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Carbohydrate Polymers 71 (2008) 550–558
Carbohydrate Polymers
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Interactions of polysaccharides extracted by mild acid hydrolysis from the leaves of *Sphagnum papillosum* with either phenylhydrazine, *o*-phenylenediamine and its oxidation products or collagen

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Received 28 February 2007; received in revised form 14 June 2007; accepted 29 June 2007
Available online 29 August 2007

Abstract

The purpose of this research was to evaluate if pectin-like polysaccharides, collectively known as sphagnan, extracted by acid hydrolysis from the leaves of *Sphagnum* moss have a unique ability to react with phenylhydrazine, *o*-phenylenediamine or collagen. A previous assay for determination of carbonyl groups in sphagnan by reaction with phenylhydrazine was disproved due to spectrophotometric interference from furans. The actual carbonyl content of sphagnan is estimated to be much less than previously thought. NMR spectroscopy showed that small amounts of *o*-phenylenediamine and/or its oxidation products bind to sphagnan probably via imine formation, but evidence of quinoxaline formation was inconclusive. Sphagnan–gelatin mixtures formed complex coacervates at pH 2.0–4.8 at low ionic strength, which is typical of electrostatic polyelectrolyte interactions, rather than covalent carbonyl-amine reactions. Measurements of hydrothermal stability and collagenase-degradation of sphagnan-treated hide powder collagen suggest that sphagnan is a poor tanning agent. The results indicate the suggested preservative properties of sphagnan are not related to tanning.

Keywords: Gelatin; Tanning; Bog body; Preservation; Maillard, 5-KMA

1. Introduction

It has been claimed that pectin-like polysaccharides in the leaves of *Sphagnum* moss, known collectively as sphagnan, have a unique potential to react with certain amines (Børsheim, Painter, & Christensen, 2001; Painter, 1991). This notion stems from the alleged presence in sphagnan of ~25 M % novel ketouronic acid residues in the form of 5-keto-D-mannuronic acid (5-KMA) which could exist in the form of either pyranose (5-KMA*p*) or furanose (5-KMA*f*), of which the latter contains an α-keto-carboxylic acid group (Painter, 1983; Painter, 1991). Two predominant reactions between amines and 5-KMA have been proposed (Painter, 1991). The first is mild acid-catalysed imine (C=NR) formation via nucleophilic addition of an amine to the α-keto group of 5-KMA*f* to form a carbinolamine, followed by acid-catalysed elimination of water. The second is glycosylamine formation by reaction of a semi-protonated diamine with 5-KMA*p* (Painter, 1991). Glycosylamine formation is promoted by electrostatic interaction between the deprotonated carboxyl group in 5-KMA and the protonated amine. The amine then forms a hydrogen bond with the hemiketal hydroxyl group of 5-KMA*p*, followed by spontaneous elimination of water to yield a glycosylamine (Painter, 1991). The hypothesis of imine formation was based on observations that sphagnan readily reacts with aqueous phenylhydrazine (calculated pK_a 5.29) to form orange-coloured polymeric phenylhydrazones, whereas sphagnan treated with $NaBH_4$, a carbonyl-reducing agent, is inert (Painter, 1983). Hydrolysis of the orange-coloured complexes with concentrated

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doi:10.1016/j.carbpol.2007.06.025

HCl followed by filtration and dilution led to the indirect determination of 5-KMA by measuring the absorbance of the released phenylhydrazine (Painter, 1983; Smidsrød & Painter, 1984). Observation of a fluorescent quinoxalinol formed when the free acid-form of sphagnan was mixed with *o*-phenylenediamine (OPD) (calculated pK_a 4.46) was presented as further evidence for imine formation (Painter, 1991).

The hypothesis of glycosylamine formation was based on observations that lower concentrations ($\leqslant$20 mM) of diamines (e.g. lysine, arginine or guanidine) than of simple amines (>100 mM) inhibits the precipitation of a gelatin–sphagnan complex (Painter, 1991). Furthermore, prior treatment of sphagnan with 20% $NaBH_4$ rendered this polymer ineffective to precipitate gelatin (Painter, 1991).

The practical significance of these various amine–sphagnan interactions is the potential for sphagnan, via its 5-KMA residues, to cross-link and ultimately tan, via Maillard browning, the surface of proteinaceous materials (Børsheim et al., 2001; Painter, 1991). It was also suggested that the same reactions lead to the immobilisation and inactivation of saprogenic enzymes (Painter, 2003). Analogy was made with the tanning abilities of various periodate-oxidised polysaccharides such as dialdehyde starch (Fein & Filachione, 1957; Nayudamma, Joseph, Rao, & Hemalatha, 1967), and other carbonyl-tanning agents such as formaldehyde and glutaraldehyde (Painter, 1991). It was proposed that tanning by sphagnan could explain the preservation of proteinaceous materials and their appearance. This includes the brown leathered skin on human bodies preserved in *Sphagnum*-dominated peat bogs (Painter, 1991; Painter, 1995), the browning of the dermis and yellowing of the scales of small fresh fish embedded in *Sphagnum* leaves (Børsheim et al., 2001), and the browning and bio-resistance of strips of pig and mackerel skin repeatedly exposed to sphagnan in a series of 'tanning', washing and drying cycles (Børsheim et al., 2001). The wound healing properties of *Sphagnum* mosses were also attributed to their tanning effect (Painter, 2003). Given the potentially high environmental and biotechnological significance of these claims, we deemed them to warrant further investigation.

