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Effect of partial desulfation and oversulfation of sodium spirulan on the potency of anti-herpetic activities

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Abstract

The aim of the present study is to evaluate the effect of partial desulfation and oversulfation of sodium spirulan (Na-SP) isolated from *Spirulina platensis* on the exhibition of anti-herpes simplex viruses type 1 and 2 (HSV-1 and -2) activities. Partially desulfated (PDS-SPs) and oversulfated derivatives (OS-SPs) were obtained by solvolytic desulfation and sulfation with dicyclocarbodiimide-sulfuric acid, respectively. When PDS-SPs were subjected to anti-HSV-1 assay, antiviral potency was dependent on their sulfate content, and PDS-SPs with lower sulfate content than 8.6% were found to be inactive. Some derivatives showing anti-HSV-1 effect also showed anti-HSV-2 activity. Anti-HSV-1 effect of OS-SPs was equivalent to that of Na-SP when they were added to the medium during viral infection and throughout the incubation thereafter, while they were enhanced as compared with Na-SP when they were added to the medium immediately after viral infection. The results of time-of-addition experiments suggested that the most sensitive phase of OS-SP-2 and -5 might be the early steps of viral adsorption and penetration.

Keywords: Sodium spirulan; *Spirulina platensis*; Partial desulfation; Oversulfation; Antiviral activity

1. Introduction

A broad series of polysaccharides have emerged as an important class of bioactive molecules that occur naturally in a great variety of animals, plants, and microorganisms. Heparin is one of glycosaminoglycans and has been used as a drug for prevention and treatment of thromboembolic disorders. Besides anticoagulant and antithrombotic activities, it was found to show antiviral activity (Witvrouw, Desmyter, & De Clercq, 1994). In recent years, the use of heparin has been accompanied with much danger of infectious diseases such as bovine spongiform encephalopathy (BSE). Therefore, it would be one of important fields of research to develop substitutes for heparin. From this point of view, various polysaccharides isolated from natural resources and their derivatives have been targeted for evaluation as alternatives to heparin.

Among a number of infectious agents, herpesviruses are major opportunistic pathogens. Infections caused by herpes simplex viruses (HSVs) are common throughout the world and characterized by latency and recurrence of symptomatic diseases. Some clinical observations and investigations have suggested that HSVs might play a role as one of cofactors by interacting with human immunodeficiency virus type 1 (HIV-1) at the cellular and/or molecular level to promote HIV-1 infection in vivo and to accelerate AIDS progression (Griffiths, 1998; Palú, Benetti, & Calistri, 2001). Furthermore, HSV could determine an alteration of the HIV-1 tropism as the result of phenotypic mixing between HIV-1 and HSV (Calistri et al., 1999; Heng, Heng, & Allen, 1994). This would allow infection of cells that are not normally susceptible to HIV-1 infection, such as CD4-negative cells. Of all the sexually transmitted diseases (STDs), there appears to be true "epidemiologic synergy" between HSV and HIV-1, in that

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HIV-1 incidence would be increased in parallel with HSV-2 prevalence among HIV-1-negative and -positive persons, and HIV-1 prevalence would increase HSV-2 incidence. Although the prevalence of genital herpes simplex virus infection has been associated with HSV-2, changes in sexual practices have led to an increase in the incidence of genital HSV-1 infections (Lafferty, 2002).

So far, several sulfated polysaccharides have been shown to inhibit the replication of various enveloped viruses including HSV, human cytomegalovirus, and HIV (Witvrouw et al., 1994; Witvrouw & De Clercq, 1997). It is noteworthy that sulfated polysaccharides could be antiviral candidates since they prevent virus replication by interfering with early steps of virus–host cell interaction. This antiviral mechanism is quite different from that of commercially available drugs. In addition, the sulfated polysaccharides showed synergistic antiviral effect when they were administrated in combination with acyclovir, an anti-herpetic agent (Lee, Hayashi, Hayashi, Sankawa, & Maeda, 1999).

