Anal. Chem. **2004,** *76,* 7387−7390

Technical Notes

LC/MS Characterization of Meningococcal Depolymerized Polysaccharide Group C Reducing Endgroup and Internal Repeating Unit

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Hydrogen peroxide has been used to cleave the native *Neisseria meningiditis* polysaccharide (PS) from mega-Dalton molecular weight to a smaller size (∼20 kDa) depolymerized polysaccharide. The polysaccharide was examined after partial peroxide depolymerization to verify the presence of the carboxyl group at position 1 and the intactness of the internal sialic acid repeating units. The reducing end group of meningococcal polysaccharide type C was also examined after derivatization by L-tyrosine hydrazide. Partial peroxide depolymerization did not result in loss of the position 1 carboxyl group at the reducing end of the polysaccharide. In addition, no loss of structural integrity was noted for the internal sialic repeat units.

Purified, native *Neisseria meningiditis* serogroup C polysaccharide (MnC-PS) is a large ($M_r \sim 2 \times 10^5$ or greater), unbranched polymer composed of repeating units of 2 → 9 linked sialic acid residues (Figure 1a).[1,2] Hydrogen peroxide (H_2O_2) was used to partially depolymerize native to smaller molecular size polysaccharide chains (mean ∼ 20 kDa) prior to conjugation to protein molecules.[3] To understand the H_2O_2 treatment for the polysaccharide and the integrity for the reducing endgroup, experiments were designed to characterize the end group residue during the depolymerization process, as well as to characterize the internal repeating units by mass spectrometry.

EXPERIMENTAL SECTION

List of Samples. *(1) Depolymerized (20 kDa) Sample.* MnC-PS was depolymerized to 20 kDa mean molecular size (relative to dextran molecular size standard) by H_2O_2 treatment, followed by a diafiltration step with a 1000-Da MWCO (molecular weight cutoff) membrane against MilliQ water to remove unwanted chemicals and smaller sized PS.

(2) Purified 1-kDa Permeate Sample from the Hydrogen Peroxide Depolymerized Polysaccharides. H_2O_2 treated ∼20-kDa-sized MnC-PS was concentrated by tangential flow ultrafiltration using a 1000-Da MWCO cellulose spirally wound cartridge. The permeate sample was further concentrated by rotary evaporation. The concentrate of the permeate sample was resuspended in Milli-Q water, and the resuspended material was further purified to remove salt and H_2O_2 by size exclusion chromatography on a (62 cm × 2.5 cm) BioGel P2 column eluted with water.

(3) L-Tyrosine Hydrazide-Labeled Depolymerized Polysaccharide. Labeling of the 20-kDa depolymerized PS was accomplished by reductive amination at the reducing end sugar, using a 5-fold molar excess of L-tyrosine hydrazide, as compared to the reducing end sugar. The hydrazone was stabilized by reduction using sodium cyanoborohydride. The structure of the labeling reagent L-tyrosine hydrazide is shown in Figure 1b. L-Tyrosine hydrazide (CAS 7662-51-3, irritant) was from Aldrich.

A final concentration of 0.2 M acetic acid was added to 1 mg/mL depolymerized MnC-PS, either with or without L-tyrosine hydrazide end-labeling (samples 1 and 3 listed above), and then subjected to 15-min microwave irradiation with a power of 320 W.[4] The hydrolyzed sample was cooled to room temperature, diluted to 100 μg/mL, and then analyzed by the LC/MS method. The purified permeate sample listed in sample 2 was analyzed directly by LC/MS. As a reference for structure elucidation, sialic acid was analyzed, and a molecular weight of 310 Da $[SA + H]^+$ was observed for the intact structure (Figure 1c).

