Further Purification and Structural Analysis of Calcium Spirulan from *Spirulina platensis*

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An antiviral sulfated polysaccharide, calcium spirulan (Ca-SP), isolated from *Spirulina platensis*, was subjected to further purification. Ca-SP was found to be composed of rhamnose, 3-*O*-methylrhamnose (acofriose), 2,3-di-*O*-methylrhamnose, 3-*O*-methylxylose, uronic acids, and sulfate. The backbone of Ca-SP consisted of 1,3-linked rhamnose and 1,2-linked 3-*O*-methylrhamnose units with some sulfate substitution at the 4-position. The polymer was terminated at the nonreducing end by 2,3-di-*O*-methylrhamnose and 3-*O*-methylxylose residues.

In a previous paper, we reported the isolation of a sulfated polysaccharide named calcium spirulan (Ca-SP) from the blue-green alga *Spirulina platensis* (Nordst.) Getil. (Oscillatoraceae) as an antiviral component.[1] Ca-SP inhibits the replication of enveloped viruses including Herpes simplex virus type 1 (HSV-1) and human immunodeficiency virus type 1 (HIV-1). The main targets for antiviral activity of Ca-SP are considered to be the early steps of viral-cell attachment and virus-cell fusion, on the basis of the results of time-of-addition experiments and HIV-induced syncytium assays. Furthermore, the calcium ion in the molecule was shown to be essential for the dose-dependent inhibition of the cytopathic effect and syncytium formation induced by HIV-1.[2] In addition, Ca-SP enhanced the antithrombin activity of heparin cofactor II (HC II) more than 10^5-fold, and the binding site of HC II for Ca-SP was suggested to be different from the heparin- or dermatan sulfate-binding site.[3] Moreover, tissue-type plasminogen activator production was enhanced by Ca-SP.[4] However, Ca-SP had very low anticoagulant activity and showed a much longer half-life in the blood of mice when compared with that of dextran sulfate (Na-salt).[2] Consequently, Ca-SP has become an attractive candidate therapeutic agent for viral infectious diseases. The present paper reports the results of the further purification of Ca-SP and linkage analysis of its component sugars.

Results and Discussion

In a previous report,[1] the isolation of Ca-SP was performed with successive chromatographic systems using Sepharose 6B, DEAE-cellulose, and Sepharose 6B columns. In the present work, further purification was achieved by column chromatography over Sepharose 6B, DEAE-Toyopearl 650M, and Toyopearl HW-65(S). The 10% trichloroacetic acid (TCA)-soluble fraction of the hot water extract form the alga was separated over a Sepharose 6B column (Figure 1), and the second eluate (SP-TSH-2) showing the highest anti-HSV-1 activity (IC_{50} 0.78 μg/mL) was further fractionated using a DEAE-Toyopearl 650M column. As shown in Figure 2, SP-TSH-2 was separated to form fractions SP-TSH-2-1 and SP-TSH-2-2. Since the latter fraction showed anti-HSV-1 activity (IC_{50} 0.68 μg/mL), it was then subjected to gel filtration over Toyopearl HW-65. The eluate was observed as a single and sharp peak. This result indicated that the eluted polysaccharide was homogeneous on the basis of molecular size distribution. The apparent molecular weight of the polysaccharide is estimated to be 7.46×10^4 by HPLC gel filtration chromatography.

The component sugar analysis of Ca-SP was performed by GC and GC−MS of the derived sugar alditol acetates form its hydrolysates to reveal the presence of rhamnose (52.3%) and a 3-*O*-methyl-6-deoxyhexose (32.5%), together with a 2,3-di-*O*-6-deoxyhexose (4.4%), a 3-*O*-methylpentose (4.8%), and trace amounts of other sugars. It was found that the 3-*O*-methyl-6-deoxyhexose, 2,3-di-*O*-methyl-6-deoxyhexose, and 3-*O*-methylpentose could be identified, respectively, as 3-*O*-methylrhamnose (acofriose), 2,3-di-*O*-methylrhamnose, and 3-*O*-methylxylose from their retention times relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol. Additional evidence for the presence of 3-*O*-methylrhamnose was obtained after its isolation after preparative paper chromatography of the acid hydrolysates. Its ^{1}H NMR spectral data were consistent with reported values.[5] A positive specific rotation indicated the obtained sugar to be of the L-series. Furthermore, it was shown that uronic acids (16.5%) were also component sugars of Ca-SP by the *m*-hydroxydiphenyl method. These uronic acids were identified as glucuronic acid and galacturonic acid by GC analysis after reduction of the carboxyl groups of Ca-SP.

