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Short Communication

Clean-up procedure for partially methylated alditol acetate derivatives of polysaccharides

David M. Gibeaut and Nicholas C. Carpita*

Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907 (USA)

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ABSTRACT

After derivatization of polysaccharides for glycosyl linkage analysis, considerable contamination is often present. We have devised a simple phase partition system which extracts virtually all phthalate esters and baseline contaminants into a carbon tetrachloride phase, but yields greater than 95% recovery of partially methylated alditol acetate derivatives in a methanol–water (40:60) phase.

INTRODUCTION

Gas chromatography of partially methylated alditol acetate (PMAA) derivatives used in linkage analysis of polysaccharides often requires injection of dilute samples. As a result, a high concentration of contaminants, introduced during the derivatization procedure, compromises chromatograms with low signal-to-noise ratios. In an effort to remove these contaminants, particularly abundant when using *n*-butyllithium in hexane to form the Li^+-methylsulfinylmethanide carbanion, we explored several clean-up and solvent extraction schemes. Carbon tetrachloride is a useful solvent for phthalate esters, known contaminants of methylation procedures [1], and PMAA derivatives of sugars are soluble in methanol. However, carbon tetrachloride and methanol are miscible. Therefore, we sought an immiscible second phase of methanol–water which would still carry the sugar derivatives. We chose a two-phase system of methanol–water (40:60), saturated with carbon tetrachloride. This system separated derivatives from contaminants with greater than 95% recovery of PMAA derivatives in the methanol–water phase after a single extraction. The carbon tetrachloride phase contained phthalate esters of known origin, and unknown contaminants contributing to a high baseline. A portion of the PMAA derivatives of terminal sugars from sucrose and the hexa- and pentaacetate derivatives of neutral sugars were also found in the carbon tetrachloride phase, but good recovery of these could be obtained by adjusting the solvent ratio.

EXPERIMENTAL

Preparation of plant oligo- and polysaccharides

Cell walls from cariocha bean (*Phaseolus vulgaris* L. cv. Cariocha) were prepared from flour made by pulverizing the seeds in a coffee grinder. The flour was homogenized in 50 m*M* N-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES)–NaOH, 30 m*M* ascorbic acid, 1.5% sodium dodecyl sulfate (SDS), pH 7.2, and washed sequentially in SDS buffer followed by water. Starch was removed by three successive extractions with 90% dimethyl sulfoxide

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(DMSO) [2] until the wall preparations were iodine-negative. The chelator-soluble pectins were extracted with trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA) [3], and esterified pectins and some hemicelluloses were extracted under nitrogen atmosphere with 1.0 *M* NaOH, containing 3 mg/ml $NaBH_4$ to prevent end elimination [4]. The majority of the hemicelluloses were extracted under nitrogen atmosphere with 4 *M* NaOH containing 3 mg/ml $NaBH_4$ for 18 h, neutralized with glacial acetic acid, dialyzed against running deionized water, and lyophilized. The 4 *M* NaOH fraction was used in this analysis.

Radioactive cell wall samples of maize seedlings were obtained as described [5]. Radioactive sucrose from ^{14}C-labeled tissue slices from Jerusalem artichoke (*Helianthus tuberosus* L. cv. Bianca) tubers were isolated and characterized as described [6].

Preparation of partially methylated alditol acetate derivatives

Simplified procedures were developed for methylation of polysaccharides with the Li^+-methylsulfinylmethanide carbanion made from *n*-butyllithium and DMSO [7–9]. About 1 mg of carbohydrate and a small stir bar were placed in 15-ml thick-walled glass tubes and stored in a vacuum dessicator over P_2O_5 overnight. The next day, the tubes were sealed with serum sleeve stoppers, evacuated, and 1.0 ml of anhydrous DMSO was added by syringe. The tubes were sonicated for 1 h in a water bath that warmed to 50°C. Nitrogen was introduced by means of a syringe needle with a second needle inserted for escape flow. The solutions were stirred, and 0.5 ml of 2.5 *M* *n*-butyllithium in hexanes was added slowly. After the solution had cleared, but before the color darkened (about 1 h) 0.5 ml of CH_3I was added. Nitrogen flow was stopped and stirring was continued for 1 h in the sealed tubes. Reactions were stopped with 5 ml of water, and the derivatives were extracted two times from the mixture with 1.0 ml chloroform. The chloroform phase was washed five times with water and then dried in 1-dram borosilicate glass vials sealed with PTFE-lined caps.

