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Composition and anti-inflammatory effect of polysaccharides from *Sargassum horneri* in RAW264.7 macrophages

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ABSTRACT

Sulfated polysaccharides extracted from brown marine algae have been shown to possess a variety of biological activities. We assessed the potential activity of the sulfated polysaccharide from *Sargassum horneri* (SP) and its isolated two major components (fraction-1 (F1) and fraction-2 (F2)), on anti-inflammatory activity in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. In the present study, analysis of polysaccharide chemical composition found that the constituent ratios of sulfate ester and fucose in SP and F1 were 4.95% vs 7.6%, and 4.48% vs 55.9%, respectively, suggesting that F1 may be a major sulfated polysaccharide containing fucose. Meanwhile, our findings demonstrated that TNF-α secretion levels were significantly ($P < 0.05$) decreased by SP and F1 treatments in LPS-stimulated RAW264.7 cells in a dose-dependent manner under the preventive and repair experimental models. Pro-/anti-inflammatory (TNF-α/IL-10) cytokines secretion ratios by LPS-stimulated RAW264.7 macrophages were significantly ($P < 0.05$) inhibited by SP and F1 treatments, particularly by F1 (at high dose, 200 μg/ml). Moreover, NO release and iNOS activity were significantly ($P < 0.05$) inhibited by F1. Collectively, the present study suggested that purified component, F1 from SP, had strong anti-inflammatory effects on LPS-stimulated RAW264.7 macrophages in the preventive and repair manner through inhibiting TNF-α secretion levels and NO release.

1. Introduction

Inflammation have been linked with pathogenesis of many disease such as atheriosclerosis, arthritis, cardiovascular disease and other deadly diseases [1–4]. Moreover, inflammation is a complex process initiated by several factors ranging from bacterial infection and chemical injury to environmental pollution that result in cell injury or death. Tissue injury induced by this trauma results in the release of inflammatory mediates including the cytokines and tumor necrosis factor (TNF-α), interleukin-6 (IL-6) from leukocytes, monocytes and macrophages [5]. During inflammation, macrophages constitute a principal component of the innate immune system and play an important role in resistance against pathogens, inflammation resolution and in the initiation and amplification of inflammation which influences the acquired immune response as well as host survival via producing various inflammatory mediators and cytokines [6,7]. Lipopolysaccharide (LPS) is one of the most potent microbial initiators of inflammation, macrophages are highly sensitive to LPS stimulation and respond by inducing the inflammatory gene expression and the release of mediators/cytokines such as TNF-α, interleukin-1 (IL-1), IL-6, and nitric oxide (NO), all of which are largely responsible for many of the pathophysiological events associated with inflammatory diseases [8,9].

In these mediators, NO, known to be an inflammatory mediator, is produced by the activation of inducible nitric oxide synthase (iNOS) from L-arginine in macrophages [10,11]. NO is an example of reactive species that participates in normal physiological processes such as vasodilation and neurotransmission; however, overproduction of NO may result in disease as observed in inflammation, asthma, cardiovascular disorders and organ transplant rejection [12]. Furthermore, iNOS expression and NO production, both of which are stimulated by LPS, have been demonstrated to contribute

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to septic shock [13]. Therefore, macrophages in the presence of LPS under different experimental models have been used widely for evaluating the anti-inflammatory potential of samples in vitro [14]. Some studies have been conducted in-vivo in order to investigate the anti-inflammatory potential of sulfated polysaccharide using gamma/LPS induced RAW 264.7 macrophage [15–17]. In the present study, a RAW264.7 macrophage, which is the most commonly used mouse macrophage cell line in medical research, was selected to evaluate anti-inflammatory activity of *Sargassum horneri* polysaccharides (SP) and two major components, fraction-1 (F1) and fraction-2 (F2).

Many species of seaweed (marine macroalgae) are used as food and they have also found use in traditional medicine because of their perceived health benefits. Sulfated polysaccharides from marine algae are known to possess various biological activities including anti-inflammatory [18–21], anticoagulant [22], and antioxidant activity [23,24], which may be relevant in nutraceutical, pharmaceutical, and cosmetic applications. In the provious studies, sulfated polysaccharides from the marine brown algae such as *Lobophora variegate*, *Turbinaria ornate*, *Sargassum hemiphyllum* have been reported to have anti-inflammatory activities [19,25,26]. Recent studies have reported that fucoidan (a marine sulfated polysaccharide) inhibits the release of NO from LPS-stimulated RAW264.7 cells [15,27]. Fucose-riched sulfated polysaccharide obtained from hot water extraction of *S. hemiphyllum* was reported to reduce pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) profiles, NO production, expressions of IL-1β, iNOS, and COX-2 and NF-κB (p65) in LPS induced RAW 246.7 macrophage [26]. Moreover, our previous results suggested that isolated a *S. Horneri* polysaccharides (SP) and its purified component F1 may be sulfated polysaccharide containing fucose (data not shown). Therefore, we surmise that SP and its purified components, F1 and F2, may have potential against LPS-stimulated inflammation on RAW264.7 macrophage cells.