In two recent studies (Ballance, Børsheim, & Christensen, 2004; Ballance, Børsheim, Inngjerdingen, Paulsen, & Christensen, 2007) we set out on the quest to verify or refute previous claims regarding the structure of sphagnan with particular emphasis on obtaining direct evidence of 5-KMA in *Sphagnum* moss. In the first study, a review of the literature, we only found indirect evidence to support the existence of 5-KMA in *Sphagnum* (Ballance et al., 2004). In the second follow-up study we then found this indirect evidence to be irreproducible (Ballance et al., 2007). In this study we aim to further examine the reactions between the free acid-form of sphagnan and either phenylhydrazine, OPD or collagen. Sphagnan was extracted by mild acid hydrolysis from acetone/methanol- or chlorite-treated *Sphagnum papillosum* leaves, of which the latter has been characterised in a previous study (Ballance et al., 2007). First we examined the interaction between sphagnan and phenylhydrazine, which was previously used as the basis of an assay to quantify the content of carbonyl groups in sphagnan (Painter, 1983; Smidsrød & Painter, 1984). Second we examine the interaction between OPD and its oxidation products with sphagnan in aqueous solution and look for evidence of quinoxaline formation. Finally we study the interaction of sphagnan with collagen in the form of gelatin or hide powder. Complex coacervate formation in sphagnan–gelatin mixtures were studied under a range of pH conditions. The tanning ability of sphagnan was assessed by measuring the hydrothermal stability and collagenase-degradation of hide powder collagen treated with sphagnan.

2. Materials and methods

2.1. Preparation of acetone/methanol-treated leaves

Sphagnum papillosum plants were collected, identified, processed and treated with acetone/methanol as previously described (Ballance et al., 2007).

2.2. Extraction of sphagnan from acetone/methanol-treated leaves

Twenty-five grams of acetone/methanol-treated *Sphagnum* leaves (H^+-form) were suspended in 1.5 l degassed water and autohydrolysed at 95 °C for 24 h. At daily intervals over 3 days the liquid was separated from the residual solid by vacuum filtration through a Whatman GF/D microfibre glass filter. The residual solid was then suspended in new degassed water and the autohydrolysis repeated in two more cycles. The liquid solutions were pooled (4.5 l) and concentrated by rotary evaporation at 30 °C to ca. 200 ml prior to further filtration through 0.22 μm membranes. The filtrate was then repeatedly dialysed against distilled water. Finally the dialysate was sterile filtered through 0.22 μm membrane and freeze-dried. The yield of light brown crude solid in its acid-form was 1.2 g. This material was characterised by some of the methods used to investigate the structure of sphagnan extracted from chlorite-treated leaves (Ballance et al., 2007).

2.3. Extraction of sphagnan from chlorite-treated leaves

Sphagnan was extracted by mild acid hydrolysis from chlorite-treated leaves as previously described (Ballance et al., 2007) and stored in the fridge in its acid-form.

2.4. Reaction with $NaBH_4$

Polysaccharides were treated with $NaBH_4$ as previously described (Ballance et al., 2007). In some cases the $NaBH_4$ concentration was increased to 20% (w/v) with a reaction time of 11 days. These samples were converted into their

H^+-form by dialysis against cold 0.02 M HCl followed by distilled water and freeze-dried.

2.5. Preparation of phenylhydrazine and o-phenylenediamine and hydroxylamine derivatives

Phenylhydrazine derivatives were prepared by reaction of 100 mg polysaccharide with 20 mg phenylhydrazine hydrochloride in 10 ml distilled water at a pH between 4 and 4.5 for 2 h at 60 °C. OPD derivative was prepared by reaction of 250 mg sphagnan (H^+-form) with 50 mg OPD in 50 ml distilled water for 24 h at 22 °C (Painter, 1991). Hydroxylamine derivative was prepared by reaction of 20 mg sphagnan with 8 mg hydroxylamine hydrochloride at pH 4–4.5 in 3 ml distilled water for 24 h at 22 °C (Calvini, Conio, Princi, Vicini, & Pedemonte, 2006; Kim & Kuga, 2000). All samples were dialysed first against 0.5 M NaCl and then against water until the conductivity was <2 μS cm^{-1}, and finally freeze-dried. Samples were also prepared where sphagnan was substituted by 2% or 20% w/v $NaBH_4$-treated sphagnan. All samples were stored in the dark at 4 °C until analysed.

