Calcium Spirulan, an Inhibitor of Enveloped Virus Replication, from a Blue-Green Alga *Spirulina platensis*

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Bioactivity-directed fractionation of a hot H_2O extract from a blue-green alga *Spirulina platensis* led to the isolation of a novel sulfated polysaccharide named calcium spirulan (Ca-SP) as an antiviral principle. This polysaccharide was composed of rhamnose, ribose, mannose, fructose, galactose, xylose, glucose, glucuronic acid, galacturonic acid, sulfate, and calcium. Ca-SP was found to inhibit the replication of several enveloped viruses, including Herpes simplex virus type 1, human cytomegalovirus, measles virus, mumps virus, influenza A virus, and HIV-1. It was revealed that Ca-SP selectively inhibited the penetration of virus into host cells. Retention of molecular conformation by chelation of calcium ion with sulfate groups was suggested to be indispensable to its antiviral effect.

Spirulina [*Spirulina platensis* (Nordst.) Geitl., Cyanophyceae] is a blue-green alga growing in some African and Central and South American lakes rich in salts. Because spirulina contains much good-quality protein as well as carotenoids, vitamins, and minerals, it has received attention as a most promising and nutritious food source. In addition, it is expected to find use as a function food because of its various biological activities such as cholesterol lowering and hypotensive effects.[1–3] Thus, in recent years, it has become popular to produce this alga by artificial culture in the open air.

In our previous paper, we reported that the hot H_2O-soluble extract from *Spirulina platensis* achieved remarkable inhibition of the replication of Herpes simplex virus type 1 (HSV-1) in HeLa cells.[4] In this continuing study, bioactivity-directed fractionation of the active extract from spirulina has led to the isolation of a novel acidic polysaccharide termed calcium spirulan (Ca-SP) as an antiviral principle. This note deals with the purification, chemical nature, and antiviral effect of Ca-SP.

Freeze-dried powder of spirulina grown in outdoor open tanks was extracted with boiling H_2O, and the hot H_2O extract was treated with 10% trichloroacetic acid (TCA). The crude polysaccharide (SP-H) was obtained from the TCA-soluble fraction by dialysis against distilled H_2O followed by lyophilization. Gel filtration of SP-H on Sepharose 6B gave three fractions as shown in Figure 1. Among these fractions, only SP-H-2 had anti-HSV-1 activity. SP-H-2 was then subjected to an ion-exchange CC on DEAE cellulose (Figure 2). After elution with H_2O, the second eluate (SP-H-2b) with 0.5M NaCl was further purified by a CC on Sepharose 6B to give a colorless polysaccharide (Ca-SP). This purified polysaccharide was homogenous on HPLC analysis (Figure 3) and showed a positive specific rotation $[\alpha]_D$ (+14.7°, *c* 0.59, H_2O). The molecular weight of Ca-SP was estimated to be 2.6×10^5 and 3.1×10^5 by gel filtration on Sepharose 6B and light scattering experiments, respectively.

