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Immuno-stimulatory Effects of Sulfated Polysaccharides Isolated from *Codium fragile* on Interleukin-1β Gene Expression in Olive Flounder, *Paralichythys olivaceus*

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Sulfated polysaccharides are known to be immune-stimulators in mammals and can be used as food additives to enhance immunity. In this study, the immune-stimulating activity of water-soluble anionic macromolecules F_2 fractionation isolated from *Codium fragile* using ion-exchange chromatography was tested in olive flounder, *Paralichythys olivaceus*, in vitro and in vivo. The gene expression of interleukin (IL)-1β was adopted to check the immune-affection. As a result, in vitro study revealed that the expression of IL-1β was significantly upregulated in head kidney cells by 1 and 5 μg/ml of polysaccharides 4 h and by 5 μg/ml of polysaccharides at 24 h. In vivo, IL-1β gene expression in head kidney was significantly upregulated by 20 and 100 μg of the polysaccharides at day 1 post-i.p. injection, while downregulated at day 3 but not significant. Meanwhile, in peritoneal cells, it was upregulated by 20 μg of the polysaccharides at day 1 but the upregulation was sustained until day 3 though it was not significant. These results indicate that the sulfated polysaccharides from *C. fragile* are an immune-stimulator and might be potential feed additives for olive flounder.

Keywords: *Codium fragile*, Sulfated polysaccharides, Olive flounder, Interleukin-1β

Introduction

Sulfated polysaccharides are anionic macromolecules that contain sulfates. They are the predominant constituents in marine algae including both red and green algae. The sulfated polysaccharides are complex and heterogeneous biopolymers with species-specific macromolecular structures vary in their monosaccharide units, glyosidic patterns and sulfate contents (Costa et al., 2010; Mehdi et al., 2012; Pereira et al., 2002). They are known to have the biological functions of antioxidant, anti-allergic, anti-virus, anticancer, anticoagulant activity (Ngo et al., 2013).

Sulfated polysaccharides extracted from algae are found to be an immune-stimulator in many mammalian studies both in vitro and in vivo. Kim et al. (2011) demonstrated that sulfated polysaccharides extracted from *Enteromorpha prolifera* can activate the expression of immune related cytokine genes in murine macrophage cell line RAW 264.7 and promote the nitric oxide (NO) production. Sulfated polysaccharides extracted from red seaweed, *Champia feldmannii* could stimulate the production of specific antibodies (Lins et al., 2009). It was also found that the fucoidan extracted from seaweed *Fucus vesiculosus* have functions of up-regulating immunity and promoting the maturation of dendritic cells (Kim et al., 2007).

IL-1β is a cytokine which is known to be an important mediator of the inflammatory response during infection or injury. It is the most studied and best characterized in the interleukin-1 family (Gloria et al., 2013). It can be produced in different kinds of cells including macrophage that plays a crucial role in both innate and adopted immunity. So IL-1β can be served as a good indicator of the immune response. Tabarsa et al. (2013) and Kim et al. (2011) have reported the stimulatory effects of the sulfated polysaccharides from *Codium fragile* and *Ulva rigida* on the gene expression of IL-1β in RAW264.7 cells, respectively.

In this study, we have targeted head kidney and peritoneal cells to test the immune-activity of the sulfated polysaccharides in olive flounder. Head kidney is chosen because it is a main hemopoietic

and immune organ in fish and contains a large amount of macrophages and other kinds of immune cells (Uribe et al., 2011). Meanwhile peritoneal cells were chosen to assess immune response in the direct administered site after i.p. injection. In a previous study, response of peritoneal cell in fish has been a hot research topic as vaccines are frequently administered by intraperitoneal injection. At the same time, it could cause inflammation, during that time the macrophages can be as much as 60% in all the cell contents (Jørgensen et al., 1993).

In this study, we used the most immune-enhancing fraction F_2 fractionations the sulfated polysaccharides extracted and then fractionated using ion-exchange chromatography *from Codium fragile*, a dark green alga which is widely distributed along the shores of East Asia, Oceania, and North Europe (Tabarsa et al. 2013). We did research on the cytokine IL-1β in vitro and in vivo on olive flounder (*Paralichthys olivaceus*) to see whether the sulfated polysaccharides of *Codium fragile* can be the potential feed additive of fish.

Materials and Methods

1. Polysaccharides

The polysaccharides are extracted from *Codium fragile* and fractionated using ion-exchange chromatography, and the most immune-enhancing fraction F_2 was used in this study (Tabarsa et al., 2013). The polysaccharides were diluted with phosphate buffer saline (PBS, PH = 7.6, WelGene, Korea) to the concentration of 1 mg/ml, autoclaved at 121℃ for 15 min and stored at -20℃ before use.

