Effects of Structural Modification of Calcium Spirulan, a Sulfated Polysaccharide from *Spirulina Platensis*, on Antiviral Activity

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Calcium ion binding with the anionic part of a molecule was replaced with various metal cations and their inhibitory effects on the replication of herpes simplex virus type 1 were evaluated. Replacement of calcium ion with sodium and potassium ions maintained the antiviral activity while divalent and trivalent metal cations reduced the activity.

Depolymerization of sodium spirulan with hydrogen peroxide decreased in antiviral activity as its molecular weight decreased.

Key words calcium spirulan; sulfated polysaccharide; antiviral activity; HSV-1; metal cation; depolymerization

Calcium spirulan (Ca-SP) is a sulfated polysaccharide isolated from a blue-green alga, *Spirulina platensis* as a potent antiviral inhibitor of the replication of enveloped viruses such as herpes simplex virus type 1 (HSV-1) and human immunodeficiency virus type 1 (HIV-1).[2)] The main targets of this polysaccharide have been thought to be the early steps of virus-cell attachment, and virus-cell or cell-cell fusion, on the basis of the results of time-of-addition experiments and HIV-induced syncytium assays.[3)] However, unlike dextran sulfate, Ca-SP was suggested to interfere with later steps of replication after penetration of the virus into the host cells.[3)]

Ca-SP was found to be composed of 1,3-linked rhamnose and 1,2-linked 3-*O*-methylrhamnose (acofriose) units in a ratio of about 5 : 3.[4)] In addition, uronic acids, in the form of glucuronic acid and galacturonic acid, were also shown to be component sugars of Ca-SP. The sulfur content was determined as 5.7% by the flask combustion method, and the degree of substitution of the sulfate ester was calculated as 0.34 mol per anhydro sugar residue. Recent structural analysis of Ca-SP-derived oligosaccharides using electrospray ionization mass spectrometry indicated that Ca-SP was composed of two types of disaccharide repeating units, *O*-rhamnosyl-acofriose and *O*-hexuronosyl-rhamnose (aldobiuronic acid).[5)] When calcium ion (Ca^{2+}) of Ca-SP was exchanged with sodium ion (Na^{+}), the sodium salt (Na-SP) showed comparably potent anti-HSV-1 activity to that of Ca-SP, while removal of Ca^{2+} and desulfation remarkably reduced its antiviral activity.[3)] Thus, metal cation binding with anionic sites such as sulfate groups was suggested to play an important role in exerting antiviral activity. In this paper, we describe the effects of further structural modification of Ca-SP on anti-HSV-1 activity.

Effect of Metal Cation on Anti-HSV-1 Activity The previous findings[3)] prompted us to examine other effects of exchanging Ca^{2+} with other metal cations on anti-HSV-1 activity. Exchange of metal cations was performed by passing through a cation exchange column on Dowex 50W×8 resin equilibrated beforehand with a salt of corresponding metal cation. Each derivative thus obtained was assayed for cytotoxicity against cultured Vero cells as well as inhibitory activity against replication of HSV-1. The antiviral potency of each derivative was evaluated on the basis of a selectivity index expressed as a ratio of 50% cell growth inhibitory concentration (CC_{50}, μg/ml) to 50% viral replication inhibitory concentration (IC_{50}, μg/ml). As shown in Table 1, Na-SP and K-SP exhibited potent anti-HSV-1 activity, while the potency of other derivatives remarkably decreased because of an increase in cytotoxicity. Particularly, replacement of Ca^{2+} with Ag^{+} or Cd^{2+} almost completely eliminated the antiviral activity as a result of a marked decrease in CC_{50}. In contrast, the decrease in selectivity index of Cr-SP was due to a significant increase in IC_{50}. It is noteworthy that Pb-SP showed relatively stronger anti-HSV-1 activity than Mg-SP, although Pb^{2+} was assumed to be more toxic than Mg^{2+} in living cells.

Our previous experiments comparing inhibitory effects of Na-SP on HIV-induced syncytium formation with those of Ca-SP suggested that a divalent metal cation such as Ca^{2+} could build up a certain molecular form essential for exerting an antiviral effect even at a concentration lower than 1 μg/ml.[3)] However, present data indicate that Ca^{2+} might be a specific metal cation which can form a potent antiviral molecule, although its three dimensional structure is not clear.

