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Note

Addition of glycerol for improved methylation linkage analysis of polysaccharides

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Abstract—A presolubilization procedure with the use of glycerol is shown to be applicable for the structural analysis of polysaccharides. Neutral, acidic, high-molecular-weight and low-molecular-weight polysaccharides were solubilized in glycerol prior to methylation and subsequent linkage analysis by GC–MS. All four types of polysaccharides showed significant increases in derivatization following presolubilization as measured by recovery of partially methylated alditol acetates.

Keywords: Linkage analysis; Polysaccharide; Gas chromatography; Methylation; PMAA

Monosaccharide linkage analysis has been used for many years for structural elucidation of polysaccharides.[1,2] Typically, polysaccharides are sequentially methylated, hydrolyzed, reduced, and acetylated to form partially methylated alditol acetates (PMAAs) that can be chromatographed and analyzed by gas chromatography–mass spectrometry (GC–MS). By far the most critical step is the initial methylation of the polysaccharide. Methylation must occur efficiently and completely to generate meaningful linkage data; undermethylation can lead to lower recovery of PMAAs and/or the generation of misleading results due to PMAA derivatives that undergo undermethylation and subsequent overacetylation. Various methylation methods have been developed and typically employ the use of the dimsyl anion[1] or alkali-metal hydroxide[2] (e.g., NaOH) to deprotonate free hydroxyls on the saccharide prior to methylation. Dimethyl sulfoxide (Me_2SO) is a commonly used solvent for the methylation reaction due to the solubility of many saccharide species in this anhydrous solvent. However, solubility of high-molecular-weight (HMW) polysaccharides is limited in Me_2SO, and often these polysaccharides are chemically or enzymatically degraded prior to methylation and linkage analysis. An example of a common chemical degradation method is the Smith degradation[3] reaction, which involves periodate oxidation of carbohydrate residues containing diol groups, followed by reduction and mild acid hydrolysis for specific cleavage of periodate-oxidized residues. The resulting oligosaccharides are readily soluble in Me_2SO and can undergo linkage analysis for detailed structural characterization and sequence determination. It would be advantageous, however, to perform linkage analysis directly on native HMW polysaccharides without degradation in order to obtain information on the native structure or to monitor the polysaccharide during processing (e.g., vaccine development) for characterization studies. To this end, glycerol was used to improve solubilization of HMW polysaccharides for methylation and linkage analysis. Dextrans and pneumococcal polysaccharides were used as model

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polysaccharide samples and were methylated using an $NaOH–CH_3I$ method with and without the addition of glycerol. Dextrans of two different molecular weights were used for the study to evaluate the effects of polysaccharide size during methylation. Also, two pneumococcal (Pn) polysaccharides (serotypes 6B and 14) were tested to examine the methylation of acidic versus neutral polysaccharides. The use of glycerol prior to derivatization allowed for efficient and complete methylation of polysaccharides leading to high recovery of PMAAs. In addition, minimal sample (100–300 μg) was required in this shortened and optimized linkage analysis procedure.

Polysaccharide repeating unit structures for the samples tested are depicted in Figure 1. The Pn 6B polysaccharide[4] was used as an example of an acidic pneumococcal polysaccharide containing phosphate. Pn 14[5] was used as an example of a neutral pneumococcal polysaccharide. The molecular weights of native Pn 6B and Pn 14 polysaccharides are similar: 1000–2000 kD.[6,7] Dextran, composed of 6-linked α-D-Glc*p* with minor branching at O-3, was used in two sizes for this study: 40 and 2000 kD. The resulting PMAAs from the polysaccharide samples are tabulated in Table 1.

