Structural Analysis of Calcium Spirulan (Ca−SP)-Derived Oligosaccharides Using Electrospray Ionization Mass Spectrometry

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Detailed structural analyses of calcium spirulan (Ca−SP)-derived oligosaccharides were performed by ESI-MS and collision-induced dissociation tandem mass spectrometry. This study indicates that Ca−SP is composed of two types of disaccharide repeating units, *O*-rhamnosyl-acofriose and *O*-hexuronosyl-rhamnose (aldobiuronic acid).

Calcium spirulan (Ca−SP) is a sulfated polysaccharide isolated from the blue-green alga *Spirulina platensis* (Nordst.) Getil. (Oscillatoraceae). This polysaccharide has shown potent antiviral activities against enveloped viruses, including herpes simplex virus type 1 and human immunodeficiency virus type 1.[1] Furthermore, Ca−SP has exhibited additional biological activities such as an inhibitory effect on tumor invasion and metastasis,[2] antithrombin activity via heparin cofactor II,[3] and enhancement of tissue-type plasminogen activator production.[4] In a previous paper, Ca−SP was revealed to be composed of 1,3-linked rhamnose and 1,2-linked 3-*O*-methylrhamnose (acofriose) units in a ratio of about 5:3.[5] Furthermore, uronic acids, in the form of glucuronic acid and galacturonic acid, were also found to be component sugars of Ca−SP. However, its detailed structural features, including the sugar sequence, remained uncertain.

Mass spectroscopic analysis has been performed to characterize oligosaccharides from glycoproteins.[6,7] Although various ionization techniques have been applied to the carbohydrate analysis, the electrospray ionization (ESI) method is particularly suitable because of its low limit of detection.[8,9] Furthermore, tandem mass spectrometry (MS−MS) with collision-induced dissociation (CID) provides considerable information on the carbohydrate structure.[8,10,11] In this paper, we describe the carbohydrate sequences of Ca−SP elucidated by using ESIMS and MS−MS of the Ca−SP-derived oligosaccharides.

To obtain Ca−SP-derived oligosaccharides, partial acid hydrolysis with 0.1 M H_2SO_4 was carried out. The hydrolysates were fractionated to afford neutral (fraction N) and acidic oligosaccharide fractions (fraction A) using a Dowex 1 × 8 resin. So far, methylated oligosaccharides have been reported to be suitable for analysis using ESIMS and MS−MS.[8,10,11] However, methylation of oligosaccharides derived from Ca−SP was not appropriate, because it contained a large amount of methylated sugars such as acofriose. Therefore, each fraction was deuteriomethylated with [$^2H_3$] methyl iodide. In Figure 1(A), a series of ions corresponding to the sodium-cationized oligosaccharides were clearly observed as deuteriomethyl ethers, and their degree of polymerization (d.p.) values ranged from 2 to 7. These ions were presumed to be composed of rhamnose and acofriose. On the other hand, it is suggested that oligosaccharide ions

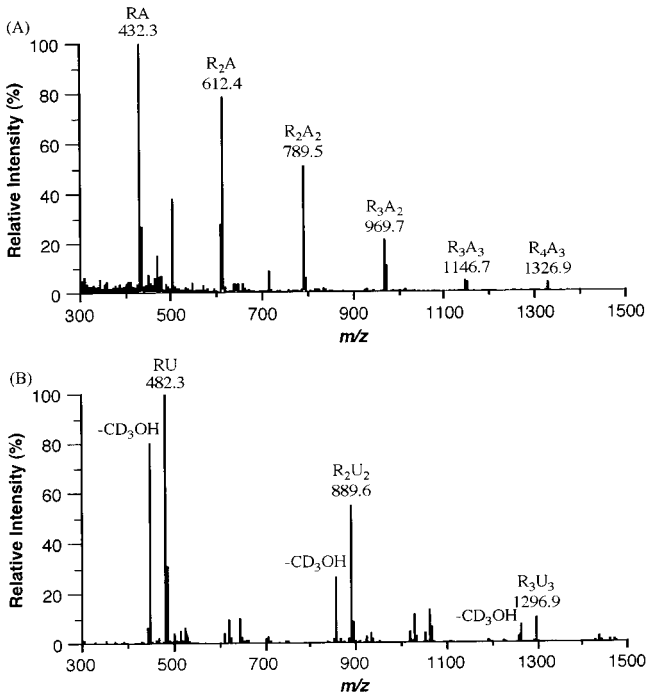

Figure 1. Positive ion ESIMS of per-*O*-deuteriomethylated oligosaccharides in fractions N (A) and A (B). R, A, and U refer to rhamnose, acofriose, and uronic acid, respectively.

