Isolation of Sulfated Galactan from *Codium fragile* and Its Antiviral Effect

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A sulfated galactan (FG) was isolated from the green alga, *Codium fragile*. Chemical analysis revealed that FG mainly consisted of o-galactose with pyruvic acid (12.3%) and sulfate (11.0%). Methylation and NMR analyses showed that FG was composed of →3)-β-o-Galp-(1→, β-o-Galp-(1→ and →3,6)-β-o-Galp-(1→ residue. In addition, pyruvic acid was suggested to be present as (1'-carboxy)-ethylenic cyclic ketal at O-3 and O-4 of non-reducing terminal galactose residues, whereas sulfate was substituted at O-4 of other galactose residues. When the antiviral potency of FG was evaluated, it inhibited the replication of herpes simplex virus type 2 (HSV-2), and the mechanism of action was suggested to be interference in the early steps such as virus adsorption to and penetration into host cells. Furthermore, it was shown that FG directly reduced virus infection rates. In a genital infection model using HSV-2-infected mice, FG improved mortality and lesion scores and reduced virus yields by intravaginal administration. These results suggest that FG might be a potent candidate as a prophylactic agent for HSV-2 infection.

Key words  *Codium fragile*; pyruvylated-sulfated galactan; herpes simplex virus type 2

Exploration of antiviral substances as novel drug candidates is important since there are increasing risks of emerging and re-emerging viral infectious diseases. AIDS caused by human immunodeficiency virus (HIV) is one of the most serious emerging infectious diseases worldwide. The number of HIV carriers and AIDS patients has been increasing not only in developing countries but also in Japan, and unprotected sexual contact is thought to be the most prominent cause of the spread of HIV. In addition, it is well known that infection with other sexual transmitted diseases including genital herpes increases the risk of HIV transmission during unprotected sex among infected and uninfected partners. There are several substantial evidences that herpes simplex virus type 2 (HSV-2) is a co-factor for HIV infection and progression in HIV-infected individuals. Therefore, the management of HSV-2 infection is regarded as one effective approach for the prevention of HIV spreading. Although various antitherpes drugs have been developed, drug-resistant mutations have often been observed, which reduce the efficiency of available antiviral drugs. Therefore, new anti-HSV-2 drugs are required and still more have to be researched and developed.

Marine algae produce various metabolites and have been recognized as promising targets in the search for biologically active compounds. So far, we have performed the screening studies on marine edible algae, and isolated some antiviral sulfated polysaccharides such as sodium hornan and rhamnan sulfate from *Sargassum horneri* and *Monostroma latissimum*, respectively. Therefore, marine algae could be important resources in the discovery of antiviral compounds. *Codium fragile* (SURINGAR) HARIOT is a green alga belonging to Codiales, and is widely distributed along the shore of Japan. In Japan, *C. fragile* is one of familiar seaweeds and has been used as edible one from ancient times. Furthermore, it was recorded in the Law of Taiho (AD701) that it was paid as tax to the Court. Its use has been recorded in the treatment for enterobiasis, dropsy and dysuria in Oriental medical textbooks. In the present study, we report the isolation, structural characterization and antiviral properties of a sulfated polysaccharide from *C. fragile*.