2. Fish

Olive flounder, *Paralichythys olivaceus*, (around 10 g) was raised in circular 200 ℓ tanks supplied with seawater at an ambient temperature of approximately 15℃ with 12/12 h illumination and fed with a commercial pellet diet (Suhyup feed, Korea) in the Marine Biology Center for Research and Education of Gangneung-Wonju National University. Fish was fed twice a day (at 9:00 and 17:00 h).

3. In vitro analysis of gene expression in head kidney

Four healthy olive flounder (10 g) were anesthetized, sacrificed by cutting the spinal cord and aseptically taken head kidney. The head kidney cells were obtained by passing through a 100 μm nylon mesh (BD Biosciences, USA) and suspending in L-15 medium (Gibco, USA) containing 10% Fetal Bovine Serum (FBS, Gibco), 100 U/ml of penicillin (Gibco) and 100 μg/ml of streptomycin (Gibco). The cells were washed by centrifugation at 800 g at 4℃ for 10 min and adjusted to 2 × 10^6 cells/ml. One milliliter of head kidney cells were transferred to a well of 6-well plate and incubated with 0, 0.2, 1, 5 μg/ml of polysaccharides for 4, 8, 24 h at 25℃. The cells were lysed with QIAzol (Qiagen, Germany) and stored at -80℃.

4. In vivo analysis of gene expression in peritoneal cells and head kidney

Ten healthy olive flounder (10 g) were anesthetized and intraperitoneally (i.p.) injected with the sulfated polysaccharides at the doses of 0, 4, 20, 100 μg per fish. The fish was sacrificed at 1 or 3 days after injection by over dose anesthetization and cutting spinal cord. To collect peritoneal cells, fish was i.p. injected with 200 μl of PBS. The PBS was recollected and centrifuged at 800 g for 10 min to collect peritoneal cells. The liquid was discarded and the remaining cells were added with 1 ml of QIAzol. Head kidney was asceptically taken and stored in 1 ml of QIAzol. All the samples were snap frozen in liquid nitrogen and stored at -80℃ before used.

5. Total RNA extraction and cDNA synthesis

Total RNA was isolated from cells or tissues and reversed transcribed following the protocols of QIAzol and the reverse transcription kit (Thermo scientific, USA). Briefly, the cells or the tissues were homogenized in a 2 ml RNase-free tube and added with 0.2 ml chloroform after incubation for 5 min at room temperature, and then shaking vigorously for 15 s. The tubes were put at room temperature for 2~3 min and then centrifuged at 12,000 × g for 15 min at 4℃. The aqueous phase, around 0.45 ml, was transferred to a new tube, with an equal amount of isopropanol added to the tube, and then the tubes were placed at room temperature for 10 min. After incubation at room temperature, the tubes were centrifuged at 12,000 × g for 10 min at 4℃. The supernatant was discarded and the tubes were washed with 1 ml 75% ethanol with the way of centrifuging at 7,500 × g for 5 min at 4℃ for twice and let the RNA air-dry. The RNA was dissolved in RNase-free water (Gibco). Electrophoresis and Nanodrop 1000 (Thermo Scientific) were used to check the quality and quantity of the RNA. As to

Table 1. Oligonucleotide primers used for Q-PCR analysis

Gene	Accession no.	Primer	Sequence (5' - 3')	Size
Elongation factor-1α	AB017183	EF1α F EF1α R	CTCCACTGAGCCCCCTTACA GTCTCCGTGCCAACCAGAGA	235 bp
Interleukin 1β	AB070835	IL-1β F IL-1β R	GACAGTGAGATGGTGCGATTTC ACCATCACTGGCCTGTTGTCT	128 bp

the reverse-transcription, 1 μg of RNA was used, with the way as follows, 1 μg of RNA together with 0.5 μg of Oligo(dT)$_{18}$ (Thermo Scientific) and added with amount of RNase-free water to a final volume of 13 μl in a nuclease-free 0.2 ml tube; The mixture was incubated at 65℃ for 5 min and then chilled in ice for 2 min; After got out the tubes from ice, the tubes were added with 5 × Reaction Buffer 4 μl, dNTP Mix, 10 mM each (#R0191) 2 μl, Thermo Scientific^{TM} RiboLock^{TM} RNase Inhibitor (#E00381) 0.5 μl and 0.5 μl of RevertAid H Minus Reverse Transcriptase to a total reaction volume of 20 μl; the mixture was incubated at 42℃ for 2 hours followed by heating at 70℃ to terminate the reaction. 480 μl of TE buffer was added to make the final volume 500 μl.