in Figure 1(B) consist of rhamnose and uronic acid. Uronic acids are often involved in acid-resistant glycosidic linkages, particularly in the aldobiuronic acid type of linkage.[12] Therefore, it is postulated that the ions at *m*/*z* 482.3, 889.6, and 1296.9 are due to aldobiuronic acid-type oligosaccharides corresponding to disaccharides, tetrasaccharides, and hexasaccharides, respectively.

To obtain more detailed structural information, CIDMS−MS of the major observed ions in Figure 1(A) and (B) were measured. In CIDMS−MS, we have adopted the nomenclature proposed by Domon and Costello.[13] Figure 2(A) shows the CIDMS−MS spectrum of the ion of *m*/*z* 612.4 in Figure 1(A), corresponding to a trisaccharide composed of two rhamnose and one acofriose residues. In this spectrum, the fragment ions of *m*/*z* 238 and 415 correspond to Y_1 and Y_2 ions, respectively. Furthermore, the ions of *m*/*z* 220 and 397 were assigned to B_1 and B_2 ions, respectively. These fragment ions indicate the trisaccharide to be *O*-rhamnosyl-acofriosyl-rhamnose, as shown in Figure 2(A). Similarly, the CIDMS−MS spectra of oligosaccharide ions at *m*/*z*

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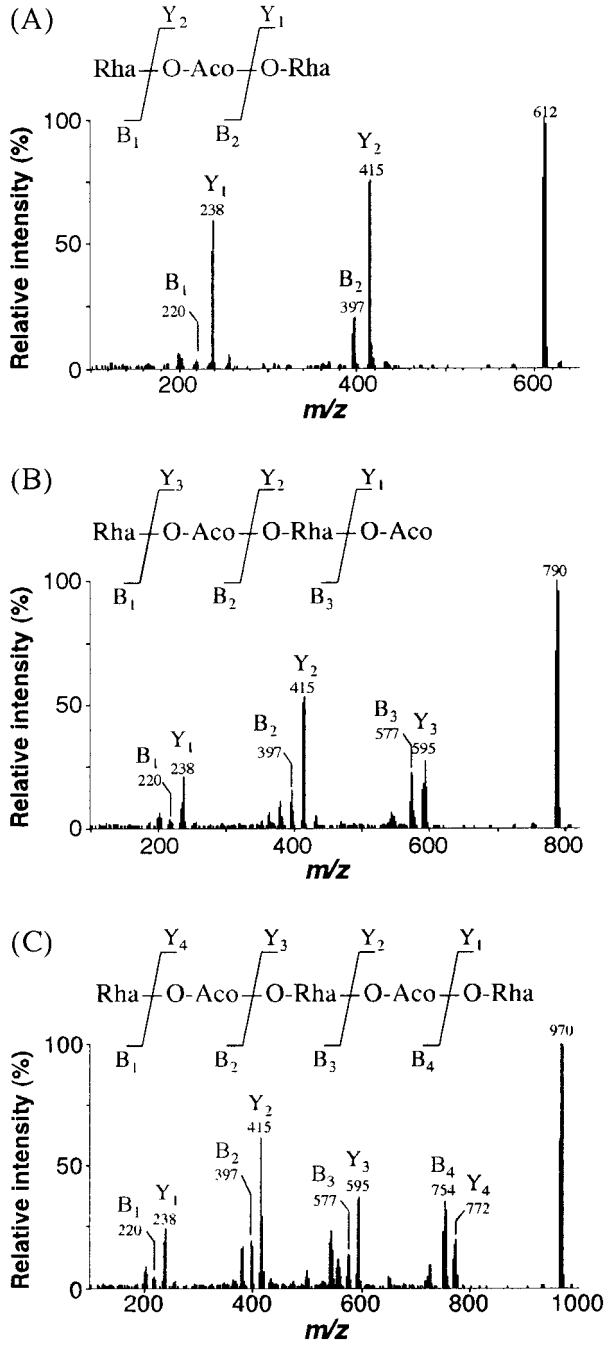