Sulfur was detected in Ca-SP by X-ray analysis. Furthermore, in the IR spectrum, an absorption band

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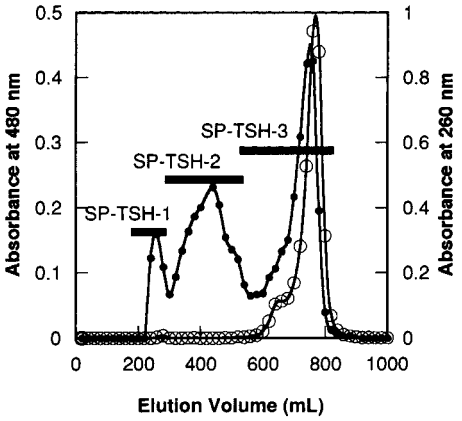

Figure 1. Elution profile of SP-TSH by Sepharose 6B column chromatography: (●): absorbance at 480 nm (phenol−H_2SO_4); (○) absorbance at 260 nm.

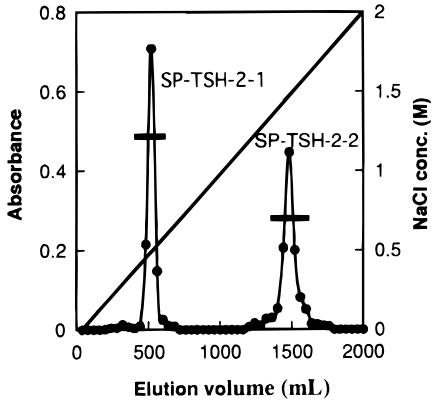

Figure 2. Elution profile of SP-TSH-2 by DEAE−Toyopearl 650M column chromatography. Detection was performed by the phenol−H_2SO_4 method.

at 1240 cm^{-1} resulting from a S=O stretching vibration indicated the presence of a sulfate ester in 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. The value of the optical rotation, $[\alpha]_D$ −24.5° (*c* 0.22, H_2O), indicated that Ca-SP has the α-L-configuration.

The glycosidic linkages and the positions of the sulfate groups in Ca-SP were determined by methylation analysis of the native (TEA-SP) and desulfated (DS-SP) polysaccharides, respectively. The amounts of the neutral sugar compositions of the permethylated DS-SP were in good agreement with the analytical data for the unmethylated polymer. For TEA-SP, however, the results were less quantitative probably because of loss of degradation products under methylation during dialysis of the derivatized sample. As shown in Table 1, permethylated DS-SP contained 1,2- and 1,3-linked rhamnose as the main components. To elucidate the linkage position of the 2,3-di-*O*-methylrhamnose, 3-*O*-methylrhamnose, and 3-*O*-methylxylose units, DS-SP was methylated with [$^2H_3$]methyl iodide. The mass spectrum of the 1,2-linked rhamnose derivative is shown in Figure 4A. In this spectrum, 1,2,5-tri-*O*-acetyl-3-*O*-methyl-4-*O*-[$^2H_3$]methylrhamnitol was detected from the main peaks at *m*/*z* 134 and 189. Furthermore, the observation of a mass peak with weak intensity at *m*/*z* 192 indicated the presence of 1,2,5-tri-*O*-acetyl-3,4-di-