6. Real-time PCR assay

Gene expression was analyzed by quantitative real-time PCR (Q-PCR) using LightCycler 96 real-time thermocylcler (Roche, Switzerland). The Q-PCR reaction was performed in a 20 μl reaction volume containing 10 μl of SYBR Green Real time PCR Master Mix (TaKaRa, Japan), 0.4 mM of each forward and reverse primers, and 4 μl of cDNA in duplicate using the following protocol: 60 s at 95℃; the template was amplified for 45 cycles of denaturation for 15 s at 95℃, annealing and extension for 1 min at 60℃. All the experiments were performed in duplicate. Gene-specific primers are listed in Table 1. Serially diluted references were used for absolute quantification analysis. In some cases, efficiency was obtained using serial dilutions of references and used for quantification. After normalization to the expression level of the house keeping gene, i.e. elongation factor (EF)-1α, fold change was calculated by dividing the ratio to EF-1α by negative control sample at each time point. Negative controls were unstimulated samples.

7. Statistical analysis

Spss16.0 (SPSS Inc.) was used to analysis the Q-PCR results. Data are presented as the mean values of the duplicate samples. One way-analysis of variance (ANOVA) and the LSD post hoc test were used to analyze the expression data, with $p < 0.05$ between treatment groups and control groups considered significant.

Results

1. In vitro analysis of gene expression in head kidney

Freshly prepared primary cultures of olive flounder head kidney cells were stimulated with various doses of the sulfated polysaccharides (0, 0.2, 1, 5 μg/ml) for 4, 8, or 24 h to analyze IL-1β gene expression (Fig. 1). There was dose dependent upregulation of IL-1β gene expression in head kidney cells by the sulfate polysaccharides stimulation at 4 h and 24 h. The significant upregulation was found when the head kidney cells were stimulated by 1 μg/ml and 5 μg/ml of the sulfated polysaccharides for 4 h and 5 μg/ml of the sulfated polysaccharides for 24 h.

2. In vivo analysis of gene expression in head kidney and peritoneal cells

To analyze the immuno-stimulating effect of the sulfated polysaccharides in vivo, IL-1β gene expression was analyzed in head kidney and peritoneal cells at day 1 and 3 post-stimulation. In head kidney, IL-1β gene expression was upregulated in all the three groups at day 1. The IL-1β gene expression level in the fish injected with 20 μg of the polysaccharides was up-regulated as much as 4.2 times compared to the control group. However, 3 post-stimulation, the IL-1β gene expression level was slightly down-regulated in all the 3 groups (Fig. 2).

In peritoneal cells, IL-1β gene expression was up-regulated in a dose and time dependent manner while it was getting decreased in the highest dose (100 μg) group at day 3. When the fish was stimulated with 20 μg of the polysaccharides, the expression was

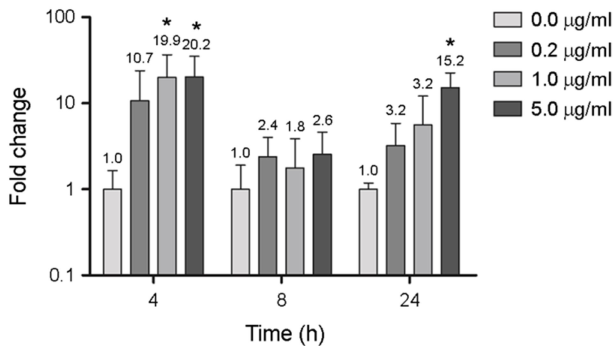

Fig. 1. Effects of polysaccharides on IL-1β expression in head kidney cells in vitro. Expression of IL-1β gene in head kidney cells stimulated with various doses of polysaccharides (0, 0.2, 1, 5 μg/ml) for 4, 8, 24 h at 25℃. The data are normalised relative to the expression of EF-1α and fold change was calculated by dividing the ratio to EF-1α by negative control sample at each time point. Negative controls were unstimulated samples. The results represent the mean ± SEM of 4 fish. The mean fold changes are shown above the bars. The asterisks (*) above the mean value are marked when the p values of ANOVA LSD post hoc test between stimulated samples and their time-matched controls are below 0.05.

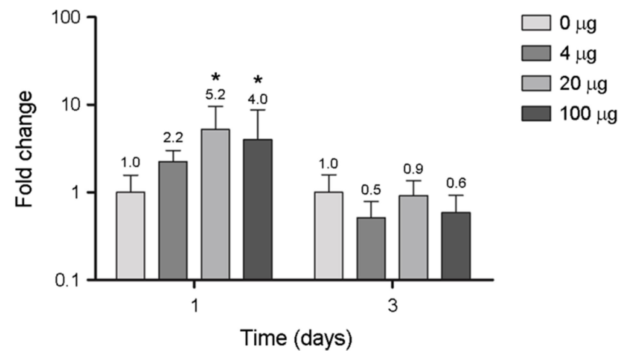

Fig. 2. Effects of polysaccharides on IL-1β expression of head kidney in vivo. Expression of IL-1β gene in head kidney of rock bream stimulated with polysaccharides (0, 4, 20, 100 μg) by i.p. injection for 1 or 3 days at 25℃. The data are normalised relative to the expression of EF-1α and fold change was calculated by dividing the ratio to EF-1α by negative control fish injected with PBS at each time point. The results represent the mean ± SEM of 4 fish. The p values of independent samples t test between stimulated samples and their time-matched controls are shown above the bars as: *$p < 0.05$.