The HPLC system used here consisted of a Finnigan MAT (San Jose, CA) Spectra system P4000 pump and an AS3500 autosampler. Samples were injected at 10 μL onto a Zorbax (Agilent, Santa Clarita, CA) RX-C18 column (150 mm × 2.1 mm) operated at ambient temperature. The mobile phase, consisting of 0.05% (v/v) formic acid in water/acetonitrile (99/1, solvent A), (0.05% (v/v) formic acid in acetonitrile/water (99/1, solvent B) was delivered at a flow rate of 0.2 mL/min. with 5 min 80:20 A/B isocratic elution prior to the MS analysis. The mass spectrometer was set at full m/z detection range (100−2000 Da), with electrospray voltage set to 4 kV and heated capillary set to be 200 °C.

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10.1021/ac0491760 CCC: $27.50
Published on Web 11/03/2004

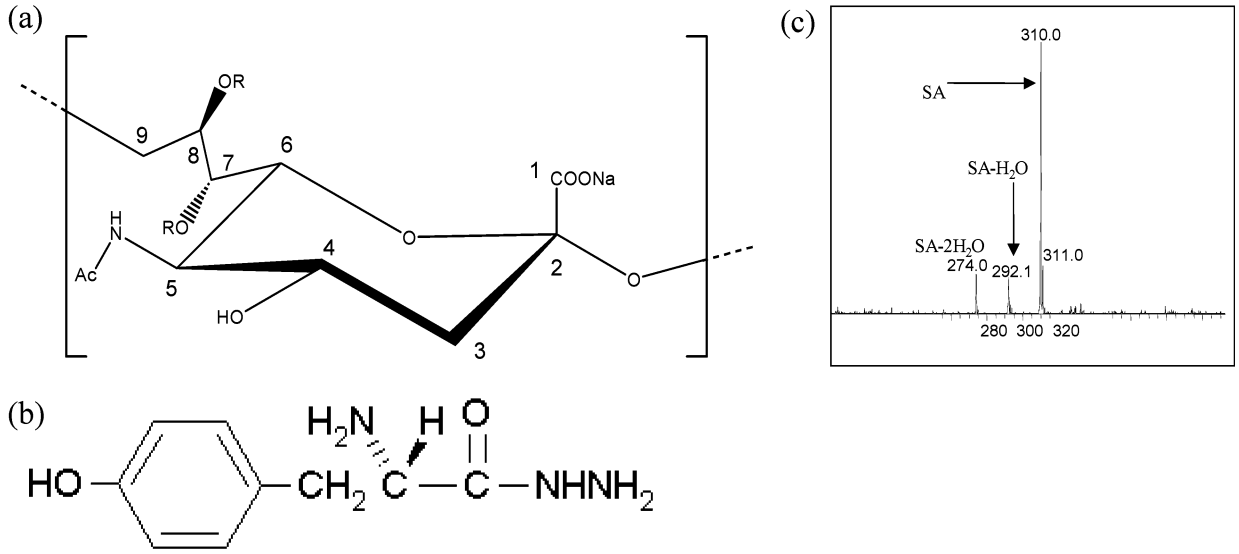

Figure 1. (a) Repeating unit structure for meninge polysaccharide group C. (b) Structure of the labeling material L-tyrosine hydrazide. (c) Mass spectrum of sialic acid.