2.6. Preparation of N-acetylneuraminic acid-quinoxalinol derivative

Fifty milligrams of *N*-acetylneuraminic acid (NANA) Type IV from *Escherichia coli* was dissolved in 50 ml 1 mg/ml OPD, and stirred in the dark at 22 °C for 24 h. The solution was then concentrated by rotary evaporation, and applied to a 10 g Sep-Pak C^{18} solid-phase extraction column. Bound material was eluted with one column volume of 40% methanol (v/v) in water. The methanol was then removed by distillation and the sample freeze-dried to yield about 45 mg solid off-white material. The product was stored in the dark at 4 °C until analysed.

2.7. Phenylhydrazine assay for determination of carbonyl groups

Native and 20%-$NaBH_4$-treated sphagnan from chlorite-treated leaves (acid-form) were reacted with phenylhydrazine hydrochloride as described above with incubation for 2 h at 60 °C followed by dialysis and freeze-drying. Prior to spectrophotometric analysis 2 mg of these samples were dissolved in 2 ml distilled water, or dissolved in 2 ml concentrated HCl, incubated for 24 h and then diluted to 4 ml with distilled water. As controls, native polysaccharide samples not exposed to phenylhydrazine were dissolved in concentrated HCl, incubated and diluted in the same way. The UV spectrum of 200–400 nm was obtained in a quartz cell in a calibrated Perkin-Elmer Lambda 25 UV/VIS spectrometer. In a separate analysis, the absorbances of each sample at 250, 275 and 300 nm were recorded. These values were then used to calculate the absorbance at 275 nm, assuming a linear baseline fit between 250 and 300 nm. Matrix matched phenylhydrazine standards ($n = 3$) of 0–5 mM were used to determine the fraction of incorporated phenylhydrazine per monosaccharide in the polymer. In our calculations the average molecular weight of sugar monomers was assumed to be 200.

2.8. Total nitrogen and carbon analysis

Total elemental nitrogen and carbon in freeze-dried samples (5–10 mg) was analysed by the flash combustion method (Kirsten, 1979) on a Thermo Finnigan FlashEA 1112 automatic elemental analyser (detection limit 100 ppm N). Phenanthrene was used as a calibration standard to yield linear fits ($R^2 = 0.98$–1).

2.9. Infrared spectroscopy

Samples were placed in KBr discs and analysed in a Nicolet 20SXC FT-IR spectrophotometer.

2.10. NMR spectroscopy

Samples were proton-exchanged with D_2O in two cycles prior to further preparation. Thirty miiligrams of NANA-OPD derivative was dissolved in 600 μl methyl alcohol-d_6, and spiked with 5 μl tetramethylsilane (TMS) as internal standard. Approximately 30 mg sphagnan-OPD was dissolved in 600 μl D_2O, and 5 μl 1% 3-(trimethylsilyl)propionic-2,2,3,3,-d_4 Na salt (TSP) in D_2O was added as internal standard. 1D ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance DPX 300 or 400 MHz spectrometer at room temperature (NANA-OPD derivatives) or 90 °C (polysaccharide samples).

2.11. Electrophoretic mobility and diffusion measurements of sphagnan–gelatin mixtures

Type A gelatin from porcine skin (approximately 300 Bloom, p*I* 7.4) or Type B gelatin from bovine skin (approximately 225 Bloom, p*I* 4.5) was purchased from Sigma. The p*I* was determined as described below for sphagnan–gelatin mixtures. Gelatin was soaked in distilled water for 2–3 h at room temperature, followed by mechanical stirring for 10–15 min at ~70 °C (Gilsenan, Richardson, & Morris, 2003) to give a dissolved gelatin concentration of 2 mg/ml. After cooling, some solutions were supplied with 40 mM arginine-, lysine- or guanidine hydrochloride. 2.5 ml gelatin solution was pipetted into a tube, followed by 2.5 ml 1 mg/ml sphagnan, 20% $NaBH_4$-treated sphagnan (both H^+-form), polygalacturonic acid (Na^+-form) or oximated-sphagnan dissolved in a weak phosphate stock solution. The final solutions comprised a 2:1 (w/w) mixture of gelatin and polysaccharide, and in some samples 20 mM amino acid, or just gelatin, all with a pH of 6.0–7.0 in 0.01 M phosphate. Ionic strength was prior adjusted with NaCl in the buffer stock solutions to give an added *I* of 0.03 (0.05 with amino acids) in all gelatin–polysaccharide mixtures. The electrophoretic mobility (an estimate of net

charge through the calculated zeta potential) and diffusion coefficient of the mixtures were then measured by laser Doppler velocimetry and dynamic light scattering, respectively, at pH intervals of 0.5 (within an interval threshold of ±0.2) from 6.0 to 2.0 at 25 °C using a Malvern Nano-ZS Zetasizer equipped with an acid/base autotitrator. Conductivity was also measured at the same time, and related to the corresponding ionic strength of a series of NaCl solutions. The calculated diffusion coefficient is only a qualitative measure of complex coacervate formation since the raw data (correlation function versus time) obtained from such polydisperse and sedimenting samples are inaccurate (Malvern Instruments, 2006).