S. horneri (Turner) C. Agardh (abbreviated as *S. horneri*), a Sargassaceae brown alga, is one of the main components in the subtidal seaweed flora along the Chinese warmer water coast. *S. horneri* has been used as fundamental source of human food and drug in traditional Chinese medicine for centuries. One particularly interesting feature of the seaweed is its richness in sulfated polysaccharies. So far, little study of the anti-inflammatory activity of the sulfated polysaccharides isolated from *S. horneri* has been performed. The existing synthetic molecule like nonsteroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors are known for their side-effect in increasing the incidence of adverse cardiovascular thrombosis. An alternative therapeutic with minimum side effect from natural plant extract should be a focus in scientific research. Therefore, the present study is designed to investigate the anti-inflammatory activity of the sulfated polysaccharides isolated from marine brown algae *S. horneri* in RAW264.7 macrophages with LPS induced NO and cytokine TNF-α pro-inflammatory markers.

2. Materials and methods

2.1. Isolation and purification of the polysaccharide from S. horneri

S. horneri (Turner) C. Agardh (abbreviated as *S. horneri*) was collected at Zhoushan islands in Zhejiang province, China in May, 2012. Dried algae were dipped into 20 volumes of distilled water and kept at room temperature for 2 h. The algae were then homogenized and the solution was refluxed at 60 °C for 3 h. After cooling, the supernatant was separated from the algae residues by centrifugation. The supernatants were concentrated under reduced pressure, dialyzed in cellulose membrane tubing (molecular weight cut off 8000) against distilled water for 24 h. The retained fraction was recovered, concentrated under reduced pressure, and precipitated by the addition of fourfold volume of 95% (v/v) ethanol and washed twice with absolute ethanol, followed by drying at 40 °C to obtain a polysaccharide. During extraction of polysaccharides, proteins were removed by the method of Staub [28]. The polysaccharide was fractionated by a Q Sepharose Fast Flow column with distilled water, 0.5, 1.0 and 1.5 mol/l NaCl. Total sugar content of the eluate was determined by the phenol–sulfuric acid method [29].

The polysaccharides were sterilized by passing it through a 0.22-μm Millipore filter to remove any contaminant and then analyzed for endotoxin level by a gel-clot Limulus amebocyte lysate assay (Zhejiang A and C Biological, Zhejiang, China). The endotoxin level in the stock soln. was less than 0.5 EU/ml.

2.2. Chemicals and reagents

Anion-exchanger Q-Sepharose Fast Flow (Amersham Pharmacia Biotech, Uppsala, Sweden), Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, and the other materials required for culture of cells were purchased from Gibco BRL, Life Technologies (Grand Island, NY, USA). Lipopolysaccharide (LPS), dimethylsulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT), Vitamin C, and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade or of the highest grade available commercially.

2.3. Composition analysis

Sulfate ester content was estimated according to the method reported by Therho and Hartiala [30]. The composition of monosaccharide was determined by gas chromatography after converting them into acetylated aldononitrile derivatives. Briefly, 10 mg of polysaccharide was hydrolyzed in a sealed glass tube with 2 mol/l trifluoroacetic acid (TFA) at 105 °C for 10 h. The hydrolysate was evaporated to dryness. TFA was removed under reduced pressure by repeated co-evaporations with methanol. The hydrolysates were then converted into alditol acetates according to conventional procedures [30]. After adding 10 mg of hydroxylammonium and 3 mg of inositol (as internal reference), the mixture was dissolved in 0.5 ml of pyridine and incubated at 90 °C for 30 min. The mixture was cooled to room temperature. Acetic anhydride (0.5 ml) was then added to the mixture and the solution was incubated at 90 °C for another 30 min. Sugar identification was done by comparison with reference sugars (mannuronate, mannose, fucose, glucuronide acid, guluronic acid, glucose). Calculation of the molar ratio of the monosaccharide was carried out on the basis of the peak area of the monosaccharide. Gas chromatography was performed on an HP 5890 II instrument (Agilent, Waldbronn, Germany).

2.4. Cell culture and treatment

Mouse macrophages RAW 264.7 cell line was obtained from the Shanghai Institute of Cell Biology (Shanghai, China) and maintained in DMEM, supplemented with heat-inactivated 10% fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. When the cells reached sub-confluence, they were pretreated for 24 h with culture medium containing different concentrations of SP, two major components, fraction-1 (F1) and fraction-2 (F2) (50, 100, and 200 μg/ml), LPS (1 μg/ml), or DEX (10 ng/ml) that were tested in the experiments. Following this, the culture supernatant was collected and cells were washed three times with phosphate-buffered saline (PBS, pH 7.2). Subsequently, the cells were exposed to LPS (1 μg/ml) diluted in