Total-ion chromatograms are shown in Figure 2 for the four samples that were analyzed for monosaccharide linkage with and without presolubilization in glycerol. In the case of Pn serotype 6B, the addition of glycerol prior to derivatization was the only way to achieve recovery of PMAAs. Pn serotype 14 yielded minimal levels of PMAAs when derivatized by the $NaOH–CH_3I$ method with drastically improved recovery upon the addition of glycerol. The dextran samples both yielded PMAAs by the $NaOH–CH_3I$ method with a significantly decreased recovery for the HMW sample. All four polysaccharide samples prepared by the glycerol-solubilized method gave significantly improved PMAA yields. Additionally, methylation using the current method appeared to be complete, as PMAA derivatives due to undermethylation could not be detected. For example, undermethylation of the tested polysaccharides would result in partial formation of monomethylated (e.g., 1,2,3,5,6-penta-*O*-acetyl-1-deuterio-4-*O*-methyl-D-glucitol) PMAAs with retention times in the range of 20–25 min. These additional peaks were not detected using the current optimized procedure (data not shown).

Relative PMAA recoveries are listed in Table 2. Linkage analysis of Pn 6B did not yield PMAAs by the

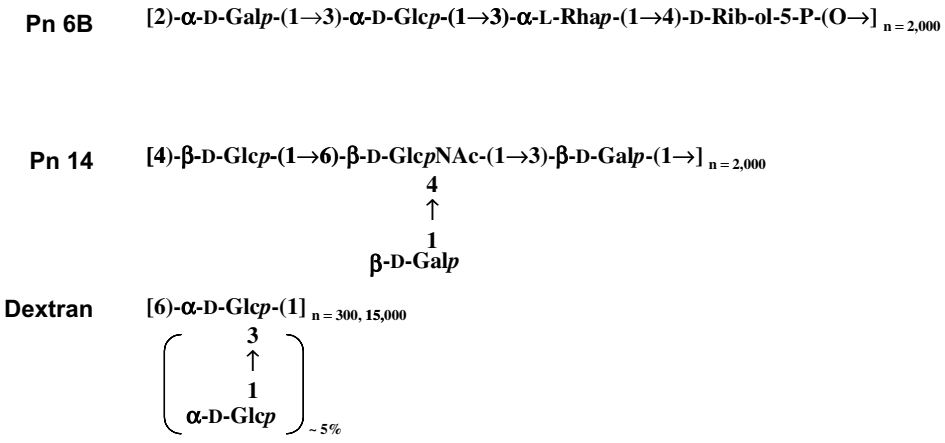

Figure 1. Repeating unit structures for Pn 6B, Pn 14, and dextran polysaccharides.

Table 1. Partially methylated alditol acetates (PMAAs) from Pn 6B, Pn 14, and dextran

Polysaccharide	PMAAs
Pn 6B	2-Gal (1,2,5-tri-*O*-acetyl-1-deuterio-3,4,6-tri-*O*-methyl-D-galactitol) 3-Glc (1,3,5-tri-*O*-acetyl-1-deuterio-2,4,6-tri-*O*-methyl-D-glucitol) 3-Rha (1,3,5-tri-*O*-acetyl-1-deuterio-6-deoxy-2,4,-di-*O*-methyl-L-mannitol) 4-Rib-ol (4,5-di-*O*-acetyl-1,2,3-tri-*O*-methyl-D-ribitol)
Pn 14	4-Glc (1,4,5-tri-*O*-acetyl-1-deuterio-2,3,6-tri-*O*-methyl-D-glucitol) 4,6-GlcNAc (1,4,5,6-tetra-*O*-acetyl-2-(acetylmethylamino)-2-deoxy-1-deuterio-3-*O*-methyl-D-glucitol) 3-Gal (1,3,5-tri-*O*-acetyl-1-deuterio-2,4,6-tri-*O*-methyl-D-galactitol) t-Gal (1,5-di-*O*-acetyl-1-deuterio-2,3,4,6-tetra-*O*-methyl-D-galactitol)
Dextran	6-Glc (1,5,6-tri-*O*-acetyl-1-deuterio-2,3,4-tri-*O*-methyl-D-glucitol) 3,6-Glc (1,3,5,6-tetra-*O*-acetyl-1-deuterio-2,4-di-*O*-methyl-D-glucitol) t-Glc (1,5-di-*O*-acetyl-1-deuterio-2,3,4,6-tetra-*O*-methyl-D-glucitol)