In the course of our screening for antiviral molecules from algae, we discovered a novel sulfated polysaccharide, calcium spirulan (Ca-SP), from *Spirulina platensis* (Cyanophyta) (Hayashi, Hamada, & Hayashi, 1996; Hayashi, Hayashi, & Kojima, 1996; Hayashi, Hayashi, Maeda, & Kojima, 1996). Previous studies revealed that the polysaccharide consists of 1,3-linked rhamnosyl, 1,2-linked 3-*O*-methyl-rhamnosyl, 1,4- and 1,3,4-linked hexuronosyl residues and sulfate groups substituted at C-2 of 1,3-linked rhamnosyl and at C-4 of 1,2-linked 3-*O*-methyl-rhamnosyl residues (Lee et al., 1998, Lee, Hayashi, Hayashi, & Sankawa, 2000). Sodium spirulan (Na-SP) was easily obtained from Ca-SP by replacement of calcium ion with sodium ion, and its antiviral potency is almost the same as that of Ca-SP. Furthermore, Na-SP has been shown to exhibit antithrombin activity by activation of heparin cofactor II with a different mechanism from that of heparin (Hayakawa, Hayashi, Lee, Ozawa, & Sakuragawa, 2000). It also stimulated the release of anticoagulant heparin and dermatan sulfate proteoglycans (Kaji et al., 2002) and inhibited the plasminogen activator inhibitor type 1 from vascular endothelial cell layers (Yamamoto et al., 2003). These results indicate that Na-SP might have beneficial effects as an anticoagulant agent on the blood-coagulation–fibrinolytic system through not only activation of heparin cofactor II but also exhibiting a fibrinolytic property based on differential effects on endothelial fibrinolytic protein secretion. These observations suggest that Na-SP might have a preventative effect on arteriosclerosis. In addition to antiviral effects, these biological effects have been thought to be dependent on molecular weight and/or sulfate content. However, the relationships between the chemical structures of sulfated polysaccharides and their biological effects remain unclear.

In this study, a series of Na-SP derivatives with different sulfate content prepared by solvolytic desulfation and chemical oversulfation were evaluated for their anti-herpetic activities in order to make clear the contribution of sulfate groups in Na-SP to exhibition of antiviral effect.

2. Materials and methods

2.1. Materials

Isolation of sodium spirulan (Na-SP) was performed as described previously (Lee et al., 1998). Eagle's minimal essential medium (MEM) was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Other reagents were from Wako Pure Chemicals (Osaka, Japan).

2.2. Partial desulfation of Na-SP

Partial desulfation of Na-SP was carried out by the solvolytic method (Nagasawa, Inoue, & Tokuyasu, 1979). Briefly, Na-SP (500 mg) was dissolved in distilled water (25 ml), followed by passing the solution through a Dowex $50W \times 8$ column (H^+ form) at 4 °C. The eluate was neutralized with pyridine and lyophilized to give pyridinium salt of Na-SP (513 mg). The pyridinium salt of Na-SP (40 mg) was dissolved in 10% MeOH/DMSO at room temperature, and incubated under indicated condition. After various reaction time points, the reaction mixture was cooled immediately in ice-water bath and then diluted with distilled water (2 ml). The mixture was adjusted to pH 9 with 0.1 M NaOH, dialyzed with the dialysis tubing whose molecular weight cutoff was 1000, and lyophilized to give partially desulfated polysaccharides (PDS-SPs).

2.3. Oversulfation of Na-SP

Oversulfation of Na-SP was carried out by the method previously reported (Takano et al., 1996). Dicyclohexylcarbodiimide (DCC, 2 mmol) was dissolved in dimethylformamide (DMF, 6 ml). Pyridinium salt of Na-SP (30 mg) was suspended in DMF (4 ml) for 15 h, and mixed with the DCC solution. To the mixture, sulfuric acid (0.6 mmol) in DMF (4 ml) was added over 10 min at 0 °C under N_2 atmosphere. Reaction temperature was kept at 0 °C for the indicated time. The reaction mixture was poured into ice and neutralized with 0.2 M NaOH. It was dialyzed against 1 M NaCl and then against distilled water. The dialysate was lyophilized to give oversulfated derivatives of Na-SP (OS-SPs).