2.12. Tanning assay

One hundred milligrams of standard bovine hide powder made from alkali-extracted hide (BLC Leather Technology Centre, UK) was soaked in 2 ml distilled water for 30 min at 30 °C. The sample was then adjusted to 0.01 M acetate ($I = 0.03$ with NaCl) prepared at pH 4.2 with a 10× stock solution, and incubated a further 30 min at room temperature. The measured pH of the mixtures was 4.0–4.5. Thirty milligrams of solid test tannage was then added over a period of 2 h while the sample was rolled. Mixing was continued for a further 5 h, followed by incubation overnight at room temperature. All samples were then rolled for a further 1 h and washed in distilled water, followed by drying overnight in a weighing boat. 10–15 mg dried sample was soaked for 72 h in 1 ml distilled water, and excess water was carefully removed by blotting the sample on tissue paper. The hydrated material was then sealed into an aluminium pan, and the denaturation temperature (onset of triple helix to random coil transition temperature) of the hide powder collagen was determined by differential scanning calorimetry (DSC) in a Perkin-Elmer DSC 7 instrument, using a punctured empty pan as reference. Samples ($n = 3$) were heated from 20 to 110 °C at a scan rate of 5 °C min^{-1} (Covington, Hancock, & Ioannidis, 1989). Commercial vegetable tannins (polyphenols) used as positive controls were seta-sun mimosa-extract from Brazil (72.5% tanning content) and unsweetened/normal chestnut-extract (75–77% tanning content).

2.13. Collagenase assay

Collagenase Type I from *Clostridium histolyticum* (Sigma) was incubated with 15 mg hide powder in 0.05 M Tris–HCl (pH 7.0) and 0.04 M $CaCl_2$ at a maintained ratio of 1:50 enzyme to substrate at 37 °C. Hydroxyproline content in the supernatant was measured after 7 days incubation by a colorimetric assay (Woessner, 1961).

3. Results and discussion

An explicit step-by-step procedure for the assay previously used to determine carbonyl groups in sphagnan is lacking. For example, in the assay described by Painter (1983) and Smidsrød and Painter (1984) it states that polymeric orange-coloured complexes (phenylhydrazones) are formed upon reaction of sphagnan with excess 0.1% phenylhydrazine for 2 h at 60 °C, but does not state the reaction pH or how they are isolated. Although it is mentioned in one paper (Smidsrød & Painter, 1984) that orange-coloured complexes were recovered by centrifugation, this was probably only in the case of insoluble holocellulose–phenylhydrazine complexes. In our experiments, no precipitation or flocculation was observed upon reaction of sphagnan with phenylhydrazine under mildly acidic conditions, either before or after centrifugation. Polymeric orange-coloured complexes were, however, recovered by dialysis against 0.5 M NaCl and water, followed by freeze-drying. Mildly acidly conditions were chosen as optimum as to have enough acid to catalyse the reaction and enough free amine for the nucleophilic attack (Kim & Kuga, 2000; McMurry, 1988).

In the next step of Painter's assay, the orange-coloured complexes were hydrolysed with concentrated HCl, followed by 'filtration and appropriate dilution', and determination of released phenylhydrazine by spectrophotometric absorbance at 275 nm (Painter, 1983; Smidsrød & Painter, 1984). In our experiments, such treated samples had absorbance bands at 210–230 and 260–300 nm irrespective of whether they contained orange-coloured complexes or not (Fig. 1). These bands are diagnostic of furfural, hydroxymethylfurfural and other furans (Martinez, Rodriguez, York, Preston, & Ingram, 2000; Taher & Cates, 1974), which are readily formed by dehydration of pentose and hexose monomers even under mildly acidic conditions (Popoff & Theander, 1972). The furans therefore make a substantial contribution to the absorbance measured at 275 nm. According to Painter's assay, sphagnan from chlorite-treated leaves (not treated with phenylhydrazine) seemingly contained 0.17 carbonyl groups per monosaccharide, while sphagnan from acetone/methanol-treated leaves were calculated to be only marginally higher at 0.21 carbonyl groups per monosaccharide. Further analysis of biopolymer preparations not exposed to concentrated acid revealed no significant absorbance above background at either 210–230 or 260–300 nm (Fig. 1). Taken together, these results cast serious doubt over the validity of Painter's assay of carbonyl groups in sphagnan, but may explain how the figure of 27 M % 5-KMA in sphagnan (Painter, 1983; Smidsrød & Painter, 1984) was reached.

An assay for the determination of carbonyl groups has been developed for neutral polysaccharides such as cellulose via oxime formation which makes use of fluorescence detection (Röhrling et al., 2002) or total N analysis (Maekawa & Koshijima, 1991). Since at present, and to our knowledge, there are no such corresponding assays in existence using fluorescent labels to detect carbonyls for anionic polysaccharides we opted for the total nitrogen analysis approach. After reaction of sphagnan with hydroxylamine at pH 4–4.5 the N content was 0.63% ± 0.085 ($n = 4$).