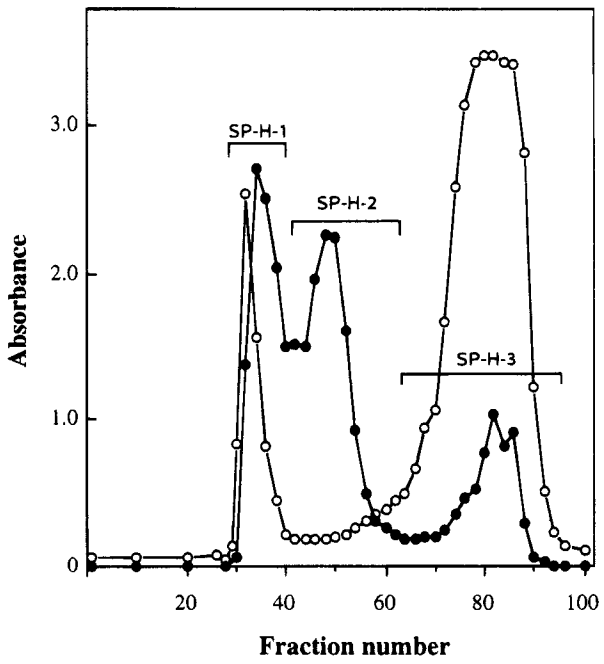

Figure 1. Elution profile of SP-H by Sepharose 6B column chromatography. The sample was dissolved in 0.01 M citrate buffer (pH 7.0) containing 0.1 M NaCl and the column was eluted with the same buffer. Each fraction was 10.1 mL. (○): absorbance at 260 nm; (●): absorbance at 480 nm (phenol-H_2SO_4 method).

Acid hydrolysates of Ca-SP were suggested to contain rhamnose, ribose, mannose, fructose, galactose, xylose,

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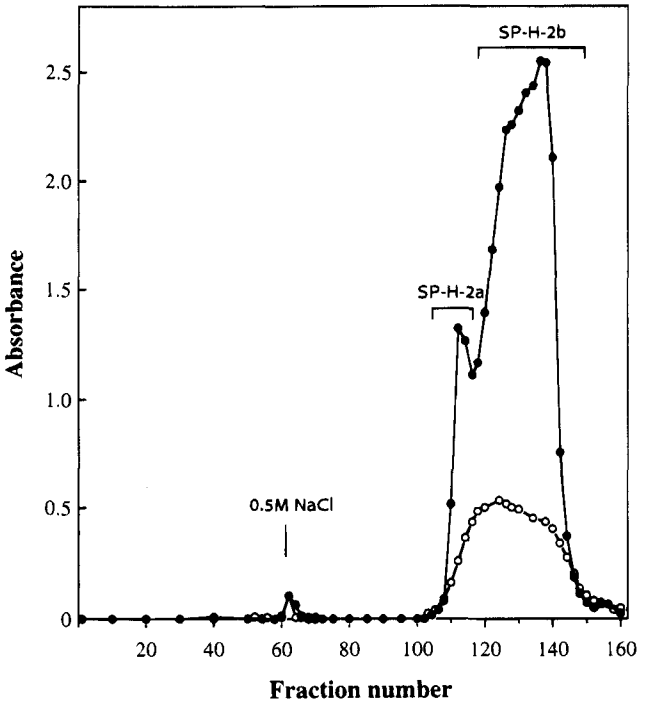

Figure 2. Elution profile of SP-H-2 by DEAE cellulose CC. The sample was dissolved in H_2O, and the column was eluted with H_2O and then 0.5 M NaCl. Each fraction was 5.0 mL. (○): absorbance at 260 nm; (●): absorbance at 480 nm (phenol–H_2SO_4 method).

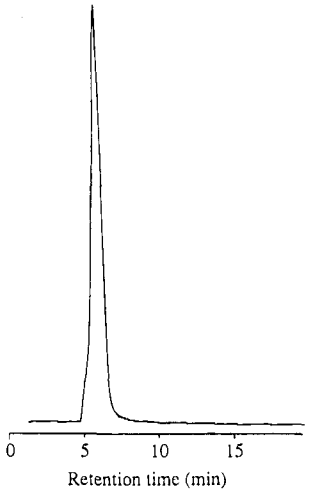

Figure 3. HPLC chromatogram of Ca-SP. The sample was dissolved in H_2O, and 5 μL was injected. The column (YMC Diol 300) was eluted with H_2O at room temperature and flow rate of 1.0 mL/min.

glucose, glucuronic acid, and galacturonic acid by HPLC analysis (Figure 4). The molar ratio of these component sugars was 60.0:3.0:1.1:46.0:3.3:0.8:6.4:9.6:5.4. A high ash content (12.17%) and detection of sulfur (3.24%) in Ca-SP suggested the presence of sulfate ester, which was confirmed by an S=O stretching absorption band at 1265 cm^{-1} in its IR spectrum. The absence of nitrogen on elemental analysis of Ca-SP indicated the absence of protein or hexosamine. X-ray microanalysis of Ca-SP revealed the presence of calcium as well as sulfur.

