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The analysis of agarose by the reductive cleavage method

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ABSTRACT

Agarose was structurally characterised by permethylation and subsequent reductive cleavage. Treatment of the fully methylated polysaccharide with triethylsilane and a mixture of trimethylsilyl methanesulfonate and boron trifluoride etherate followed by a newly developed, acid-catalysed, in situ acetylation yielded two main products: the expected 4-*O*-acetyl-1,5:3,6-dianhydro-2-*O*-methyl-L-galactitol and 3-*O*-acetyl-1,5-anhydro-2,4,6-tri-*O*-methyl-D-galactitol in the molar ratio 1:1, and traces of 1,5-anhydro-2,3,4,6-tetra-*O*-methyl-D-galactitol. Reductive cleavage using triethylsilane and trimethylsilyl trifluoromethanesulfonate as the catalyst yielded the same anhydroalditols as well as a smaller amount of 1,4,5-tri-*O*-acetyl-3,6-anhydro-2-*O*-methyl-L-galactitol due to ring-opening of 3,6-anhydrogalactopyranosyl residues during reductive cleavage. In this paper, results from reductive cleavage are compared with results using standard methylation analysis.

INTRODUCTION

Standard methylation analysis[1], involving permethylation of a polysaccharide, hydrolysis, and subsequent acetylation to form partially methylated alditol acetates which are analysed by GLC/GLC–MS, is a widely used method for the structural characterisation of polysaccharides. However, this standard methylation analysis procedure is not applicable for red-algal polysaccharides because they contain up to 50% of 3,6-anhydrogalactosyl residues, a very acid-labile sugar that is rapidly destroyed under the harsh acidic conditions commonly used.

Several attempts have been made to obtain quantitative information about the 3,6-anhydrogalactosyl residues during acid-catalysed cleavage by application of methanolysis[2], mercaptolysis[3], formolysis[4], or bromine oxidation[5]. However, none of these techniques yielded satisfactory results for the analysis of red-algal polysaccharides.

Recent reports of the greater stability of the 3,6-anhydrogalactose under mild acidic conditions[2,6] led to the development of more suitable procedures: the "double hydrolysis–reduction" method[7] and the "reductive hydrolysis"[7] method. The "double hydrolysis–reduction" method employs a preliminary mild hydrolysis for various lengths of time, subsequent reduction of the liberated 3,6-anhydrogalactosyl residues, and a second hydrolysis step in order to fully hydrolyse the polysaccharide. The results showed that conditions for the preliminary hydrolysis must be optimised for each polysaccharide. The "reductive hydrolysis" method uses the acid-stable 4-methylmorpholine–borane to reduce the 3,6-anhydrogalactose end-groups as they are released during acid hydrolysis. Compared to earlier methods, these methods are an improvement for the structural characterisation of carrageenans and agars.

With the introduction of the reductive cleavage method for structural studies of polysaccharides[8], some of the disadvantages of standard methylation analysis could be circumvented.

Recently, we described the application of the reductive cleavage method to di- and poly-saccharides containing acid-labile pentosyl residues[9,10]. Encouraged by these studies and other investigations concerning the structural characterisation of polysaccharides bearing acyl substituents[11,12] or pyruvic acid acetals[13,14], we envisioned that this method might be applicable to polysaccharides containing acid-labile 3,6-anhydrogalactosyl residues directly after permethylation. In this study, we describe its successful application to permethylated agarose.

EXPERIMENTAL

General.—Commercial agarose (Merck, Darmstadt) was used without further purification.

Methylation was carried out as described by Ciucanu and Kerek[15]. Partially methylated alditol acetates were produced by hydrolysis (2 M CF_3CO_2H, 120°C) for various times, reduction ($NaBD_4$), and acetylation (Ac_2O–*N*-methylimidazole) of the permethylated polysaccharide, performed as described[16].

Reductive cleavage of permethylated material was performed as described by Gray and co-workers[17] with the exception that, in reactions catalysed by $Me_3SiOSO_2Me–BF_3 \cdot Et_2O$, subsequent acetylation was performed in situ with a mixture of $CF_3CO_2H–Ac_2O$ (1:10, 50°C, 15 min).