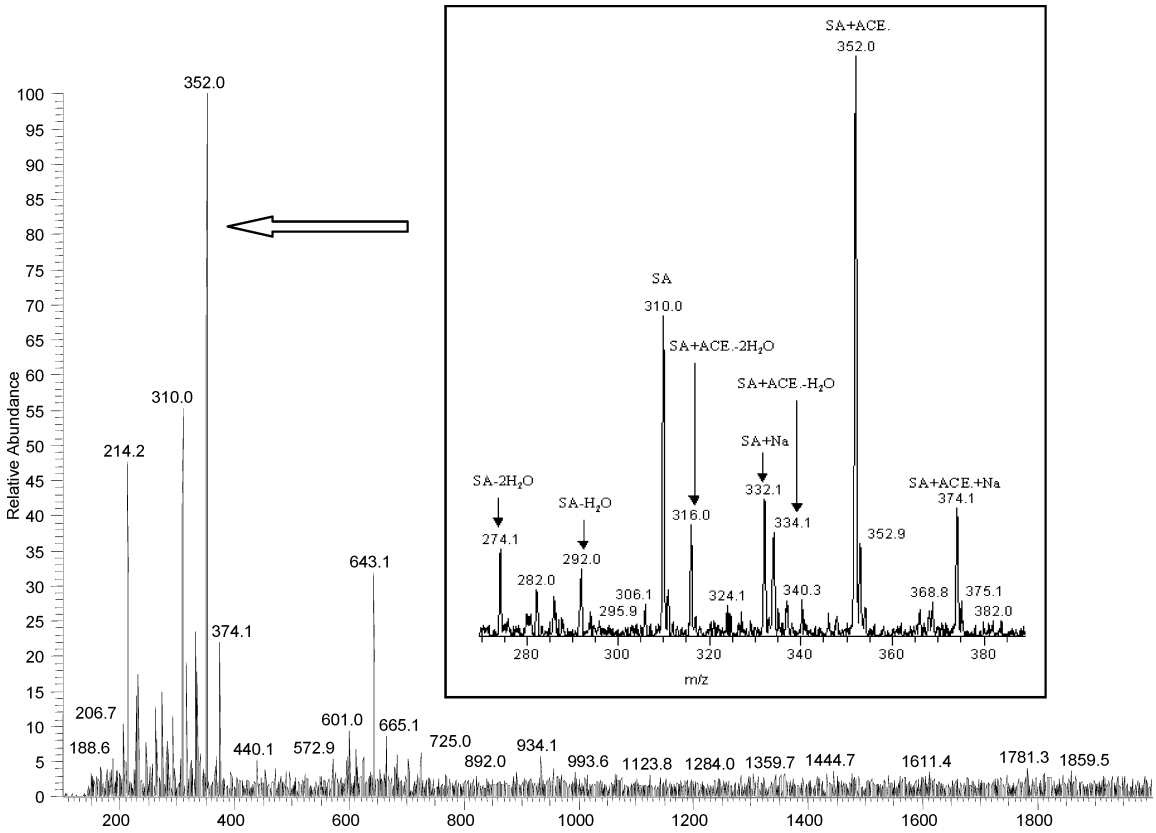

Figure 2. Full mass range scan (*m*/*z* 100−2000 Da) of purified depolymerized MnC permeate samples.

RESULTS AND DISCUSSION

Characterization of the Small Mass Fragments from the Hydrogen Peroxide Depolymerization. The mass spectra of the permeate sample (sample 2) from the hydrogen peroxide depolymerized polysaccharides are shown in Figure 2. (MnC-PS is very stable under these conditions in the absence of peroxide (our unpublished data)). This sample consists of very short chain oligosaccharide fragments from the depolymerization reaction. The *m*/*z* pattern of the sample supports the conclusion that cleavage of the serogroup C polysaccharides occurs at the 2 → 9 linkage and that the reducing end sugar group remains intact with O-acetylation. The mass assignments of these fragments are summarized in Table 1. The mass fragment pattern of the depolymerized/acid-hydrolyzed polysaccharide compares closely