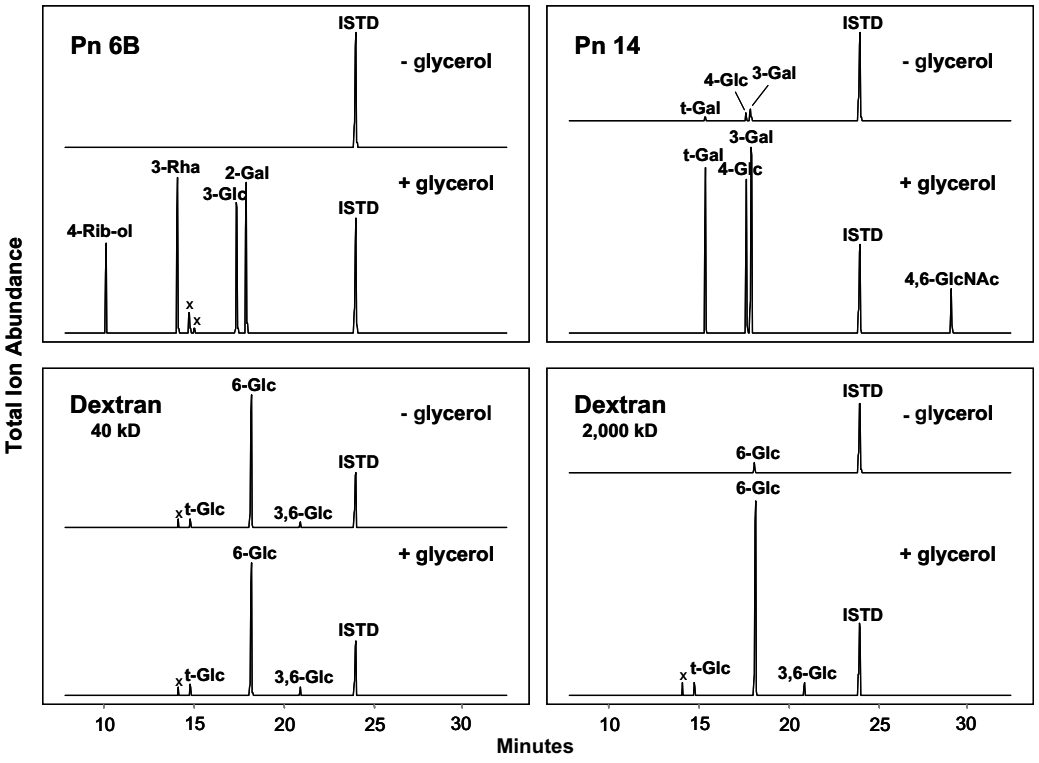

Figure 2. Total-ion chromatograms of PMAAs generated from Pn 6B, Pn 14, and dextran derivatized with (+) and without (−) presolubilization in glycerol. ISTD = internal standard, × = non-PMAA derivative.

Table 2. Relative percent recovery of PMAAs based on total peak areas

Polysaccharide	+Glycerol[a]	−Glycerol[a]
Pn 6B	100	0
Pn 14	100	4.0
Dextran 40 kD	100	85
Dextran 2000 kD	100	4.6

[a] Polysaccharides were treated with (+) or without (−) glycerol prior to methylation.

NaOH–CH_3I method. Similarly Pn 14 and the HMW dextran yielded only marginal recoveries of their respective PMAAs (4.0% and 4.6%, respectively). The LMW dextran, however, could be analyzed by the NaOH–CH_3I method and the glycerol-solubilized method, indicating that presolubilization may not be necessary for linkage analysis of LMW polysaccharides. Even so the LMW dextran PMAA recovery using the NaOH–CH_3I method was slightly lower (85%) relative to the recovery based on the glycerol-solubilized method. All four of the polysaccharide samples that were presolubilized in glycerol could be easily analyzed for monosaccharide linkage regardless of molecular weight or charge differences.

The efficient methylation of HMW polysaccharides has been demonstrated based on subsequent linkage analysis and recovery of PMAAs. The glycerol-solubilized method opens the door for re-evaluation of the methylation procedure for many classes of polysaccharides for which linkage analysis was dismissed due to issues with insolubility. Additional benefits to the field of carbohydrate analysis may also be realized for these polysaccharides by the application of presolubilization-aided derivatization, followed by detailed structural analysis using NMR or MS methods.

