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A re-examination and partial characterisation of polysaccharides released by mild acid hydrolysis from the chlorite-treated leaves of *Sphagnum papillosum*

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Abstract

Mild acid hydrolysis was used to release polysaccharides from the chlorite-treated leaves of *Sphagnum papillosum*. These polysaccharides, collectively known as sphagnan, were physically and chemically characterised. No evidence was found of the presence of a previously described 5-keto-D-mannuronic acid (5-KMA) monomer in sphagnan, or in the chlorite-treated leaves from which sphagnan was extracted. After fractionation by anion-exchange chromatography the majority of polysaccharides were similar to rhamnogalacturonan I-type pectins. They were highly branched and had a weight average molecular weight of 3.9×10 g/mol.

Keywords: 5-keto-D-mannuronic acid; Sphagnan; *Sphagnum* moss; Peat

1. Introduction

Between 1978 and 2003 the late Terence John Painter investigated the structural and functional properties of polysaccharides extracted from the leaves of *Sphagnum* mosses. In 1983 he claimed to have isolated polysaccharides from *Sphagnum quinquefarium* that contained a previously unidentified ketouronic acid (Painter, 1983). The new monomer was systematically named D-*lyxo*-5-hexosulopyranuronic acid. It became known trivially as 5-keto-D-mannuronic acid (5-KMA), and the polysaccharide population in which it was enriched was later given the name sphagnan. This polysaccharide was thought to reside in the hyaline cell wall of all peat forming *Sphagnum* species (Painter, 1991).

5-KMA was thought to exist in sphagnan in two interconvertible, isomeric ring forms (pyranose and furanose, Fig. 1). The latter ring-form contains a reactive carbonyl group at C-5 in the form of an α-keto carboxylic acid. It is the presence of this reactive carbonyl group, with its supposed propensity to condense with primary amino-groups, which gave rise to the idea that sphagnan possessed unique functional properties that distinguished it from other plant polysaccharides. Indirect evidence was offered to suggest that this mechanism, involving the initial formation of a Schiff base and/or glycosylamine complex, which leads to eventual Maillard browning, had the ability to inhibit and suppress the microbial decay of proteinaceous material. It was believed that sphagnan could cross-link and tan the surface of protein-containing material in which it came into contact, and thus form a barrier to protect the proteinaceous material from degradation. Furthermore, it was suggested that 5-KMA in hyaline cell wall-bound sphagnan could directly deactivate saprogenic enzymes, chelate essential metal ions and sequester amino-nitrogen.

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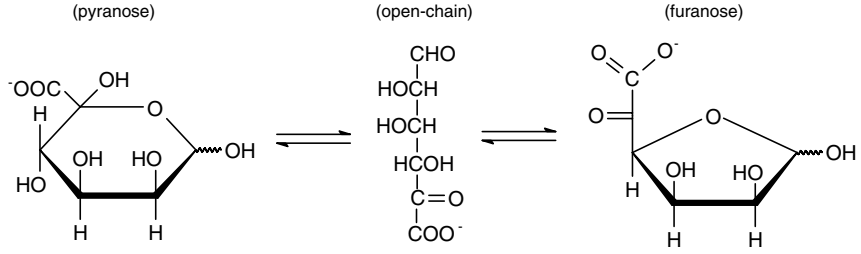

Fig. 1. 5-Keto-D-mannuronic acid (5-KMA) in its pyranose and furanose-form.

To one extent or another all these mechanisms were thought to contribute, and to explain (Painter, 1991), the reported preservation properties of *Sphagnum* mosses and their peat. The notion of preservation by *Sphagnum* appears to be now well established (Abbott, 2002).

In its native state, sphagnan was thought to be covalently cross-linked through some of its pyranose 5-KMA residues to cellulose and a hemicellulose of xyloglucomannan in the hyaline cell wall, whilst others in their furanose-form were also available to react with amines. According to Andresen, Grasdalen, Holsen, and Painter (1987) approximately every fourth carbohydrate monomer in sphagnan comprises 5-KMA (at this time sphagnan was referred to as crude-pectin). The rest comprised a ratio of carbohydrate monomers typical of pectins with an abundance of Rha and GalA together with smaller amounts of Gal, Glc, Man and Xyl (see Table 1).

5-KMA, it was claimed, was detected as a free monomer after its stabilisation by selective reduction to yield 2-keto-D-gluconic acid (2-KGA). The same monomer, either free or part of an oligosaccharide, was also released from the polymer when crude sphagnan, or chlorite-treated leaf material, was treated with aqueous sodium borohydride, together with the products of its reverse aldol condensation (Andresen et al., 1987). Identification of 2-KGA was based, after its acid catalysed de-carboxylation, as D-Ara (Painter, 1983), and by direct comparison to an authentic 2-KGA specimen (Børsheim, Christensen, & Painter, 2001). Isolation and identification as its quinoxalinol complex has also been used to directly detect the presence of 5-KMA and 2-KGA in borohydride-treated moss polysaccharides (Painter, 1991; Ballance, Børsheim, & Christensen, 2004). Further evidence, however, of the essentially pectin-like character of sphagnan was afforded by partial acid hydrolysis of a crude preparation to yield a mixture of oligosaccharides, from which α-D-Gal*p*A-(1 → 4)-D-Gal*p*A and α-D-Gal*p*A-(1 → 2)-L-Rha were isolated (Andresen et al., 1987). These dimers are characteristic of the backbone of rhamogalacturonan I which are common to the primary cell wall of plants (Albersheim, Darvill, O'Neil, Schols, & Voragen, 1996). Following uronic acid degradation of the polymer backbone and analysis by thin-layer chromatography it was also suggested that some of the 4-linked Gal*p*A carried Gal and Ara substituents on position 3. Some of the

Table 1
Monosaccharide composition of sphagnan released from the chlorite-treated leaves of *S. papillosum* and *quinquefarium* before and after treatment with sodium borohydride

	S. papillosum				*S. quinquefarium*[a,d]		
	Before $NaBH_4$ treatment		After $NaBH_4$ treatment		Before $NaBH_4$ treatment		After $NaBH_4$ treatment
	M%	M ratio	M%	M ratio	M%	M ratio	M%
Rha	22	0.6	22	0.5	19	0.75	n.d.[f]
Xyl	9	0.2	8	0.2	6	0.2	n.d.[f]
Man	5	0.1	3	0.1	4	0.2	n.d.[f]
Gal	10	0.3	8	0.2	10	0.4	n.d.[f]
Glc	15	0.4	13	0.3	7	0.3	n.d.[f]
GalA	38	1	41	1	25	1	n.d.[f]
GlcA	1	<0.1	1	<0.1	n.d.[e]		n.d.[f]
5-KMA[b,c]	n.d.[e]		n.d.[e]		27	1.1	n.d.[f]
Rha-ol	0		3	0.1	n.d.[f]		n.d.[f]
Glc-ol	0		1	<0.1	n.d.[f]		n.d.[f]
Unknown compounds	Trace		Trace		n.d.[f]		n.d.[f]

[a] Various methods (Painter, 1983).
[b] For *S. papillosum* as either TMS-methyl glycosides of 2-KGA or gluconic acid.
[c] For *S. quinquefarium* determined by UV absorption at 275 nm of phenylhydrazine released from the polymer preparation by acid hydrolysis (Painter, 1983).
[d] The same data is presented in Table I of Andresen et al. (1987) and referred to as 'pectin.' Rather it should have been labelled 'crude pectin' when reference is made to Scheme I in the same article.
[e] Not detected.
[f] Not determined.

