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Antiviral Activities of Sulfated Derivatives of a Fucosamine-Containing Polysaccharide of Marine Bacterial Origin

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Sulfated derivatives of a polysaccharide prepared by chlorosulfonic acid treatment of a fucosamine-containing polysaccharide from a marine *Pseudomonas* showed strong antiviral activity against herpes simplex type 1 (HSV-1). This sulfated polysaccharide inhibited the cytopathic effect of HSV-1 at 0.72 μg/m*l*. No cytotoxic effects on Vero cells were detected with concentrations of sulfated polysaccharide up to 1,000 μg/m*l*.

Since inhibitory effects of polyanionic substances such as sulfated polysaccharides on viral attachments were reported more than twenty years ago,[1,2] much effort has been directed at trying to adapt these substances to the treatment of patients. In recent years many reports on the effects of sulfated polysaccharides such as dextran sulfate on several viruses *in vitro*, including human immunodeficiency virus, have been published.[3-6] It is known that antiviral effects shown by sulfated polysaccharides generally appear to require a high degree of sulfation (S>10%, W/W) of the molecule.[6,7] Despite the urgent need for an effective chemotherapy for virus induced diseases, clinical trials of sulfated polysaccharides have as yet been unsuccessful in the therapy of virus infections in humans because of their blood anticoagulant activity caused by the high sulfate contents of the polysaccharide, of its large molecule that cannot easily cross membranes, and of its poor absorption when given orally.[4,8]

This paper describes antiviral activities of a sulfated derivatives of a fucosamine-containing polysaccharide produced by a marine *Pseudomonas*. The characteristic feature is the polysaccharide of a lower degree of sulfation than the sulfated polysaccharide such as dextran sulfate.

Materials and Methods

Bacterial Strain

Pseudomonas sp., strain HA-318, was isolated from the intestinal contents of the sea cucumber *Stichopus japonicus*, collected from the Seto Inland Sea, Japan. The seawater medium used for bacterial isolation and polysaccharide production contained 30 g of sucrose, 5 g of peptone, 1 g of yeast extract, and 15 g of agar per liter of seawater.

Isolation of the Polysaccharide

An actively growing culture was used to inoculate 10 m*l* of seawater medium (as above, but without agar) which was incubated for 24 h at 28°C with shaking. The culture medium was poured onto a seawater agar plate (18×26 cm). After incubation for 3 days at 25°C, the mucoid growth was scraped from the agar surface using 1% phenol solution. After stirring the mucoid material vigorously, bacterial cells were removed by high-speed centrifugation (70,00×g for 2 h). The polysaccharide was isolated and purified from the supernatant by precipitations with ethanol and Cetavlon.[9] The polysaccharide thus obtained was dissolved in water and the resulting solution was dialyzed against de-ionized water, followed by freeze-drying.

Preparation of the Sulfated Polysaccharide

The polysaccharide (1 g) was sulfated with chlorosulfonic acid (15 m*l*) in formamide (100 m*l*) in an ice-water bath with continuous stirring. The reaction mixture was incubated at 25°C for 1 h, followed by neutralization with solid Na_2CO_3. After dialysis, the sulfated polysaccharide was isolated by precipitations with Cetavlon and ethanol. The sulfated polysaccharide thus obtained was dissolved in water and the resulting solution was dialyzed against de-ionized water, followed by freeze-drying (1.4 g). This polysaccharide was used as the crude, sulfated polysaccharide preparation.

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The sulfated polysaccharide was fractionated by chromatography on a column (1.6×25.8 cm) of DEAE-cellulose ion-exchange resin equilibrated with 0.01 M sodium phosphate buffer (pH 7.0) using stepwise elution with the same buffer containing from 0 to 2 M sodium chloride. Fractions from the column were analyzed for carbohydrate by the phenol-sulfuric acid method,[10] and combined fractions were dialyzed against de-ionized water, followed by freeze-drying.

Analytical Methods

Methods for qualitative and quantitative analyses of component sugars were described previously.[11]

The ^1H NMR spectrum was obtained with a JEOL JNM GSX 500 spectrometer (500 MHz) at 75°C. The sample was dissolved in D_2O containing DSS as the internal standard.