Table 1. Anti-HSV-1 Activity of Metal Cation Exchanged Derivatives from Ca-SP

Sample	CC_{50} (μg/ml)	IC_{50} (μg/ml)		Selectivity index (CC_{50}/IC_{50})	
		A	B	A	B
Ca-SP	>10000	0.74	4.7	>14000	>2100
Na-SP	6000	0.46	4.8	13000	1300
K-SP	7700	0.88	3.5	8800	3200
Ag-SP	7.0	1.6	3.2	4.4	2.2
Mg-SP	310	1.7	4.3	180	72
Mn-SP	630	1.8	5.0	1200	380
Co-SP	100	1.1	3.1	91	32
Ni-SP	260	2.8	5.2	93	50
Cu-SP	28	0.23	3.3	38	8.5
Zn-SP	320	3.3	5.3	97	60
Cd-SP	2.2	1.0	1.1	2.2	2.0
Pb-SP	3200	1.3	1.8	2500	1800
Fe(II)-SP	3200	2.9	8.4	1100	380
Fe(III)-SP	2200	>10	>10	<220	<220
Al-SP	6200	2.6	8.5	1200	380
Cr(III)-SP	1100	>100	>100	<11	<11

A: Sample was added to the medium during viral infection and throughout the incubation. B: Sample was added to the medium immediately after viral infection.

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Table 2. Anti-HSV-1 Activity of Na-SP and Its Low Molecular Weight Derivatives (LW-SP)

Sample	M_r	CC_{50} (μg/ml)	IC_{50} (μg/ml)		Selectivity index (CC_{50}/IC_{50})	
			A	B	A	B
Na-SP	210000	6000	0.46	4.8	13000	1300
LW-SP (2)[a)]	37000	5600	0.5	5.7	11000	980
LW-SP (4)	22600	5000	0.62	7.0	8100	710
LW-SP (6)	18500	4800	1.3	16	3700	300
LW-SP (12)	12700	5200	2.1	28	2500	190
LW-SP (24)	10700	4900	5.4	>100	910	<49

a) Numbers in parentheses indicate reaction time (h) with H_2O_2. A: Sample was added to the medium during viral infection and throughout the incubation. B: Sample was added to the medium immediately after viral infection.

Table 3. Effect of Time of Addition of the Depolymerized Derivatives from Na-SP, LW-SP (12) and LW-SP (24)

Treatment	Antiviral activity (IC_{50}, μg/ml)	
	LW-SP (12)[a)]	LW-SP (24)[b)]
Pretreatment of host cells for 3 h	>500	>500
During infection	34	71
During infection and for 24 h thereafter	8.0	11
0—24 h p.i.[c)]	35	76
2—24 h p.i.	58	180
4—24 h p.i.	59	170
6—24 h p.i.	90	270

a) Sulfated polysaccharide obtained after 12 h-reaction with H_2O_2 from Na-SP. *b*) Sulfated polysaccharide obtained after 24 h-reaction with H_2O_2 from Na-SP. *c*) p.i.: postinfection.

Effect of Depolymerization on Anti-HSV-1 Activity As reported previously, the potency of anti-HIV-1 activity of synthesized sulfated polysaccharides such as dextran sulfates was highly dependent on their sulfate content and molecular weight.[6)] To obtain depolymerized derivatives of the sulfated polysaccharides heparin and fucoidan, chemical methods[7—9)] and enzymatic reaction systems[10,11)] have been used to cleave the glycosidic bonds of the polysaccharides.

In our experiments, the derivatives with lower molecular weights were obtained from Na-SP by free radical reaction with H_2O_2. As summarized in Table 2, the anti-HSV-1 activity of depolymerized molecules decreased as their molecular weights decreased. A significant decline of activity was observed between the products obtained 12 h and 24 h after initiation of the reaction, in spite of small differences in their apparent molecular weights. When component sugars of both compounds were analyzed, the contents of sulfate ion, uronic acid and acofriose in the molecule of 24-h reaction product were relatively less than that of 12-h reaction product, while the contents of rhamnose were similar. In the time-of-addition experiments, a rather great difference was observed in the antiviral effects between two compounds when added to the bioassay system at 2 h or later after virus infection (Table 3).

In our study using the biomolecular interaction assay system,[12)] Na-SP was shown to bind preferentially to virus-infected cells, compared to uninfected cells. This was also confirmed by the confocal laser scanning microscopic (CLSM) analysis, in which fluorescence-labeled Na-SP molecules could be detected more abundantly in infected cells than in uninfected ones. The internalization of Na-SP in infected cells was also observed in the CLSM analysis. To understand the anti-HSV-1 mechanism of Ca-SP and its derivatives at the molecular level, further detailed structures of the molecules should be elucidated.

So far, in addition to antiviral effect, Ca-SP has been found to exhibit inhibitory effects on tumor invasion and metastasis,[13)] antithrombin activity *via* heparin cofactor II,[14)] and enhancement of tissue-type plasminogen activator production.[15)] Therefore, Ca-SP-derived molecules obtained in this study can be valuable tools to elucidate the mechanisms of these interesting biological actions.

Experimental

Metal elements were analyzed with a Hitachi scanning electron microanalyzer X-650. HPLC was performed with a Shimadzu LC-6A HPLC system equipped with a refractive index detector (Shimadzu, model RID-6A). GLC was carried out on a Shimadzu GC-9A gas chromatograph equipped with a hydrogen flame-ionization detector.