2.4. Determination of apparent molecular weights of polysaccharides

Each chemically modified polysaccharide was analyzed by high-performance gel filtration chromatography in 0.1 M NaCl at a flow rate of 0.5 ml/min using TSK-gel $GMPW_{XL}$ columns (300×7.6 mm i.d. $\times 2$). Column calibration was performed with standard pullulans (Shodex P-52, Showa Denko K.K., Tokyo, Japan).

2.5. Chemical analyses of polysaccharides

Total neutral sugar content was determined by phenol-sulfuric acid method, using rhamnose as a standard (Dubois, Gilles, Hamilton, Revers, & Smith, 1956). Uronic acid and sulfate contents were determined by *m*-hydroxydiphenyl method (Blumenkrantz & Asboe-Hansen, 1973) or rhodizonate method (Silvestri, Hurst, Simpson, & Settine, 1982), respectively.

2.6. Sugar composition and linkage analysis of polysaccharides

Sugar composition of polysaccharides was analyzed as follows. Each sample was hydrolyzed with 2 N H_2SO_4 at 100 °C for 3 h. The hydrolysates were reduced with $NaBH_4$, followed by acetylation. The alditol acetates were analyzed by GC. For methylation analysis, sample was converted to a triethylammonium salt by passing through the Dowex 50W resin (triethylamine form) (Stevenson & Furneaux, 1991). The triethylammonium salt was methylated three times according to Hakomori method (Hakomori, 1964). The methylated polysaccharide was hydrolyzed with 2 N sulfuric acid and acetylated to partially methylated alditol acetates. The resultants were analyzed by GC and GC-MS. GC analysis was carried out using GC-353 gas chromatograph (GL Science Inc., Tokyo, Japan) with a SP-2330 fused silica capillary column (30 m × 0.32 mm i.d., Supelco Inc., PA) with a temperature program starting at 160 °C followed by 2 °C/min to 210 °C, and then 5 °C/min to 240 °C. GC-MS analysis was carried out using a DB-1MS (30 m × 0.32 mm i.d., J&W Scientific Inc., CA). The derivatives were identified by comparison of their relative retention time with 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgluciton and their mass fragmentation patterns (Carpita & Shea, 1988). Peak area was corrected using published molar response factors (Sweet, Shapiro, & Albersheim, 1975).

2.7. Cell and virus

Vero (African green monkey kidney) cells were grown in Eagle's MEM containing 5% fetal bovine serum (FBS) and kanamycin (60 mg/L). HSV-1 (HF strain) and -2 (UW264 strain) were propagated on Vero cells and stored at −80 °C until use. An aliquot of the virus stock was titered by plaque assay.

2.8. Antiviral activity and cytotoxicity

Vero cell monolayers in 48-well plates (2×10^5 cells/well) were infected with HSV-1 or -2 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 cells were incubated at an initial density of 1.2×10^4 cells/well in 96-well plates. After cells had been incubated for 8 h at 37 °C, sample was added and the incubation was continued for 3 days. Viable cell yield was determined by the trypan blue exclusion test. The 50% cytotoxic concentration (CC_{50}) was obtained from concentration-response curves. All data were expressed as mean ± SD from triplicate assays.

3. Results

3.1. Preparation and characterization of partially desulfated spirulans (PDS-SPs)

Na-SP was partially desulfated by solvolytic method under various experimental conditions. Fig. 1 shows correlation between reaction period and sulfate content. Sulfate content decreased rapidly from 17.3 to 8.6 during initial 10 min, and sulfate groups were completely removed after 120 min when incubated at 80 °C. It was found to be difficult to prepare the derivatives with sulfate content ranging from 13.8% to 17% under this reaction condition. Thus, the reaction temperature was reduced to 40, 60, or 70 °C to prepare some derivatives with sulfate content between 14.4% and 17.1%. Table 1 summarizes the reaction conditions and chemical characteristics of PDS-SPs. With extending the reaction time, the content of carbohydrate and uronic acid in the products increased while sulfate content decreased. HPLC chromatograms of PDS-SPs revealed that they were homogeneous on the basis of molecular size distribution. It was noted that the apparent molecular weight of each PDS-SP decreased in parallel with its sulfate content (Table 1). The apparent molecular weight of DS-SP, in which no sulfate was detected, was about 3.7-fold lower than that of Na-SP.

