Appl Biochem Biotechnol (2008) 148:271–276
DOI 10.1007/s12010-007-8044-8

Permethylation Linkage Analysis Techniques for Residual Carbohydrates

Neil P. J. Price

Received: 23 May 2007 / Accepted: 4 September 2007 /
Published online: 2 October 2007

Abstract Permethylation analysis is the classic approach to establishing the position of glycosidic linkages between sugar residues. Typically, the carbohydrate is derivatized to form acid-stable methyl ethers, hydrolyzed, peracetylated, and analyzed by gas chromatography-mass spectrometry. The position of glycosidic linkages in the starting carbohydrate are apparent from the mass spectra as determined by the location of acetyl residues. The completeness of permethylation is dependent upon the choice of base catalyst and is readily confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry mass spectrometry. For the permethylation of β-cyclodextrin, Hakomori dimsyl base is shown to be superior to the NaOH–dimethyl sulfoxide system, and the use of the latter resulted in selective under-methylation of the 3-hydroxy groups. These techniques are highly applicable to residual carbohydrates from biofuel processes.

Keywords Carbohydrate · Linkage analysis · Permethylation · Mass spectrometry

Introduction

Permethylation analysis of sugars is a useful technique for establishing the position of glycosidic linkages (Fig. 1) [1–3]. Free hydroxyl groups on the carbohydrate under study are initially converted to methyl ethers by application of a strongly based-catalyzed Williamson ether synthesis. Because this reaction is sensitive to the presence of water, the permethylation reaction is generally undertaken in a dry solvent such as dimethyl sulfoxide (DMSO) or dimethylformamide. The methylating reagent itself is usually methyl iodide or dimethyl sulfate, although the use of the latter is generally discouraged because of the high toxicity.

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N. P. J. Price (✉)
USDA-ARS-NCAUR, Bioproducts & Biocatalysis Research Unit, 1815 North University Street, Peoria, IL 61604, USA
e-mail: neil.price@ars.usda.gov

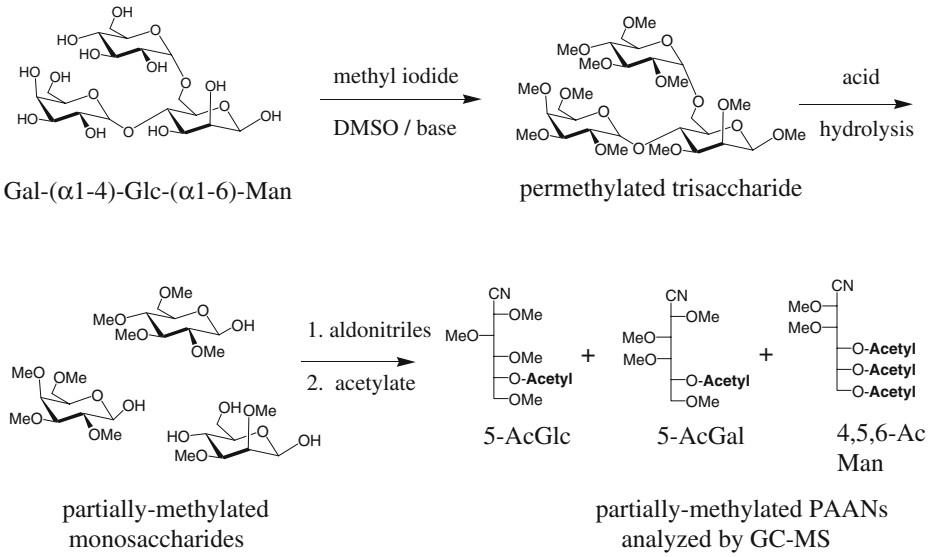

Fig. 1 Schematic for the permethylation linkage analysis, exemplified by a hypothetical trisaccharide, Gal-(α-1,4)-Glc-(α-1,6)-Man. The acid-hydrolyzed partially methylated monosaccharides generated are analyzed by GC-MS, typically as alditol or aldononitrile derivatives