When Ca-SP was eluted with distilled H_2O through an ion exchange column of Dowex 50W (X-8) followed by lyophilization, a colorless powdered polysaccharide (H-SP) free from Ca was obtained. Furthermore, treatment of H-SP with 10% MeOH in DMSO at 80 °C gave a desulfated polysaccharide. These results suggested that Ca-SP is a sulfated polysaccharide chelating calcium ion and mainly composed of rhamnose and fructose.

To determine the antiviral activity of Ca-SP, its effects on HSV-1 replication were studied. As shown in Figure 5, dose-dependent inhibition of virus replication was observed within the concentrations tested when the compound was added from 3 h before infection, while no cytotoxicity was shown toward uninfected host cells at concentrations less than 1 mg/mL. The selectivity index was approximately 8,600 (Table 1). In parallel experiments, an aqueous extract of spirulina showed an ID_{50} of 2,300 μg/mL for cytotoxicity and an ED_{50} of 2.5 μg/mL for anti-HSV activity, giving a selectivity index of 920. The purified active component thus has activity against HSV-1 replication nine times higher than that of the extract.

The effects of Ca-SP on the growth of several cell lines and viruses are shown in Table 1. Cytotoxic activity of Ca-SP was very low, with ID_{50}s of 7,900, 4,800, 6,300, 5,400, and 2,900 μg/mL for HeLa, human embryonic lung (HEL), green monkey kidney (Vero), Madin-Darby canine kidney (MDCK), and MT-4 cells, respectively. When Ca-SP was evaluated for activity against different viruses, the compound was found to have a high selectivity index for all enveloped viruses including HSV-1, human cytomegalovirus (HCMV), measles virus, mumps virus, influenza A virus, and HIV-1. However, Ca-SP was inactive against nonenveloped viruses, including poliovirus and coxsackievirus. Ca-SP was found to have a similar broad spectrum of antiviral activity against enveloped viruses to those of other sulfated polysaccharides.[5,6] Thus, this compound holds great promise for the treatment of HIV-1, HSV-1, and HCMV infections, which is particularly advantageous for AIDS patients who are prone to these life-threatening infections.[7]

As shown in Table 1, antiviral activity against enveloped viruses was more potent in the cultures treated with Ca-SP from 3 h before infection (−3 h) compared with that in the cultures treated immediately after infection (0 h). These results suggest that Ca-SP may interfere with a very early stage of viral replication such as virus adsorption and penetration. To assess the inhibitory effect of Ca-SP on the virus penetration stage, the kinetics of penetration of HSV-1 into cells were determined by inactivating the unpenetrated viruses with a low-pH citric acid buffer at 1 h and 6 h after a temperature shift from 4 to 37 °C (Table 2). The presence of Ca-SP during virus adsorption at 4 °C resulted in the dose-dependent inhibition of virus penetration. At concentrations more than 40 μg/mL, Ca-SP almost completely blocked the penetration. These results indicate that Ca-SP is responsible for the inhibition of virus penetration observed in the aqueous extract of spirulina.[4]

When calcium-free spirulan (H-SP) and a desulfated compound from Ca-SP were subjected to cytotoxicity and antiviral assays, both compounds exerted strong toxicity on the growth of HeLa cells and weak inhibition against HSV-1 replication, resulting in remarkable reduction of their selectivity indices when compared with Ca-SP