C-2-linked Rha were also substituted at position 4 by oligosaccharides containing Gal and Ara (Andresen et al., 1987).

The purpose of this study is to re-examine, by physical and chemical characterisation, the structure of polysaccharides extracted from the chlorite-treated leaves of *S. papillosum* by mild acid hydrolysis. These polysaccharides were prepared essentially as previously described to yield the light brown freeze-dried preparation collectively classed as 'soluble sphagnan' (Børsheim et al., 2001) which we now refer to as sphagnan. One of our main objectives was to definitively ascertain whether or not 5-KMA is a component of this preparation or any other component in the leaves of the moss. The second objective was to describe the polysaccharides that comprise sphagnan in more detail. We have selected to study *S. papillosum* because it is representative of a common peat-forming moss that is widely distributed in the open acid peatlands of Western and Northern Europe (Daniels & Eddy, 1985). It decomposes at a slower rate than some other *Sphagnum* species (Clymo, 1965). It has also been used in medicine as a bactericidal ingredient (Podterob & Zubets, 2002) and is one of four out of forty *Sphagnum* species that has been routinely used as a surgical dressing (Varley & Barnett, 1987).

2. Materials and methods

2.1. Preparation of chlorite-treated leaves

Sphagnum papillosum plants were harvested next to a lake (63°25, 55′N, 10°17, 22′E) in Tømmerdalen, Bymarka, Trondheim, Norway in August 2002 and 2003. Identification of the *Sphagnum* species was made by *Sphagnum* biologist Prof. Kjell Ivar Flatberg, NTNU, Trondheim, Norway. Immediately after picking, whole fronds of moss were dried over a period of 3 days in a current of air at 60 °C. Dried leaves were stripped manually from the stems and the latter discarded. The leaf material (50 g) was boiled in acetone (2 l, 57 °C, 3 min) and collected by manual filtration through a nylon filter mesh (pore size 60 μm). This was repeated three times, until the filtrate containing extracted waxes and pigments was almost colourless. Finally the leaf residue was extracted once more with dry methanol and air-dried in the fume hood at room temperature.

Once dried, acetone/methanol-treated leaves (47 g) were split into two portions and stirred mechanically in water (3 l) at 75 °C in the fume hood. Glacial acetic acid (30 ml) was then added, followed by sodium chlorite (30 g), added in small amounts over 1 h to generate chlorine dioxide. After 3 h these additions were repeated, and after a further 3 h the mixture was cooled and filtered as before through a 60 μm nylon filter mesh. The purpose of this process was to selectively oxidise all aromatic compounds with chlorine dioxide and leave behind the pure white intact leaves with apparent minimal modification to their polysaccharide structure (Wise, Murphy, & D'Addieco, 1946). The pure white chlorite-treated leaves were washed well with water followed by ice-cold 0.02 N HCl, and then with distilled water until the washings were neutral. The residue was finally washed with acetone, methanol and air-dried to give chlorite-treated leaves in their H^+-form. Previous work has referred to this preparation as 'holocellulose' – a name that specifically refers to a lignin-free carbohydrate fraction (Ritter & Kurth, 1933).

2.2. Preparation of sphagnan

Dry phenol-free leaves (33 g in their H^+-form) were made into a thick slurry with 2 l degassed distilled water (final pH was 4.5) which was then heated at 98 °C. At daily intervals for 3 days, the residual solid was collected by vacuum filtration through a Whatman GF/D glass microfibre filter and re-suspended in another 1.5 l of distilled water, which was then heated again. On the fourth day when the slurry was impossible to filter in a reasonable time it was precipitated by centrifugation at 16,264*g* for 10 min at room temperature, the supernatant decanted off and pooled with the earlier filtrates. This liquid solution (5.5 l) was concentrated in a rotary evaporator at 30 °C to 500 ml before progressive filtration through GF/D, GF/C, GF/F (Whatman glass microfibre filters), 0.45 and finally 0.22 μm membranes (Millipore nitrocellulose). The filtrate was then first dialysed (m.w.c.o. 12000–14000 for globular proteins) against 0.5 M NaCl to convert the sphagnan into its Na^+-form and then repeatedly against distilled water. Finally, the dialysate was sterile filtered through a 0.22 μm membrane and freeze-dried. The yield of light-brown crude solid (Na^+-form) was 10 g. This was stored in the refrigerator until used.

2.3. Treatment of sphagnan with sodium borohydride

One gram of sphagnan was dissolved in 500 ml of 2% w/v sodium borohydride. 2–5 mg of each pooled fraction from the round of anion-exchange chromatography (see below) was dissolved in 1 ml of 2% w/v sodium borohydride. These were all mixed and incubated at room temperature for 24 h, prior to the addition of acetic acid to destroy excess borohydride, followed by dialysis against distilled water until the conductivity was <2 μS/cm. Material recovered from the dialysis tubes was then freeze-dried and stored in the fridge until further use. The dialysate from the sodium borohydride-treated sphagnan was recovered, evaporated to dryness, and then processed as described below (starting at the distillation step) prior to its assay for 5-KMA (see below).

2.4. Assay for 5-KMA as its reduced product (2-KGA and gluconic acid)

Twenty grams of each acetone-extracted and chlorite-treated leaves were mixed with 1 l of 2% sodium borohydride for 24 h at room temperature. The solution was acidified to pH 4.5 with concentrated acetic acid and filtered

through a 3 μm membrane (removal of solids) followed by a 0.45 and finally a 0.22 μm membrane. The filtrate was recovered and evaporated to dryness followed by repeated drying/distillation in portions of 2% acetic acid in methanol to remove boric acid. The recovered dried material was dissolved in 50 ml of water and sodium ions were removed by mixing for 2 h with an H^+ exchange-capacity excess of AG-50W-X8 resin (60 mesh, H^+-form, 1.7–1.9 meq/ml wet volume). The sample was filtered to remove the resin and again evaporated to dryness. Finally the sample was dissolved in methanol evaporated to dryness, dissolved in water and freeze-dried. As a positive control and standard 50 mg of 2-keto gluconic acid (2-KGA) was dissolved in 50 ml of 2% sodium borohydride or sodium borodeuteride and treated in an identical fashion for 24 h and just 1 h.

The recovered standards were analysed in water by standard direct injection electrospray ionisation-mass spectroscopy (ESI-MS) in negative mode (Griffiths, Jonsson, Liu, Rai, & Wang, 2001). 2-KGA Ca^{2+}-form, gluconic acid Na^+-form with and without borohydride treatment were used as standards. These yielded the expected negative mode mass-to-charge ratio (m/z) of 193 $[2\text{-KGA-H}]^-$ and 195 $[\text{gluconic acid-H}]^-$. Second, and together with the material recovered from the borohydride treatment of the leaf tissue and sphagnan, 0.5–5 mg samples, respectively, were subjected to methanolysis and analysed by gas chromatography as described below. Both 2-KGA and gluconic acid were again used as standards and they were prepared for GC-analysis in an identical fashion to the other samples assayed in this study.