GLC was performed on-column with a Carlo Erba 5160 gas chromatograph equipped with a flame-ionisation detector, a Shimadzu C-R3A integrator, and a J&W Scientific DB-5 fused-silica capillary column (0.25 mm × 30 m; film thickness, 0.25 μm). The temperature program used was as follows: after 3 min at 70°C, increase to 120°C at a rate of 20°C/min. Following 5 min at 120°C, increase to 300°C at a rate of 5°C/min. The integral values of all peaks in GLC were corrected by relative molar response factors, calculated by the effective-carbon-response (e.c.r.) method[18] which has been shown[19] to be applicable to anhydroalditols.

GLC–MS analysis was performed with a VG 70-250 SE mass spectrometer and a Hewlett–Packard model 5890 gas chromatograph, equipped with a 30-m J&W

Scientific DB-5 fused-silica column. Column effluents were analysed by electron-impact mass-spectrometry (GLC–EIMS) and by chemical-ionisation mass spectrometry (GLC–CIMS) with ammonia as the reagent gas, wherein characteristic $(M + H)^+$ and $(M + NH_4)^+$ ions were detected.

HPLC was performed with a Waters 590 HPLC-pump and a Knauer differential refractometer equipped with a Lichrosorb RP-18 column (4 × 240 mm). The column was eluted with 2:8 MeOH–water at a flow rate of 1 mL/min.

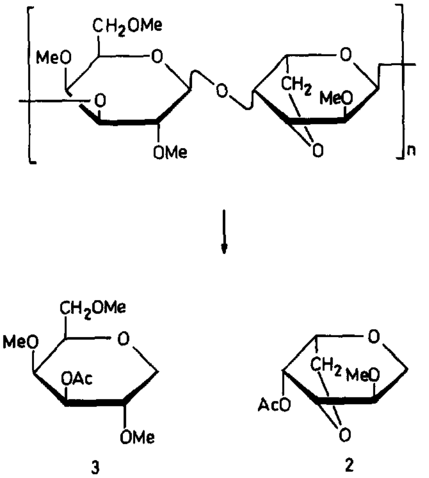
NMR spectra were recorded with a Bruker AMX-400 spectrometer. ^{1}H NMR spectra were recorded at 400 MHz with $CDCl_3$ as solvent and tetramethylsilane as internal reference; ^{13}C NMR spectra were recorded at 100 MHz with $CDCl_3$ as solvent and tetramethylsilane as internal reference.

*4-O-Acetyl-1,5 : 3,6-dianhydro-2-O-methyl-*L*-galactitol* (**2**).—Per-*O*-methylated agarose (10 mg) was reductively cleaved for 3 h in the presence of 5 equiv of Et_3SiH, 1 equiv of $BF_3 \cdot Et_2O$, and 5 equiv of Me_3SiOSO_2Me per equiv of acetal, and the products were acetylated in situ. Compound **2** was isolated by reversed-phase HPLC. ^{1}H NMR: δ 2.07 (s, 3 H, AcO), 3.41 (s, 3 H, MeO), 3.52 (dd, 1 H, $J_{2,3}$ 5.0 Hz, H-2), 3.74 (d, 1 H, H-1b), 3.77 (dd, 1 H, $J_{1a,1b}$ 13.0 Hz, H-1a), 3.98 (dd, 1 H, H-6*exo*), 4.18 (d, 1 H, $J_{6endo,6exo}$ 10.01 Hz, H-6*endo*), 4.38 (dd, 1 H, $J_{5,6exo}$ 3.0 Hz, H-5), 4.54 (d, 1 H, H-3), 5.35 (d, 1 H, $J_{4,5}$ 2.0 Hz, H-4); ^{13}C NMR: δ 21.0 (C-2 of acetyl), 57.3 (methoxy), 62.5, 68.7, 74.1, 76.5, 77.3, 79.3, (C-6, C-1, C-2, C-4, C-3, C-5), and 169.7 (C-1 of acetyl); GLC–CIMS (NH_3, positive): *m/z* 203(8), 220(100); GLC–EIMS: *m/z* 43(100), 45(21), 55(11), 58(27), 59(12), 69(99), 71(13), 73(12), 74(16), 82(11), 84(31), 85(32), 99(16), 100(11), 102(75), 111(15), 112(13), 129(25), 142(13), 159(19), 160(52), 202(12).