The partially methylated derivatives from cell wall polysaccharides were hydrolyzed in 1.0 ml 2 *M* trifluoroacetic acid (TFA) at 120°C for 90 min; whereas the derivatives of sucrose were hydrolyzed in 1.0 ml of 1 *M* TFA at 70°C for 30 min to minimize decomposition of the fructofuranosyl units. After hydrolysis, 1.0 ml of *tert.*-butyl alcohol was added and the mixtures were dried under a stream of nitrogen. The partially methylated sugars, L-[U-^{14}C]arabinose and *myo*-[U-^{14}C]inositol were reduced and acetylated as described [8,10]. Fully derivatized sugars were transfered in dichloromethane to 0.5-ml conical vials, and dried with a stream of nitrogen before the clean-up procedure.

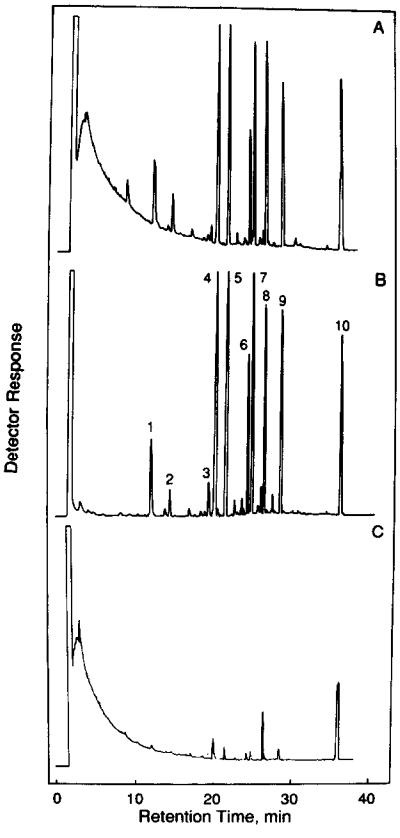

Fig. 1. Flame ionization detector response showing the removal of baseline components in the chromatographic separation of PMAA derivatives of bean cell wall. The sample was divided equally before the clean-up procedure, and samples were injected representing equal quantities of sample. The solvent volumes used for clean-up were 10 µl carbon tetrachloride and 100 µl methanol–water (40:60). (A) Before clean-up. (B) After clean-up. (C) Carbon tetrachloride phase of the clean-up from B. Glycosyl residue: 1 = terminal-arabinose; 2 = terminal-xylose; 3 = terminal-galactose; 4 = 5-arabinose; 5 = 2-xylose + 4-xylose; 6 = 2,5-arabinose; 7 = 4-glucose; 8 = arabinose pentaacetate; 9 = 4,6-glucose; 10 = *myo*-inositol hexaacetate.

Clean-up procedure

Spectrophotometric grade solvents and deionized water (18 MΩ) were used in the extraction procedures. A stock solution was prepared by adding equal volumes of carbon tetrachloride and methanol–water (40:60, v/v) in a reagent bottle. The stock solution was shaken and allowed to separate for several hours. It was necessary to saturate the two solvent phases because a considerable amount of carbon tetrachloride entered the methanol–water phase. Routinely, 10 μl of the carbon tetrachloride phase and 100 μl of the methanol–water phase were added to 0.5 ml-conical vials containing derivatives. The vials were vortex-mixed and then centrifuged at 2 000 *g* for 10 min to quickly separate the two phases. The upper, methanol–water phase containing the derivatives was pipetted into a clean vial and dried with a stream of nitrogen. For determination of extraction efficiency samples of radioactive sugar derivatives were extracted and the two phases were assayed by liquid scintillation spectroscopy.

Gas chromatography

Separations were carried out with a Hewlett-Packard 5840A gas chromatograph. Samples in ethyl acetate were injected in the split mode (split ratio *ca.* 10:1) and detected by flame ionization. The derivatives were separated on a SP-2330 fused-silica capillary column, 30 m × 0.25 mm I.D., 0.20 μm film (Supelco, Bellfonte, PA, USA) temperature programmed from 160 to 210°C at 2°C/min then to 240°C at 5°C/min with a 10-min hold at the upper temperature. The carrier gas was helium at 180 kPa.