Molecular weights were determined by gel-filtration on an Asahipak GFA 7 M column (Asahi Chemicals, 7.6×500 mm). For minimizing the association effect of the polysaccharide solution, 0.1 M NaCl was used as a mobile phase at 30°C at a flow rate of 0.4 ml/min. A shodex Standard Kit P-82 (Showa Denko, M. W.$=0.58\times10^4-85\times10^4$) was used for calibration of the molecular size. Sulfate content was determined with a Shimadzu HIC-6A ion chromatograph using a Shimpack IC-Al column (Shimadzu, 4.6×100 mm) and a conductivity detector at 40°C after hydrolysis with 2 M TFA for 12 h at 100°C. Pyruvate was determined with a Shimadzu HIC-6A ion chromatograph equipped with a UV detector (at 210 nm). The hydrolyzates of the polysaccharide which were produced with 0.05 M H_2SO_4 for 1 h at 100°C were analyzed on a Shimpak IC-101H column (Shimadzu, 7.9×300 mm) at 50°C with 5 mM perchloric acid as a mobile phase.

Assay for Viruses Induced Cytopathic Effect

The KOS strain of herpes simplex virus type 1 (HSV-1) and strain of A/PR/8/34 (HINI) of influenza virus type A (influenza A) were grown on Vero cells and MDCK cells, respectively. Vesicular stomatitis virus (VSV) and adenovirus type 3 (adeno 3) were grown on Hela S_3 cells. These were grown in Eagle's medium (MEM, Nissui Seiyaku, Tokyo) supplemented with 1.5% fetal calf serum (FBS, Flow Lab. Inc., U. S. A.). MEM containing 7.5% FBS was used for the maintenance medium.

Host cells were placed in 96-well trays at 5×10^5 cells/ml. After a 24 h incubation at 37°C in 5% CO_2, the medium was removed, and the cell monolayers were treated with various concentrations of the test compounds (0.1 ml) dissolved in the cell maintenance medium. After the addition of the virus (3×10^3 $TCID_{50}$ per cell), the well trays were incubated at 37°C in 5% CO_2 for another 72 h. The level of cell survival was estimated spectrophotometrically using crystal violet stain. Cells treated only with each compound, as well as untereated infected or noninfected cells, were incubated as controls. The 50% antivital effective dose ($CPIE_{50}$) and the 50% cytotoxic dose (CLE_{50}) of the sample were determined. The selective toxicity (ST) was based on the ratio of CLE_{50} to $CPIE_{50}$.

Results and Discussion

Fractionation of the sulfated polysaccharide gave three peaks in DEAE-cellulose column chromatography upon elution with 0.4, 0.6, and 0.8 M NaCl (Fig. 1). Each fraction (products A1, A2, and A3) was found to be homogeneous by electrophoretic and ultracentrifugal analyses. Component analyses of these products showed the presence of galacturonic acid, galactose, fucosamine, and pyruvate, in the approximate molar ratios of 3:1:1:1, together with sulfate and *N*-acetyl groups and indicated that the products had the same monosaccharide constituents and identical ^1H NMR spectra (Fig. 2) as compared with those of the unsulfated native polysaccharide described in detail previously.[11] There were no significant differences in molecular size among the purified fractions (Table 1). It seems that these sulfated polysaccharides have the original chemical structure of the native polysaccharide except for the introduction of sulfate groups.

Antiviral Effect of Polysaccharides

The effects of the sulfated polysaccharides on viral growth are shown in Table 2. The unsulfated polysccharide (native polysaccharide) had no antiviral activity. In the presence of the sulfated polysaccharides, however, the host cells were protected against the HSV-1 induced cytopathic effect. For the polysaccharides with a low degree of sulfation (crude and product A1), the inhibitory effect was noted only at higher concentrations. The most active sample was that

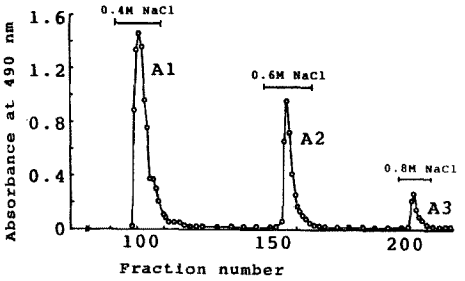

Fig. 1. Fractionation of the sulfated polysaccharide on a DEAE-cellulose column. The sample (100 mg) was dissolved in 0.01 M phosphate buffer (30 m*l*) and applied to the column (1.6×25.8 cm). Fractions (each 5.5 m*l*) from the column were assayed for sugars by the phenol sulfuric acid method.