Table 1. Partially Methylated Alditol Acetates from TEA-SP and DS-SP

partially methylated alditol acetates	deduced linkage	composition (mol %)	
		TEA-SP	DS-SP
2,3,4-Rha[a]	Rha(1-	0.2	4.2
2,4-Rha	-3)Rha(1-	6.7	44.6
3,4-Rha	-2)Rha(1-	24.0	32.6
3-Rha	-4,2)Rha(1-	17.5	
4-Rha	-3,2)Rha(1-	27.0	2.9
Rha	-4,3,2)Rha(1-	19.8	2.9
2,3-Fuc	-4)Fuc(1-	0.4	2.4
3-Fuc	-4,2)Fuc(1-		0.3
2,3,4-Xyl	Xyl(1-	0.2	5.6
2,3-Xyl	-4)Xyl(1-	4.1	2.5
2,4-Xyl	-3)Xyl(1-		0.4
2,3-Ara	-4)Ara(1-	0.1	0.4
3,4-Glc	-6,2)Glc(1-		1.2

[a] 2,3,4-Rha = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol.

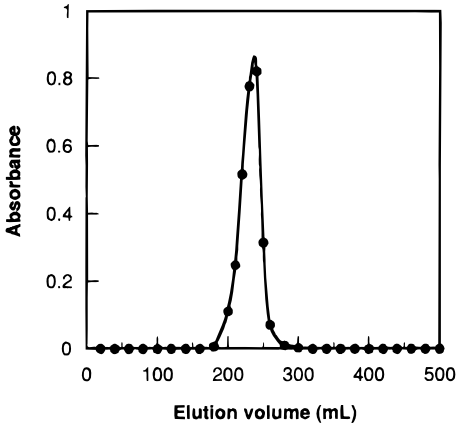

Figure 3. Elution profile of SP-TSH-2-2 by Toyopearl HW-65F column chromatography. Detection was performed by the phenol−H_2SO_4 method.

O-[$^2H_3$]methylrhamnitol. Therefore, the 1,2-linked rhamnose derivative was suggested to originate from 3-*O*-methylrhamnose (major) and rhamnose (minor). As shown in Figure 4B, the mass spectrum of the nonreducing end of the rhamnose derivative revealed the presence of 1,5-di-*O*-acetyl-2,3-di-*O*-methyl-4-*O*-[$^2H_3$]-methylrhamnitol (*m*/*z* 117, 134, 161, and 178) and 1,5-di-*O*-acetyl-3-*O*-methyl-2,4-di-*O*-[$^2H_3$]methylrhamnitol (*m*/*z* 120, 134, 164, and 178), with no 1,5-di-*O*-acetyl-2,3,4-tri-*O*-[$^2H_3$]methylrhamnitol. Since the intensity of the mass peaks from the 2,3-di-*O*-methylrhamnose derivative (*m*/*z* 117 and 161) were stronger than that of the 3-*O*-methylrhamnose derivative (*m*/*z* 120 and 164), this nonreducing end rhamnose derivative originated from 2,3-di-*O*-methylrhamnose (main) and 3-*O*-methylrhamnose (minor). In addition, the nonreducing end xylose derivative was identified solely as 1,5-di-*O*-acetyl-3-*O*-methyl-2,4-di-*O*-[$^2H_3$]methylxylitol (*m*/*z* 104, 120, and 164), suggesting that a 3-*O*-methylxylose unit was located at the terminal position of the polymer. The results of methylation analysis of TEA-SP indicated that the 4-position of 1,2-linked rhamnose and 1,3-linked rhamnose was the main sulfate substitution site (Table 1). Furthermore, some of 1,3-linked rhamnose was suggested to be substituted by sulfate groups at the 2,4-positions. Nonreducing terminal rhamnose and xylose residues were also substituted by sulfate groups.