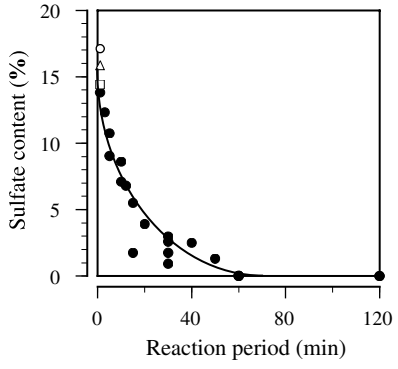

Fig. 1. Correlation between reaction period and sulfate content. Partial desulfation reactions were conducted at 80 °C (closed circle), 70 °C (open circle), 60 °C (triangle), and 40 °C (square).

Table 1
Reaction conditions for preparation of partially desulfated polysaccharides and their characteristics

Sample	Reaction		Yield (mg)	Sulfate (%)	Carbohydrate (%)	Uronic acid (%)	M_r ($\times 10^5$)
	Temp (°C)	Time (min)					
Na-SP				17.3	44.5	16.4	2.45
PDS-SP-1	40	1	34.2	17.1	47.3	16.7	2.23
PDS-SP-2	60	1	33.0	15.9	48.5	17.9	2.19
PDS-SP-3	70	1	32.2	14.4	52.1	18.3	2.12
PDS-SP-4	80	1	31.3	13.8	53.4	18.6	2.11
PDS-SP-5	80	3	30.9	12.3	54.1	19.2	2.10
PDS-SP-6	80	5	27.1	10.7	55.2	19.9	2.00
PDS-SP-7	80	10	28.4	8.6	56.3	21.6	1.98
PDS-SP-8	80	12	28.2	6.8	56.4	22.0	1.90
PDS-SP-9	80	15	25.0	5.5	57.2	22.5	1.83
PDS-SP-10	80	40	23.1	2.5	62.6	24.9	1.19
DS-SP	80	120	15.6	n.d.[a]	73.3	26.4	0.66

[a] n.d., not detected.

3.2. Linkage and substitution position analysis by methylation

In order to elucidate the stoichiometry of desulfation, we performed methylation analyses that give the information on the substitution site of sulfate groups. To avoid incomplete methylation, samples were converted to triethylammonium salts before methylation. Our previous report revealed that Na-SP mainly consists of 1,3-linked rhamnose and 1,2-linked 3-*O*-methyl-rhamnose units with sulfate substitution at the 2- and/or 4 positions (Lee et al., 1998). It was also revealed that most of rhamnosyl residues were unbranched. In the present study, these findings were confirmed on the basis of the chemical characterization of DS-SP. Therefore, as indicated in Fig. 2 and Table 2, the deduced sugars can be regarded as 1,2-linked 3-*O*-methyl-rhamnose, 1,3-linked rhamnose, 1,3-linked 2-*O*-sulfated rhamnose, 1,2-linked 4-*O*-sulfated 3-*O*-methyl-rhamnose, and 1,3-linked 2,4-di-*O*-sulfated rhamnose residues. When the data on PDS-SP-1 were compared with those on Na-SP, the mole percentage of 1,3-linked 2,4-di-*O*-sulfated rhamnose relatively decreased while 1,3-linked 2-*O*-sulfated rhamnose increased. In the cases of PDS-SP-2, -3 and –4, the mole percentages of 1,3-linked 2,4-di-*O*-sulfated rhamnose and 1,2-linked 4-*O*-sulfated 3-*O*-methyl-rhamnose residues decreased while the mole percentages of 1,3-linked 2-*O*-sulfated rhamnose and 1,2-linked 3-*O*-methyl-rhamnose residues increased. There was no significant difference in the amount of 1,3-linked rhamnose residue among these three derivatives. In PDS-SP-5, the mole percentage of 1,3-linked rhamnose residue increased. Both 1,2-linked 3-*O*-methyl-rhamnose and 1,3-linked rhamnose residues increased in PDS-SP-6, -7, -8, -9 and –10. Taken together, it was suggested that the 4-*O*-sulfate group was removed more rapidly than the 2-*O*-sulfate group by solvolytic desulfation of Na-SP.