MATERIALS AND METHODS

**Chemicals**  Eagle's minimal essential medium (MEM) and Dulbecco's MEM (DMEM) were obtained from Nissui Pharmaceutical (Tokyo, Japan). Acyclovir (ACV) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DEAE 650 M and GMPW_M columns were purchased from Tosoh (Tokyo, Japan), and Sepharose 6B was obtained from GE Healthcare (Piscataway, NJ, U.S.A.). Other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**Isolation of Sulfated Galactan from *Codium fragile***  *C. fragile* was collected at Kamakura, Japan, in June 2007. Collected seaweed was immediately washed in tap water and then oven dried at 50°C. The dried algal ground to fine powder and defatted with EtOH at 4°C twice. The residual powder was extracted twice with H2O under reflux for 30 min. The combined extract was concentrated in vacuo and lyophilized to give FH (yield: 13%). FH was dissolved in H2O and centrifuged at 5000 rpm for 15 min to remove an insoluble portion. The soluble portion was dialyzed against H2O with a seamless cellulose tube (MWCO: 14000, Wako Pure Chemicals) and the nondialysate was lyophilized to give FH4 (yield: 13%). FH4 dissolved in H2O was applied to a DEAE 650M cation exchange column (5×15 cm) equilibrated with H2O. Elution was carried out with a linear gradient system composed of H2O and 2M NaCl. Fractions of 20 ml were collected and monitored by phenol–H2SO4 method and UV absorbance at 256 nm. The yields of the fractions were 17%, 4%, 14%, and 30% for FH-1, -2, -3, and -4, respectively. FH-4 was applied to a Sepharose 6B gel filtration (4.4 i.d.×92 cm) and eluted with 0.1 M NaCl. Fractions of 15 ml were collected and monitored by phenol–H2SO4 method and UV absorbance at 256 nm to give 4 fractions, FH4A (24%), FH4B (12%), FH4C (37%) and FH4D (1%). FH4C was applied to the same column chromatography to give FG.

**Electrophoresis with Cellulose Acetate Membrane**  FG was applied to a cellulose acetate membrane (Separax; Jokoh Co., Ltd., Tokyo, Japan) in a 0.1 M pyridine/0.47 M formic acid buffer.
acid buffer (pH 3), and run at 1 mA/cm. The membrane was stained with 0.1% toluidine blue in 1% AcOH/50% EtOH.

**Estimation of Apparent Molecular Weight** The apparent molecular weight of FG was estimated by HPLC analysis. The sample was applied on TSK GMPWxl gel filtration columns (7.6×300 mm×2; Tosoh) and eluted with 0.1 M NaNO3 at 0.6 mL/min at 40 °C. Commercially available pullulans (Shodex P-52; Showa Denko, Tokyo, Japan) were used as standard molecular makers.

**Determination of Pyruvate Content** The amount of pyruvate was determined as follows: FG was hydrolyzed with 1 M trifluoroacetic acid (TFA) for 6 h at 120 °C. After removal of TFA under N2 gas, the hydrolysate was analyzed using a HPLC system equipped with UV detector at 220 nm. The absorbance at 220 nm was recorded using a spectrophotometer (JEOL) using internal DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) as reference. 1D (1H and 13C) and 2D double quantum filtered correlation spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY), nuclear Overhauser effect spectroscopy (NOESY), heteronuclear single quantum correlation (HSQC), HSQC-TOCSY, heteronuclear 2-bond correlation (H2BC), and heteronuclear multiple bonding correlation (HMBC) experiments were performed.

**Cells and Viruses** Vero and Madin–Darby canine kidney (MDCK) cells were grown in MEM containing 5% fetal bovine serum (FBS), HSV-2 (UW268 strain) and influenza A virus (A/NWS/33, H1N1) (IFV-A) were propagated on Vero and MDCK cells, respectively.

**Evaluation for Antiviral Activity and Cytotoxicity in Vitro** For cell growth inhibition studies, Vero and MDCK cells were cultured for 72 h in the presence of increasing amounts of FG. Viable cell yield was determined by the trypan blue exclusion test. The inhibition data were plotted as dose–response curves, from which the 50% cell growth inhibitory concentration (CC50) was obtained. For antiviral tests, cell monolayers in 48 well plates were infected with virus 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 maintenance medium (MEM plus 2% FBS) at 37 °C. Samples were added during infection and throughout the incubation thereafter, or immediately after virus infection. The cell cultures were harvested at 24 h post-infection (p.i.), and their virus yields were determined by plaque assay after 2 d of incubation. The antiviral activity was expressed as the 50% inhibitory concentration (IC50), which was the concentration of sample that reduced plaque numbers by 50% in the treated cultures as compared with no drug control.

**Time-of-Addition Experiment** Vero cell monolayers were infected with HSV-2 at 10 PFU per cell. FG was added at a concentration of 100 μg/ml before viral infection for 3 h, during viral infection for 1 h, during viral infection and throughout the incubation thereafter, immediately after infection, at 1 h p.i., at 3 h p.i., or at 6 h p.i. At 24 h p.i., the cell cultures were harvested and subjected to plaque assay.

