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Increase in anti-inflammatory activities of radical-degraded porphyrans isolated from discolored nori (*Pyropia yezoensis*)

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ABSTRACT

The anti-inflammatory properties of porphyrans (D1–D4) obtained from four discolored nori (*Pyropia yezoensis*) with different growth backgrounds were studied to examine possible variations in their bioactivities. Elution profiles of the porphyrans on Sepharose 4B indicated that D2-porphyran had relatively lower-molecular-size porphyrans than the other porphyrans. Inhibitory activities of the four porphyrans against nitric oxide (NO) and tumor necrosis factor-α (TNF-α) secretion by lipopolysaccharide (LPS)-stimulated RAW264.7 cells were different, whereas no significant differences were observed in the sulfate and anhydrogalactose levels. D2-porphyran showed the highest inhibitory activity against NO and TNF-α secretion by LPS-stimulated RAW264.7 cells, whereas D3- and D4-porphyrans had almost no activity. All porphyrans were efficiently degraded by free radical generated with ascorbate and hydrogen peroxide. The free-radical degradation resulted in a significant increase in the inhibitory activities of the four porphyrans against NO and TNF-α secretion, with varying rates depending on the porphyrans. The ability of D2-porphyran to suppress the receptor activator of nuclear factor κB ligand (RANKL)-induced osteoclastogenesis in RAW264.7 cells was also significantly enhanced after degradation. Our results suggest that molecular size is an important factor affecting the anti-inflammatory activity of porphyrans, and radical degradation might be a promising procedure to obtain active low-molecular-size porphyrans.

1. Introduction

The *Pyropia* genus classified as red algae is mainly cultivated and consumed in Japan, China, and Korea. *Pyropia yezoensis* is an important raw material for the production of sheet-like dried foodstuff commonly known as "nori," which is traditionally used in the Japanese cuisine sushi. Along with the recent trend of sushi becoming an extremely popular Japanese food, the market for nori is now spreading worldwide. Nori contains several nutritionally valuable components, such as dietary fibers, taurine, polyunsaturated fatty acids, carotenoids, and mycosporine-like amino acids (porphyra-334), as well as minerals, vitamins, and relatively high amounts of proteins. Porphyran, one of the major constituents of *P. yezoensis*, is a linear sulfated polysaccharide comprising D-galactose, 3,6-anhydro-L-galactose, 6-*O*-methyl-D-galactose, and L-galactose-6-sulfate [1–3]. In addition to its health benefit as a major indigestible dietary fiber in nori, porphyran has diverse physiological activities, including antitumor, immunomodulating, antioxidant, antihyperlipidemic, and hypercholesterolemic effects [4–8]. Our previous study demonstrated that porphyran prepared from *P. yezoensis* inhibits nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW264.7 mouse macrophages in a concentration-dependent manner through the prevention of nuclear factor kappa-light-chain enhancer of activated B cell (NF-κB) activation [9].

On the other hand, cultivation of *P. yezoensis* often suffers from lack of nutrition because of the deficiencies in nitrogen, phosphorus, and trace elements during cultivation, resulting in the deterioration of the quality of nori, which is called discolored nori and has no commercial value. To identify the beneficial uses of discolored nori, we previously isolated porphyran from discolored waste nori and compared its bioactive features with the porphyran isolated from normal high-quality nori. We found that porphyrans obtained from discolored nori had a relatively smaller mean molecular size than did those isolated from regular nori, and showed greater antioxidant and anti-inflammatory effects [10]. In fact, porphyran isolated from discolored nori exhibited protective effect against LPS-induced endotoxin shock in mice model [11]. Furthermore, porphyran isolated from discolored nori showed suppressive effect on the receptor activator of nuclear factor κB ligand (RANKL)-

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induced osteoclastogenesis in RAW264.7 cells without any cytotoxic effects [12]. Real-time PCR analyses indicated that porphyran significantly attenuated RANKL-induced increase in the mRNA levels of osteoclastogenesis-related marker genes, such as nuclear factor of activated T cells (*NFATc1*), *TRAP*, cathepsin K, and matrix metalloproteinase 9 (*MMP-9*) in RAW264.7 cells [12]. These results suggested that porphyran can be used as a safe therapeutic agent to improve osteoclast-related pathological conditions.

The structural features and biological activities of seaweed-derived polysaccharides, including porphyran are generally known to be different depending on the preparation procedures and the sources of raw materials. Harvest season and local climate conditions can influence the structures and bioactivities of the polysaccharides, even in the same algal species [13,14]. Our preliminary analysis indicated that the bioactivities of porphyran isolated from discolored nori significantly differed depending on the lot of nori used as raw material. To further clarify this point, we isolated four porphyrans from different discolored nori with different growth backgrounds (D1–D4) by the same procedure and compared their bioactivities in this study. In Japan, nori is generally graded from 1 as the lowest to 9 as the highest quality based on certain evaluation criteria including the color, which are considered to reflect the growth background. Discolored nori D1, D2, D3, and D4 used in this study is graded as 5, 7, 6, and 5 grade, respectively. Hence, all the discolored nori samples are inferior ones, which have no commercial value, and D2 is the lowest quality nori.