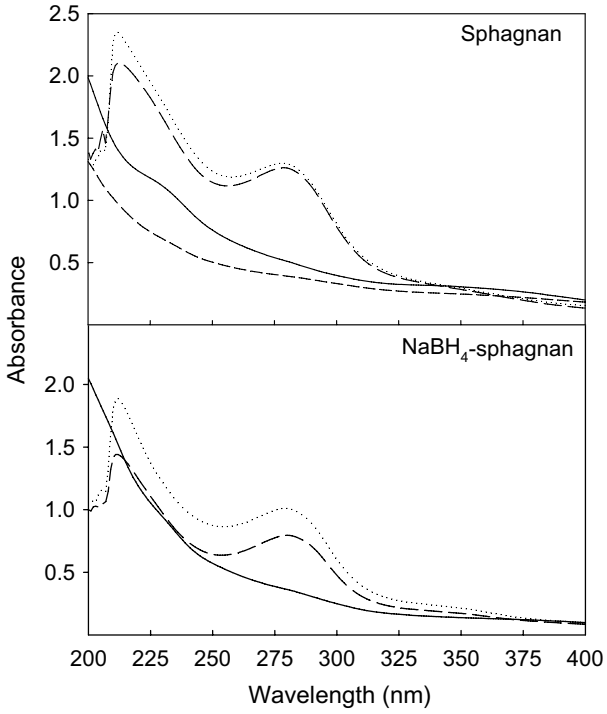

Fig. 1. UV spectra (200–400 nm): of native and phenylhydrazine derivatives of sphagnan and 20%-$NaBH_4$ sphagnan from chlorite-treated leaves. Dotted lines are samples incubated with phenylhydrazine at 60 °C for 2 h, hydrolysed in concentrated HCl for 24 h, and diluted to 6 M HCl. Solid lines are samples treated with phenylhydrazine, but not with acid, and dissolved in water. Long dashed lines are native samples hydrolysed in concentrated HCl for 24 h, and diluted to 6 M HCl. Short dashed lines represent spectra of native samples dissolved in water.

Similar values were also obtained for the sphagnan extracted from acetone/methanol-treated leaves and reacted with hydroxylamine or from corresponding samples reacted with phenylhydrazine. Since the N content of untreated sphagnan was about 0.2% this means that <1 in 18 sugars contain a reactive carbonyl group in addition to the reducing end rather than about 1 in 4 as suggested earlier (Painter, 1983; Smidsrød & Painter, 1984). As of yet, however, a more accurate measurement of the carbonyl content of sphagnan cannot be made with confidence simply because the nitrogen analysis method is not sensitive enough and its resolution is too low.

It has been suggested that sphagnan in its free acid-form reacts with OPD to form a quinoxaline (Painter, 1991), which in turn was considered diagnostic of an α-keto acid group in sphagnan. NMR analysis lends some weight to suggest that OPD or its common oxidation products such as 2,3-diaminophenazine binds to sphagnan from chlorite- or acetone/methanol-treated *Sphagnum* (Fig. 2a). The two broad resonances at 7.3–7.9 ppm and at 118/128 ppm in the ^{1}H and ^{13}C NMR spectra, respectively, are all characteristic of an aromatic compound coupled to a polymer (Fig. 2a, b and d). There are no aromatics in sphagnan extracted from chlorite-treated leaves because any originally present are destroyed by the chlorite treatment (Ballance et al., 2007). The aromatics present in the native sphagnan extracted from acetone/methanol-treated leaves resonate at 6.5–7 ppm (Fig. 2c) and the identity of these is discussed below. In addition since any free OPD or its oxidation products remaining in the sample would have appeared in the same chemical shift range as a sharp resonance rather than a broad one. This observation indicates OPD or its oxidation products with a free amino group are bound to sphagnan. No characteristic aromatic resonances from OPD or its oxidation products were observed in the NMR spectra of sphagnan prior treated with the carbonyl-reducing agent $NaBH_4$ (not shown). These results therefore suggest that OPD or its oxidation products reacted with the free acid-form of sphagnan via nucleophilic addition to form an imine (C=NR). Nitrogen analysis of OPD reacted samples also supports this contention. Attempts by 2D NMR, IR and fluorescence spectroscopy to identify any quinoxalines or vicinal diones in sphagnan though were inconclusive. This is possibly because they either comprise just a minor fraction and hence their signals were too weak, or the resonances were masked by others from the carbohydrate polymer. Quinoxalines were formed as expected when OPD was reacted with sialic acid under the same conditions as sphagnan to give characteristic signals in both NMR and IR spectroscopy (Table 1).