**Virus Adsorption Assay** The effect of FG on HSV-2 adsorption to host cells was evaluated by an infectious center assay. Briefly, Vero cell suspensions, HSV-2 (1 PFU/cell) and FG were pre-cooled on ice for 3 h before viral infection for 1 h. After 1 h of viral infection at 4 °C, the cell suspensions were washed three times with ice-cold PBS to remove any unbound viruses and free compounds. The cell pellets were diluted 10-fold with ice-cold PBS and immediately added to Vero cell monolayers in 35-mm dishes to be plaque-assyayed.

**Virus Penetration Assay** Virus penetration into host cells was evaluated according to the method reported by Huang and Wagner with some modifications. Vero cell monolayers in 12-well plates pre-cooled for 3 h on ice were infected with HSV-2 (approximately 100 PFU/well) at 4 °C for 1 h in the absence of compound. After washing three times with ice-cold PBS, cell monolayers were incubated at 37 °C in the media containing FG. At 0, 0.5, 1, 2, 3, and 6 h after the temperature shift to 37 °C, the cell monolayers were treated with a 40 mM citrate buffer (pH 3.0) for 1 min to inactivate any unpenetrated viruses, and then overlaid with media containing 0.8% methylcellulose and 2% FBS to be plaque-assyayed.
**Virucidal Assay** To determine the effect of FG on direct inactivation of virus particles, HSV-2 (2×10⁴ PFU/100 µl) was treated with an equal volume of FG at 37 °C. After 0, 1, 2, 3, or 6 h, 100-fold dilutions of the mixture were added to Vero cell monolayers for 1 h at room temperature. The cell monolayers were overlaid with media containing 0.8% methylcellulose and 2% FBS to be plaque-assayed.

**Evaluation for Anti-HSV-2 Activity in Vivo** Female BALB/c mice (5—6 weeks old) were obtained from Japan SLC, Shizuoka, Japan. All experiments were conducted in accordance with the animal experimentation guidelines of the University of Toyama. Mice were subcutaneously injected with 3 mg medroxyprogesterone 17-acetate at 6 d before virus inoculation, and then were infected vaginally with HSV-2 (1×10⁵ PFU). FG (1 mg/20 µl) or ACV (0.2 or 1 mg/20 µl) was administered intra-vaginally 5 times, that is, 1 h before infection, 10 min, 6 h, 24 h and 48 h after infection. In the control group, mice were treated with 20 µl PBS. Clinical signs of infection were graded according to a five-point scale: 0, no sign of infection; 1, slight genital erythema and edema; 2, moderate genital inflammation; 3, severe exudative genital lesion; 4, hind limb paralysis; and 5, death. Viral shedding was determined by washing the vaginal cavity with 100 µl PBS on day 3 after infection and titrating the virus on Vero cell monolayers.

**Statistical Analysis** The data are presented as the mean±S.D. The differences between groups were analyzed by one-way analysis of variance (ANOVA), and correction for multiple comparisons was made by using Dunnett’s multiple-comparison test. A comparison between the two groups was made by using Student’s t-test.

**RESULTS**

**Isolation of Sulfated Galactan from Codium fragile**
The defatted algal powder, obtained by treatment with EtOH, was extracted with hot water to give hot-water extract (F). Non-dialyzable portion (FH) was subjected to a DEAE 650M anion exchange column chromatography with a linear gradient elution to give four fractions (FH-1 to -4). The most abundant fraction (FH-4) was subjected to gel filtration on Sepharose 6B, and it gave four fractions. The most abundant fraction (FH-4C) was re-chromatographed to give sulfated galactan (FG). FG was detected as a single band on the cellulose acetate membrane electrophoretic pattern (Fig. 1). Therefore, FG was regarded to be a highly purified polysaccharide. Its molecular weight (Mw) was estimated to be 1.4×10⁵ (Mw/Mn=1.17) by HPLC analysis.