2.5. Monosaccharide analysis

Samples (1 mg) were dried in vacuum over P_2O_5 for 24 h prior to methanolysis for 24 h at 80 °C in 4 M methanolic-HCl spiked with 100 μg of mannitol as internal standard. Samples were then dried with nitrogen gas at 35 °C and dried for a further three times following repeated additions of anhydrous methanol. Samples were then stored in vacuum over P_2O_5 for at least 1 h prior to the addition of 200 μl of a mixture of pyridine–hexamethyldisilazane–chlorotrimethylsilane (5:2:1) followed by incubation for 30 min at room temperature (Barsett & Paulsen, 1992; Samuelsen et al., 1995). Quantitative analysis was then carried out by gas chromatography on a 25 m DB-5 capillary column calibrated with carbohydrate standards common to the polysaccharides of plants that were treated in the same way as the samples. The quantity of rhamnitol, in samples treated with sodium borohydride, was estimated using rhamnose as standard because no commercial sample of its alditol was available. As a further estimate of the reducing rhamnose content of sphagnan, alditol acetates were prepared from the borohydride-treated sphagnan, as described below, and analysed with EI-MS. The ratio of m/z 289 and m/z 290 were used to estimate the ratio of total reducing and non-reducing rhamnose in the polysaccharide sample.

2.6. Determination of glycosyl-linkage/methylation analysis

Carboxyl groups were activated with carbodiimide at the polymer level (H^+-form) and reduced with sodium borodeuteride to yield 6,6′ dideuterio neutral sugars. Hydroxyl groups were then de-protonated with a mixture of sodium hydroxide and dimethylsulphoxide followed by methylation with methyl iodide. These methylated polysaccharides were hydrolysed for 2 h at 110 °C in 2.5 M TFA, subsequently reduced with sodium borodeuteride to yield partially methylated alditols, and finally acetylated with acetic anhydride to yield partially methylated alditol acetates (Kim & Carpita, 1992). These were then dissolved in anhydrous methanol, separated by gas chromatography and analysed online by EI-mass spectrometry. Data was processed with Fisons Masslab software. The relative proportion of uronic acid to corresponding neutral sugar and 2- and 4-linked pentopyranosyl units was determined as described previously (Carpita & Shea, 1989). Effective carbon-response factors were applied for quantification (Sweet, Shapiro, & Albersheim, 1975).

2.7. NMR

Samples of 60 mg were dissolved in 600 μl of D_2O and spiked with 100 μl of an internal reference standard of 1% 3-(trimethylsilyl)propionic-2,2,3,3,-d_4 Na salt (TSP) in D_2O. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance DPX 300 spectrometer. For ^1H NMR, spectra were obtained at 90 °C using a 30° pulse angle, a spectral width of 3591 Hz and a data-block size of 32 K. After 4 dummy scans 64 more were accumulated. For ^{13}C NMR, spectra were recorded at 90 °C with power-gated proton decoupling using a 45° pulse angle, a spectral width of 18,832 Hz and a data-block size of 64 K. After 8 dummy scans 20,000 more were accumulated.

2.8. Determination of molecular weight and intrinsic viscosity

Sphagnan, pullulan standards and alginate (positive control) were dissolved in 0.05 M sodium sulphate, 0.01 M EDTA, pH 6 to a concentration of 2 mg/ml prior to size-exclusion chromatography (SEC) on 3 HPLC columns connected in series (TSK 6000, 5000 and 4000 PWXL). Borohydride-treated sphagnan and, pooled fractions of sphagnan from preparative anion-exchange chromatography were chromatographed on 2 HPLC columns in series (TSK 4000 and 3000 PWXL). These columns were eluted at room temperature at a flow of 0.4 ml/min in the same buffer used to dissolve the samples. Effluent was monitored by multi-angle laser light scattering (MALLS) followed by refractive index (RI) detection on a DAWN DSP instrument. The refractive index increment dn/dc was 0.150 except for pullulan were it was 0.148. All data was processed with ASTRA software. After RI-detection, online intrinsic viscosity $[\eta]$ was also monitored using a

Viscotek 301 Triple array detector. Data was collected and processed on TriSEC GPC Triple detector software.

2.9. Ion-exchange capacity

Five replicates of 20 mg sphagnan, Sigma P-3850 polygalacturonic acid; 85% GalA (control) and alginate from *Laminaria hyperborea* stipe (control) were dissolved in 5 ml of distilled water and placed into a pre-washed dialysis sack. Repeated dialysis was carried out against daily renewal of 0.2 M magnesium nitrate for 5 days, followed by changes of distilled water until a conductivity <2 μS/cm outside the dialysis bag was achieved. Each individual dialysis tube was then dialysed against 25 ml 0.2 M nitric acid to release bound Mg^{2+}. The volume of the dialysate was recorded each day (via weight assuming a density of 1 g/ml) for a total of 4 days with daily renewal of acid. Each day 10 ml of dialysate was collected and stored until analysis of Mg^{2+} by flame atomic absorption spectrometry over a linear range of 0–1 ppm Mg^{2+} against matrix matched standards. Polysaccharide dry weight was determined by thermo-gravimetrical analysis on a Netzsch STA 449 instrument at 160 °C over a 30 min period. An aluminium oxide standard was used for calibration. Ion-exchange capacity was expressed as meq/g dry weight polysaccharide corrected against replicate water blanks.

2.10. Fractionation of sphagnan by preparative anion-exchange chromatography

Sphagnan was dissolved to a concentration of 5 mg/ml in 20 ml 0.02 M sodium phosphate, pH 7. This sample was then subjected to anion-exchange chromatography on a column of DEAE-Sepharose CL-6B (2.7 × 33 cm) at a flow rate of 0.5 ml/min. Buffer A was 0.02 mM sodium phosphate, pH 7, and buffer B was 0.5 M NaCl in 0.02 M sodium phosphate, pH 7. After elution in 160 ml buffer A, a linear gradient of chloride was produced from 0% to 100% in 2 l of eluent. Fractions of 10 ml were collected and then every second fraction assayed for total carbohydrate, 6-deoxy-hexoses and uronic acids by standard colorimetric methods (see below). Material that remained bound to the anion-exchange resin at the end of the chloride gradient was removed and collected by washing the column with a reverse-flow of 1 M NaOH. Fractions were then combined into one of six pools, and together with the material eluted by the NaOH wash were dialysed against distilled water until the conductivity was <2 μS/cm. Material recovered from dialysis tubes was then freeze-dried and stored in the fridge until further use.

2.11. Colorimetric assays

Total carbohydrate was determined by the phenol–sulphuric acid reaction (Doubois, Giles, Hamilton, Rebers, & Smith, 1956) using D-glucose as the standard. 6-deoxy-hexoses were determined by the cysteine–sulphuric acid reaction (Dische, 1962) using L-rhamnose as the standard. Uronic acids were determined by the carbazole–sulphuric acid reaction (Blumenkrantz & Asboe-Hansen, 1973) using D-galacturonic acid as the standard.