Complete methylation (termed, "per"methylation) is essential to achieving good linkage analysis results because under-methylation of the hydroxyl groups can lead to linkage artifacts. Two factors are important here; firstly, the choice of base catalyst, and secondly, to ensure good solubility and miscibility of the carbohydrate and methylation reagent [4–9]. DMSO is generally the solvent of choice because it may be commercially acquired and stored water-free and has a high solvation for most oligosaccharides and polysaccharides. In addition, those polysaccharides that are insoluble can sometimes be analyzed as a fine suspension in DMSO. The use of DMSO as solvent has also specified the choice of either sodium hydride of dry sodium hydroxide as the base catalyst [4, 5]. Sodium hydride has the advantage that it actually reacts with the DMSO solvent to form its anion, methyl sulfinyl carbanion, also called the dimsyl ion, first reported by Corey and Chaykovsky [10]. Dimsyl base is highly suited to ether preparation, but is difficult to handle and must be stored under oil. This led to the introduction of suspended sodium hydroxide as base catalyst [4]. However, sodium hydroxide is hygroscopic, and because it is not soluble in DMSO, it must be ground up to form a suspension. This can result in non-miscible methylation conditions, especially with DMSO-insoluble polysaccharides, and may therefore result in under-methylation.

In this paper, we have compared two commonly used permethylation methods [4, 5] and have applied them to a model carbohydrate compound. The completeness of the methylation steps was established by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The methylated carbohydrate products were acid-hydrolyzed and converted to methylated partially acetylated aldononitrile derivatives (Me PAANs) for linkage analysis by gas chromatography-mass spectrometry (GC-MS) [11].

Methods

All reagents were obtained commercially from Sigma-Aldrich, St. Louis. Permethylations were undertaken as described, using either NaOH–DMSO suspension [4] or dimsyl base [5] as the catalyst. The acid hydrolysis (2.0 M trifluoroacetic acid) was undertaken at 121 °C, and aldononitrile acetates were prepared as described [11]. GC-MS analyses of the derivatized samples employed an Agilent 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an HP 7683 autoinjector interfaced with an HP 5973 series mass spectrometer configured in electron impact mode. Chromatography was accomplished with a capillary HP-1 column (25 m long; 0.2-mm diameter) using helium as the carrier gas at a flow rate of 0.8 ml/min. The oven temperature was ramped over a linear gradient from 150 to 250 °C at 4 °C/min. Mass spectra were recorded in positive-ion mode over the range 50 to 500 m/z. MALDI-TOF MS spectra were obtained on a Bruker–Daltonic Omniflex instrument (Bruker, Billerica, MA, USA) operating in reflectron mode. Samples were dried under a lamp on a conventional 49-place stainless steel target. The matrix used was 2,5-dihydroxybenzoic acid. A 200-ns pulsed ion extraction was used, with matrix suppression up to 200 Da. Excitation was at 337.1 nm, typically at 60% of a 150-μJ maximum output, and 80 shots were accumulated. Ion sources 1 and 2 were 19.0 and 14.0 kV, with lens and reflector voltages of 9.20 and 20.00 kV, respectively.

Results

A comparison study was undertaken of two bases commonly used for the permethylation analysis of sugars [4, 5]. A model carbohydrate, β-cyclodextrin (β-CD) was chosen because it contains a single type of sugar residue, β-1,4-linked glucose and because of its comparatively poor solubility in DMSO [12, 13]. β-CD was dissolved in DMSO and methylated using methyl iodide, essentially as described by Hakomori (using dimsyl base [5]) or Kerek and Ciucanu (using NaOH base [4]). At the end of the reactions, the methylated β-CDs were extracted into hexane and split into three aliquots. Two of these aliquots were analyzed by either MALDI-TOF mass spectrometry or thin layer chromatography (TLC) to determine the completeness of methylation (Fig. 2). The third aliquots were acid-hydrolyzed to their component sugar residues, converted to the corresponding partially methylated aldononitriles (PMANs), and analyzed by GC-MS (Fig. 3). This was undertaken to confirm that the linkages present for the β-CD were correctly identified and also to ascertain the positions of under-methylated hydroxyl groups.