Figure 2. Positive ion CIDMS−MS spectra of (A) per-*O*-deuteriomethylated R_2A $[M + Na]^+$ at *m/z* 612.4, (B) per-*O*-deuteriomethylated R_2A_2 $[M + Na]^+$ at *m/z* 789.5, and (C) per-*O*-deuteriomethylated R_3A_2 $[M + Na]^+$ at *m/z* 969.7 in Figure 1(A).

789.5 and 969.7 show a series of Y_n- and B_n-type ions [Figure2(B) and (C)], respectively. These fragmentation patterns indicate the oligosaccharides to consist of rhamnose and acofriose, alternately. The ions at *m/z* 1146.7 and 1326.9 were also assigned to analogous structures (data not shown). In contrast, the ion at *m/z* 482.3 in Figure 1(B) did not show abundant fragmentation in the CIDMS−MS [Figure 3(A)]. However, a fragment ion at *m/z* 238 with weak abundance was observed and is suggested to be a Y_1 ion. Therefore, this ion was deduced to be derived from an aldobiuronic acid-type disaccharide composed of uronic acid and rhamnose. As shown in Figure 3(B), fragment ions at *m/z* 447 and 465 were distinctly observed in the CIDMS−MS ion at *m/z* 889.6 in Figure 1(B). Because this precursor ion corresponds to a tetrasaccharide composed of two uronic