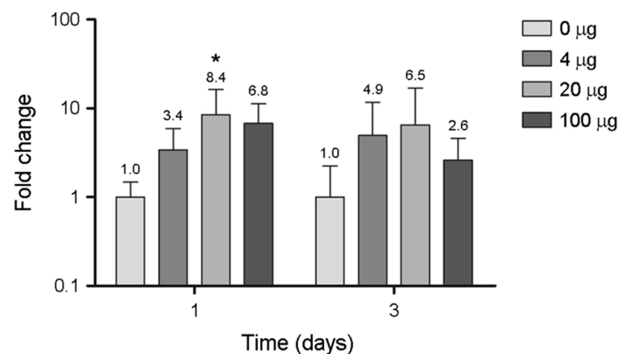

Fig. 3. Effects of polysaccharides on IL-1β expression of peritoneal cells in vivo. Expression of IL-1β gene in peritoneal cells of rock bream stimulated with polysaccharides (0, 4, 20, 100 μg) by i.p. injection for 1 or 3 days at 25℃. The data are normalised relative to the expression of EF-1α and fold change was calculated by dividing the ratio to EF-1α by negative control fish injected with PBS at each time point. The results represent the mean ± SEM of 4 fish. The p values of independent samples t test between stimulated samples and their time-matched controls are shown above the bars as: *$p < 0.05$.

the highest as seen 8.4 times higher expression level than negative control (0 μg) group (Fig. 3).

Discussion

In this study, the immunological function of polysaccharides extracted from *Codium fragile* was studied by observing the regulation of IL-1β gene expression and it was found that this gene was upregulated by the polysaccharides in vitro and in vivo. This is in agreement with many mammalian studies which demonstrated that sulfated polysaccharides extracted from algae are found to be an immune-stimulator in both in vitro and in vivo. For example, Kim et al. (2011) have implied that sulfated polysaccharides extracted from *Enteromorpha prolifera* can activate immune related gene expression in RAW264.7 and promote the production of NO and relative cytokines. The sulfated polysaccharides of *Codium fragile* and *Ulva rigida* showed the similar effect on IL-1β in RAW 264.7 (Tabarsa et al., 2013; Kim et al., 2011). This paper seems to be the first one to focus on the regulation of IL-1β in the fish or the cells of fish stimulated with sulfated polysaccharides. The result shows that the sulfated polysaccharides have the same effects on fish or the cells of fish as on mammalian cells.

The sulfated polysaccharides from *C. fragile* have reported the stimulatory effects on the gene expression of IL-1β in RAW264.7 cells via nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPK) pathways. It has been know that the expression of Iκ-Bα can be lowered and the expression of JNK, ERK and p38

which belong to the MAPK family were up-regulated when the polysaccharides were added into the RAW264.7 cells (Tabarsa et al., 2013). It was also reported that the fucoidan extracted from *Fucus vesiculosus* have up-regulated immunity and promote maturation of dendritic cells via at least the NF-κB pathway (Kim et al., 2007). While Wang et al. (2004) demonstrated that the expression of pro-inflammatory cytokine interleukin-1 can be reduced when the MEK/ERK 1/2 pathway is inhibited. Ohta et al. (Ciancia et al., 2007) elucidated that the sulfated polysaccharides from *C. fragile* are composed of D-galactan with pyruvates and sulfates; D-galactan can be the trigger of NF-κB and MAPK pathways through binding to Toll-like receptor (TLR)-4 (Capiralla et al., 2012; Meng et al., 2013).

In peritoneal cells, high-level expression of IL-1β gene was sustained until day 3. This might be because the cells are exposed to the sulfated polysaccharides directly and the cells can be stimulated continuously. However, unlikely in head kidney, when the cells are exposed to the sulfated polysaccharides indirectly, the expression of IL-1β can first up-regulate and then comes back to normal level. The similar expression pattern was observed when the rats were i.p. injected with lipopolysaccharide (Quan et al., 1998).

In conclusion, we studied the immunological effect of the F_2 fraction of sulfated polysaccharides isolated from *C. fragile* on the cytokine IL-1β in vitro and in vivo on flounder. In the in vitro experiment, the IL-1β was upregulated in dose dependent manner. When the fish was i.p. injected with polysaccharides, the gene was first upregulated at day 1 and then downregulated at day 3. However, in peritoneal cells, IL-1β was upregulated in all the stimulated and both the 1 and 3 day groups.

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