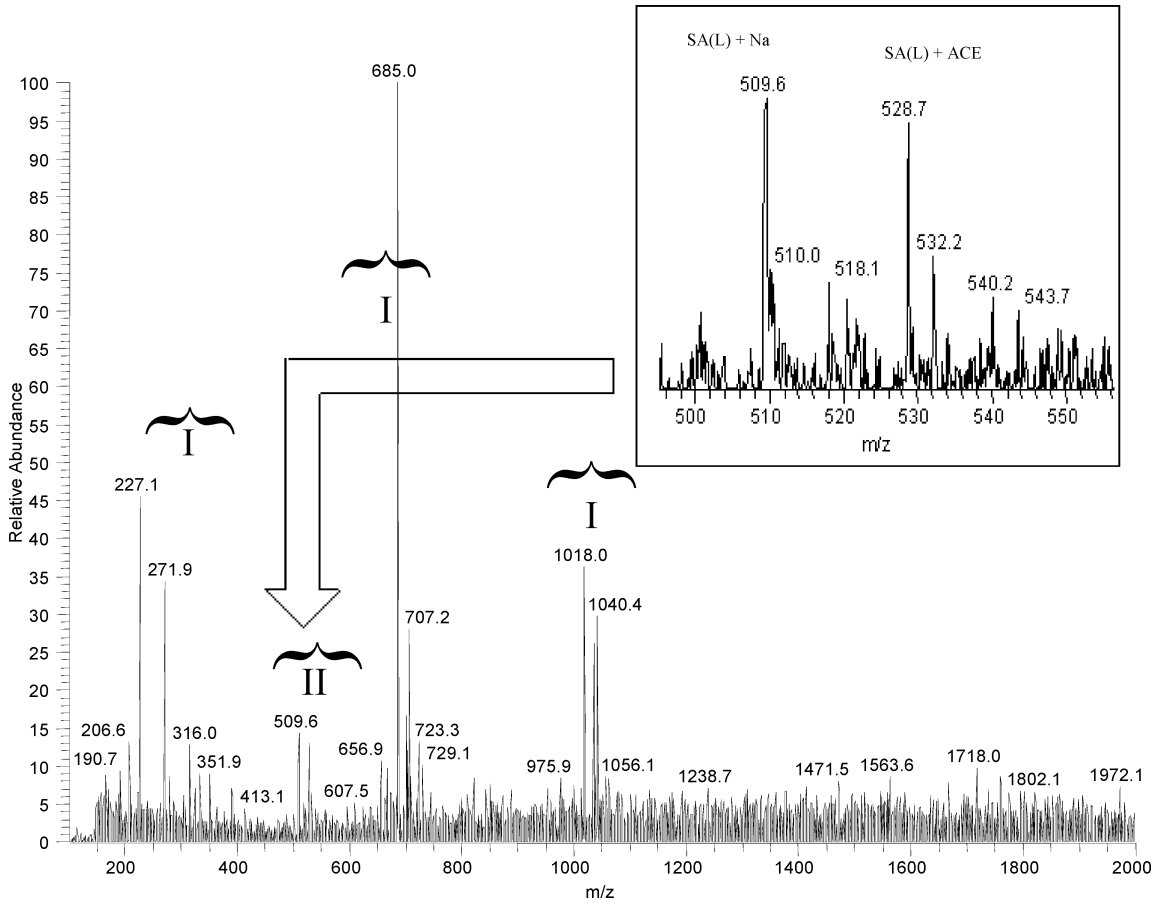

Figure 3. A full mass range scan (100−2000 Da *m*/*z*) of acetic acid-hydrolyzed labeled MnC depolymerized polysaccharide. Protonated peaks were labeled directly on the figures with the name or abbreviation without adding H; SA, sialic acid (*N*-acetylneuraminic acid); Ace or ACE, acetylation; SA(L), sialic acid labeled with L-tyrosine hydrazide.

Table 1. Summary of the Peaks Detected in the Full Range Scan (*m*/*z* 100−2000) of the Permeate Sample for MnC-PS after the Hydrogen Peroxide Depolymerization Treatment from Figure 2 and the Peaks Detected in the Full Range Scan (*m*/*z* 100−2000) of the Hydrolyzed L-Tyrosine Hydrazide-Labeled MnC Polysaccharide from Figure 3