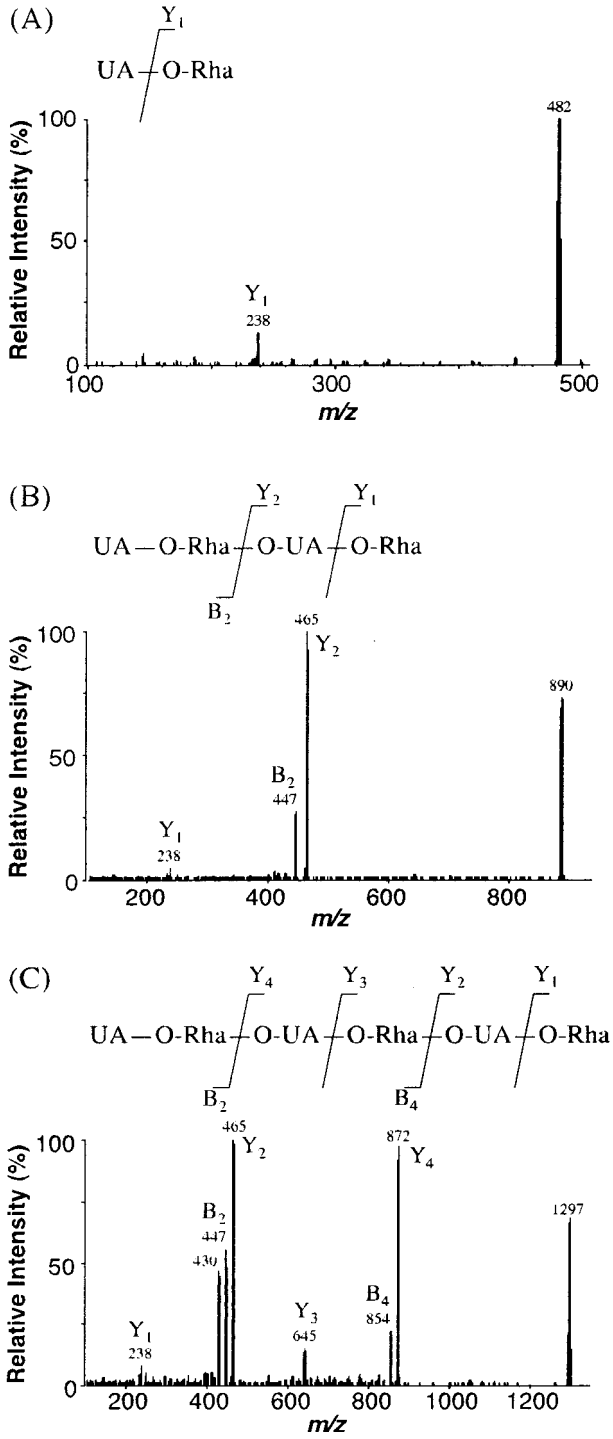

Figure 3. Positive ion CIDMS−MS spectra of (A) per-*O*-deuteriomethylated RU $[M + Na]^+$ at *m/z* 482.3, (B) per-*O*-deuteriomethylated R_2U_2 $[M + Na]^+$ at *m/z* 889.6, and (C) per-*O*-deuteriomethylated R_3U_3 $[M + Na]^+$ at *m/z* 1296.9 in Figure 1(B).

acids and two rhamnose residues, the observed fragment ions at *m/z* 447 and 465 were assigned as the B_2 and Y_2 ion, respectively. Furthermore, the fragment ion at *m/z* 238 corresponding to the Y_1 ion was also observed with weak abundance. Therefore, it is suggested that this tetrasaccharide consists of two units of an aldobiuronic acid-type disaccharide. Similarly, the ion at *m/z* 1296.9 shown in Figure 3(C) probably corresponded to three units of an aldobiuronic acid-type disaccharide.

In a previous paper, we reported that Ca−SP contained rhamnose and acofriose and were mainly 1,3-linked and 1,2-linked, respectively.[5] Therefore, the disaccharide unit

observed in fraction N is suggested to be →3)-α-L-Rha-(1→2)-α-L-Aco-(1→. The acidic oligosaccharides from Ca−SP were also revealed to be composed of repeating structures of aldobiuronic acid-type disaccharides.

In our previous study, the sulfate groups were indicated to be substituted at the C-2 or C-4 position of 1,3-linked rhamnose and at the C-4 position of acofriose.[5] Because polysaccharide sulfate groups have been found to be important for biological activity, the sulfated units of →3)-α-L-Rha-(1→2)-α-L-Aco-(1→ might be essential for activity of Ca−SP. This paper is the first report of the fine structural elucidation of sulfated polysaccharides from a blue-green alga.

Experimental Section

General Experimental Procedures. The isolation of Ca−SP was performed as reported elsewhere.[5] ESIMS analysis was performed on a Sciex (Thornhill, Ontario, Canada) API-III triple-quadrupole mass spectrometer equipped with an electrospray ion source.

Partial Acid Hydrolysis of Ca−SP and Fractionation. Ca−SP (180 mg) was hydrolyzed with 0.1 M H_2SO_4 at 100 °C for 150 min. After neutralization with $BaCO_3$, the filtrate was collected and concentrated to dryness. The hydrolysates were subjected to column chromatography over Dowex 1 × 8 (acetate form, 2 × 14 cm, Dow Chemical). The neutral fraction (fraction N, 71.9 mg) and acidic fraction (fraction A, 19.2 mg) were obtained by elution with H_2O and 4 M AcOH, respectively.

Deuteriomethylation of Fractions N and A. Each fraction was methylated with [$^2H_3$] methyl iodide by Anumula and Taylor's method.[14]

ESIMS Analysis. The deuteriomethylated oligosaccharides were dissolved in 70% MeOH containing 5 mM NaOAc. The sample solution was introduced into the mass spectrometer with a model 2400-001 syringe pump (Harvard Apparatus, South Natick, MA) at rate of 2 μL/min. The mass spectrometer was operated in the positive mode; the electrospray voltage was set to 4500 V, and the interface plate voltage was 650 V. The orifice voltage was 100 or 120 V. CIDMS−MS were acquired by passing the oligosaccharide ions into the second quadrupole when they were dissociated by collision with argon gas. The argon collision target gas thickness was used at 2.2×10^4 atoms/cm^2 and collision energies of 35−75 eV (collision energies measured as the voltage difference between Q_0 and Q_2).

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