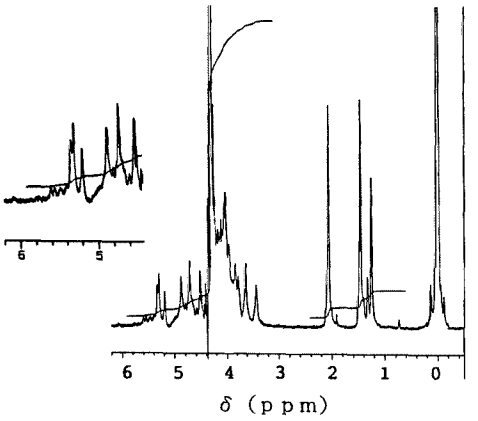

Fig. 2. ^1H NMR spectrum of the sulfated polysaccharide (product A2). The spectrum for the sample in D_2O was obtained at 500 MHz and 75°C with DSS as the internal reference. Chemical shifts (δ ppm) were expressed downfield from the value of DSS.

possessing the highest sulfate content (product A3). The antiviral index (ST value) for A3 was more than 1,389 as defined by the concentration of the compound that produces a 50% inhibition of cell growth divided by the concentration that confers 50% protection from the cytopathic effect. Furthermore, the purified sample with the lowest sulfate content (product A1) showed the lowest activity, perhaps being related to the low degree of sulfation.

It should be noted that the native polysaccaharide contained no sulfate group at all, whereas products A1, A2, and A3 contained 0.08, 0.43, and 0.79 sulfate groups per sugar residues, respectively. These sulfate values are lower than those for other sulfated polysaccharides with antiviral activity, such as heparin[7] (1.25 sulfate groups/sugar residue) and dextran sulfate 7,000[12] (2–3 sulfate groups/sugar residue). The inhibitory effects of various polyanions on viruses have been reported,[7] and it has been suggested that these substances influence the primary electrostatic attachment of the virus to the cell prior to viral penetration. Although the antiviral effects generally appear to require a high degree of sulfation

Table 1. Characteristics of the sulfated polysaccharide

Samples	Yields (mg)	M.W.	S (%, W/W)
Native[1]	—	1.50×10^6	0
Crude[2]	—	—	2.2
A1	51.4	8.91×10^5	1.2
A2	28.6	5.76×10^5	3.5
A3	7.6	4.07×10^5	6.4

The sulfated polysaccharide (100 mg) was fractionated on a DEAE-cellulose colum (1.6×25.8 cm) as described in Fig. 1.
1) Unsulfated native polysaccharide.
2) Sulfated polysaccharide before fractionation on DEAE-cellulose column.

Table 2. Antiviral activities of the sulfated polysaccharide

Samples	HSV-1			Influenza A			VSV			Adeno 3		
	$CPIE_{50}$	CLE_{50}	ST	$CPIE_{50}$	CLE_{50}	ST	$CPIE_{50}$	CLE_{50}	ST	$CPIE_{50}$	CLE_{50}	ST
Native	>70	>70	—	>88	88	—	>43	43	—	43	43	—
Crude*	3.5	650	186	>1,000	>1,000	—	410	560	1.4	>560	560	—
A1	>10	>1,000	<100	>1,000	>1,000	—	>1,000	>1,000	—	>1,000	>1,000	—
A2	2.5	>1,000	>400	>1,000	>1,000	—	>1,000	>1,000	—	>1,000	>1,000	—
A3	0.72	>1,000	>1,389	>1,000	>1,000	—	>1,000	>1,000	—	>1,000	>1,000	—

Samples were the same as those described in Table 1.
$CPIE_{50}$: μg/ml, 50% cytopathic inhibition effect.
CLE_{50}: μg/ml, 50% cell lethal effect.
ST: selective toxicity.
—: No activity was detected.
* Sulfated polysaccharide before fractionation on a DEAE-cellulose column.

of the molecule, the present sulfated polysaccharide had anti HSV-1 effects at a lower degree of sulfation than heparin and dextran sulfate.

As regards the antiviral spectrum of this sulfated polysaccharide, it was active against enveloped virus such as HSV-1, but it was devoid of any activity against VSV and influenza A. It had no inhibitory effect against naked viruses like adeno 3 (Table 2). The results suggest that product A3 has a narrow antiviral spectrum.

In sum, the introduction of sulfate group into this polysaccharide from *Pseudomonas* sp. appears to be essential for its anti HSV-1 activity and the level of that activity seems to be related to the degree of sulfation.

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