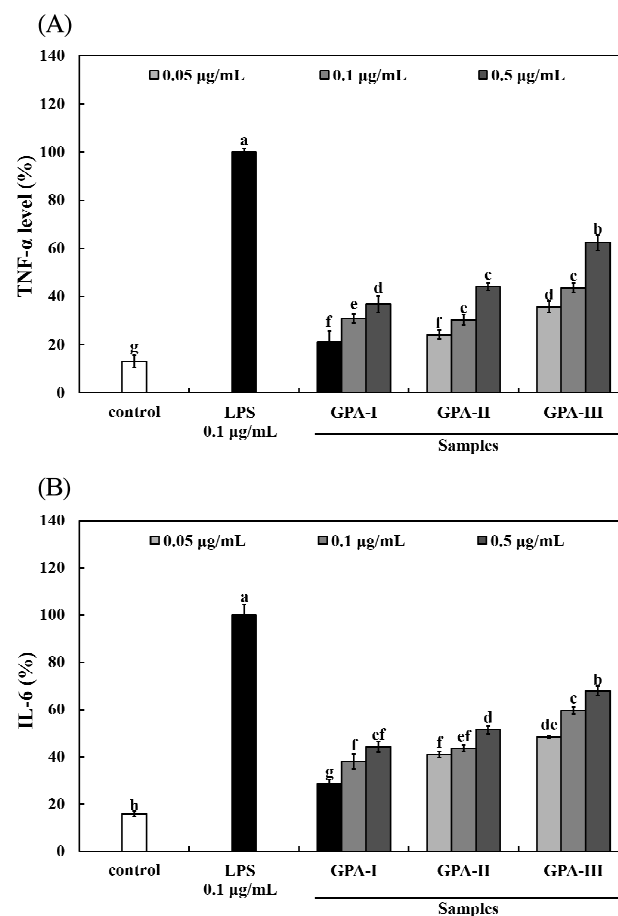


Fig. 3. Effect of polysaccharides with different molecular weights from *G. furcata* on the cytokine of RAW264.7 cell.

(A), TNF- α ; (B), IL-6.

GPA- I, polysaccharides with molecular weight ≤ 10 kDa; GPA- II, polysaccharides with molecular weight 10-100 kDa; GPA-III, polysaccharides with molecular weight ≥ 100 kDa.

Mean \pm SD (n=3) followed by the same letter are not significantly different (p<0.05).