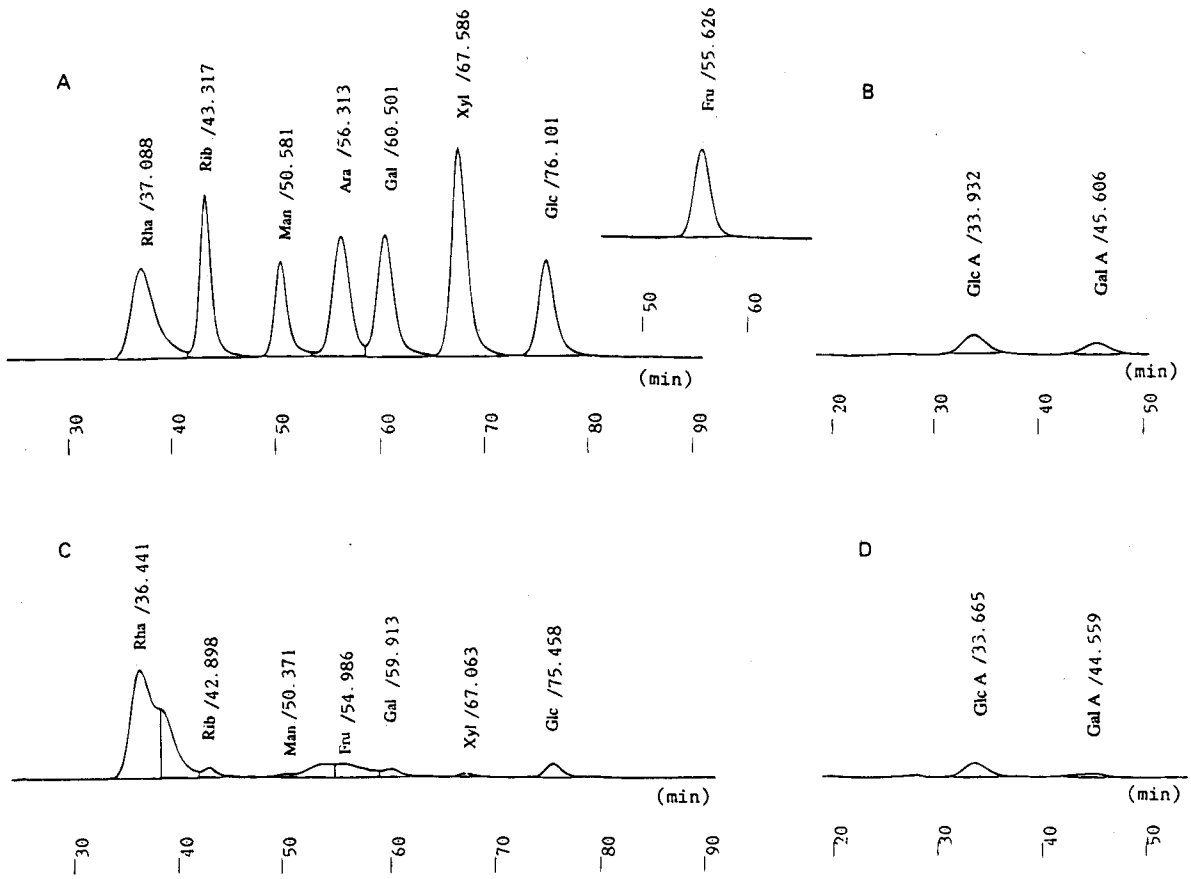

Figure 4. HPLC chromatograms of standard neutral sugars (A) and uronic acids (B), and acid hydrolysates of Ca-SP (C, D).

Table 1. Inhibitory Effect of Ca-SP on the Replication of Different Viruses

virus	cell culture	cytotoxicity (ID_{50}, μg/mL)[a]	antiviral activity (ED_{50}, μg/mL)[b]		selectivity index (ID_{50}/ED_{50})	
			0 h[c]	−3 h[d]	0 h	−3 h
HSV-1	HeLa	7900	16.5	0.92	479	8587
HCMV	HEL	4800	41	8.3	117	578
Measlesvirus	Vero	6300	39	17	162	371
Mumpsvirus	Vero	6300	92	23	68	274
Influenzavirus	MDCK	5400	230	9.4	23	574
Poliovirus	Vero	6300	2300	2200	2.7	2.9
Coxsackievirus	Vero	6300	2600	1850	2.4	3.4
HIV-1	MT-4	2900	11.4	2.3	254	1261

[a] Concentration required to reduce cell growth by 50%; mean value for two experiments. [b] Concentration required to reduce virus replication by 50%; mean value for two experiments. [c] Ca-SP was added to the medium immediately after viral infection. [d] Ca-SP was added to the medium 3 h before viral infection.