no. SA repeating units in hydrolyzed PS	no. O-acetylation	expected *m*/*z*	observed *m*/*z*	peak assigned
monosaccharide	0 acetylation	310.1	274.1	(1) sialic acid − 2 H_2O
			292.0	(1) sialic acid − H_2O
			310.0	(1) sialic acid
			332.1	(1) sialic acid + Na
	1 acetylation	352.1	316.0	(1) sialic acid + Ace − 2H_2O
			334.1	(1) sialic acid + Ace − H_2O
			352.0	(1) sialic acid + Ace
			374.1	(1) sialic acid + Ace + Na
			509.6	(1) sialic acid + tyrosine + Na
			528.7	(1) sialic acid + tyrosine + Ace
disaccharide	0 acetylation	601.2	601.0	(2) sialic acid
	1 acetylation	643.2	625.2	(2) sialic acid + Ace − H_2O
			643.1	(2) sialic acid + Ace
			665.1	(2) sialic acid + Ace + Na
	2 acetylation	685.2	667.1	(2) sialic acid + 2Ace − H_2O
			684.9	(2) sialic acid + 2Ace
			707.3	(2) sialic acid + 2Ace + Na
			725.0	(2) sialic acid + 2Ace + Na + H_2O

to the mass fragment pattern of the permeate sample, as shown in Figure 2, in the mono- and disaccharide region of the mass spectrum.

The L-tyrosine hydrazide-labeled 20 kDa depolymerized polysaccharide was subjected to further hydrolysis using acid. Following this treatment, the sample was analyzed by LC/MS, and the mass fragments are shown in Figure 3. The group I peaks are from nonlabeled repeating units that arose during the acid hydrolysis treatment step, and the group II peaks are the end group sugar fragments labeled with L-tyrosine hydrazide. Group II peak intensities are low overall due to the smaller population of the labeled reducing end group in the hydrolyzed polysaccharide

relative to the newly end group sugars that formed as a result of acid hydrolysis, which are summarized in Table 1. The bond between the L-tyrosine hydrazide and the reducing end group sugar was found to be less susceptible toward acid hydrolysis in comparison to the glycan bonds between the sialic acid subunits, since free L-tyrosine hydrazide was not observed.

On the basis of the mass fragment patterns of the 20-kDa hydrogen peroxide depolymerized polysaccharide permeate sample, the acid-hydrolyzed 20-kDa hydrogen peroxide-depolymerized polysaccharide, and the acid-hydrolyzed 20-kDa hydrogen peroxide-depolymerized polysaccharide labeled with L-tyrosine hydrazide, the sialic acid repeating unit and endgroup sugar remain intact during depolymerization by hydrogen peroxide. There is no evidence of decarboxylation during the hydrogen peroxide depolymerization or during the acid hydrolysis step. Loss of the carboxyl group, if it occurred, would not be expected to hinder ionization of the sialic molecule, since protonation occurs at the amino containing group. The integrity of the internal repeat unit sugars can be assessed by analyzing the fragmentation pattern of the unlabeled, acid-hydrolyzed polysaccharides. The intact sialic acid repeat units are detected at 310.0 Da $[SA + H]^+$, 601.0 Da $[2SA + H]^+$, etc., with O-acetylation at 352.0 Da, 643.2 Da, etc. The ions detected with Na^+ adduction or the loss of water molecules may be attributed to the ubiquitous existence of sodium ions in biological samples and chemically induced lactone formation from the electrospray ionization process. These results demonstrate that the carboxyl, *N*-acetyl, and *O*-acetyl functional groups are present on the internal repeating units.

In conclusion, by using LC/MS the integrity of the reducing end group and the repeating sialic acid units of depolymerized serogroup C polysaccharide were found to remain intact upon depolymerization using hydrogen peroxide. The results from this study support that hydrogen peroxide depolymerization of serogroup C polysaccharide occurs by cleavage at the 2 → 9 glycosidic linkage, yielding intact sialic acid units without loss of *O*-acetyl or carboxyl groups.

Received for review June 4, 2004. Accepted September 16, 2004.

AC0491760