RESULTS AND DISCUSSION

Reductive cleavage analysis.—Agarose is a neutral, linear molecule consisting of repeating units of the disaccharide agarobiose, which is composed of alternating units of 4-linked 3,6-anhydro-α-L-galactopyranose and 3-linked β-D-galactopyranose[20].

The structures of permethylated agarose and of its expected reductive cleavage products are shown in Scheme 1. Reductive cleavage of permethylated agarose was carried out in the presence of 5 equiv of triethylsilane (Et_3SiH) as the reducing agent and either a mixture consisting of 5 equiv of trimethylsilyl methanesulfonate (Me_3SiOSO_2Me) and 1 equiv of boron trifluoride etherate ($BF_3 \cdot Et_2O$) or 5 equiv of trimethylsilyl trifluoromethanesulfonate ($Me_3SiOSO_2CF_3$) followed by in situ acetylation. Normally, after reductive cleavage with BF_3-containing catalysts, quenching with methanol, deionisation with mixed-bed ion-exchange resin, and evaporation is required before base-catalysed acetylation. However, we observed that this additional, time-consuming procedure could result in the discrimination of higher substituted derivatives, probably through the interaction of more acidic alditol borate esters with the ion-exchanger. In addition, evaporation of the



Scheme 1.

methanol mixture is critical for more volatile derivatives. We observed that less careful evaporation leads to losses of terminal, permethylated derivatives. As borate complexes do not form under acidic conditions, we tried to overcome these problems by developing an acid-catalysed acetylation. Harris et al.[21] recommended perchloric acid in ethyl acetate for quantitative acetylation of partially methylated alditol acetates. We found that a modified acid-catalysed acetylation procedure of Garegg et al.[22], using CF_3CO_2H–Ac_2O (1 : 10, 50°C, 15 min), is suitable for in situ acetylation of partially methylated anhydroalditols in BF_3-containing solutions. The mixture of products was analysed by GLC combined with chemical-ionisation mass spectrometry (CIMS) and electron-ionisation mass spectrometry (EIMS).

Reductive cleavage with Et_3SiH and Me_3SiOSO_2Me–$BF_3 \cdot Et_2O$.—The gas–liquid chromatogram obtained when permethylated agarose was subjected to reductive cleavage in the presence of Et_3SiH and a combination of Me_3SiOSO_2Me and $BF_3 \cdot Et_2O$ is shown in Fig. 1. Reductive cleavage was complete after 3 h and two main products could be identified. Peak 2 was identified as 4-*O*-acetyl-1,5 : 3,6-dianhydro-2-*O*-methyl-L-galactitol (**2**) derived from the 4-linked 3,6-anhydro-L-galactopyranosyl residues. Its CI mass spectrum (see Experimental section) indicated, as expected, a molecular mass of 202. The EI mass spectrum (see Experimental section) of this component showed a molecular ion at *m/z* 202 and fragment ions at *m/z* 160 and 142, due to loss of ketene or acetic acid, respectively. The occurrence of the secondary fragment ion with *m/z* 69 is characteristic for substances having a 3,6-anhydro-ring system[23]. In order to confirm the structure of **2**, reductive cleavage was carried out using larger amounts of permethylated agarose (10 mg), followed by in situ acetylation. The resulting anhydroalditols were separated using reversed-phase HPLC (Fig. 2). The ^1H NMR spectrum of **2** displayed typical resonances for the acetoxyl group at δ 2.07 (s, 3 H), the methoxyl group δ 3.41 (s, 3 H), and the expected number of ring-hydrogen