**Chemical Characterization of FG**

**Sugar composition** analysis revealed that FG consisted of large amounts of galactose (98%) with trace amounts of arabinose (2%). The galactose residues were identified to be α-configurations by comparing with authentic α- and β-galactoses after derivatization to corresponding (+)-2-butyryl galactosides. A trace amount of protein and uronic acid in FG were detected by using a Bradford assay and m-hydroxydiphenyl method. The IR spectrum of FG contained an intense absorption band at 1257 cm⁻¹ which is common to the sulfate esters, therefore, FG was suggested to be a sulfated polysaccharide. Furthermore, the presence of a sulfate absorption band at 848 cm⁻¹ (secondary axial sulfate) indicated that the majority of sulfate groups occupy the 4-O of galactose residues. Since the sulfur content was found to be 4.4% by elemental analysis, the sulfate content of FG was calculated to be 11%. On the other hand, carboxylic acid stretching absorption (1645 cm⁻¹) was observed in its IR spectrum. In addition, singlet methyl signal (1.68 ppm) was also observed in its 1H-NMR spectrum. Therefore, it was suggested that FG carried another functional group such as pyruvate ketal ester in the polymer, and pyruvate content was estimated to be 12.3% by HPLC analysis.

In order to determine the sugar linkages and the position of substituents, FG was subjected to solvolytic desulfation to obtain desulfated FG (DSFG). Pyruvate esters were removed by weak acid hydrolysis to give desulfated and depyruvated FG (DSPFG). As shown in Table 1, methylation of DSFG revealed that it mainly consisted of Galp-(1→3)-Galp-(1→ and -3,6)-Galp-(1→. On the other hand, DSFG was suggested to be composed of 3-substituted, 3,4-disubstituted and 3,6-disubstituted galactose residue. Therefore, pyruvic acid residue was suggested to be present at 3-O- and 4-O-positions of nonreducing terminal galactose residue. Furthermore, it was found that FG was composed of 3-substituted, 3,4-disubstituted and 3,4,6-trisubstituted galactose residue. On comparing data of methylation analyses of FG and DSFG, 3,4- and 3,6-substituted residues were increased,

![HPLC Chromatogram (a) and Cellulose Acetate Membrane Electrophoretic Pattern (b) of FG](image)

**Table 1. Methylation Analysis of Galactan Sulfate (FG) and Its Chemically Modified Preparations**

<table>
<thead>
<tr>
<th>Methyalted sugar</th>
<th>Deduced linkage</th>
<th>FG (mol%)</th>
<th>DSFG (mol%)</th>
<th>DSPFG (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4,6-Me₄Gal⁺</td>
<td>Galp-(1→</td>
<td>n.d.⁵)</td>
<td>3.5</td>
<td>25.8</td>
</tr>
<tr>
<td>2,4,6-Me₃Gal</td>
<td>→3)-Galp-(1→</td>
<td>16.2</td>
<td>34.1</td>
<td>36.5</td>
</tr>
<tr>
<td>2,3,4-Me₃Gal</td>
<td>→3)-Galp-(1→</td>
<td>n.d.</td>
<td>n.d.</td>
<td>5.8</td>
</tr>
<tr>
<td>2,6-Me₂Gal</td>
<td>→3,4)-Galp-(1→</td>
<td>40.2</td>
<td>33.1</td>
<td>3.6</td>
</tr>
<tr>
<td>2,4-Me₂Gal</td>
<td>→3,6)-Galp-(1→</td>
<td>6.0</td>
<td>27.1</td>
<td>28.2</td>
</tr>
<tr>
<td>2-MeGal</td>
<td>→3,4,6)-Galp-(1→</td>
<td>37.6</td>
<td>2.4</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

a) 2,3,4,6-Me₄Gal was 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylgalactitol; b) n.d., not detected.
whereas, 3-substituted residue was decreased. Therefore, sulfate esters were suggested to be present at 4-O-positions of →3)-β-D-Galp-(1→ and →3,6)-β-D-Galp-(1→ residues.