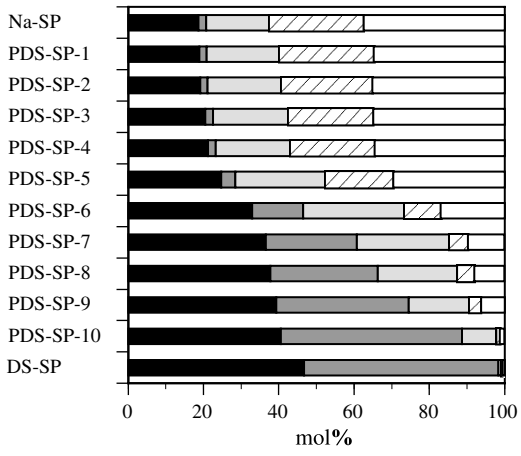

Fig. 2. Deduced sugar linkages in Na-, PDS-, and DS-SPs. Black bar: →2)-3-*O*-Me-Rha-(1→, Gray bar: →3)-Rha-(1→, Light gray bar: →3)-2 – *O*-SO_3-Rha-(1→, Stripe bar: →2)-3-*O*-Me-4-*O*-SO_3-Rha-(1→, White bar: →3)-2,4-di-*O*-SO_3-Rha-(1→.

3.3. Preparation and characterization of oversulfated Na-SPs (OS-SPs)

Although sulfation of polysaccharide is usually carried out by SO_3-DMF and $ClSO_3$-DMF (Yoshida et al., 1995), fatal depolymerization was frequently observed during sulfation reactions. In the present study, sulfation was performed with sulfuric acid and dicyclohexylcarbodiimide (DCC) because just slight depolymerization was observed under this condition (Takano et al., 1996). In order to prepare oversulfated Na-SPs (OS-SPs), the ratio of sulfuric acid to sugar residue was set to be 3, and the reaction time were varied from 30 to 180 min. Table 3 summarizes the reaction conditions and characteristics of OS-SPs. Sulfation with H_2SO_4-DCC method achieved the preparation of OS-SPs with sulfate content ranging from 21.1% to 28.1%. HPLC analyses revealed their apparent molecular weight to range from 1.60 to 2.18×10^4. In spite of the increase in their sulfate content, only slight change in apparent molecular weight of OS-SPs was observed. These findings indicated that the resultant polysaccharides might be slightly depolymerized during oversulfation. On the other hand, the content of neutral sugar and uronic acid relatively decreased due to the increase in sulfate content.

Table 2
Results of methylation analyses of partially desulfated spirulans

	Deduced linkages (mol%)				
	→2)-3-Me-Rha-(1→	→3)-Rha-(1→	→3)-2S-Rha-(1→	→2)-3-Me-4S-Rha-(1→	→3)-2,4-di-S-Rha-(1→
Na-SP	18.6	2.1	17.0	24.9	37.4
PDS-SP-1	18.9	1.9	19.4	25.0	35.0
PDS-SP-2	19.2	1.9	19.6	24.2	35.2
PDS-SP-3	20.5	2.0	20.0	22.6	34.9
PDS-SP-4	21.2	2.0	20.0	22.3	34.5
PDS-SP-5	24.7	3.7	24.0	18.0	29.7
PDS-SP-6	32.9	13.6	26.9	9.6	17.1
PDS-SP-7	36.5	24.3	24.5	4.9	9.7
PDS-SP-8	37.8	28.5	21.3	4.3	8.0
PDS-SP-9	39.3	35.2	16.1	3.2	6.2
PDS-SP-10	40.6	48.1	9.2	0.8	1.2
DS-SP	46.7	51.7	0.8	0.3	0.5

Table 3
Reaction conditions for preparation of oversulfated polysaccharides and their characteristics

Sample	Reaction time (min)	Yields (mg)	Sulfate (%)	Carbohydrate (%)	Uronic acid (%)	M_r ($\times 10^5$)
Na-SP			17.8	46.3	16.5	2.28
OS-SP-1	30	41.0	21.1	45.5	14.0	1.98
OS-SP-2	60	35.6	23.0	41.3	12.9	1.60
OS-SP-3	90	34.7	25.6	42.4	10.8	1.81
OS-SP-4	120	34.3	27.8	38.5	11.5	2.18
OS-SP-5	180	33.8	28.1	40.8	12.0	2.10