Dynamic light scattering measurements at low ionic strength of freshly prepared 1:2 (w/w) sphagnan (from chlorite-treated leaves)–gelatin mixtures ($I = 0.03$) titrated from pH 6.0 to 2.0 indicated complex coacervation (as the inverse of the calculated diffusion coefficient) between the two polymers at pH 2.0–4.8 (Fig. 3a). No complex formation was detected at any pH for single polymer solutions (results not shown). These patterns of complex coacervation are typical for polyelectrolyte complexes formed by electrostatic interaction of oppositely charged polymers rather than a covalent reaction between carbonyl groups in sphagnan and amino groups in gelatin. Below pH 5 all the basic amino acids in gelatin are fully charged, and the association of gelatin and sphagnan is increasingly favoured as the net positive charge density along the gelatin chain increases with protonation of the carboxyl groups down to the pH where maximum coacervation occurs at the so called electrical equivalence pH (3.5 for a 2:1 [w/w] gelatin–sphagnan mixture). Here the total charges on gelatin and sphagnan are opposite and equal (Fig. 3b).

Dissolution of the coacervate starts to occur as the majority of carboxyl groups in sphagnan become protonated as the pH drops from 3.5 to 2.5 (Fig. 3). This results in charge screening with a consequent loss of negative charge (Fig. 3b). Titration with NaCl has a similar dissociating effect (not shown) but is rather driven by the counterion-dictated entropy of mixing (de Kruif, Weinbreck, & de Vries, 2004; Piculell, Bergfeldt, & Nilsson, 1995). Similar results have been obtained for gelatin–acacia mixtures (Burgess & Carless, 1984).

It is interesting to note that coacervation of Type A gelatin–sphagnan occurs over a wider pH range than that with Type B gelatin (Fig. 3a). This difference in pH profiles can