**NMR Analyses of DSPFG and DSFG** In general, sulfate substitutions cause signal broadening and perturbation of the substitution pattern which give complicated spectrum. As shown in Fig. 2, DSPFG (a) gave more simple 13C-NMR spectrum than those of DSFG (b) and FG (c). Three anomeric proton and carbon signals ranging from 4.68 to 4.52 ppm and from 107.0 to 105.8 ppm were mainly observed in the 1H- and 13C-NMR spectrum of DSPFG, respectively. From these results, all galactose residues were suggested to be β-configuration. These three anomeric signals were designated to A (δH 4.68/δC 106.9), B (δH 4.62/δC 107.0) and C (δH 4.52/δC 105.8) and signal assignments were performed using several 2D NMR experiments such as DQF-COSY, TOCSY, HSQC, HSQC-TOCSY, H2BC, and HMBC experiments as shown in Table 2. When 13C chemical shifts of residues A, B and C were compared with those of methyl-β-D-galactopyranoside, significant downfield shifts were observed at C-3 of residues A and C (data not shown). Thus, these residues were suggested to be substituted at O-3 position at the residues. Additionally, C-6 signal (δ 72.4) of residue C was also deshielded and it indicated that the residue C was disubstituted residue at O-3 and O-6 positions.

On the other hand, three extra carbon signals at δ 26.1, δ 110.5, and δ 180.2 were observed in the DSFG when compared with that of DSPFG (Fig. 2). Therefore, these signals were suggested to be originated from pyruvate ester and their chemical shifts were consistent with those of literatural data.11,12) In addition, chemical shifts of residues B, which was assigned to be a non-reducing terminal residue, was solely changed when compared with those of DSPFG (Table 2). In particular, C-3 and C-4 were shifted downfield which suggests that the pyruvate was substituted at these positions (Fig. 3). In addition, HMBC correlation between δH 4.21 and δC 110.5 revealed the presence of long-range coupling between H-3 of residue B and quaternary carbon in pyruvate group. By combining the data of methylation analyses, pyruvate was suggested to be present as a five-membered cyclic ketal including O-3 and O-4 of the non-reducing terminal galactose residues.

The sequence of glycosyl residues of DSPFG was determined by NOESY and HMBC experiments. NOE and HMBC correlations between H-1 (δ 4.68) and H-3 (δ 84.7) of residue A were observed and the residue was suggested to be linked at O-3 of the adjacent

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**Table 2.** 1H and 13C Chemical Shifts (ppm) of DSPFG and DSFG Recorded in D2O at 30 °C

<table>
<thead>
<tr>
<th>Residue</th>
<th>Chemical shift δ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C-1</td>
</tr>
<tr>
<td>DSPFG</td>
<td></td>
</tr>
<tr>
<td>→3)-β-D-Galp-(1→3)</td>
<td>106.9</td>
</tr>
<tr>
<td>A</td>
<td>4.68</td>
</tr>
<tr>
<td>β-D-Galp-(1→3)</td>
<td>107.0</td>
</tr>
<tr>
<td>B</td>
<td>4.62</td>
</tr>
<tr>
<td>→3,6)-β-D-Galp-(1→6)</td>
<td>105.8</td>
</tr>
<tr>
<td>C</td>
<td>4.52</td>
</tr>
<tr>
<td>DSFG</td>
<td></td>
</tr>
<tr>
<td>→3)-β-D-Galp-(1→3)</td>
<td>106.7</td>
</tr>
<tr>
<td>A</td>
<td>4.68</td>
</tr>
<tr>
<td>β-D-Galp-(1→3)</td>
<td>106.0</td>
</tr>
<tr>
<td>B</td>
<td>4.67</td>
</tr>
<tr>
<td>→3,6)-β-D-Galp-(1→6)</td>
<td>105.8</td>
</tr>
<tr>
<td>C</td>
<td>4.52</td>
</tr>
</tbody>
</table>

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**Fig. 2.** 13C-NMR of DSPFG (a), DSFG and FG (b, c)

NMR spectra were recorded in D2O at 30 °C (a, b) or 40 °C (c). The value of chemical shift is relative to internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at 0 ppm for 13C.