Table 2. Effect of Ca-SP on HSV-1 Penetration

time of treatment (h), (post infection)	Ca-SP (μg/mL)					
	0	0.32	1.6	8	40	200
0	0.8[a]					
1	97	88	67	31	0.8	0.8
6	100	89	70	33	0.8	0.8

[a] Percent penetration, taking the maximum penetration of virus on untreated control cells at 6 h postinfection as 100%.

(Table 3). These results confirmed the importance of sulfate groups for the antiviral activity of sulfated polysaccharides.[5,8−10] However, in spite of the presence of sulfated groups in H-SP, no significant antiviral activity was detected in the compound. Therefore, it is suggested that molecular conformation by chelation of calcium ion with sulfate groups might play a crucial role in the sulfated polysaccharide. This is the first report of the importance of the metal salt of a sulfated polysaccharide for antiviral activity. We are now examining the detailed structure as well as the relationship between molecular conformation and bioactivity of Ca-SP.

Experimental Section

General Experimental Procedures. CC was monitored by determination of absorbance at 260 nm as well as phenol-H_2SO_4 method (480 nm). Gel filtration was performed on a column of Sepharose 6B (Pharmacia) with 0.01 M citrate buffer, pH 7.0, containing 0.1 M NaCl as eluent. Ion exchange chromatography was carried out on a column of DEAE-cellulose (Wako Pure Chemical Industries Ltd., OH^- form) or Dowex 50W (X-8, H^+ form, 100−200 mesh). Optical rotation was measured for an aqueous solution of sample at 23 °C with a JASCO DIP-140 polarimeter. UV absorptions were measured with a Hitachi 220S spectrophotometer. IR spectra were recorded in KBr disk using a JASCO IR-700 IR spectrophotometer. Metal elements were

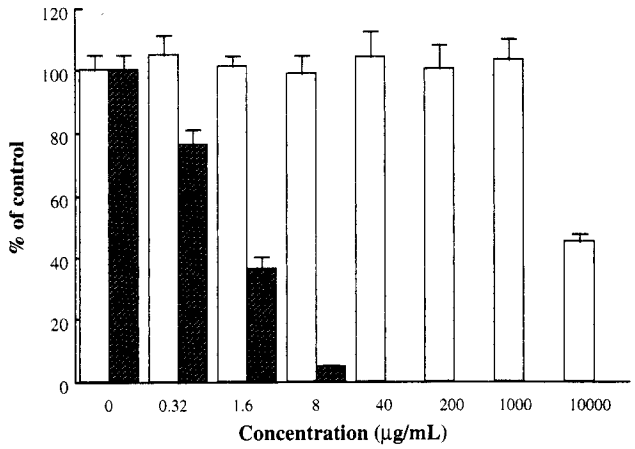

Figure 5. Effect of Ca-SP on the cell growth and HSV-1 replication. Uninfected (open column) and virus-infected (hatched column) HeLa cells were incubated in the presence of varing concentrations of Ca-SP. The cell viability was assayed after 72 h of incubation. In the assay of antiviral activity, Ca-SP was added to HeLa cells from 3 h before infection. The cells were infected with 0.2 plaque-forming units of HSV-1 per cell and incubated for 24 h. The virus titer was determined by plaque assay.