3. Results and discussion

Comparison of the sugar composition of sphagnan extracted from the chlorite-treated leaves of *S. papillosum* with those extracted earlier from *S. quinquefarium* (Painter, 1983) show certain similarities but also a striking difference (Table 1). The presence and molar ratio of the common monosaccharides, typically found in pectins, was confirmed but we found no evidence of 5-KMA, and only trace amounts of other unidentified compounds in the analysis (Table 1). Furthermore, we determined the ion-exchange capacity of sphagnan to be 2.35 ± 0.05 meq Mg^{2+}/g dry weight, which indirectly corresponds to a content of uronic acid of about 46%. The ion-exchange capacity for sphagnan treated with sodium borohydride was only slightly less at 2.00 ± 0.05 meq Mg^{2+}/g, which corresponds to a uronic acid content of 40%. The respective directly determined uronic acid content of both these samples was 39 and 42 M%. Although a similar ion-exchange capacity for Mg^{2+} of 2.5 ± 0.15 was found earlier (Smidsrød & Painter, 1984) less than half of it was attributed to the content of GalA. Within a confidence limit of ±15%, as were both the positive control samples, we now find the ion-exchange capacity of sphagnan and borohydride-treated sphagnan is accounted for by its content of GalA (Table 1). The same conclusion has been reached before by Schwarzmaier and Brehm (1975) following analysis of *S. magellanicum* plant tissue.

It has previously been claimed that free 5-KMA was extremely unstable in acid (Painter, 1983). Still it might have been expected that when sphagnan was subjected to mild methanolysis (0.05 M HCl–MeOH, 1 h, 80 °C) one would have perhaps stabilised some of it as its respective methyl glycoside derivatives. This is especially so given it was claimed that some 5-KMA liberated from chlorite-treated leaves was at least transiently stabile under the condition of mild acid hydrolysis (Painter, 1983). In earlier studies, 5-KMA was assayed by mixing the polysaccharides with phenylhydrazine (Painter, 1983; Smidsrød & Painter, 1984; see Table 1). The subsequent hydrazones formed were released from the polymer by its treatment with concentrated acid, diluted and assayed by UV spectroscopy. However, after repeating this assay we discovered some fundamental flaws in it (Ballance et al., Unpublished results). We therefore could not use this assay for the detection or determination of 5-KMA. Instead we opted to assay for 2-KGA; the apparent product of 5-KMA after its partial reduction.

First we treated 2-KGA with an aqueous solution of 2% sodium borohydride. Under these conditions it was previously claimed that 2-KGA was stabile (Painter, 1983; Andresen et al., 1987). We found, however, this was not the case. 2-KGA was reduced to its corresponding aldonic acid even after incubation in 2% sodium borohydride at room

temperature for just 1 h. Evidence for this was obtained by negative mode ESI-mass spectroscopy which afforded the major molecular ion of *m*/*z* 195 [gluconic acid]$^-$ and *m*/*z* 196 [2-*deutero*-gluconic acid]$^-$ after treatment of 2-KGA with $NaBH_4$ and $NaBD_4$, respectively. Methanolysis and analysis of the corresponding TMS-methyl glycosides of these two borohydride/borodeuteride treated 2-KGA preparations also yielded three peaks all with identical retention times on the DB-5 column as their TMS methyl-glycosides prepared from a standard of gluconic acid. No evidence for the presence of 2-KGA retro-aldol products (3-hydroxypyruvic acid or glycerol) were observed either by mass spectroscopy or by GC analysis. Alone, this evidence does not prove that 5-KMA is not a major component of *Sphagnum* moss polysaccharides. It merely suggests that perhaps Painter's previous assays, which he used to detect any 2-KGA, as a proof of 5-KMA, were inaccurate.

It appears the correct assay to use which would unequivocally determine the presence of 5-KMA in *Sphagnum* mosses after its stabilisation by reduction, would rather be that for gluconic acid. It was claimed by Andresen et al. (1987) that, "treatment of *Sphagnum* holocellulose [10 g] with dilute aqueous borohydride [2% w/v in 500 ml water] solubilises −10% of it. The soluble products consist of a mixture of 2-KGA, [the products of reverse aldol condensation] and oligosaccharides glycosidically linked to 2-KGA". A repetition of these experiments with the additional inclusion of sphagnan and the moss leaves subjected only to acetone and methanol-, and not chlorite-treatment was therefore carried out. Neither gluconic acid nor 2-KGA were detected as their corresponding methyl glycoside derivatives in any of these samples and only traces of unknown sugar components were observed (Table 2). The total yield of recovered material was not determined directly. Although the calculated molecular weight of the residual polymer (3.0×10^4 g/mol) was about 25% less than sphagnan (Table 3) it is probably just better dissolved/dispersed in solution rather than actually depolymerised. Samples not treated with borohydride seem to be slightly aggregated throughout the light scattering profile.

Treatment of sphagnan, by Andresen et al. (1987), with borohydride also yielded a residual polymer containing very

Table 2
Monosaccharide composition of recovered dialysable material from acetone/MeOH-treated leaves, chlorite-treated leaves and sphagnan released after treatment with sodium borohydride

	Acetone/MeOH-extracted leaves		Chlorite-treated leaves		Sphagnan	
	M%	M ratio	M%	M ratio	M%	M ratio
Ara	6	0.3	3	0.1	0	
Rha	4	0.2	11	0.4	22	0.6
Fuc	3	0.1	2	0.1	0	
Xyl	7	0.4	14	0.6	9	0.2
Man	9	0.5	10	0.4	5	0.1
Gal	21	1.1	23	0.9	10	0.3
Glc	31	1.7	12	0.5	16	0.4
GalA	19	1.0	25	1.0	38	1.0
GlcA	Trace		Trace		Trace	
5-KMA[a]	n.d.[b]		n.d.[b]		n.d.[b]	
Alditols	Trace		Trace		Trace	
Unknown compounds	Trace		Trace		Trace	

[a] As either TMS-methyl glycosides of 2-KGA or gluconic acid.
[b] Not detected.

Table 3
Number (M_n) and weight average (M_w) molecular weight, polydispersity (M_w/M_n), intrinsic viscosity ([η]) and MHKS exponent of sphagnan, borohydride reduced ($NaBH_4$-) sphagnan, aliginate (positive control), pullulan and pooled fractions of sphagnan after fractionation by preparative anion-exchange chromatography

Sample	*Sphagnan*	$NaBH_4$-sphagnan	*Alginate*	*Pullulan* (Cal. Std.)			
M_n ($\times 10^4$ g/mol)	1.7	1.3	11.2	86.1			
M_w ($\times 10^4$ g/mol)	3.9	3.0	17.2	120.8			
M_w/M_n	2.3	2.3	1.54	1.4			
$[\eta]_w$ (ml/g)	12	12	812	200			
MHKS exponent	0.54	0.53	0.89	0.55			
Pool	I	II	III	IV	V	VI	Column wash
M_n ($\times 10^4$ g/mol)	0.7	0.5	0.7	0.8	1.9	3.0	n.d.
M_w ($\times 10^4$ g/mol)	2.0	1.3	1.6	1.9	3.4	5.3	n.d.
M_w/M_n	2.88	2.36	2.20	2.29	1.79	1.79	n.d.
[η] (ml/g)	22	18	15	12	14	19	n.d.
MHKS exponent	0.65	0.74	0.72	0.69	0.63	0.88	n.d.

n.d. Not determined.