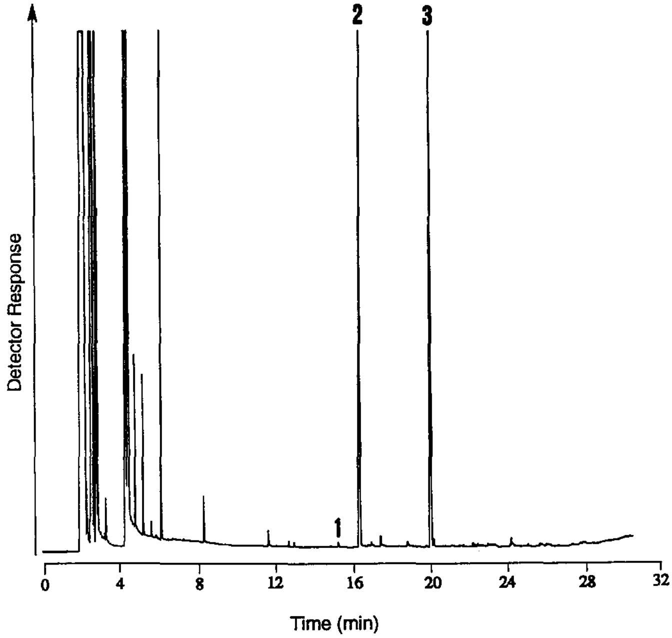


Fig. 1. Gas–liquid chromatogram of the partially methylated anhydroalditol acetates derived by $Me_3SiOSO_2Me-BF_3 \cdot Et_2O$-catalysed reductive cleavage of per-*O*-methylated agarose. Numbering of peaks corresponds to compound designations described in the text. Unnumbered peaks were present in a reagent-only control.

atoms (see Experimental section). The ^{13}C NMR data (see Experimental section) are also consistent with the proposed structure.

The CI mass spectrum of the other main product, peak 3, revealed that its molecular mass was 248 [$(M + NH_4)^+ = m/z$ 266]. Upon comparing its EI mass spectrum with published spectra[24], it could be concluded that peak 3 is 3-*O*-acetyl-1,5-anhydro-2,4,6-tri-*O*-methyl-D-galactitol (**3**). In addition to the major components **2** and **3**, a minor component eluted initially was observed. Its GLC retention time in combination with its CI or EIMS data identified this peak as 1,5-anhydro-2,3,4,6-tetra-*O*-methyl-D-galactitol (**1**) arising from terminal nonreducing galactopyranosyl groups.

From the corrected peak areas, a molar ratio of ca. 1:1 was calculated,for **2** and **3**. This result is in agreement with the proposed structure of agarose.

Reductive cleavage with Et_3SiH and $Me_3SiOSO_2CF_3$.—When reductive cleavage was carried out with $Me_3SiOSO_2CF_3$ as the catalyst, small amounts of a fourth peak were observed (Fig. 3). Peak 4 exhibited in CIMS a molecular mass of 304 [$(M + NH_4)^+ = m/z$ 322], which can only correspond to that of a tri-*O*-acetyl-mono-*O*-methyl-anhydrogalactitol. Surprisingly, its EI mass spectrum was consistent[25] with the EI mass spectrum of 1,4,5-tri-*O*-acetyl-3,6-anhydro-2-*O*-methyl-L-galactitol (**4**). This derivative is usually formed from 4-*O*-linked 3,6-anhydro-L-galactopyranosyl residues in standard methylation analysis. We concluded that the