The dimsyl base was seen to be highly effective for the permethylation of β-CD. A molecular $[M+Na]^+$ adduct ion was observed at m/z 1451, which corresponds to the molecular mass of β-CD with all of the 21 free hydroxyls replaced by methyl groups (Fig. 2). Except for a single $[M-14+Na]^+$ at m/z 1437, there was little evidence for partially methylated β-CDs. Moreover, the fully methylated β-CD was also observed as a single band by the TLC analysis (Fig. 2). By contrast, MALDI-MS analysis of the NaOH-catalyzed reaction showed a series of ions incrementally 14 mass units less than the fully methylated m/z 1451 molecular ion. This ion series is evidence of a mixture of under-methylated β-CDs. Hence, under-methylated ions are observed at m/z 1437, 1423, 1409, etc. (Fig. 2) where the observed 14 mass unit differentials are due to the mass difference between –H and a $–CH_3$. Furthermore, under-methylated β-CDs formed under the NaOH-catalyzed conditions are also apparent as a less motile "ladder" when analyzed by TLC (Fig. 2).

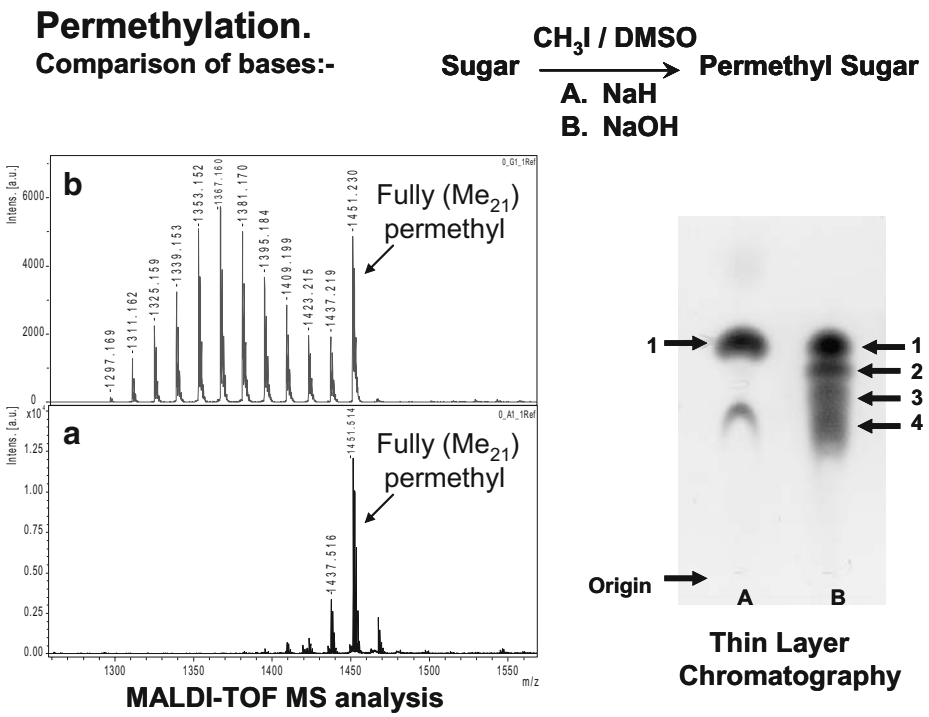

Fig. 2 Comparison of the effectiveness of the base catalysts used in permethylation of β-cyclodextrin. The catalyst used was Hakomori dimsyl base (**a**) or NaOH–DMSO suspension (**b**). The analysis was by MALDI-TOF MS (*left*) and thin layer chromatography (*right*)