3.4. Evaluation of PDS- and OS-SPs for their antiviral activity

3.4.1. Selective effect of PDS- and OS-SPs against viral replication

Antiviral activities of PDS- and OS-SPs were tested to evaluate the contribution of sulfate group to the suppression of HSV-1 replication (Table 4). All samples were assayed for cytotoxicity to host cells as well as inhibition of viral replication in the host cells. Anti-HSV-1 potency was estimated from the selectivity index expressed as the ratio of CC_{50} to IC_{50}. In the experiment A, sample was added to the medium during viral infection and throughout the incubation thereafter, while in the experiment B, sample was added immediately after viral infection. In a series of experiments, acyclovir, a clinically used anti-HSV-1

Table 4
Anti-HSV-1 activity of OS- and PDS-SPs

Sample	Sulfate (%)	Cytotoxicity (CC_{50}, μg/ml)	Anti-HSV-1 activity (IC_{50}, μg/ml)		Selectivity index (CC_{50}/IC_{50})	
			A[a]	B[b]	A	B
Na-SP	17.8	7100 ± 350	0.63 ± 0.32	3.1 ± 0.61	13,000 ± 4600	2300 ± 320
OS-SP-1	21.1	4400 ± 260	0.64 ± 0.056	0.69 ± 0.16	6900 ± 450	6400 ± 850
OS-SP-2	23.0	2500 ± 440	0.46 ± 0.079	0.75 ± 0.055	5400 ± 720	3300 ± 400
OS-SP-3	25.6	3100 ± 440	0.61 ± 0.11	0.64 ± 0.11	5100 ± 260	4800 ± 210
OS-SP-4	27.8	3900 ± 360	0.74 ± 0.070	0.84 ± 0.10	5300 ± 290	4600 ± 300
OS-SP-5	28.1	3300 ± 360	0.84 ± 0.074	0.76 ± 0.13	3900 ± 670	4300 ± 290
PDS-SP-1	17.1	6500 ± 310	1.7 ± 0.76	4.1 ± 0.61	4200 ± 1500	1600 ± 180
PDS-SP-2	15.9	5300 ± 210	1.7 ± 0.70	3.8 ± 0.67	3500 ± 1400	1400 ± 220
PDS-SP-3	14.4	4500 ± 200	1.8 ± 0.10	12 ± 4.4	2500 ± 28	390 ± 130
PDS-SP-4	13.8	4500 ± 210	2.6 ± 0.38	19 ± 3.5	1800 ± 180	240 ± 36
PDS-SP-5	12.3	5100 ± 200	3.2 ± 0.26	20 ± 2.3	1600 ± 100	260 ± 26
PDS-SP-6	10.7	5100 ± 250	5.9 ± 1.00	34 ± 3.8	880 ± 100	150 ± 12
PDS-SP-7	8.6	3800 ± 150	20 ± 4.6	150 ± 51	200 ± 32	26 ± 6.6
PDS-SP-8	6.8	3700 ± 150	49 ± 110	710 ± 110	7.9 ± 1.6	5.4 ± 0.7
PDS-SP-9	5.5	3900 ± 100	>1000	>1000	<5	<5
PDS-SP-10	2.5	4900 ± 210	>1000	>1000	<5	<5
DS-SP	n.d.[c]	4900 ± 200	>1000	>1000	<5	<5

[a] Sample was added to the medium during viral infection and throughout the incubation thereafter.
[b] Sample was added to the medium immediately after viral infection.
[c] n.d., not detected.

drug, was used as a positive control, showing a selectivity index of about 1000 (data not shown).