**Fig. 3.** HSQC Spectra of DSPFG (a) and DSFG (b)

The spectra were recorded in D2O at 30 °C. The assignments were based on 2D NMR experiments. The values of chemical shifts are relative to internal DSS at 0 ppm for 1H and 13C. The signals are denoted by A for →3)-β-D-Galp-(1→, by B for β-D-Galp-(1→ and by C for →3)-β-D-Galp-(1→ residues. The Arabic numerals refer to proton/carbon positions in the galactose residues.
residue. Moreover, NOE correlation between H-1 (δ 4.62) of residue B and H-3 (δ 3.84) of residue A was observed and the non-reducing terminal galactose residue was suggested to be linked at O-3 of the 3-substituted galactosyl residue. HMBC correlation between H-1 (δ 4.62) of residue B and C-3 (δ 85.1) of residue C was observed and the non-reducing terminal galactose residue was suggested to be linked to the 3,6-di-substituted galactosyl residue. Similarly, NOE contact and HMBC correlation between H-1 (δ 4.52) and H-6 (δ 3.94, 4.03) or C-6 (δ 72.4) of residue C were observed and it revealed the 3,6-di-substituted residue was connected to the 6-O position of the adjacent 3,6-di-substituted galactosyl residue.

**Antiviral Activities of FG** The antiviral potency of FG was evaluated against HSV-2 and IFV-A and shown at Table 3. Cytotoxicities of FG were quite low, with CC50 values of 3700 and 4300 µg/ml for Vero and MDCK cells, respectively. The IC50 values for HSV-2 and IFV-A replication were 4.7 and >1000 µg/ml, respectively, under conditions in which the polyaaccharide was added at the same time as viral infection (experiment A). The resulting selectivity indices (SI, CC50/IC50) were 790 and <4 for HSV-2 and IFV-A, respectively. Therefore, it was suggested that FG had a potent anti-HSV-2 effect whereas it did not possess anti-IFV-A effect. In addition, FG showed anti-HSV-2 activity when it was added after viral infection (experiment B). However, the value of SI was lower than that in experiment A. These experimental results suggested that antiviral target(s) of FG might be the inhibition of virus binding and/or penetration step(s) onto the host cells.

In order to delineate the most sensitive phase of HSV-2 replication to FG, time-of-addition experiments were performed. In these experiments, Vero cells were infected with HSV-2 at a high multiplicity of infection of 10. As shown in Fig. 4, FG suppressed virus production most efficiently when added at the same time as virus infection and throughout the incubation thereafter. On the other hand, pretreatment of host cells with FG showed no inhibitory effects, and less anti-HSV-2 effect was observed when added to the medium only during viral infection. It is noteworthy that FG maintained antiviral activity at higher levels when added to the medium even after 6 h of virus infection.

From the results of the time-of-addition experiments as described above, the most sensitive phase of HSV-2 replication to FG was suggested to be the very early events: virus adsorption to the host cell surface and/or virus penetration into host cells. At first, the effect of FG on HSV-2 adsorption into host cells was evaluated by an infectious center assay, which determined the number of cells binding the virus particles at low temperature (4°C) in the presence of different concentrations of the compound. As shown in Fig. 5a, FG showed a concentration-dependent interference with virus adsorption. Therefore, one of the antiviral targets of FG was suggested to be the interference of virus–cell interaction. An additional study investigated whether or not FG inhibited the virus penetration into host cells or not, since the virus penetration process was one possible target of FG. As shown in Fig. 5b, FG also showed the inhibitory effects on virus penetration in a concentration-dependent manner. Therefore, it was suggested that the virus penetration step was also involved in the antiviral target of FG.

As described above, while main antiviral target(s) of FG was suggested to be early stage(s) of HSV-2 replication, there are possibilities to possess another antiviral mechanism since FG showed antiviral effects by its addition to the medium after 6 h post infection when virus binding and penetration steps had completed (Fig. 4). If FG could exert virucidal effect on progeny viruses released from infected cells, this effect might contribute to the antiviral activity of delayed addition of the compound. Thus, we attempted to elucidate whether FG had the effect on HSV inactivation or not. As shown in Fig. 6, preincubation of HSV-2 with FG resulted in time- and concentration-dependent reduction of remaining infectivity. For example, the remaining infectivity was reduced to 42% and 5.8% of the no-drug control by 2- and 6-h
treatment, respectively, with FG at 10 μg/ml.