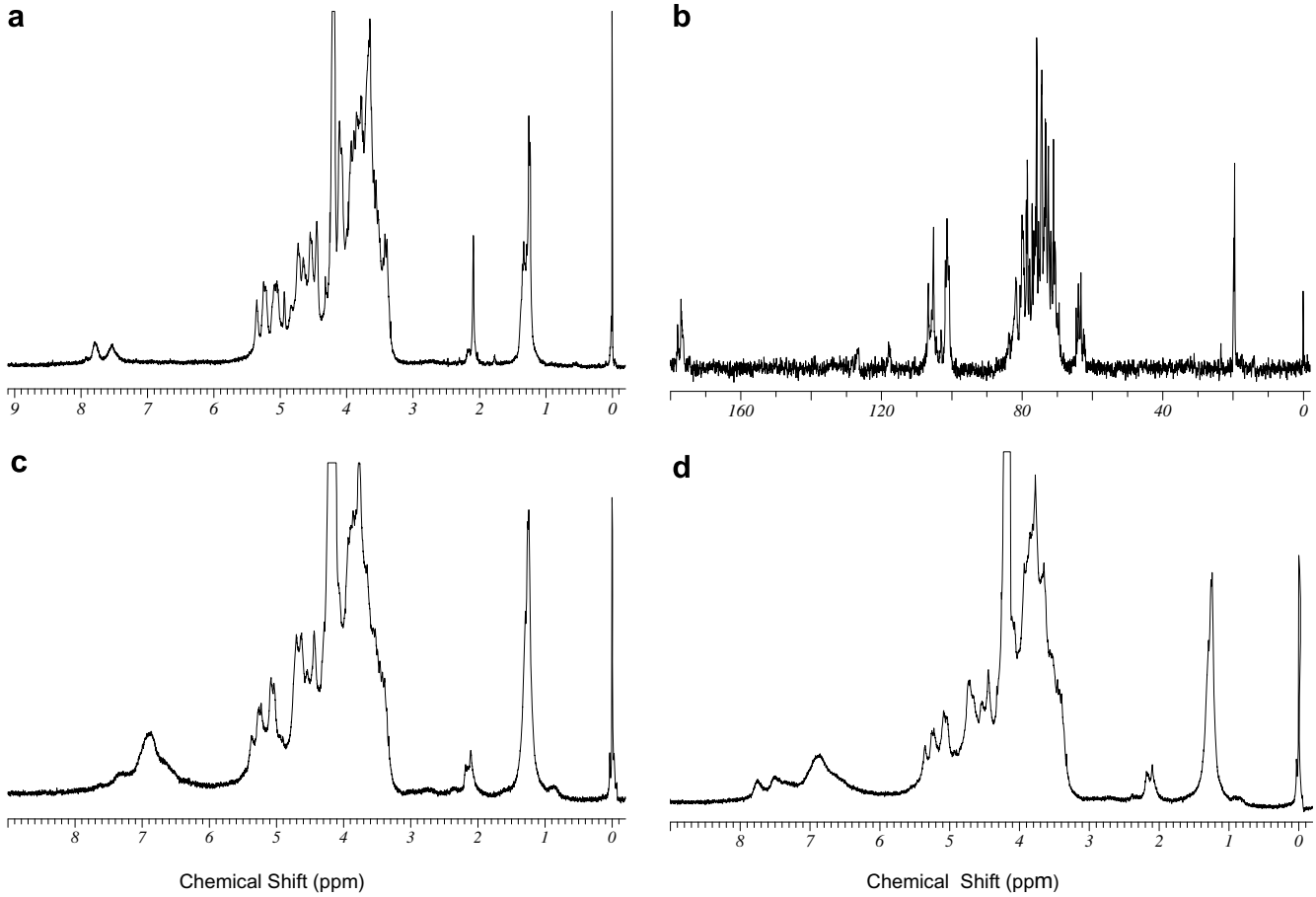

Fig. 2. Three hundred MHz ^{1}H (a) and 75 MHz ^{1}H-decoupled ^{13}C (b) NMR spectra of sphagnan from chlorite-treated leaves after reaction with OPD, and 300 MHz ^{1}H NMR of sphagnan from acetone/methanol-treated leaves before (c) and after (d) reaction with OPD with TSP as reference. In ^{1}H-spectra the residual water peak is at 4.2 ppm.

Table 1
Spectroscopic data for quinoxaline of sialic acid

^{1}H NMR (d_4-MeOD): 1.98 (s, 3H), 2.03 (br, 1H), 2.97–3.12 (m, 1H), 3.43 (d, 1H, $J = 8.8$ Hz), 3.61 (q, 1H, $J = 5.8$ and 10.8 Hz), 3.68–3.75 (m, 1H), 3.77–3.81 (dd, 1H, $J = 3.0$ and 8.8 Hz), 3.98 (q, 2H, $J = 8.8$ and 10.3 Hz), 4.64 (t, 1H, $J = 6.3$ and 13.4 Hz), 7.18 (m, 2H), 7.48 (m, 2H)

^{13}C NMR (d_4-MeOD): 23.0 (CH_3), 35.7, 55.7, 65.6, 69.2, 70.1, 71.7, 72.9, 103.5 (phenyl), 118.2 (phenyl), 121.2 (phenyl), 123.6 (2× phenyl), 128.5 (phenyl), 129.0 (quinoxaline), 154.5 (quinoxaline), 175.0 (C=O)

IR (KBr disc): 3315 (s), 1728 (w), 1650 (s) (quinoxaline, C=N), 1504 (s), 1434 (s), 1375 (m), 1336 (m), 1274 (m), 1225 (m), 1073 (m), 1035 (m), 746 (s) cm^{-1}

s, singlet; m, multiplet; br, broad; d, doublet; q, quartet; t, triplet; dd, doublet of doublets.

be explained by the different positive charge densities of the gelatins. Since alkali is used in the production of Type B gelatin, all glutamine and asparagine residues are deamidated to form glutamic and aspartic acid, respectively. Consequently, there are fewer carboxyl groups in Type A gelatin, and its p*I* is higher (Fig. 3b). Type A gelatin therefore has a higher positive charge density at pH 3.8–5.0 than Type B gelatin, and can thus form complex coacervates with sphagnan at a slightly higher pH (Fig. 3). Dissolution of Type B gelatin–sphagnan complexes occurs at a slightly higher pH because there are more carboxyl groups which are protonated in this gelatin at a given pH below its p*I*. As mentioned in the methods, the diffusion coefficients obtained from the dynamic light scattering correlogram are not quantitative, but sufficient for qualitative comparative purposes. The results are also consistent with previous studies of mixtures of Type A and B gelatin and acacia (see Figs. 1 and 2 in Burgess & Carless, 1984). Polygalacturonic acid, 20% $NaBH_4$-treated sphagnan and sphagnan in the presence of 20 mM arginine, lysine or guanidine all formed complex coacervates when mixed with Type B gelatin at low ionic strength. We could therefore not substantiate the earlier claims made by Painter (1991) that $NaBH_4$ treatment, or the addition of basic amines at low concentration, inhibits gelatin precipitation. There was neither any evidence to suggest that the coacervates that form with gelatin and sphagnan are denser than with gelatin and other acidic polysaccharides.

A true tannage elevates hydrothermal stability with a consequent rise in shrinkage temperature, whereas 'leathering', typical of oil, brains and smoke-treated collagen, just renders a material non-putrescible and changes its physical appearance. Measurements of hydrothermal stability as