Table 4
Monosaccharide composition of fraction P-2 isolated after anion-exchange chromatography on DEAE-cellulose and after subsequent treatment of fraction P-2 with sodium borohydride

	S. acutifolium[a]			
	Before $NaBH_4$ treatment		After $NaBH_4$ treatment	
	M%	M ratio	M%	M ratio
Rha	19	0.8	38	0.9
Xyl	3	0.1	0	
Man	4	0.2	0	
Gal	10	0.4	9	0.2
Glc	7	0.3	2	<0.1
GalA	25	1	44	1
GlcA	n.d.[c]		n.d.[c]	
Ara	3	0.1	5	0.1
5-KMA[b]	27	1.1	0	
Alditols	n.d.[c]		n.d.[c]	
Unknown compounds	n.d.[c]		n.d.[c]	

Previously unpublished data obtained in 2002 from Terence John Painter.
[a] Fractionation, conditions of chromatography and assays used are described in Andresen et al. (1987).
[b] Determined by UV absorption at 275 nm of phenylhydrazine released from the polymer preparation by acid hydrolysis (Painter, 1983).
[c] Not determined.

little Man, Xyl or Glc (see also Table 4). Yet we found that the monosaccharide composition of the carbohydrates released by borohydride treatment in our analysis (Table 2) was almost identical to those of the starting polymer (Table 1) with a significant contribution from both Rha and GalA. Although the monosaccharide composition of borohydride-treated sphagnan was never previously published (Table 1), by inference of what Andresen et al. (1987) claim to have observed, their residual polymer must have become further enriched with Rha and GalA. Within the bounds of analytical accuracy our results show there is little difference in the sugar composition of sphagnan treated or untreated with borohydride (Table 1) except the latter treatment forms alditols from the reduction of exposed reducing-end monomers of which the contribution from Rha-ol is notable (Table 1). Looking at Table 2 again it is also apparent that a small amount of Fuc and Ara is present in the acetone/MeOH-extracted and chlorite-extracted leaves. The former residue is not detected in the soluble sphagnan probably because it is terminal and very easily detached from the polymer during its preparation by mild acid hydrolysis.

Examination of sphagnan by standard 1D ^{1}H NMR (Fig. 2A and C) revealed an absence of typical aromatic

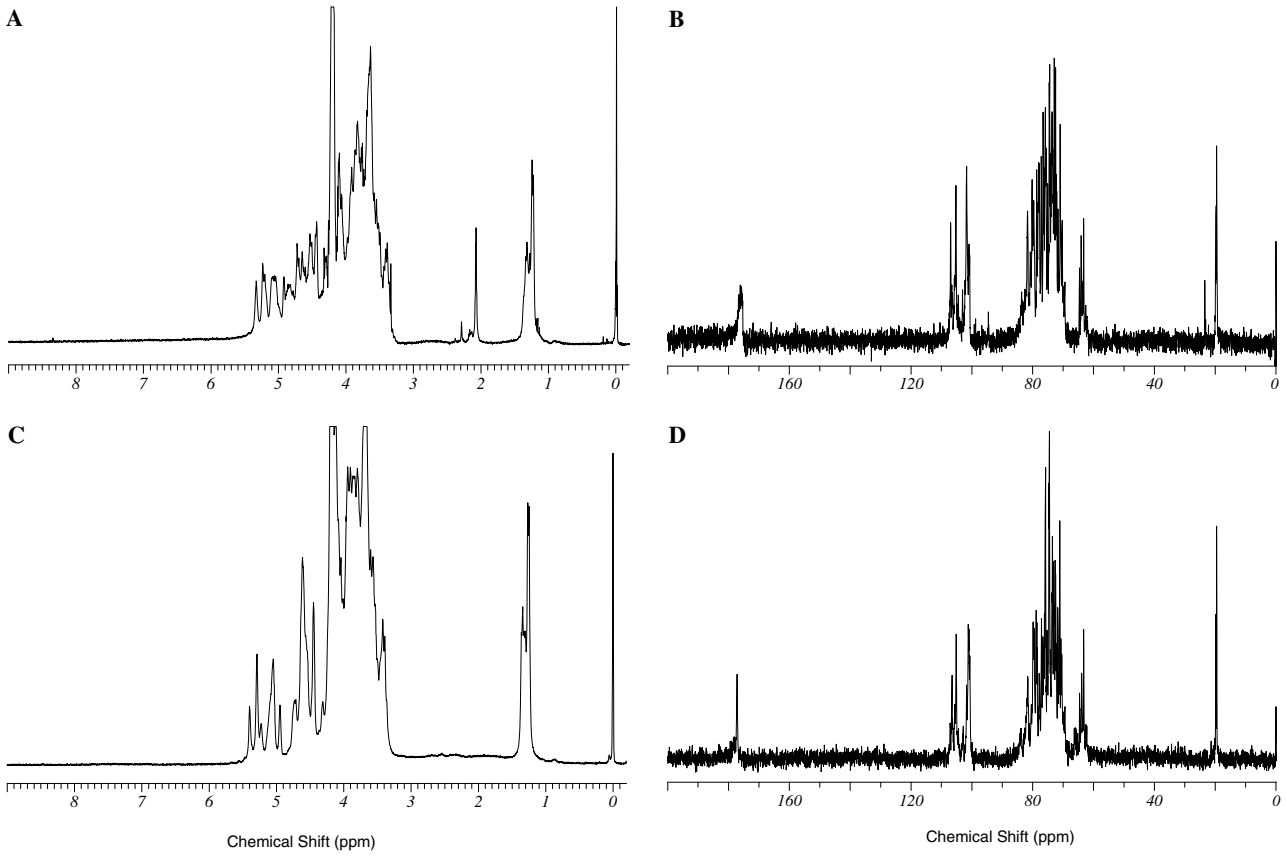

Fig. 2. 300 MHz ^{1}H (A and C) and 75 MHz ^{1}H-decoupled ^{13}C (B and D) NMR spectra of sphagnan (Na^{+}-form) released from the chlorite-treated leaves of *S. papillosum* before (A and C) and after treatment with sodium borohydride (B and D). Reference standard TSP = 0 ppm. In ^{1}H-spectra the residual water peak is at 4.2 ppm.

Table 5
Glycosyl-linkage composition and physical parameters deduced from methylation analysis of acid labile polysaccharides in soluble sphagnan (Na^+-form, treated and untreated with sodium borohydride ($NaBH_4$-), italics) and pooled fractions generated by preparative anion-exchange chromatography of sphagnan (Na^+-form) released from the chlorite-treated leaves of *S. papillosum*

