International Journal of Biological Macromolecules 49 (2011) 1051–1058

Contents lists available at SciVerse ScienceDirect

International Journal of Biological Macromolecules

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

In vitro and *in vivo* immunomodulatory activity of sulfated polysaccharides from *Enteromorpha prolifera*

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ARTICLE INFO

Article history:
Received 4 March 2011
Received in revised form 25 August 2011
Accepted 27 August 2011
Available online 2 September 2011

Keywords:
Enteromorpha prolifera
Immunomodulation
Sulfated polysaccharide
Green seaweed
Th-1 cells

ABSTRACT

Water-soluble sulfated polysaccharides extracted from *Enteromorpha prolifera* and fractionated using ion-exchange chromatography (crude, F_1, F_2 and F_3 fractions) were investigated to determine their *in vitro* and *in vivo* immunomodulatory activities. The sulfated polysaccharides, especially the F_1 and F_2 fractions, stimulated a macrophage cell line, Raw 264.7, inducing considerable nitric oxide (NO) and various cytokine production via up-regulated mRNA expression. The *in vivo* experiment results show that the sulfated polysaccharides (the crude and F_2 fractions) significantly increased Con A-induced splenocyte proliferation, revealing their potential comitogenic activity. In addition, IFN-γ and IL-2 secretions were considerably increased by the F_2 fraction without altering the release of IL-4 and IL-5. This implies that the F_2 fraction can activate T cells by up-regulating Th-1 response and that Th-1 cells might be the main target cells of the F_2 fraction. These *in vitro* and *in vivo* results suggest that the sulfated polysaccharides are strong immunostimulators.

1. Introduction

Sulfated polysaccharides are complex, heterogeneous and bioactive macromolecules in which some of the hydroxyl groups in sugar residues are substituted with sulfate groups. These anionic polysaccharides are ubiquitous in nature and occur in a wide variety of organisms, including mammals, invertebrates and flora [1,2]. Seaweeds are the most important source of non-animal derived sulfated polysaccharides. Studies on sulfated polysaccharides from marine algae have been mainly carried out on brown algae (Phaeophyta), including fucoidans, ascophyllan, sargassan and glucuronoxylofucan, as well as red algae (Rhodophyta), including agar and carrageenan, because of their potent biological activities [3–7]. Green algae belonging to Ulvaceae are a rich source of sulfated polysaccharides that are referred to as ulvans. Recently, ulvans have attracted increasing attention because they exhibit unique structural features containing rhamnose and uronic acid with major repeating disaccharide units of α-L-Rha*p*-(1→4)-D-Xyl and (→4)-β-D-Glc*p*A-(1→4)-α-L-Rha*p* [8,9]. They also possess various effective biological and pharmacological activities such as anticoagulant, anticancer, antiviral and anti-hyperlipidemia activities [10–13].

In a recent study, ulvans from *Ulva rigida* stimulated macrophages to secrete nitric oxide (NO) and prostaglandin-2 (PGE_2) by inducing an increase of inducible nitric oxide synthases (iNOS) and cyclooxygenase-2 (COX-2) mRNA expression [6]. Macrophages are known to be important components of host defense systems against bacterial infections and various types of invading cells, including cancer cells [14]. Activated macrophages release cell factors such as NO, cytokines, reactive oxygen intermediates, and other substances to remove pathogens and inhibit cancer cell growth. In a more recent study, Lee et al. [15] report that ulvans from *Codium fragile* enhance the production of NO and various cytokines by activating macrophages. The authors observed enhanced production of pro-inflammatory (interleukins [IL]-1, IL-6, IL-12 and tumor necrosis factor ([TNF]-α) and anti-inflammatory (IL-10) cytokines, suggesting that ulvans might possess potent immunostimulating activities by activating macrophages while preventing potential detrimental inflammatory effects from excessive macrophage activation. In our previous work, sulfated polysaccharides from *Enteromorpha prolifera*, one of the most popular green seaweeds in Asian countries, were extracted and fractionated into three fractions, F_1, F_2 and F_3, using ion-exchange chromatography and their structures were characterized [16]. These polysaccharides have no significant direct cytotoxicity to AGS and DLD-1 cancer cells. However, a considerable amount of NO from macrophages is produced by these polysaccharides, especially in the F_2 fraction.