As shown in Table 4, there was no significant cytotoxicity among all polysaccharides tested. It was found that anti-HSV-1 activity was dependent on their sulfate content in a series of PDS-SPs, whereas there was no correlation between antiviral activity and sulfate content in a series of OS-SPs. In a series of PDS-SPs, anti-HSV-1 activity was vanished when their sulfate content decreased to 6.8% or less. These results suggested that the degree of sulfation of Na-SP might be important for the interference with virus binding onto host cell surface. However, it was of interest that in the experiment B the IC_{50}s of the OS-SPs decreased by approximately one-fourth as compared with Na-SP, suggesting that OS-SPs might be more toxic in the later steps of virus replication than Na-SP.

Several samples were assayed to evaluate their anti-HSV-2 activity (Table 5). As a results, all polysaccharides tested showed higher antiviral potency for HSV-2 than those for HSV-1 except for OS-SP-3.

3.4.2. Time-of-addition experiments of Na-SP and OS-SPs

Since OS-SPs were suggested to exert potent inhibitory activity even in the later steps of virus replication, time-of-addition experiments were carried out using Vero cells infected with virus at a high PFU of 10 per cell to delineate the drug-sensitive phase (Table 6). There was no remarkable difference in the antiviral potency between OS-SPs and Na-SP when sample was added to the medium during infection and for 24 h thereafter. The IC_{50} values of OS-SPs, however, showed a tendency to decrease as compared with those of Na-SP when samples were added to the medium at later times after viral infection. It was noteworthy that lower concentrations of both OS-SPs were required for 50% inhibition of virus replication as compared with Na-SP even when these samples were added 8 h postinfection.

4. Discussion

Seventeen modified polysaccharides with sulfate content on ranging from 0% to 28.1% could be obtained by chemical modification of Na-SP. In the course of preparation of PDS-SPs, no serious side reaction was suggested to occur since the apparent molecular weight of derivatives were kept identical with DS-SP when the reaction was performed for less than 120 min. We should emphasize that these PDS-SPs are useful to clarify the correlation between biological activity and degree of sulfation. Although a large number of studies have been performed from a similar viewpoint, chemical sulfation was mostly applied. As mentioned below, chemical sulfation strategies often lead to depolymerization of polysaccharides, which may cause confusion in the interpretation of results solely depending on degree of sulfation or concerning degree of polymerization.

The reaction scheme of desulfation of Na-SP could be explained by the results of methylation analysis of

Table 5
Anti-HSV-2 activity of OS- and PDS-SPs

Sample	Cytotoxicity (CC_{50}, μg/ml)	Anti-HSV-2activity (IC_{50}, μg/ml)		Selectivity index (CC_{50}/IC_{50})	
		A[a]	B[b]	A	B
Na-SP	6900 ± 640	0.41 ± 0.032	2.1 ± 0.15	17,000 ± 1000	3200 ± 490
OS-SP-2	3900 ± 400	0.26 ± 0.042	0.72 ± 0.11	14,000 ± 1600	5400 ± 560
OS-SP-3	2600 ± 380	0.58 ± 0.046	1.5 ± 0.11	4600 ± 950	1700 ± 86
PDS-SP-2	5700 ± 1100	0.52 ± 0.050	1.4 ± 0.23	11,000 ± 1000	4100 ± 1200
PDS-SP-4	4700 ± 560	1.5 ± 0.23	2.2 ± 0.74	3300 ± 920	2300 ± 610
PDS-SP-7	4300 ± 820	5.0 ± 0.60	12 ± 0.44	860 ± 59	380 ± 79

[a] Sample was added to the medium during viral infection and throughout the incubation thereafter.
[b] Sample was added to the medium immediately after viral infection.

Table 6
Time-of-addition experiments of Na-SP and OS-SPs

Treatment	Na-SP		OS-SP-2		OS-SP-5	
	IC_{50}[a]	SI[b]	IC_{50}	SI	IC_{50}	SI
During infection	>200		>200		>200	
During infection and for 24 h thereafter	0.57 ± 0.16	12456	0.87 ± 0.38	2874	0.90 ± 0.19	3667
0–24 h postinfection	2.9 ± 1.0	2448	0.92 ± 0.34	2717	1.3 ± 0.61	2538
2–24 h postinfection	13 ± 4.8	546	11 ± 4.8	227	8.7 ± 4.6	379
4–24 h postinfection	25 ± 7.0	284	14 ± 8.6	179	15 ± 10	220
6–24 h postinfection	50 ± 10	142	27 ± 8.4	93	32 ± 12	103
8–24 h postinfection	82 ± 16	87	38 ± 16	66	37 ± 11	89

[a] The 50% inhibitory concentration (μg/ml).
[b] Selectivity index as shown in Table 3.