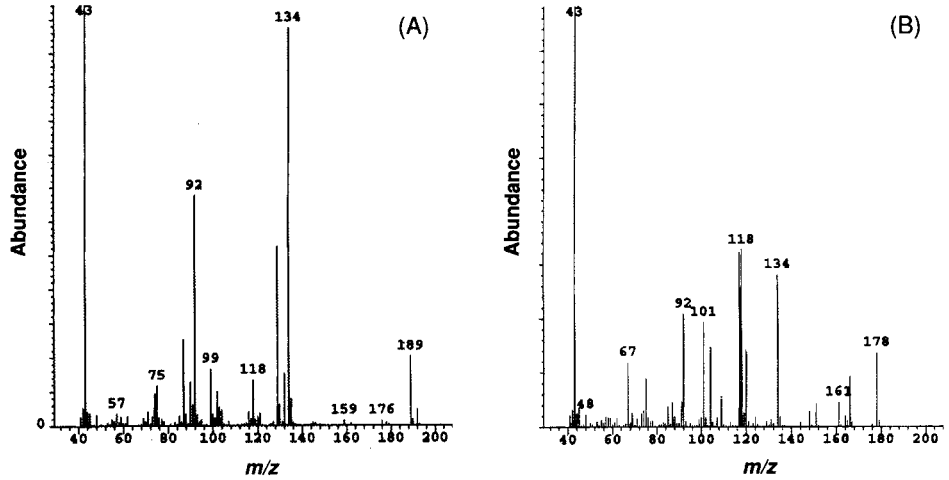

Figure 4. Mass fragmentation patterns of 1,2-linked rhamnose derivative (A) and nonreducing end-rhamnose derivative (B).

These findings indicate that the Ca-SP from *Cyanophyta* is a novel sulfated polysaccharide. So far, rhamnan sulfates have been isolated from the family Monostromaceae in the *Chlorophyta*.[6,7] However, none of partially methylated rhamnose residues such as 3-*O*-methylrhamnose have been found as a constituent in rhamnan sulfates from the *Chlorophyta* or in the sulfated polysaccharides from the other taxa. 3-*O*-Methylrhamnose has been reported to be present in quite limited quantities in lipopolysaccharides from *Pseudomonas aeruginosa* IID 1008 (ATCC 27584)[8] and *Campyrobacter fetus*.[9] Similarly, 2-*O*-methylrhamnose and 3-*O*-methylrhamnose were identified as minor component sugars of acidic polysaccharides from *Chlorella vulgaris* K 2210 and *S. platensis*.[11] The present study shows that Ca-SP contained 3-*O*-methylrhamnose, 2,3-di-*O*-methylrhamnose, and 3-*O*-methylxylose, but no 2-*O*-methylrhamnose. Since *O*-methyl sugars have been proposed to contribute to the lipophilic character of the cell surface,[12] if Ca-SP carries hydrophilic sulfate groups in the molecule it might show amphipathic properties.

Experimental Section

General Experimental Procedures. Solvent evaporation was performed below 40 °C under reduced pressure. Total sugar content was determined by the phenol−H_2SO_4 method.[13] Uronic acid content was quantified by the *m*-hydroxydiphenyl method.[14] GC was carried out on a GL-Science GC-353 gas chromatograph equipped with a FID, and N_2 was used as carrier gas. GC−MS analysis was carried out on a Hewlett-Packard HP-5890 series II gas chromatograph combined with a Hewlett-Packard HP-5972MSD mass spectrometer or a JEOL JMS-AX505HAD mass spectrometer, and the ionization potential was 70 eV. The ^{1}H NMR spectrum was measured in D_2O solution at 50 °C using a Varian Unity 500 NMR spectrometer, with chemical shifts (δ) reported in ppm. Tetramethylsilane (TMS) was used as an internal standard. IR spectra were recorded with a Shimadzu FT-IR4200 spectrophotometer. The optical rotation was determined in H_2O using a 1 cm light path length cell with a JASCO DIP-1000 digital polarimeter. Metal elements were analyzed using 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).

Isolation and Purification of Polysaccharide. Powdered *S. platensis* was extracted with boiling H_2O for 1 h twice. The combined supernatant was dialyzed and lyophilized. The extract was treated with 10% TCA, and the TCA-soluble portion was dialyzed. Then, the nondialyzate (SP-TSH, 500 mg) was dissolved in 0.1 M citrate buffer containing 0.1 M NaCl (pH 7) and applied to a Sepharose 6B column (4 × 72 cm, Pharmacia). The column was eluted with the same buffer, and fractions of 10 mL were collected according to the results of monitoring by the phenol−H_2SO_4 method with UV detection at 260 nm.[15] The second eluate (SP-TSH-2, 400 mg), which showed potent anti-HSV-1 activity, was dissolved in H_2O and loaded onto the DEAE-Toyopearl 650M column (2.2 × 76 cm, Tosoh). The column was eluted with a linear gradient solvent system (0−2 M NaCl). Carbohydrate-positive fractions were combined, dialyzed, and lyophilized to yield fractions SP-TSH-2-1 (142.0 mg) and SP-TSH-2-2 (150.7 mg). The obtained antiviral fraction (SP-TSH-2-2, 53.6 mg) was further purified over a Toyopearl HW65(S) column (2.2 × 95 cm, Tosoh) eluting with 0.1 M NaCl to give a colorless solid (Ca-SP) with a white cotton-like appearance (44.3 mg).