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0141-8130/$ – see front matter © 2011 Published by Elsevier B.V.
doi:10.1016/j.ijbiomac.2011.08.032

Thus, in the present study, we further evaluated the immunomodulatory activity of these sulfated polysaccharides by investigating the production of other cytokines and their mRNA expressions through *in vitro* and *in vivo* studies.

2. Materials and methods

2.1. Materials

Green seaweed, *E. prolifera*, collected in the spring of 2007 from the coast of Wando, Chunnam, Korea, was purchased from a wholesaler. The raw material was washed with fresh water and air-dried at 60 °C. The dried raw material was milled using a blender, sieved (<0.5 mm), and stored at −20 °C. The crude polysaccharide was extracted from the milled biomass by using the method of Cho et al. [16]. Briefly, 5 g dried biomass was rehydrated in 100 mL distilled water at 65 °C for 2 h and centrifuged at 18,500× *g* for 10 min. Ethanol (99%) was added to the supernatant to obtain a final ethanol concentration of 30%. After centrifugation at 18,500× *g* for 10 min, an additional 150 mL ethanol was added to the collected supernatant to obtain a final ethanol concentration of 70% (v/v), and the solution was stored at 4 °C overnight. Crude polysaccharide was obtained by filtering the ethanol solution with a nylon membrane (0.45 μm pore size; Whatman International Ltd., Maidstone, UK), washing with ethanol (99%) and acetone, and then drying at room temperature overnight.

Crude polysaccharide (100 mg) dissolved in 10 mL distilled water was fractionated using ion-exchange chromatography on a DEAE Sepharose fast flow column (17-0709-01; GE Healthcare Bio-Science AB, Uppsala, Sweden). The column was washed with distilled water, and the bound polysaccharide was eluted with a solution of distilled water and different concentrations of NaCl increasing stepwise from 0.5 to 1.0 M. The three fractions obtained are referred to as F_1, F_2 and F_3.

2.2. Macrophage proliferation assay

Raw 264.7 cells (ATCC, Rockville, MD, USA) were seeded in a 96-well microplate (1×10^4 cells/well, in a volume of 100 μL) and incubated in RPMI-1640 medium (Hyclone, Logan, UT, USA) containing 10% FBS with 100 μL sample polysaccharides at a concentration of 12.5, 25, or 50 μg/mL. The experiment was performed in triplicate. After cells were incubated at 37 °C for 72 h, WST-1 reagent was used to determine the proliferation of macrophage cells as described previously [16]. Absorbance at 450 nm was determined by spectrophotometry. The absorbance (A) was translated into the macrophage proliferation ratio (%) = $A_t/A_c \times 100$, where A_t and A_c are the absorbances of the test and control groups, respectively.

2.3. Endotoxin test

The concentration of endotoxin was determined by using the limulus amebocyte lysate (LAL) method obtained from E-TOXATE assay kit (Sigma, St. Louis, MO, USA). The experiment was performed according to the manufacturer's instructions. In brief, the sterile endotoxin-free water was used to dissolve and dilute the test samples and endotoxin standard. The triplicate samples and standard solutions were contained in a sterile glass tube, and 100 μL E-TOXATE working reagent was added to each tube. The mixtures of samples and E-TOXATE reagent were incubated at 37 °C for 1 h and then observed for evidence of gelation. The samples were interpreted as "+" (presence of a hard gel; i.e., sample contains endotoxin) and "−" (absence of a hard gel; i.e., sample does not contain endotoxin).