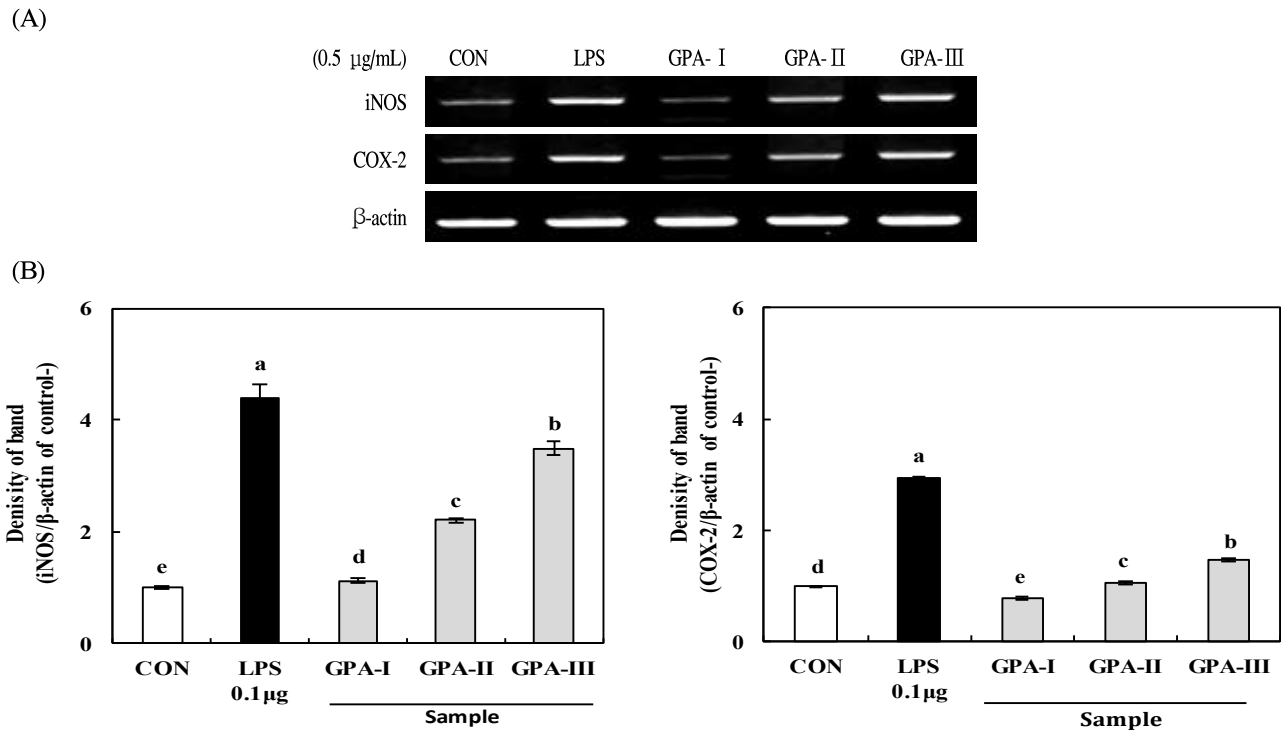


Fig. 4. Effect of polysaccharides with different molecular weights from *G. furcata* on the RT-PCR of RAW264.7 cell.

(A) The RT-PCR products were separated by electrophoresis using a 1.2% agarose gel, DNA bands were visualized by ultraviolet light, and (B) all samples were documented with image J software, were normalized against a β -actin control band.

GCP, crude polysaccharide; GPA-I, polysaccharides with molecular weight ≤ 10 kDa; GPA-II, polysaccharides with molecular weight 10-100 kDa; GPA-III, polysaccharides with molecular weight ≥ 100 kDa.

Mean \pm SD (n=3) followed by the same letter are not significantly different ($p < 0.05$).

cell surface receptors, they can exert their effects on cell proliferation, differentiation, as well as exerting other effects. Once released into the blood they behave like hormones and affect different tissues and organs, often distant from the original site of release (19). TNF- α is a cytokine that has both antitumor and immunoregulatory functions. It has been recognized to be an important host defense cytokine that can affect tumor cells. IL-6 is also considered to be an important mediator of acute inflammatory responses and functions as a differentiation and growth factor for hematopoietic precursor cells, B cells, T cells, keratinocytes, neuronal cells, osteoclasts, and endothelial cells (20).

RT-PCR analysis of mRNA expression

Although host defense mechanisms are complex, it is known that macrophages are key participants in the innate immune response to the invasion of pathogenic organisms (21). RAW264.7 macrophage cells were treated with the different subfractions of polysaccharides with different molecular weights, and RNA was harvested from the cells and subjected to RT-PCR. As shown in Fig. 4, the sulfated polysaccharides with different molecular weights stimulated

the induction of the mRNAs encoding iNOS and COX-2. Moreover, the data revealed that polysaccharides with a molecular weight ≥ 100 kDa might be immunostimulatory polysaccharides that could activate macrophages. The sulfated polysaccharides with a GPA-III (≥ 100 kDa) also stimulated the production of the effector molecule NO in macrophages. It has been found that NO inhibits the early stages in viral replication, and thus prevents viral spread, promoting viral clearance and recovery of the host (22). These results were supported by studies by Lee et al. (23), who demonstrated that sulfated galactans from *Codium fragile* had macrophage stimulating effects.

요 약

본 연구에서는 불등폴가사리(홍조류) 다당류를 기능성 소재로의 활용 가능성을 확인하기 위하여 분자량이 다른 다당류 3종을 제조하여 다양한 면역증강 효과를 확인하였다. GPA-III(≥ 100 kDa)는 nitric oxide 및 cytokine(TNF- α 및 IL-6)분석에서 농도 유의적으로 함량이 증가하였으며, RT-PCR 분석을 통한 유전자 발현에서 iNOS 및 COX-2에서

높은 발현을 유도하여 면역증강 효과를 확인하였다. 따라서, GPA-III는 기능성식품 및 의약품의 잠재적 응용 가능성을 가지는 면역 증강제임을 나타내었다.

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