Pool	I	II	III	IV	V	VI	Column wash	*Sphagnan*	*$NaBH_4$-sphagnan*
T-Rha*p*	0.5	0.4	0.2	0.7	0.7	0.6	0.9	0.5	0.8
1,2-Rha*p*	0.5	0.5	3.9	7.7	13.2	13.8	12.1	11.2	12.2
1,2,4-Rha*p*	Trace	0.1	0.9	5.5	14.1	18.5	8.0	10.3	9.0
T-Xyl*p*	6.2	7.4	6.9	4.9	1.3	0.3	3.7	2.9	2.6
1,4-Xyl*p*	4.1	5.6	5.7	4.2	2.1	1.1	4.2	4.0	2.1
1,2,4-Xyl*p*	0.8	1.0	2.4	2.9	0.6	0.6	1.1	2.1	3.2
T-Gal*p*	12.1	4.4	3.7	3.4	4.6	1.2	2.9	6.4	3.7
1,2-Gal*p*	0.6	0.7	0.5	0.2	0.1	0.1	0.2	0.4	0.6
1,3-Gal*p*	0.6	0.7	0.5	0.2	0.1	0.1	0.2	0.4	0.6
1,4-Gal*p*	13.6	7.7	7.1	2.3	0.5	1.2	1.4	1.7	2.5
1,6-Gal*p*	2.4	3.7	0.7	0.3	0.5	0.2	1.1	0.4	0.5
1,3,6-Gal*p*	0.7	0.7	0.3	0.1	Trace	Trace	Trace	0.5	0.1
1,3,4,6-Gal*p*	Trace	Trace	0.3	0.2	Trace	Trace	Trace	0.1	Trace
T-GalA*p*	9.6	11.9	16.0	9.8	10.0	9.9	18.3	8.3	8.5
1,2-GalA*p*	0.3	0.5	0.6	1.1	2.3	1.8	2.3	1.3	0.3
1,4-GalA*p*	0.1	0.6	2.2	19.9	25.8	21.7	5.2	22.4	26.0
1,3,4-GalA*p*	Trace	Trace	1.0	3.2	10.9	15.6	11.2	5.6	6.2
1,2,4 GalA*p*	Trace	1.0	1.1	0.7	0.9	0.9	1.0	0.5	1.1
T-Glc*p*	6.8	5.8	2.6	1.3	1.3	3.8	1.0	0.9	1.5
1,4-Glc*p*	3.9	12.4	16.0	12.7	4.2	1.4	9.5	10.5	5.9
1,4,6-Glc*p*	14.3	13.7	14.4	12.0	4.5	0.8	10.5	8.2	5.6
1,6-Man*p*	6.4	7.0	3.9	1.2	0.8	1.5	1.0	3.0	0.8
1,4,6-Man*p*	16.6	14.0	9.1	2.8	1.2	1.5	1.0	2.0	2.2
T-GlcA	Trace	Trace	Trace	1.0	1.0	2.0	2.0	1.0	1.0
Physical Parameters									
$\sum$ Terminals ($\sum$ Ter)	35.2	30.0	29.4	21.1	19.0	17.8	28.8	20.0	18.2
$\sum$ Branches ($\sum$ Br)	32.4	29.6	27.9	21.4	18.3	19.5	24.9	18.5	17.4
Ratio ($\sum$Ter:$\sum$Br)	1.1	1.0	1.1	1.0	1.0	0.9	1.2	1.1	1.0
ø *L* (*average linearity*)	1.0	1.4	1.5	2.7	3.4	3.2	1.9	3.3	3.7

The prefix T refers to non-reducing terminal residues.

resonances (Abraham, Fisher, & Loftus, 1988). The absence of any resonances between 55 and 61 ppm in the ^{13}C NMR spectra (Perlin & Casu, 1982; Westerlund, Åman, Andersson, & Andersson, 1991; Perrone et al., 2002) also indicates that both methyl esters and ethers, common to many plant pectins, are not a component of sphagnan (Fig. 2B). However, the ^{1}H and ^{13}C NMR methyl resonance observed in sphagnan at about 2 ppm and 21.5 ppm, respectively (Fig. 2A and B) both disappear after exposure to weak alkali (borohydride solution, ~pH 10.5) (Fig. 2C and D). This strongly indicates the presence of *O*-acetyl functionalities (Perlin & Casu, 1982; Perrone et al., 2002), but as yet their location remains unknown. Coincidently, after borohydride treatment of sphagnan, only one major resonance remained between 175 and 180 ppm in the ^{13}C spectra (Fig. 2D). This is probably C-6 of GalA. The identity of additional resonances observed in the spectra of sphagnan for this chemical shift region (Fig. 2B) are unknown but may well be associated with carbonyl functions either reduced of removed during the borohydride treatment. No attempt was made to assign ^{13}C and ^{1}H monosaccharide ring resonances because these often overlapped (Fig. 2) which is a combined product of the complexity of the polysaccharide and signal broadening of which the latter phenomena is typical for polysaccharides (Perlin & Casu, 1982).

In terms of molecular weight the molecules in sphagnan are not that large, and this is probably a consequence of their extensive branching and their extraction by mild acid hydrolysis. Their weight average radius of gyration was less than 20 nm with a weight average molecular weight (M_w) of 3.9×10^4 g/mol and polydispersity (M_w/M_n) of 2.4. These molecules also have a low intrinsic viscosity [η] of 12 ml/g (Table 3) (independently determined by both off- and on-line capillary viscometery). Overall the intrinsic viscosity of sphagnan is slightly less than pullulan, but 1–1.5 orders of magnitude less than alginate (linear polysaccharide reference analogue); of the same molecular weight (Fig. 4). A similar significant difference is also seen for these molecules in a radius of gyration v molecular weight plot (result not shown). This indicates that sphagnan has a compact structure in solution which is probably attributed to the extensive branching revealed by the methylation analysis (Table 5).

The linear region of the intrinsic viscosity–molecular weight plot (solid white lines, Fig. 4) corresponds to a Mark-Houwink-Kuhn-Sakurada (MHKS) exponent of 0.54. This exponent is typical (0.5–0.8) of a random coil (Smidsrød & Moe, 1995). When compared to an unbranched random coil such as alginate (exponent = 0.89), it would seem that the branching in sphagnan has only a moderate effect on hydrodynamic structure, which is explained by regularly distributed short branches. In fact, it turned out to be almost overlapping with data for soy bean rhamnogalacturonan (data not shown). Alternatively, the degree of branching may decrease with increasing molecular weight, producing qualitatively the same effect. This remains to be investigated.

In taking these observations and associated conclusions into account we deemed it necessary to re-examine the structure of polysaccharides in sphagnan in more detail, especially given their likely heterogeneity and claimed ability to retard the decay of certain organic materials (Børsheim et al., 2001). We therefore fractionated sphagnan by anion-exchange chromatography as shown in Fig.3. The majority of the material (pool IV–VI) was retained on the column and eluted between 0.25 and 0.5 M of the applied salt gradient (Fig. 3). This material had a similar overall molecular weight (Table 3) and monosaccharide composition to the starting sphagnan (Tables 1 and 6), the corresponding borohydride-treated fractions and pool P-2 'pectin' (Table 4) isolated by Painter as described by Andresen et al. (1987) after DEAE-cellulose anion-exchange chromatography. It was especially rich in GalA and Rha (>50%). In general, the stronger the retention on the anion-exchange column, the greater the proportion of linked acid components 1,4-GalA*p* (>20%), 1,3,4-GalA*p* (3–15%), 1,2,4-GalA*p* (0.7–0.9%), (Fig. 3; Tables 5 and 6). A similar relationship was observed for Rha with an abundance of 1,2- and 1,2,4-residues (Table 5). In line with previous findings (Theander, 1954; Andresen et al., 1987; Kremer, Pettolino, Bacic, & Drinnan, 2004) it is reasonable to assume some of these linked residues are associated in a pectin-like polysaccharide structure similar to that referred to now as rhamnogalacturonan I (Albersheim et al., 1996). If one assumes most of the linked Rha and GalA is in such a structure, then this structure must be quite heavily branched since 25–50% of these linked residues in fractions IV–VI have branch points (Table 6). The branches stemming from some of these GalA and Rha residues are therefore likely to comprise and account for the majority of remaining neutral sugars (Tables 5 and 6). On average about one out of five sugars comprises a branch point and the average degree of linearity is no greater than 3.4 (Table 5). This evidence is supported by MHKS-exponents (Table 3) for these fractions in the similar 'random-coil' range to crude sphagnan. It would therefore appear that these polysaccharides share the compact characteristics associated with the 'hairy-regions' of pectin.