Estimation of Apparent Molecular Weight. The apparent molecular weight of *S. platensis* carbohydrate was estimated by HPLC analysis. The sample was applied on a TSK $GMPW_{XL}$ gel filtration column (7.6 mm × 30 cm, Tosoh) and eluted with 0.2 M NaCl at 0.5 mL min^{-1}. Commercially available pullulans (Shodex P-52, Showa Denko, Tokyo, Japan) were used as standard molecular markers.

Sugar Composition Analysis. A sample of Ca-SP (3 mg) was hydrolyzed with 1 M H_2SO_4 (2 mL) at 100 °C for 3 h. The hydrolysates were converted to the corresponding alditols by addition of $NaBH_4$. After excess $NaBH_4$ was destroyed by acidification with AcOH solution, it was removed as methanolic borate by repeated codistillation with anhydrous MeOH. The obtained alditols were then acetylated with the mixture of anhydrous acetic acid and 1-methylimidazole at room temperature for 45 min,[16] and the derivatives obtained were analyzed by GC and GC−MS. GC and GC−MS were carried out using a SP-2330 fused silica capillary

column (30 m × 0.32 mm i.d., Supelco Inc., Bellefonte, PA) with a temperature program starting at 160 °C followed by 2 °C min^{-1} to 210 °C and then 5 °C min^{-1} to 240 °C. Identification of alditol acetates was carried out from the observed mass fragmentation patterns and from relative retention times to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol.[16]

Isolation and Identification of 3-*O*-Methylrhamnose. Acid hydrolysates of Ca-SP were separated as bands by preparative paper chromatography (Toyo No. 50 paper) using *n*-BuOH-pyridine−H_2O (6:4:3, v/v) as developing solvent. After the guide-strip detection of sugars (R_f 0.66) by $AgNO_3$−NaOH, the corresponding bands were extracted with H_2O. The extract was concentrated and then lyophilized to yield 3-*O*-methylrhamnose.

Reduction of Carboxyl Groups in Ca-SP. The carboxyl groups in Ca-SP were reduced according to Taylor and Conrad's method.[17]

Desulfation of Ca-SP. Desulfation of Ca-SP was performed by solvolytic desulfation with 10% MeOH in DMSO.[18] After dialysis and lyophilization, a colorless white powder was obtained (DS−SP).

Microdetermination of Sulfur. Sulfur was determined by a flask combustion method under O_2 gas in the presence of H_2O_2.[19] After the sample in the combustion flask was ignited under the O_2 atmosphere with an aliquot of H_2O_2, the solution was titrated with standard solution of 50 mM $BaCl_2$ using 0.1% dimethylsulfonazo III reagent.

Methylation Analysis. Ca-SP was converted to the triethylamine salt (TEA-SP) by passing through a Dowex 50W × 8 column (TEA-form, 1 × 15 cm).[20] Methylation was performed by Hakomori's method[21] with methyl iodide or [$^2H_3$]methyl iodide. The methylated polysaccharides were hydrolyzed and converted to alditol acetates.[16] The partially methylated alditol acetates were analyzed by GC and GC−MS using a SP-2330 (30 m × 0.32 mm i.d., Supelco Inc., Bellefonte, PA) fused silica capillary column with the following temperature program: 160−210 °C, 2 °C min^{-1}, 210−240 °C, 5 °C min^{-1}, 240 °C 14 min. Peak areas were corrected using published molar response factors.[22] The derivatized compounds were identified by comparison of their 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol and their GC−EI-MS fragmentation patterns.[16]

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