1. Experimental

1.1. General methods

Pneumococcal polysaccharides were obtained from American Type Culture Collection (ATCC, Manassas, VA), and dextrans were obtained from Sigma–Aldrich (St. Louis, MO). All chemicals were of analytical grade, and all samples were prepared and derivatized in Teflon screw-cap test tubes. All dryings were achieved using a steady stream of nitrogen at 40 °C. *myo*-Inositol (20 μg) was included in all samples prior to hydrolysis as an internal standard (ISTD).

1.2. Addition of glycerol

For routine analysis, 5 μL of anhyd glycerol was added to 100–300 μL of 1 mg/mL polysaccharide solutions and dried prior to derivatization.

1.3. Preparation of partially methylated alditol acetates (PMAAs)

1.3.1. Preparation of NaOH–Me_2SO slurry. CH_3OH (400 μL) and 50% NaOH (100 μL) were added to a Teflon screw-cap test tube and vortexed until clearly mixed. Me_2SO (2 mL) was added, followed by vortexing and centrifugation at 3500 rpm for 1 min to pellet the NaOH. The supernatant was decanted and the pellet was washed an additional five times with 2 mL of Me_2SO. The final pellet was resuspended in 2 mL of Me_2SO yielding enough slurry for two samples.

1.3.2. Methylation. The following steps were performed quickly in order to minimize introduction of moisture to the samples. To dried samples, with and without presolubilization in glycerol, was added 100 μL of Me_2SO, and the mixture was vortexed until the material was clearly dissolved. NaOH slurry (0.5 mL) was added, followed by vortexing. CH_3I (0.1 mL) was then added, and the samples were vortexed intermittently for 10 min until clear. Samples were blown ~10 min with a steady stream of N_2 to remove excess CH_3I (caution: use fume hood).

1.3.3. Sample cleanup. The samples were partitioned with 2 mL of H_2O and 2 mL of CH_2Cl_2 by vortexing and centrifugation at 3500 rpm for 1 min. The samples were then washed an additional three times with H_2O, and the final organic phase was dried. The methylation and cleanup steps were repeated to ensure complete methylation.

1.3.4. Hydrolysis. CF_3CO_2H (250 μL of a 2 N solution) was added, followed by incubation at 121 °C for 2 h. The samples were dried, followed by two additional dryings with a few drops of CH_3OH.

1.3.5. Reduction. Samples were reduced with 0.1 mL of 10 mg/mL $NaBD_4$ for 1 h at room temperature. The reduction was quenched with one drop of glacial HOAc, and the samples were dried. The samples were then dried an additional three times by addition of a few drops of CH_3OH, followed by evaporation under a stream of N_2.

1.3.6. Acetylation. AC_2O (100 μL) and CF_3CO_2H (80 μL) were added to the samples, followed by a 10 min incubation at 50 °C. Acetone (0.5 mL) was added, and the samples were dried. The samples were dried an additional two times by the addition of a few drops of acetone, followed by evaporation under N_2. The samples were then cleaned up by partition as described above, and the final organic phase was dried. The final samples were solubilized in 2 mL of CH_2CL_2, and 1 μL of sample was injected onto the GC–MS.

1.4. Gas chromatography–mass spectrometry (GC–MS)

The GC–MS parameters have been previously described.[8] Briefly, the system used was an Agilent 6890 gas chromatograph/5973 mass selective detector with a 30 m HP-5 capillary column. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The oven conditions included an initial temperature of 50 °C and an initial time of 2 min, 30 °C/min to 150 °C, 3 °C/min to 220 °C and finally 30 °C/min to 300 °C for a 10 min bakeout. The inlet temperature was kept constant at 250 °C, and the MS transfer line was set at 300 °C. MS acquisition parameters included scanning from m/z 50–550 in the electron impact (EI) mode for routine analysis. Peak assignments were made based on retention times and mass spectra against PMAA standards as generated by the method of Doares et al.[9]

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