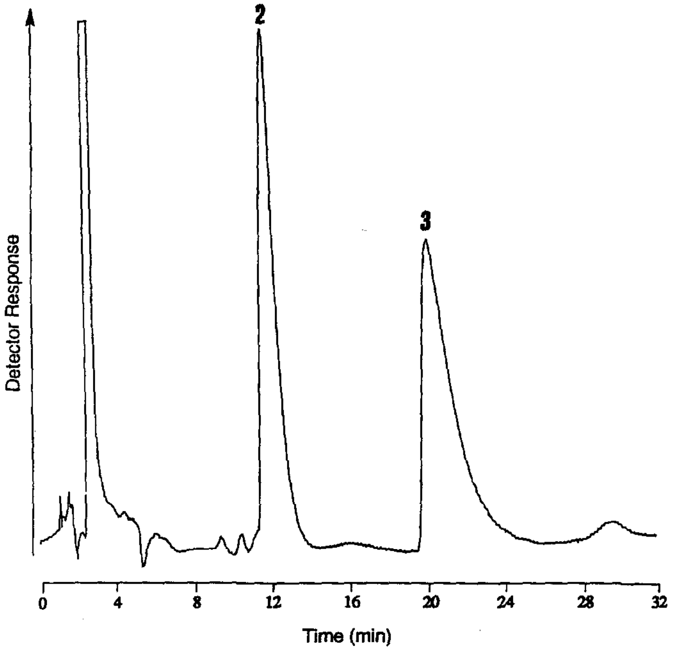
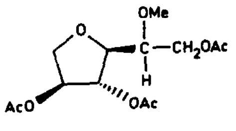


Fig. 2. High-performance liquid chromatogram of the partially methylated anhydroalditol acetates derived by reductive cleavage of per-*O*-methylated agarose. Numbering of peaks corresponds to compound designations described in the text.

occurrence of **4** is due to ring-opening during reductive cleavage with $Me_3SiOSO_2CF_3$. In fact, integration of the GLC profile and correction for molar response showed that the molar ratio of **2** to **3** had decreased, but the combined amounts of **2** + **4** to **3** was again 1:1 (see Table I).

The fact that **4** was not formed when reductive cleavage was performed with $Me_3SiOSO_2Me–BF_3 \cdot Et_2O$ led us to assume that its formation is artifactual.



4

Standard methylation analysis.—As discussed in the introduction, a major problem with conventional methylation analysis applied to red-algal polysaccharides is the degradation of methylated 3,6-anhydrogalactosyl residues. In order to demonstrate the advantage of the reductive cleavage method for the structural characterisation of agarose, we subjected the permethylated agarose to standard methylation analysis.

Portions of per-*O*-methylated polysaccharide were hydrolysed for different times. The resulting monomers were subjected to reduction ($NaBD_4$) and acetyla-

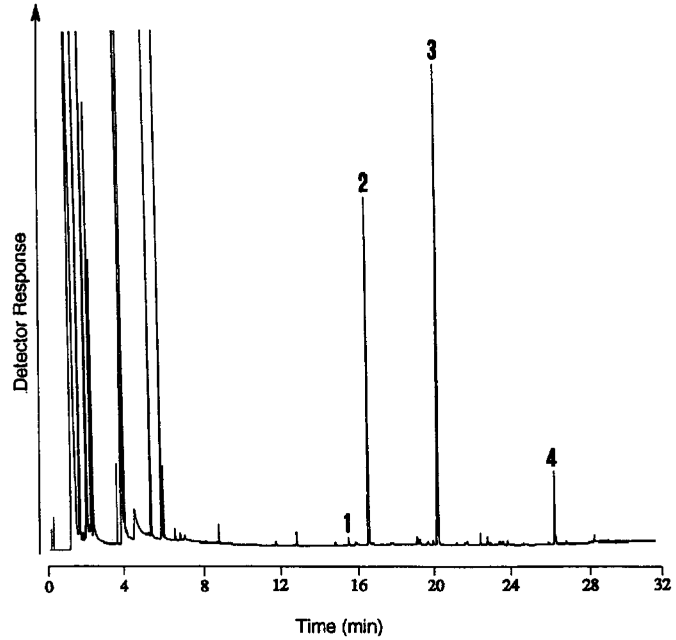


Fig. 3. Gas–liquid chromatogramm of the partially methylated anhydroalditol acetates derived by $Me_3SiOSO_2CF_3$-catalysed reductive cleavage of per-*O*-methylated agarose. Numbering of peaks corresponds to compound designations described in the text. Unnumbered peaks were present in a reagent-only control.

