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Structure of rhamnan sulfate from the green alga *Monostroma nitidum* and its anti-herpetic effect

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ABSTRACT

"A sulfated polysaccharide from *Monostroma nitidum* Wittrock (Ulvophyceae)" was purified by anion-exchange and gel filtration column chromatographies. The isolated polysaccharide consisted of large amount of L-rhamnose with small amount of D-glucose, and it was regarded to be a rhamnan sulfate (RS). Methylation analysis of the native and desulfated polysaccharide suggested that this polymer was mainly composed of 1,2- and 1,3-linked rhamnose residues with a ratio of *ca* 1:2. In addition, it was found the presence of 1,2,3-linked rhamnose and 1,4-linked glucose residues. Sulfate groups were suggested to be mainly located at C-2 and C-3 of 1,3- and 1,2-linked rhamnose residues, respectively. NMR analyses including 1D and 2D experiments indicated that RS consisted of sugar linkage units as follows:

1: →3)-α-L-Rha-(1 → 3)-α-L-Rha-(1→
2: →3)-α-L-Rha-(1 → 2)-α-L-Rha-(1→
3: →3)-α-L-Rha-(1 → 3)-α-L-Rha-(1→

2
β-D-Glc-(1- ↑

Moreover, RS showed potent antiviral activity against herpes simplex virus type 2 virus, whereas it has no effect on the replication of influenza A virus. Anti-HSV-2 target(s) of RS was suggested to be virus adsorption and penetration steps onto host cell surface.

1. Introduction

Herpes simplex virus type 2 (HSV-2) is an ubiquitous pathogen and major cause of genital herpes. Immunocompetent people with genital HSV infection can have frequent, painful, and recurrent genital lesions associated with much psychosocial distress. Over the past two decades, HSV-2 infection has also been linked to three times higher risk of sexually acquired HIV (Freeman et al., 2006). Therefore, treatment and prophylaxis of the infection of HSV-2 should reduce the likelihood of HIV infection. Acyclovir is the most commonly used chemotherapy as a very effective treatment for HSVs, however, it is not always tolerated and drug-resistant mutants are rapidly emerging, particularly in immunocompromised patients. Thus, it is demanded to develop novel type of anti-HSV drugs with novel mode of action.

So far, many polysaccharides from algae have been studied vigorously on their structures and biological activities. In especial, fucoidans (fucan sulfates) from brown algae have been focused on those studies, because they have various biological activities such as anticoagulant and antiviral activities (Berteau & Mulloy, 2003; Hayashi, Nakano, Hashimoto, Kanekiyo, & Hayashi, 2008; Hoshino et al., 1998). On the other hand, some algae produce specific sulfated polysaccharides which are mainly composed of α-L-rhamnose moiety. An α-L-rhamnose is a rare monosaccharide in human and animals, whereas it is widely distributed in plants and microorganisms as a component of glycosides or polysaccharides. However, distribution of a polymer composed of large amounts of α-L-rhamnose, so-called "rhamnan", is quite limited in the nature. Our studies have demonstrated that some rhamnan-type sulfated polysaccharides, such as sodium spirulan (Na-SP) from *Spirulina platensis* (cyanophyta) and rhamnan sulfate (RS) from *Monostroma latissimum* (chlorophyta), had potent antiviral activities against enveloped viruses including human immunodeficiency virus type 1 (HIV-1) and herpes simplex viruses (HSVs) (Hayashi & Hayashi, 1996; Lee, Hayashi, Hayashi, Sankawa, & Maeda, 1999). The green alga *M. nitidum* grows in upper part of intertidal zone, and widely cultivated to use as food resources at Mie and Oki-

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nawa in Japan. There are several reports about the structure of sulfated polysaccharides from *Monostroma* sp. (Harada & Maeda, 1998; Lee, Yamagaki, Maeda, & Nakanishi, 1998), however, fine chemical structure of rhamnan sulfate from *Monostroma* sp. is not elucidated. In the present paper, we describe the fine structural investigations of the sulfated rhamnan from *M. nitidum* and the evaluation of their antiviral activities.

2. Materials and methods

2.1. Isolation of rhamnan sulfate

Crude rhamnan sulfate (CRS) extracted from *Monostroma nitidum* was kindly gifted from Mr. Shigeo Tanaka (Konan Chemical Manufacturing Co., Ltd., Mie, Japan). Briefly, dried seaweed was washed with H_2O and then extracted with 24 vol. of H_2O for 6 h at 95–100 °C. Then, celite (300 g) was added to the extract, and then centrifuged to remove algal fronds. Thus obtained hot water extract was lyophilized to give CRS (153 g). CRS (45 g) dissolved in H_2O was dialyzed against H_2O to fractionate to dialyzate (ML) and non-dialyzate (MH) fractions, and then both were freeze-dried (Yield: ML, 13.5 g; MH, 26.1 g). MH was dissolved in H_2O and applied to a Toyopearl DEAE 650 M anion-exchange chromatography (5 i.d. × 14 cm; Tosoh, Tokyo, Japan), which was successively eluted with H_2O, 0.5 M NaCl, 1.0 M NaCl, and 2.0 M NaCl. The yields of the eluates were 23 (MH-1), 13 (MH-2), 32 (MH-3), and 3% (MH-4), respectively. MH-3 obtained as the most abundant fraction was applied to a Toyopearl DEAE 650 M column (5 i.d. × 14 cm), which was eluted with a linear gradient system prepared by H_2O and 1.5 M NaCl. Fractions of 20 ml were collected and monitored by the phenol–H_2SO_4 method (Dubois, Gilles, Hamilton, Revers, & Smith, 1956) and UV detection at 280 nm. MH-3A (3%), MH-3B (25%), MH-3C (41%), and MH-3D (14%) were obtained on the basis of their elution profiles. The most abundant fraction, MH-3C, was applied to a Toyopearl HW-65S gel filtration (2.2 i.d. × 96.5 cm) and eluted with 0.1 M NaCl. Fractions of 5 ml were collected and monitored by the phenol–H_2SO_4 method to obtain a rhamnan sulfate (RS, yield = 62.5%).