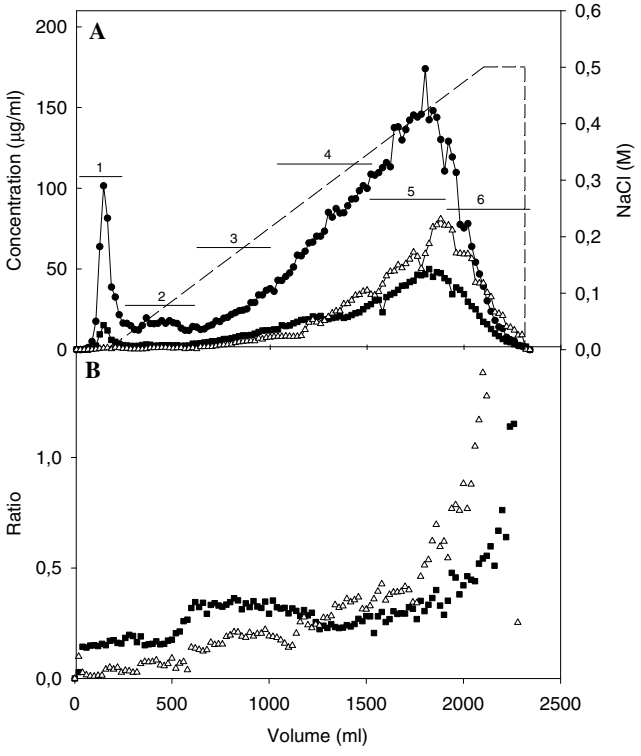

Fig. 3. Preparative DEAE-Sepharose CL-6B anion-exchange chromatography of sphagnan released from the chlorite-treated leaves of *S. papillosum*. Fractions of 10 ml (A) were assayed for total carbohydrate (solid circles), uronic acids (open triangle) and rhamnose (solid squares). The ratio of uronic acids and rhamnose to total carbohydrate is shown in (B). Fractions were combined as indicated by the bars in (A) to yield six pools. The dashed line is the NaCL gradient.

Some of the material (ca. 10%, pool 1) was not retained on the column, whilst another population (ca. 10%, pool 2 and 3) was only weakly retained and eluted by 0.2 M salt (Fig. 3). Molecules in both of these populations were relatively small, with an average molecular weight $\leqslant 2 \times 10^4$ g/mol (Table 3; Fig. 5), and highly branched, with about one out of every three residues comprising a branch point (Table 5). MHKS-exponents were similar to that of the crude sphagnan (Table 3). All these fractions were rich in neutral sugar (80–90%) while the remainder consisted of mostly terminal GalA (Table 5). The neutral sugars comprised mainly Man, Gal, Glc and Xyl, but only a trace of Rha (Table 6). This is further evidence to suggest Rha is largely associated with linked-GalA. Inspection of Table 5 shows that the majority of residues with branch points were 1,4,6-Glc*p* and 1,4,6-Man*p*. Most of these polysaccharides were probably a mixture of cellulose and hemicellulose perhaps similar in composition and origin to those described previously (Andresen et al., 1987; Kremer et al., 2004). Significant amounts of 1,4 Gal*p* (Table 5) would also indicate the presence of a hemicellulose fraction that contained galactoglucomanan. Polysaccharides in the weakly retained fraction only contained traces of the linked and branched GalA and Rha residues (Table 5) characteristic of rhamnogalcturonan I.

It is interesting to note that the relative high content of non-reducing terminal residues of GalA in all fractions

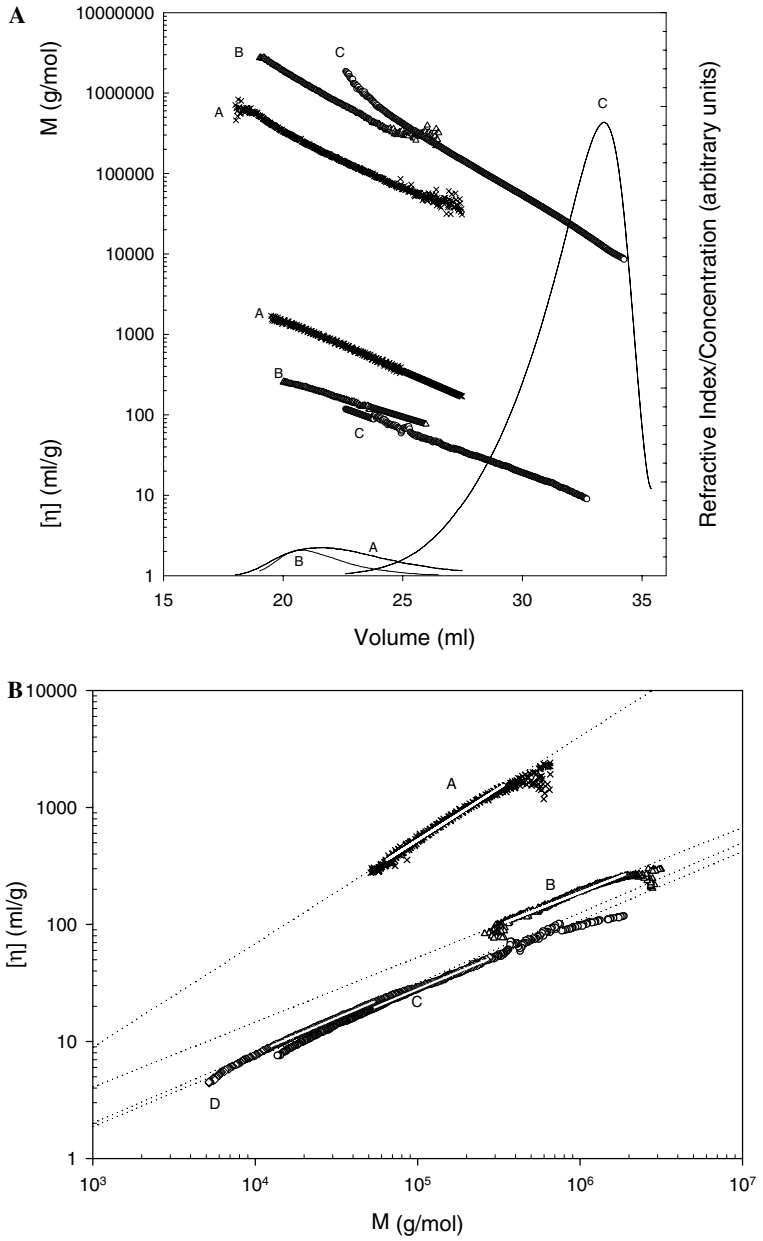

Fig. 4. (A) Size-exclusion chromatography (SEC, 3 columns in series) with on-line multi-angle laser light scattering and refractometry (RI, solid lines) to determine molecular weight (upper symbols) and viscometry to determine intrinsic viscosity (lower symbols) of alginate (crosses, small caps A), pullulan (open triangles, small caps B) and, sphagnan (open circles, small caps C). (B) Relationship between intrinsic viscosity ([η]) and molecular weight (MHKS plot) of the same samples with the addition of sphagnan treated with 2% sodium borohydride (open diamonds, small caps D). Dotted lines are extrapolations of $[\eta] = KM^a$ fits (white solid lines) to the respective data sets. N.B., Chromatogram of SEC-MALLS-VISC data for sphagnan treated with 2% sodium borohydride is not shown because one less in-series SEC column was used which gave a characteristic smaller total column volume. All data are typical examples of repeated measurements.