Effect of FG on Experimental Genital Herpetic Infection In order to investigate whether FG could protect mice from HSV-2 infection, the animals pretreated with medroxyprogesterone were given FG intravaginally 5 times beginning 1 h before vaginal challenge with the virus. No toxicity was observed due to the administration of the compound. The mean titers of virus shed on day 3 were reduced from 17.5 × 10^2 PFU/100 μl in untreated control mice to 0.66 × 10^2, 1.1 × 10^2 and 0.26 × 10^2 PFU/100 μl in mice that received 1 mg FG (p < 0.05), 0.2 mg (p < 0.05) and 1 mg (p < 0.05) ACV, respectively (Fig. 7a). The mice treated with FG also showed significantly suppressed herpetic lesions as compared with untreated ones (Fig. 7b). As predicted from viral shedding and severity of infection, all mice (5/5) treated with FG and ACV (1 mg/20 μl) survived the virus challenge, while 60 (3/5) and 40 (2/5) % of mice untreated or treated with ACV (0.2 mg/20 μl), respectively, died by day 10 after infection (Fig. 7c).

DISCUSSION

In the present study, we isolated a sulfated galactan (FG) from C. fragile possessing anti-HSV-2 activity in vitro and in vivo. FG consisted of a large amount of galactose residues with trace arabinose, and the presence of pyruvate and sulfate as substituents were observed. Chemical and spectroscopic analyses revealed that FG consisted of β-d-Galp-(1→3)-β-d-Galp-(1→ and →3,6)-β-d-Galp-(1→ with a ratio of ca. 2 : 3 : 2. Together with these residues, a small amount of →6)-Galp-(1→ and →3,4)-Galp-(1→ residues was also detected by methylation analysis. In addition, sulfates were suggested to be mainly linked at O-4 of →3)-Galp-(1→ and →3,6)-Galp-(1→ residues on the basis of the results of methylation analyses. Also, FG was suggested to be highly branched, and 3-O- and 4-O-positions of non-reducing termini were masked by 3,4-O-(1’-carboxy)-ethylenedic cyclic ketal. By considering the biosynthesis of polysaccharides, the pyruvic acid ketal were suggested to block the chain elongation in the biosynthesis of FG. So far, sulfated galactans have been isolated from Codium sp. such as C. yezoence and C. isthmoacladum.11,12 Our study revealed that structural features of FG was closely resembled those of sulfated galactan from C. yezoence. However, 4,6-O-(1’-carboxy)-ethylenedic cyclic ketal was not present in the case of FG as reported by previous report.13 Furthermore, Ciancia et al. reported that sulfated galactan or sulfated arabinoxylogalactan was extracted from C. fragile.14 The polysaccharide is composed of 3-linked β-D-galactopyranose and it is highly sulfated and substituted with pyruvic acid ketals, however, detailed structure is still unknown. Therefore, this paper is the first report concerning the isolation and determination of the fine chemical structure of sulfated galactan from C. fragile.

To date there are several studies on biological activities of sulfated polysaccharides from Codium sp., such as anticoagulant activity, induction of platelet aggregation, antiangiogenesis and antiviral activities.13–18 However, the antiviral effect of galactan sulfates from Codium sp. have not been reported previously. In the present study, it was shown that FG possesses antiviral activity against HSV-2. The mechanisms by which sulfated polysaccharides inhibit can be explained in general by the inhibition of virus binding to host cells and subsequent virus–cell fusion step.19 The main antiviral target of FG was also found to be the interference of virus–host cell interaction such as virus binding and penetration steps (Figs. 4, 5). In addition, FG was found to make the virion to lose its infectivity (Fig. 6). So far, there are several reports that some sulfated polysaccharides such as λ-carrageenan from Gigartina skottsbergii (1T1) exerted virucidal effect.20 In general, the virucidal effect is thought to be favorable in preventing from virus infection. In the present study, in vivo anti-HSV-2 effect of FG was evaluated using HSV-2 genital infection model. The high therapeutic efficacy of FG was confirmed in this murine model (Fig. 7). The in vivo anti-HSV-2 effect of FG might reflect its in vitro antiviral actions exerted by the interference of viral attachment and virucidal activity.
Thus, FG could be a candidate for use as a prophylactic agent against HSV-2 infection.

REFERENCES