To ascertain the positions of the under-methylated hydroxyl groups and also to confirm the 1,4-linkages of β-CD, the methylation products from the above reactions were acid-hydrolyzed, converted to aldononitrile acetates, and analyzed by GC-MS (Fig. 3). The dimsyl-catalyzed reaction gave rise to a single chromatographic peak, which, in the mass spectrometer, was identified as due to 2,3,6-trimethyl-4-*O*-acetyl-glucose. Because the position of *O*-acetyl groups corresponds to the original linkage position, the 2,3,6-trimethyl-4-*O*-acetyl-glucose peak (peak 1, Fig. 3) confirmed that the only linkage present in the original β-CD is the expected 1,4. Other glycosidic linkages are absent, as evident from the lack of other GC peaks. Moreover, the lack of other GC peaks also shows that the original β-CD sample was fully methylated under dimsyl-catalyzed conditions. By contrast, two peaks were observed for the NaOH-catalyzed reaction corresponding to 2,3,6-trimethyl-4-*O*-acetyl-glucose peak (peak 1) plus 2,6-dimethyl-3,4-di-*O*-acetyl-glucose (peak 2, Fig. 3). Integration results indicates peak 1/peak 2 ratio of ~3:1. The more intense peak 1 confirmed the expected 1,4-linkage, and also indicates ~66% of the β-CD was in fact fully methylated. However, the 3,4-di-*O*-acetylated derivative (peak 2) shows that 33% under-methylation of β-CD occurs with NaOH–DMSO. Furthermore, the under-methylated hydroxy groups all occur selectively at the 3-position, with no evidence of under-methylation at the 6-hydroxyl or 2-hydroxyl groups. Importantly, when taken together, the MALDI-TOF MS data (Fig. 2) and GC-MS data (Fig. 3) indicate that the under-methylated β-CDs occur as a complex mixture in which