(Table 5). This may suggest it was the selective cleavage of a glycosidic linkage involving this monomer which contributed to the release of sphagnan from the chlorite-treated leaves. It is known that under the conditions of mild acid hydrolysis (pH 2–4), unionised carboxyl groups can readily donate a proton to cleave glycosidic bonds attached at C-4 of uronic acids (Smidsrød, Larsen, Painter, & Haug, 1969). Indeed this mechanism may explain why a series of homologous oligomers, released by partial acid hydrolysis (0.1 M HCl, 80 °C, 72 h) of apple, beet and citrus pectins, comprised a repeating sequence of α-D-Gal*p*A-(1 → 2)-L-Rha where Rha was at the reducing end (Renard, Crépeau, & Thibault, 1995). This is the same as Andresen et al. (1987) found after the partial acid hydrolysis of sphagnan (see Section 1). Similarly, using two independent methods we indirectly estimated *after* borohydride reduction that between 2 and 4 M% of total monomers was Rha-ol (Tables 1 and 6). We cannot directly confirm whether any Rha in sphagnan was reducing but it would seem a strong possibility. Since a pure polysaccharide can only have one reducing end per molecule (see Smith & Montgomery, 1956) it therefore means either: (1) the quantity

Table 6
Monosaccharide compositions of pooled fractions of sphagnan released from the chlorite-treated leaves of *S. papillosum* after (A) fractionation by preparative anion-exchange chromatography and (B) subsequent treatment with sodium borohydride

Pool	I		II		III		IV		V		VI		Column wash	
	M%	M ratio	M%	M ratio	M%	M ratio	M%	M ratio	M%	M ratio	M%	M ratio	M%	M ratio
A														
Rha	1	0.1	1	<0.1	5	0.3	14	0.4	28	0.6	33	0.6	21	0.6
Xyl	11	1.0	14	1.0	15	0.7	12	0.4	4	0.1	2	0.1	9	0.2
Man	23	2.3	21	1.5	13	0.6	4	0.1	2	<0.1	3	0.1	2	0.1
Gal	30	2.9	18	1.3	13	0.6	9	0.3	6	0.1	4	0.1	7	0.2
Glc	25	2.5	32	2.3	33	1.6	26	0.8	10	0.2	6	0.1	21	0.6
GalA	10	1.0	14	1.0	21	1.0	34	1.0	49	1.0	50	1.0	38	1.0
GlcA	0		0		0		1	<0.1	1	<0.1	2	<0.1	2	<0.1
5-KMA[a]	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
Unknown compounds	Trace		Trace		Trace		Trace		Trace		Trace		Trace	
B														
Rha	2	0.3	1	0.1	3	0.1	15	0.4	28	0.6	31	0.6	20	0.5
Xyl	11	1.7	15	0.1	12	0.5	11	0.3	4	0.1	2	<0.1	9	0.2
Man	20	3.2	18	1.7	10	0.4	2	0.1	2	0.1	3	0.1	2	0.1
Gal	37	6.1	24	2.2	15	0.7	11	0.3	6	0.1	4	0.1	7	0.2
Glc	24	3.8	30	2.7	35	1.6	20	0.5	7	0.2	5	0.1	19	0.5
GalA	6	1.0	11	1.0	23	1.0	37	1.0	48	1.0	48	1.0	38	1.0
GlcA	n.d.		n.d.		n.d.		1	<0.1	2	<0.1	3	0.1	2	<0.1
5-KMA[a]	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
Rha-ol	Trace		1		2	0.1	3	0.1	3	0.1	4	0.1	3	0.1
Glc-ol	Trace		Trace		Trace		Trace		Trace		Trace		Trace	
Unknown compounds	Trace		Trace		Trace		Trace		Trace		Trace		Trace	

n.d. Not detected.
[a] As either TMS-methyl glycosides of 2-KGA or gluconic acid.

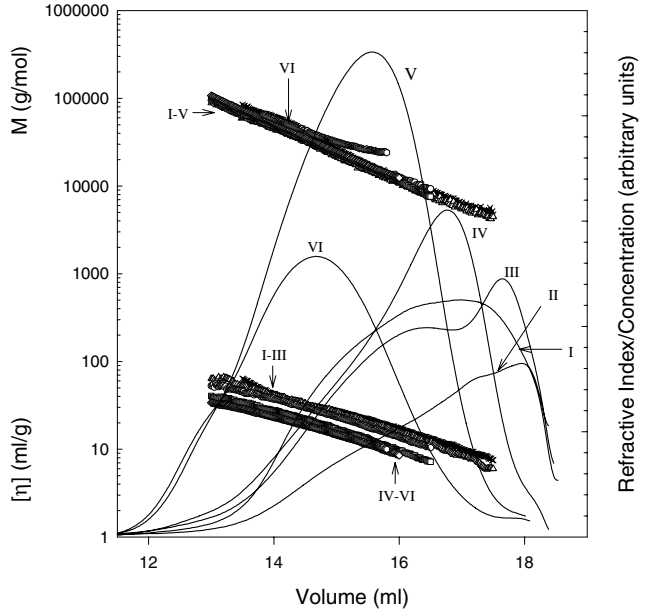

Fig. 5. Size-exclusion chromatography (SEC, 2 columns in series) with on-line multi-angle laser light scattering and refractometry (RI, solid lines) to determine molecular weight (upper symbols) and viscometry to determine intrinsic viscosity (lower symbols) of pooled fractions of sphagnan after fractionation by preparative anion-exchange chromatography. The symbols: cross, open triangle, open circle, open square, open diamond, and open hexagon, respectively, represent pooled fractions 1–6.

of Rha-ol is slightly overestimated; assuming sphagnan was pure it should be no greater than about 1 M% or 1 reducing end per 100 monomers (Table 3) given the determined M_n, (2) polysaccharide chains are cross-linked by some as yet unknown component, or (3) reducing Rha is exposed and subsequently reduced during borohydride treatment.

Painter (1983) had previously proposed that one key role for 5-KMA*p* (Fig. 1) was to cross-link the rhamnogalacturonan I type material by a ketal bond to other insoluble structural polysaccharides. He proposed that in analogy with C-2 linked sialic acids, the C-5 linkage was the one broken, incidentally by the same mechanism of intramolecular autocatalysis observed for 1,4-linked uronic acids (Smidsrød et al., 1969). We speculate that this latter mechanism may be partly responsible for the release of polysaccharides from the chlorite-treated moss leaves. However, it is not possible to rule out that some other, as yet unidentified component has a linkage function. What we can say is that we have no evidence to support the notion that this component is 5-KMA. Future work will investigate the interaction of these polysaccharides with various micro-organisms and their interaction with amino-nitrogen.

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