RESULTS AND DISCUSSION

Removal of contaminants from PMAA derivatives of plant polysaccharides

A cell wall sample was divided equally after derivatization but before the clean-up procedure to demonstrate the removal of contaminants (Fig. 1). The chromatogram of a sample before clean-up shows an elevated baseline that does not return to the initial level until many of the PMAA derivatives have eluted. However, the cleaned sample recovered from the methanol–water phase shows baseline resolution throughout the chromatogram. The baseline contaminants, phthalate esters (gas chromatography–electron impact mass spectrometry base peak *m/z* 149), and a portion of the *myo*-inositol hexaacetate were partitioned into the carbon tetrachloride phase.

Extraction efficiency

Recovery was quantified with radioactive samples (Table I). Comparison of a first and second

TABLE I

EXTRACTION EFFICIENCY OF A TWO-PHASE, CARBON TETRACHLORIDE/METHANOL–WATER (40:60), CLEAN-UP PROCEDURE FOR THE REMOVAL OF CONTAMINANTS FROM THE DERIVATIZATION PROCEDURE OF PARTIALLY METHYLATED ALDITOL ACETATES

Radioactive samples were extracted and assayed as described in the Experimental section. Alditol acetate derivatives of arabinose and inositol do not require clean-up, but demonstrate selective partitioning. PMAA derivatives of terminal glucose and fructose from sucrose are not as well recovered as derivatives of linked sugars.

	Solvent ratio carbon tetrachloride–methanol[a]	Recovery (%)			
		Maize cell wall (PMAA)	Arabinose (pentaacetate)	Inositol (hexaacetate)	Sucrose (PMAA)
First extraction[b]	(10:100)	94.6	49.2	73.8	77.1
Second extraction[b]	(10:100)	95.4	52.0	77.7	79.9
One extraction[c]	(5:100)	97.8	70.7	84.0	89.3
One extraction[d]	(5:500)	99.6	93.0	96.4	98.5

[a] Volumes in μl.

[b] Derivatized samples were extracted ("first extraction") and the phases separated; then the methanol–water phase was re-extracted ("second extraction") with additional carbon tetrachloride.

[c,d] Equivalent samples were extracted once with different volumes of solvents.

extraction demonstrated that derivatives were recovered consistently between extraction in all samples and that loss into the carbon tetrachloride phase was not from breakdown products of the sugars. Recovery of PMAA derivatives of cell walls was quite high with each extraction. However, with a 10:100 solvent ratio there was a greater loss of both fully acetylated sugars and PMAA derivatives of terminal sugars from sucrose. We then found that the ratio of volumes was important to improving recovery. By reducing the volume of the carbon tetrachloride phase and increasing the volume of methanol–water, we showed that all derivatives could be recovered with good efficiency. Of course, rather than increasing the volume of the methanol–water phase, re-extraction of the carbon tetrachloride phase with fresh methanol–water would yield equal results with less volume of methanol–water.

This clean-up procedure is rapid and can be accomplished with minimal selective loss of PMAA derivatives. After clean-up, baseline resolution is achieved throughout the chromatogram and elimination of contaminants should greatly increase column life.

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REFERENCES

1 R. J. Henry, P. J. Harris, A. B. Blakeney and B. A. Stone, *J. Chromatogr.*, 262 (1983) 249.
2 N. C. Carpita and J. Kanabus, *Anal. Biochem.*, 161 (1987) 132.
3 M. C. Jarvis, *Plant Cell Environment*, 7 (1984) 153.
4 G. O. Aspinall, C. T. Greenwood and R. J. Sturgeon, *J. Chem. Soc.*, (1961) 3667.
5 D. M. Gibeaut and N. C. Carpita, *Plant Physiol.*, 97 (1991) 551.
6 N. C. Carpita, F. Keller, D. M. Gibeaut, T. L. Housley and Ph. Matile, *J. Plant Physiol.*, 138 (1991) 204.
7 A. L. Kverheim, *Acta Chem. Scand., Ser B*, 41 (1987) 150.
8 N. C. Carpita and E. M. Shea, in C. J. Bierman and G. A. MacGinnis (Editors), *Analysis of Carbohydrates by GLC and MS*, CRC Press, Boca Raton, FL, 1989, p. 155.
9 E. M. Shea, D. M. Gibeaut and N. C. Carpita, *Planta*, 179 (1989) 293.
10 A. B. Blakeney, P. J. Harris, R. J. Henry and B. A. Stone, *Carbohydr. Res.*, 113 (1983) 291.