It has been reported that porphyrans can be efficiently degraded by free radicals produced from ascorbate and hydrogen peroxide in combination, and the resulting degraded porphyrans showed higher antioxidant activities than the original porphyrans [15]. Hence, we prepared degraded porphyrans by using this radical degradation procedure, and investigated whether or not the bioactivities of degraded porphyrans can be improved.

2. Materials and methods

2.1. Materials

Four discolored nori samples (D1–D4) with different growth backgrounds were kindly provided by the Japan Fisheries Cooperatives (Saga and Nagasaki, Japan). recombinant human soluble RANKL was obtained from the Oriental Yeast Co., Ltd. (Tokyo, Japan). LPS was isolated from *Escherichia coli* 0111: B4 (purified by phenol extraction), and custom oligonucleotides (iNOS and β-actin primers) were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). PrimeScript® 1st strand cDNA synthesis kit for reserve transcription-polymerase chain reaction (RT-PCR) was purchased from TaKaRa Bio Inc. (Otsu, Shiga, Japan). GoTag Green Master Mix was purchased from Promega KK. (Tokyo, Japan). TNF-α capture antibody and anti-mouse TNF-α monoclonal antibody were purchased from Thermo, Inc. (Boston, MA, USA). Tartrate-resistant acid phosphatase (TRAP) stain kit was obtained from Wako Pure Chemical Industries, Ltd., (Osaka, Japan). Other chemicals used in this study were of the commercially available highest grade.

2.2. Preparation of porphyran from discolored nori

Four porphyrans (D1–D4) were prepared from four discolored nori (*P. yezoensis*) samples with different growth background (Supplementary data) as reported previously [10]. In brief, dry sheets (about 15 g) of discolored nori were homogenized in 1000 mL of 85% ethanol and heated at 75°C with constant stirring for 1 h, and then filtered to remove the ethanol-soluble substances. After repeating this extraction for three times, the residue was washed with methanol and dried to obtain decolorized polysaccharide fraction. This fraction was extracted with 2000 mL of distilled water at 95°C for 1.5 h and then centrifuged at 8000 ×*g* for 20 min, and the supernatant was concentrated. To the supernatant, sodium acetate and acetic acid were added to make a 0.5 M sodium acetate solution (pH 5.0), and then ethanol was added at a concentration of 42% (v/v). After 1 h, the precipitates in the solution were removed by centrifugation, and ethanol was added to the supernatant at a concentration of 60%. The precipitate was harvested by centrifugation (10,000 ×*g* for 30 min at 4°C), and then dissolved in distilled water, dialyzed against distilled water using 3500 cut dialysis membrane, and lyophilized. The resulting dried powder was used as porphyran. Porphyran was dissolved in 50 mg mL^{-1} of phosphate buffer saline (PBS) and passed through a filter of 0.20 μm pore size for sterilization. PBS alone (final 0.1% or lower than that in assay system) as the vehicle had no significant effects in the assay systems (data not shown).

2.3. Degradation of porphyrans

Degradation of porphyrans using ascorbate and H_2O_2 was carried out as described previously with slight modifications [15]. In brief, ascorbate (final 20 mM) and H_2O_2 (final 20 mM) were added to 0.5% porphyran solution in distilled water, and stirred at 25°C for 1 h. The reaction mixture was extensively dialyzed against distilled water using 3500 cut dialysis membranes to remove the reagents.

2.4. Chemical analyses of porphyrans

Analysis of the molecular masses of the porphyran samples and the degraded porphyrans were conducted using gel filtration chromatography with a Sepharose 4B (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) column (1.5 × 100 cm). The total sugar level in each fraction was monitored by the phenol-sulfuric acid method as described previously [10]. The content of 3,6-anhydrogalactose in the porphyran samples was determined by resorcinol-hydrochloride method [16]. The sulfate level of the porphyran samples was measured by the Dodgson-Price method, as described previously [17]. Fourier-transformed infrared spectra of porphyrans were measured by the KBr pellet method, using the Nicolet Nexus 670NT FT-IR apparatus (Thermo Fisher Scientific Inc., MA, USA).

2.5. Cell culture

RAW264.7 (mouse macrophage) cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in a CO_2 (5%) incubator at 37°C in Dulbecco's modified Eagle's minimum essential medium, supplemented with 10% fetal bovine serum (FBS), 100 IU mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin, which unless otherwise specified was used as the growth medium throughout the experiments.

2.6. Cytotoxicity

The cytotoxic effect of the porphyrans on RAW264.7 cells was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously [18]. In brief, adherent RAW264.7 cells (5×10^4 cells per well in 96-well plates) were incubated with varying concentrations of the porphyran samples in the growth medium for 24 h, and then MTT was added to the treated cells. After 30 min of incubation, the optical density (OD) of the MTT formazan reaction product was measured at 570 nm, using a Multiskan GO scanner (Thermo Fisher Scientific Inc., MA, USA).

2.7. Nitrite assay for the estimation of nitric oxide (NO)

Nitrite level, a stable reaction product of NO with O_2, in the supernatants of each treated RAW264.7 cells, was determined by a method based on the Griess assay, as described previously [19]. In brief, adherent RAW264.7 cells in 96-well plates (5×10^4 cells $well^{-1}$) were treated with the indicated concentrations of porphyran samples in the growth medium for 1 h, and then LPS was added at a