tion. Analysis of the resulting mixture of partially methylated alditol acetates by GLC–MS (CI and EI) revealed three components. The 1,4-linked 3,6-anhydro-L-galactosyl residue was converted into the expected 1,4,5-tri-*O*-acetyl-3,6-anhydro-2-*O*-methyl-L-galactitol derivative (**4**). Another component, identified as 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-galactitol (**5**), represents the 1,3-linked D-galactosyl residues of the repeating disaccharide units. Traces of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol (**6**) arising from nonreducing terminal D-galactosyl groups were also observed. The peak areas were integrated and corrected for molar response (single data not shown). Evident in the plot of the molar ratio of liberated 4-linked 3,6-anhydrogalactosyl residues to 3-linked galactosyl residues against time of hydrolysis (Fig. 4) is the rapid decrease of this ratio, calculated from the molar

TABLE I

Molar ratio of products (compounds **1**–**4**) derived by reductive cleavage of permethylated agarose

Catalyst	Mole fraction				Ratio of 1,4-linked to 1,3-linked residues *
	1	**2**	**3**	**4**	
$Me_3SiOSO_2Me–BF_3 \cdot Et_2O$	1.0	50.2	48.8		1.03:1
$Me_3SiOSO_2CF_3$	1.1	46.4	49.5	3.0	0.99:1

* Calculated from the ratio of compound **2** to **3** or compound **2**+**4** to **3**, respectively.

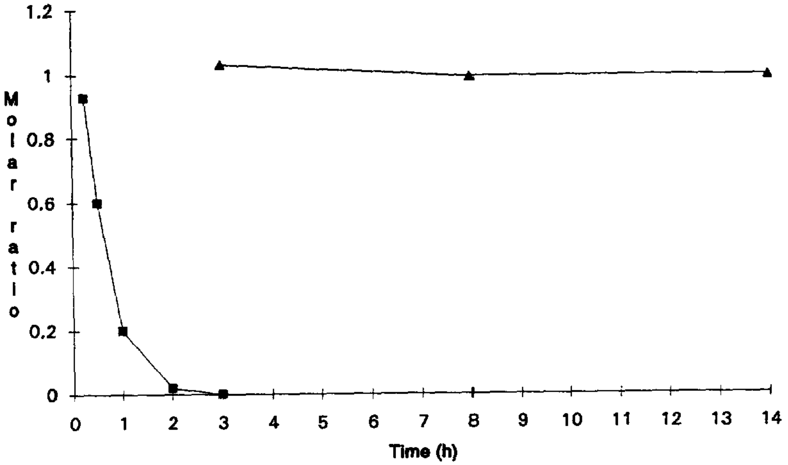


Fig. 4. Molar ratio of the 4-linked 3,6-anhydrogalactosyl residues to 3-linked galactosyl residues of permethylated agarose, calculated from the molar ratios of **2** to **3** from reductive cleavage with $Me_3SiOSO_2Me–BF_3 \cdot Et_2O$ (▲ —— ▲) or of **4** to **5** from hydrolysis (■ —— ■).

ratio of **4** to **5**, with increasing time of hydrolysis. However, permethylated agarose was fully reductively cleaved after 3 h and the molar ratio of the derivatives **2** and **3** did not change, within experimental error, with prolonged reaction time. Because of the rapid degradation of the liberated 3,6-anhydrogalactosyl residues immediately from the beginning of their release during hydrolysis, some authors have determined the molar ratio of **4** to **5** expected for a polysaccharide that is based on agarobiose repeating units by linear regression of the individual data points and extrapolation[4,26,27].

We conclude from this study that reductive cleavage, especially when it is carried out with $Me_3SiOSO_2Me–BF_3 \cdot Et_2O$ as the catalyst, will serve as a useful, complementary procedure in the structural characterisation of agarose and other red-algal polysaccharides containing 3,6-anhydrogalactosyl residues.

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