Extraction and Isolation of Ca-SP Ca-SP was extracted and isolated as previously reported.[4)]

Exchange of Metal Ions A Dowex 50W×8 (1×17 cm) column was pre-equilibrated with each metal salt solution and the solution of Ca-SP (20 mg/ml) in H_2O was passed through this column. The eluted solution was freeze-dried to give a metal-exchanged sulfated polysaccharide. Yield: Ca-SP (Ca 45%; S 43%), 22.8 mg; Na-SP (Na 42%; S 58%), 21.7 mg; K-SP (K 61%; S 39%), 20.8 mg; Ag-SP (Ag 58%; S 42%), 27.7 mg; Mg-SP (Mg 28%; S 42%), 22.1 mg; Mn-SP (Mn 38%; S 62%), 22.6 mg; Co-SP (Co 35%; S 65%), 19.6 mg; Ni-SP (Ni 35%; S 65%), 24.4 mg; Cu-SP (Cu 36%; S 64%), 22.8 mg; Zn-SP (Zn 32%; S 68%), 24.3 mg; Cd-SP (Cd 39%; S 61%), 25.2 mg; Pb-SP (Pb 51%; S 48%), 20.1 mg; Fe(II)-SP (Fe(II) 34%; S 66%), 17.9 mg; Fe(III)-SP (Fe(III) 24%; S 76%), 19.1 mg; Al-SP (Al 27%; S 73%), 21.6 mg; Cr-SP (Cr 42%; S 58%), 16.1 mg

Depolymerization of Na-SP Na-SP (100 mg) was dissolved in 0.1 M acetate buffer (pH 5.5, 25 ml). After addition of 5 ml of 30% H_2O_2, the solution was kept at 45 °C. Five ml portions were taken out of the solution after reaction for 2, 4, 6, 12, and 24 h. Each reaction product was dialyzed and freeze-dried. Yield: LW-SP (2), 17 mg; LW-SP (4), 16 mg; LW-SP (6), 12 mg; LW-SP (12), 14 mg; LW-SP (24), 18 mg.

Estimation of Apparent Molecular Weight The apparent molecular weight of each sulfated polysaccharide obtained by depolymerization of Na-SP was estimated by the HPLC method reported previously.[4)]

Analysis of Component Sugars of Depolymerized Products by GC Three mg of the depolymerization products from Na-SP (12 and 24 h reaction products) were hydrolyzed with 2 N H_2SO_4 (1 ml) at 100 °C for 3 h. After neutralization of the reaction mixture with $Ba(OH)_2$, the neutralized solution was filtered and the filtrate was concentrated under reduced pressure. The hydrolysates were converted into alditol acetates and analyzed by GC as reported elsewhere.[4)] The sugar composition (molar % of rhamnose : acofriose) were Na-SP; 59.0 : 25.9, LW-SP (12); 42.7 : 16.1 and LW-SP (24); 42.9 : 14.0.

Quantitative Analysis of Sulfate The sulfate contents of the polysaccharides were measured by the barium-rhodizonate method using H_2SO_4 for calibration.[16)] Sample (0.5 mg/10 ml) and 0.1 M NaOH (20 μl) were pipetted into borosilicate glass ignition tubes (14×100 mm) and freeze-dried. The dried residues were pyrolyzed by heating evenly for approximately 6 s in a Fischer burner and then dissolved in 0.5 ml of deionized water. Barium-containing buffer (3.0 ml) and rhodizonate reagent (1.5 ml) were added, and the solution was mixed. After 10-min incubation at room temperature, the absorbance was read at 520 nm. The sulfate content of Na-SP, LW-SP (12) and LW-SP (24) was 17.8, 22.6 and 11.39%, respectively.

Quantitative Analysis of Uronic Acid[17)] Sample was added with 0.0125 M $Na_2B_4O_7$ in H_2SO_4 (1.2 ml) and iced. After mixing, the reaction mixture was heated at 100 °C for 5 min. After cooling, 0.15% *m*-hydroxyldiphenyl in 0.5% NaOH (20 μl) was added and UV absorption at 520 nm was measured within 5 min. 0.5% NaOH was used as blank solution. D-glucuronic acid was used for preparation of a calibration curve. The uronic acid content of Na-SP, LW-SP (12) and LW-SP (24) was 14.9, 10.7 and 7.2%, respectively.

Evaluation of Anti-HSV-1 Activity CC_{50} and IC_{50} were determined as

reported elsewhere.[3] Anti-HSV-1 activity was evaluated by calculation of CC_{50}/IC_{50}.

Time-of-Addition Experiments Time-of-addition experiments were performed as reported elsewhere.[3]

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References and Notes

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