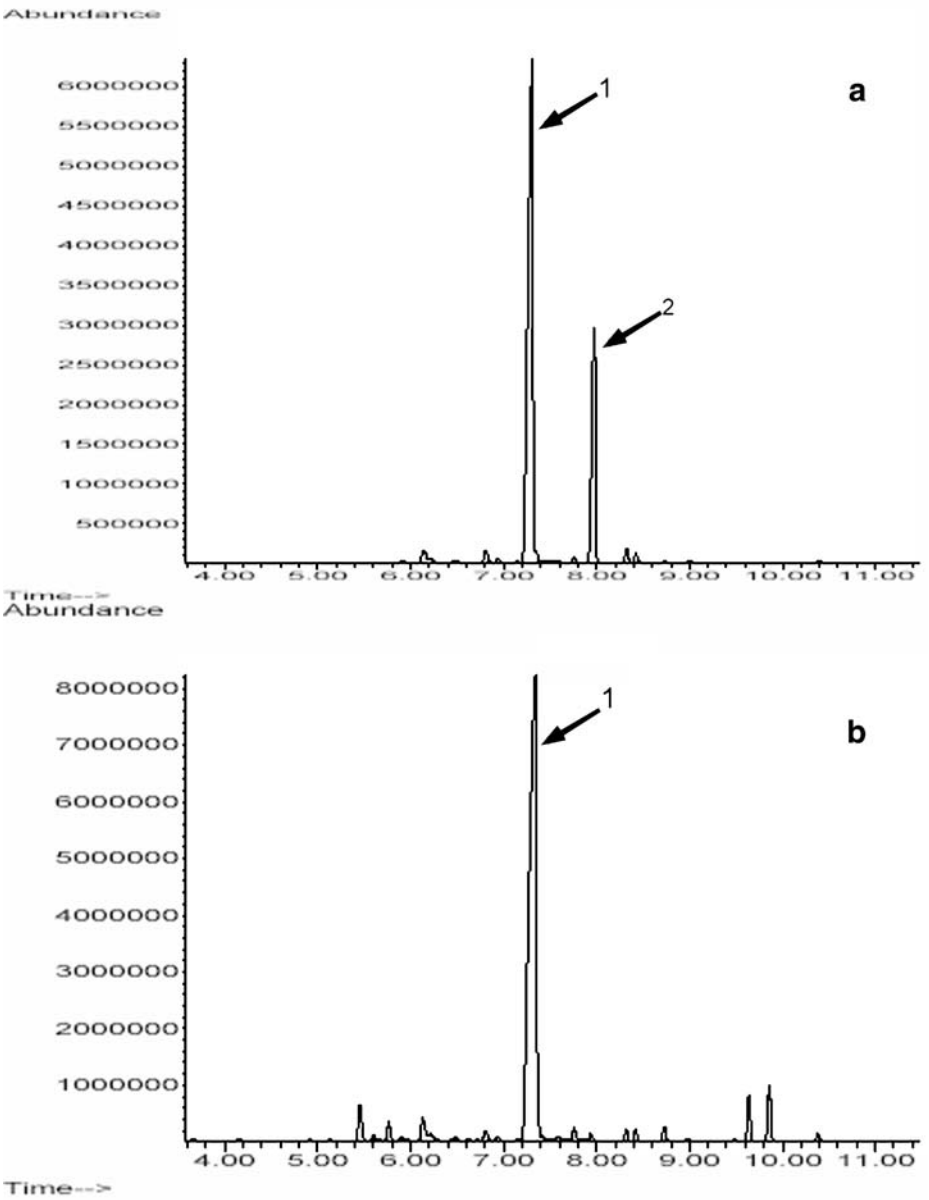

Fig. 3 GC-MS traces of PMANs derived from permethylation of β-cyclodextrin. **a** Using NaOH–DMSO base; **b** using dimsyl base. Peak 1 is due to 2,3,6-trimethyl-4-*O*-acetyl-glucose, and peak 2 to 2,6-dimethyl-3,4-di-*O*-acetyl-glucose. The latter peak is evidence of under-methylation of the β-cyclodextrin at position 3 of each glucose residue

the 3-*O*-methyl groups may be absent from one, two, three, or more of the seven glucose residues of the starting β-CD. Hence, after GC-MS, the under-3-*O*-methylated glucose residues of the mixture of partially methylated β-CD give rise to peak 2, whereas the remaining fully methylated Glc residues are identified by GC peak 1.

Discussion

This paper discussed some issues involved in permethylation linkage analysis of carbohydrates and highlights the importance of solvation of the sample and reactant and the choice of base catalyst. Complete permethylation is shown to be essential to obtaining confident results. For the example carbohydrate, β-CD, it was shown that the Hakomori dimsyl reagent is a superior base catalyst than NaOH–DMSO suspension. Under the latter condition, under-methylation of β-CD occurs selectively at 3-hydroxy groups, which might be interpreted as artifactual 3-*O*-linkages. This may occur because the 3-hydroxy groups are buried within the relatively hydrophobic torus of β-CD where they are excluded from deprotonation by the NaOH base [14]. Consistent with this, maltoheptoase, a linear form of β-CD, is permethylated equally well using either dimsyl or NaOH, and several mannose oligosaccharides are more completely permethylated using NaOH–DMSO (data not shown). The conclusion is that permethylation conditions are not universally applicable to all carbohydrate types, and it is therefore recommended that the completeness of permethylation of carbohydrate samples be checked by MALDI-TOF MS analysis before the acid hydrolysis step. Under-methylation can then be corrected either by changing the base-catalyst or by exhaustive re-methylation of the sample.

Acknowledgment Jim Nicholson and Trina Hartman (NCAUR-ARS-USDA) are both thanked for their technical assistance.

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