PDS-SPs. That is, when the temperature was 40 °C, the 4-*O*-sulfate in 1,3-linked 2,4-di-*O*-sulfated rhamnose residue was predominantly desulfated, judging from the fact that the relative amount of 1,3-linked 2-*O*-sulfated rhamnose residue increased. As the temperature was raised at 80 °C, the 4-*O*-sulfate group began to cleave, followed by the removal of 2-*O*-sulfate group in sugar residue. Previous report on the solvolytic desulfation procedure revealed that polar aprotic solvents such as DMSO caused desulfation of sulfated polysaccharides, and MeOH could improve the rate of desulfation (Nagasawa et al., 1979). Thus, it was thought that 4-*O*-sulfate group, which was in the equatorial position of rhamnosyl residues, might be easily exposed to the reaction solvent since it was quite susceptible to the reaction and was preferentially desulfated. On the other hand, oversulfation of Na-SP with H_2SO_4-DCC reagent was achieved to prepare OS-SPs of which sulfate content ranged from 21.1% to 28.1%, but slight depolymerization was observed during the reaction.

Anti-HSV-1 activity of PDS-SPs seemed to be dependent on the degree of sulfation. It means that sulfate groups might play a crucial role in exhibition of their biological activity. Interestingly, in spite of its lower degree of sulfation when compared with other bioactive sulfated polysaccharides, Na-SP showed potent anti-HSV-1 activity. Furthermore, PDS-SP-7 with much lower sulfate content (8.6%) also kept antiviral activity. So far, there are a large number of reports on the correlations between degree of sulfation (DS) and biological activity such as anticoagulant activity (Alban & Franz, 2001; Groth & Wagenknecht, 2001). It is well documented that biological activities of sulfated polysaccharides are dependent on their DS, molecular weight, and polysaccharide structure such as linear or branched one. For example, curdlan sulfates of high DS (>1.1) have been found to show potent anti-human immunodeficiency virus activity with low anticoagulant activity (Uryu, Katsuraya, & Yoshida, 1996). Other semisynthetic sulfated polysaccharides such as lentinan sulfate and dextran sulfate showed similar tendencies. All semisynthetic sulfated polysaccharides mentioned above are linear polymers, while Na-SP is a highly branched one. Therefore, Na-SP and its derivatives could yield 'cluster' or 'multivalency' effect (Lundquist & Toone, 2002), which might result in much stronger interaction with virus and consequently produce more effective inhibition of virus replication. Furthermore, Na-SP and PDS-SP-1 showed similar chemical properties except apparent molecular weight, while the antiviral potency of PDS-SP was less than Na-SP. Thus, it was suggested that the difference might be derived from lowering apparent molecular weight.

In contrast to PDS-SPs, no clear dependence of anti-HSV-1 activity on degree of sulfation was observed in the case of OS-SPs as shown in the experiment A. Thus, naturally occurring Na-SP might contain sufficient sulfates for expressing the antiviral activity when they were added to the medium at the early stage of viral replication, including virus binding to host cell membrane. OS-SPs, however, are superior to Na-SP in exerting anti-HSV-1 activity at the later stages of viral replication. It might be expected that oversulfation could give rise to novel antiviral effect although its mechanism is not yet elucidated.

S. platensis has been produced in a large scale under controlled conditions and used as nutritional and therapeutic supplement (Belay, 2002). Therefore, Na-SP might have no potential risk of viral or prion contamination as seen in animal products and could be supplied in good quality unlike other polysaccharides from plants and animals. Thus, Na-SP would be an attractive candidate as a therapeutic agent for viral infectious diseases and arteriosclerosis. Furthermore, chemical modifications of Na-SP were found to be promising for production of novel biomolecules with more valuable biological effect. Further studies are needed to improve our understanding of the antiviral mechanism of Na-SP and the critical structural features required for more elaboration of the activity.

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