2.2. Estimation of homogeneity of RS

The molecular weight of RS was estimated by HPLC analysis. The sample was applied on TSK $GMPW_{XL}$ gel filtration columns (7.6 mm × 300 mm × 2; Tosoh, Tokyo, Japan) and eluted with 0.1 M $NaNO_3$ at 0.6 ml/min. Commercially available pullulans (Shodex P-52; Showa Denko K.K., Tokyo, Japan) were used as standard molecular markers. RS was applied to a cellulose-acetate membrane (Separax; Jokoh Co. Ltd., Tokyo, Japan) in 0.1 M barium acetate and run at 1 mA/cm. The membrane was stained with 0.25% toluidine blue.

2.3. Colorimetric analyses of polysaccharide

Uronic acid content was determined by *m*-hydroxydiphenyl method (Blumenkrantz & Asboe-Hansen, 1973). Protein content was determined using a Bio-Rad protein assay kit. Sulfate content was determined by rhodizonate method (Silvestri, Hurst, Simpson, & Settine, 1982).

2.4. Sugar composition of RS

Sugar composition was determined as follows: RS (1 mg) was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120 °C for 1 h. After removal of TFA under N_2 gas, the hydrolyzates were converted to alditol acetates, which were analyzed by GC using a SP-2330 fused silica capillary column (30 m × 0.32 mm i.d.; Supelco, MA, USA) with the oven temperature of 200–240 °C (4 °C/min). Absolute configuration of monosaccharide was analyzed as (+)-2-butyl-glycoside (Gerwig, Kamerling, & Vliegenthart, 1979).

2.5. Methylation analyses of polysaccharides

Desulfation of RS was performed by solvolytic desulfation with 10% MeOH/DMSO (Nagasawa, Inoue, & Tokuyasu, 1979). After dialysis and lyophilization, a colorless polysaccharide was obtained (DS-RS). RS was converted to the triethylammonium salt (TEA-RS) by dialyzed against 0.1 M triethylammonium hydrogen chloride (Stevenson & Furneaux, 1991). Methylation of TEA-RS and DS-RS was performed by the Ciucanu's method (Ciucanu & Kerek, 1984). In the case of TEA-RS, it was methylated three times to achieve complete methylation. The methylated polysaccharides were hydrolyzed with 2 M TFA at 120 °C for 1 h, reduced with $NaBD_4$, and acetylated. The partially methylated alditol acetates were analyzed GC using a SP-2330 fused silica capillary column and GC–MS using a DB-5MS fused silica capillary column. Identification of partially methylated alditol acetates was carried out on the basis of relative retention time to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol and its mass fragmentation patterns (Carpita & Shea, 1988). Peak area was corrected using published molar response factors (Sweet, Shapiro, & Albersheim, 1975).

2.6. Spectroscopic analyses of polysaccharides

IR spectra of RS and DS-RS were recorded with a FT/IR-460plus spectrophotometer (Jasco, Tokyo, Japan). NMR spectra were recorded at 303 K on a Varian Unity 500 *plus* spectrophotometer, and sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as internal reference. The homonuclear two-dimensional experiments such as DQF-COSY, TOCSY (spinlock time of 80 ms), NOESY (mixing time of 80 ms) were performed using the Varian standard program. The heteronuclear experiments were performed using pulse field gradient program as gHSQC, gHSQC-TOCSY, and gHMBC. H2BC experiments were performed with reported pulse program (Petersen et al., 2006).

2.7. Cells and viruses

Vero and MDCK cells were grown in Eagle's MEM supplemented with 5% FBS and kanamycin (60 mg/l). RAW 264.7 cells were grown in DMEM supplemented with 10% FBS. HSV-2 (UW268 strain) and influenza A virus (A/NWS/33 strain, H1N1) were propagated on Vero and MDCK cells, respectively. Those viruses were stored at −80 °C until use. An aliquot of the virus stock was titered by plaque assay.

2.8. Antiviral activity and cytotoxicity of RS

Vero and MDCK cell monolayers (2×10^5 cells/well) were infected with HSV-2 or influenza virus, respectively, at 0.1 plaque forming unit (PFU) per cell at room temperature. After 1 h of viral infection, the monolayers were washed three times with phosphate-buffered saline (PBS) and incubated in a maintenance medium (MEM plus 2% FBS) at 37 °C. Sample was added either during infection and throughout the incubation thereafter (A) or immediately after the viral infection (B). Virus yields were determined by plaque assay at 1-day incubation point. The 50% inhibitory concentration (IC_{50}) was obtained from concentration–response curves. For cell growth inhibition study, Vero or MDCK cells were incubated at an initial density of 1.2×10^4 cells/well in 96-well plates. After cells had been incubated for 1 day at 37 °C, sample was added and the incubation was continued for 3 days.