2.4. NO production assay

NO secretion from macrophages and the nitrite concentration was measured using the Griess reaction as described by Green et al. [17]. Raw 264.7 cells were seeded in a 96-well microplate (1×10^5 cells/well in a volume of 100 μL) and incubated for 24 h at 37 °C. The cultured cells were treated with 100 μL polysaccharide solution at 12.5, 25.0, or 50 μg/mL or 1 μg/mL lipopolysaccharide solution (LPS), which was used as a positive control, in the presence or absence of the arginine analogue, *N*(G)-monomethyl-L-arginine (L-NMMA) (500 μM), in triplicate. After incubating for 24 h at 37 °C, 100 μL of cultured cell supernatant was mixed with an equal volume of Griess reagent (1%, [w/v] sulfanilamide and 0.1% [w/v] *N*-[1-naphthyl] ethylenediamine hydrochloride in 2.5% [v/v] phosphoric acid) and left at room temperature for 10 min. The absorbance at 540 nm was measured using a microplate reader. The NO secretion from the Raw 264.7 cells was calculated with reference to a standard curve obtained with $NaNO_2$ (1–200 μM in culture medium).

2.5. IL-1β production assay

To measure the concentration of IL-1β, Raw 264.7 cells (1 mL, 1×10^5 cells/well) were cultured in RPMI-1640 medium containing 10% FBS with 12.5, 25.0, or 50 μg/mL polysaccharide solution or LPS (1 μg/mL) as a positive control in a 24-well microplate for 72 h. The supernatants were collected, and the IL-1β concentration was measured with reference to the introduction of the examination agent box (R&D System, Inc., Minneapolis, MN, USA).

2.6. Reverse transcription polymerase chain reaction (RT-PCR)

Raw 264.7 cells (1×10^5 cells/well) were treated in the presence of the sample solutions or LPS at 37 °C for 18 h. The total RNA of Raw 264.7 cells treated with LPS or samples was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and kept at −80 °C until use. The RNA concentration was measured by a spectrophotometer before the construction of cDNA with an oligo-$(dT)_{20}$ primer and Superscript III RT (Invitrogen). PCR amplification was carried out using GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA) and specific primers. The primers were as follows: iNOS, 5′-CCCTTCCGAAGTTTCTGGCA GCAGC-3′ (forward) and 5′-GGCTGTCAGAGCCTCGTGGCTTTGG-3′ (reverse); IL-1β, 5′-ATGGCAACTATTCCTGAACTCAACT-3′ (forward) and 5′-CAGGACAGGTATAG ATTCTTTCCTTT-3′ (reverse); TNF-α, 5′-ATGAGCACAGAAAGCATGATC-3′ (forward) and 5′-TACAGGCTTGTCACTCGAATT-3′ (reverse); COX-2, 5′-CCCCCACA GTCAAAGACACT-3′ (forward) and 5′-GAGTCCATGTTCCAGGAGGA-3′ (reverse); IL-6, 5′-TTCCTCTCTGCAAGAGACT-3′ (forward) and 5′-TGTATCTCTCTGAAGGAC T-3′ (reverse); IL-10, 5′-TACCTGGTAGAAGTGATGCC-3′ (forward) and 5′-CATCATGTATGCT-TCTATGC-3′ (reverse); β-actin, 5′-TGGAATCCTGTGGCATCCAT GAAAC-3′ (forward) and 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′ (reverse). Reverse transcriptase amplification was conducted with initial denaturation at 94 °C for 3 min; 30 cycles of denaturation (94 °C for 30 s), annealing (56 °C for 40 s) and extension (72 °C for 1 min); and a final extension step at 72 °C for 10 min. PCR products were run on 1% agarose gel and visualized by ethidium bromide staining.

2.7. Experimental animals

Six-week-old female BALB/c mice (body weight, 17–19 g) were purchased from KOATECH (Pyeongtek, Korea). For adaption, the mice were housed in polycarbonate cages and fed a standard animal