Table 3. Comparison of Anti-HSV-1 Activity of Ca-SP, H-SP, and the Desulfated Polysaccharide Derived from Ca-SP

compd	cytotoxicity (ID_{50}, μg/mL)	antiviral activity (ED_{50}, μg/mL)[a]	selectivity index (ID_{50}/ED_{50})
Ca-SP	5200	0.86	6047
H-SP	505	155	3.3
desulfated polysaccharide	146	24	6.1

[a] Each compound was added to the medium 3 h before viral infection.

analyzed by 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) or a fluorescence HPLC monitor (Shimadzu RF-535).

Estimation of Molecular Weight. The average molecular weight of the polysaccharide was estimated by gel filtration using standard pullulans (Shodex Standard P-82, Showa Denko Co.) and light scattering method using a Wyatt mini-Multi Angle Laser Light Scattering (DAWN) equipped with a refractive index detector (Shodex RI).

Isolation of the Polysaccharide. The blue-green alga was suspended in a culture medium (0.18 g/mL) and cultured in an outdoor tank (20 m^2 × 20 cm) for 13 h at 30−35 °C. The component salts of culture medium are as follows: $NaHCO_3$ (16.8 g/L), K_2HPO_4 (0.5 g/L), $NaNO_3$ (2.5 g/L), K_2SO_4 (1.0 g/L), NaCl (1.0 g/L), $MgSO_4 \cdot 7H_2O$ (0.2 g/L), $CaCl_2 \cdot 2H_2O$ (40 mg/L), $FeSO_4 \cdot 7H_2O$ (10 mg/L), EDTA (80 mg/L), H_3BO_3 (2.85 mg/L), $MnCl_2 \cdot 4H_2O$ (1.81 mg/L), $ZnSO_4 \cdot 7H_2O$ (0.22 mg/L), $CuSO_4 \cdot 5H_2O$ (0.08 mg/L), MoO_3 (0.015 mg/L), NH_4VO_3 (0.023 mg/L), $K_2Cr_2(SO_4)_4 \cdot 24H_2O$ (0.096 mg/L), $NiSO_4 \cdot 7H_2O$ (0.048 mg/L), $Na_2WO_4 \cdot 2H_2O$ (0.018 mg/L), $Ti(SO_4)_3$ (0.04 mg/L), $Co(NO_3)_2 \cdot 6H_2O$ (0.044 mg/L). The freeze-dried powder of spirulina (4.0 kg) was extracted with boiling H_2O (20 L) for 1 h. After centrifugation (3,000 rpm, 10 min), the precipitate was again extracted with the same way. The combined supernatant was concentrated and lyophilized to yield a yellowish brown powder (520 g) with a characteristic smell. The extract (10 g) was treated with 10% TCA. After leaving the solution in a refrigerator for 1 h, the TCA-soluble fraction obtained by centrifugation was dialyzed for 24 h against distilled H_2O. The nondialyzing portion was lyophilized to give a grayish green powder (SP-H, 2.0 g). SP-H was dissolved in 0.01 M citrate buffer, pH 7.0, containing 0.1 M NaCl, and the soluble portion (1.64 g) was applied to a column (4.5 × 67.0 cm) of Sepharose 6B. The column was eluted with the same buffer, and fractions of 10.1 mL were collected. The second eluate was concentrated, dialyzed, and lyophilized to afford a colorless powder (SP-H-2, 630 mg). SP-H-2 was dissolved in H_2O and then applied to a column (2.5 × 76.0 cm) of DEAE-cellulose. After elution with H_2O, the column was eluted with 0.5 M NaCl and fractions of 5.0 mL were collected. The second eluate (SP-H-2b, 276 mg) with 0.5 M NaCl was rechromatographed on a Sepharose 6B column (3.0 × 92.0 cm) by eluting with 0.01 M citrate buffer, pH 7.0, containing 0.1 M NaCl. Fractions of 5.0 mL were collected. Carbohydrate-containing fractions were combined, concentrated, dialyzed, and lyophilized to give a colorless substance with cotton-like appearance (177 mg). The homogeneity of Ca-SP was analyzed by HPLC equipped with a YMC-packed column (Diol 300, 500 × 8.0 mm i.d.). The column was eluted with H_2O as a mobile phase at a flow rate of 1.0 mL/min. The collected fractions were monitored with refractive index.

Analysis of Component Sugars. Ca-SP (5 mg) was hydrolyzed with 4 M trifluoroacetic acid (1 mL) for 6 h at 100 °C in a sealed glass tube. After evaporation to dryness, the hydrolysate dissolved in 2 mL of H_2O were analyzed by HPLC using post-column reaction method.[11]

Removal of Calcium. Ca-SP (22 mg) dissolved in H_2O was applied to a cation exchange column (1.2 × 12.5 cm) on Dowex 50W (X-8, H^+ form) and eluted with H_2O. The polysaccharide-containing fractions were combined and lyophilized to give a colorless powder (H-SP, 16.5 mg).

Desulfation.[12] The solution of H-SP in H_2O was adjusted to pH 7.6 by addition of pyridine and lyophilized. The colorless powder of pyridinium salt thus obtained was added by 10% MeOH in DMSO (15 mL) and kept stirring for 5 h at 80 °C under dry condition. The reaction mixture was dialyzed against running H_2O (3 days), followed by further dialysis against 0.5 M EDTA (2 days). After ultrafiltration with nitrocellulose membrane, the nondialyzing fraction was freeze-dried to give a colorless powder.

Cells and Viruses. HeLa, HEL, Vero, and MDCK cells were grown in Eagle minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS). MT-4 cells, a CD4-positive cells[13] were grown in RPMI-1640 medium supplemented with 10% FBS. HSV-1 (HF strain) was grown on HeLa cells. HCMV (Towne strain) was grown on HEL cells. Measles virus (Toyoshima strain), mumps virus (EY strain), poliovirus type 1 (Sabin strain), and coxsackievirus type B1 (Conn-5 strain) were propagated on Vero cells. Influenza A virus (NWS strain, H1N1) was grown on MDCK cells. HIV-1 was obtained from the culture supernatant of persistently infected Molt-4/HTLV-IIIB cells.[14]

Cytotoxicity. For cell-growth-inhibition studies, HeLa, Vero, MDCK, and Molt-4 cells were cultured for 72 h, and HEL cells were cultured for 120 h, at 37 °C,

in the presence of increasing amounts of Ca-SP. Viable cell yield was determined by the trypan blue exclusion test. The inhibition data were plotted as dose-response curves, from which the 50% inhibitory dose (ID_{50}) was obtained.

Antiviral Activity. Except for the anti-HIV assay, the methods for antiviral assays have been described previously.[15] Cell monolayers were infected with virus at 0.2 plaque-forming units per cell for 1 h at room temperature and refed with maintenance medium (MEM plus 2% FBS). Virus yields were determined by plaque assay at 1-day incubation for HSV-1, measles virus, mumps virus, influenza A virus, poliovirus, and coxsackievirus; and at 5-day incubation for HCMV. To determine the activity of Ca-SP against HIV-1, extracellular HIV content was measured by p24 core antigen production. Briefly, MT-4 cells were infected with 100 50%-cell-culture infective doses of HIV-1. After 5 days of incubation at 37 °C, the amounts of p24 antigen in the medium were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) using p24 assay kit (Abbott).[16]

Inhibition of Virus Penetration. The procedures for virus penetration assay have been described previously.[15] Briefly, HeLa cell monolayers were inoculated with HSV-1 and incubated at 4 °C for adsorption in the presence of Ca-SP. After 1 h, the cultures were refed with drug-free medium and shifted to 37 °C for penetration into cells. At 1-h and 6-h incubation, unpenetrated viruses were inactivated by treatment with citrate buffer (pH 3.0) for 1 min. The monolayers were overlaid with 0.5% methylcellulose and incubated for 2 days to be plaque-assayed.

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