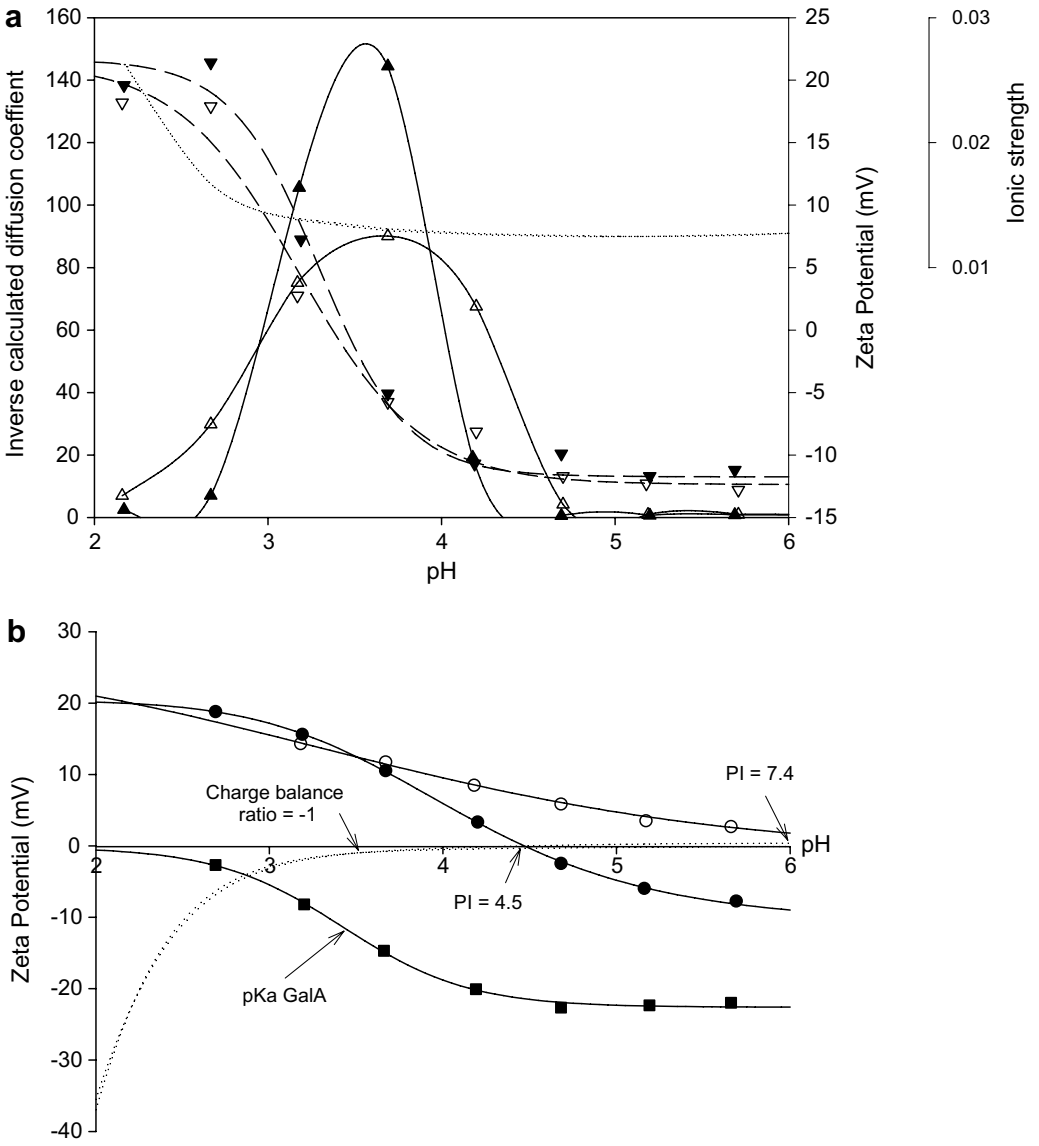

Fig. 3. Electrophoretic mobility and diffusion measurements of 2:1 (w/w) gelatin–sphagnan mixtures from pH 6.0 to 2.0. (a) Open and solid triangles represent Type A gelatin–sphagnan and Type B gelatin–sphagnan mixtures, respectively. Up triangles and fitted solid lines represent the inverse of the calculated diffusion coefficient, while down triangles and the fitted dashed lines represent the electrophoretic mobility measurements expressed as the calculated zeta potential. The dotted line is the calculated ionic strength determined from *in situ* conductivity measurements. (b) Electrophoretic mobility measurements of 0.1% (w/w) solutions of Type A gelatin (open circles), Type B gelatin (solid circles) and sphagnan (solid squares). The dotted lines represent the gelatin/sphagnan charge ratio.

denaturation temperature of bovine hide powder collagen treated with sphagnan (from chlorite-treated leaves) at pH 4.0–4.5 for 24 h showed little difference from an untreated control (Table 2). Furthermore, the sphagnan-treated collagen was readily degraded by collagenase (Fig. 4). On the other hand, positive control polyphenolic tannins, chestnut- and mimosa-extracts, increased the hydrothermal stability of collagen by 10–15 °C (Table 2) as previously found (Covington, 2001). The control tannins also imparted resistance to degradation by collagenase (Fig. 4). In earlier calorimetric analysis of mackerel skin treated repeatedly with sphagnan, the release of water at

Table 2
Denaturation temperatures ($n = 3$) of untreated hide powder collagen and hide powder collagen treated with sphagnan from chlorite-treated leaves (A), sphagnan from acetone/methanol-treated leaves (B) and two polyphenolic vegetable tannins

Hide powder treatment	Denaturation temp. (°C)
Untreated	44.0 (42.0)
Sphagnan A	46.0 (44.0)
Sphagnan B	54.0 (48.0)
Chestnut-extract	57.5 (57.5)
Mimosa-extract	63.5 (63.5)

Corresponding denaturation temperatures after 4 wetting and drying cycles are given in brackets.

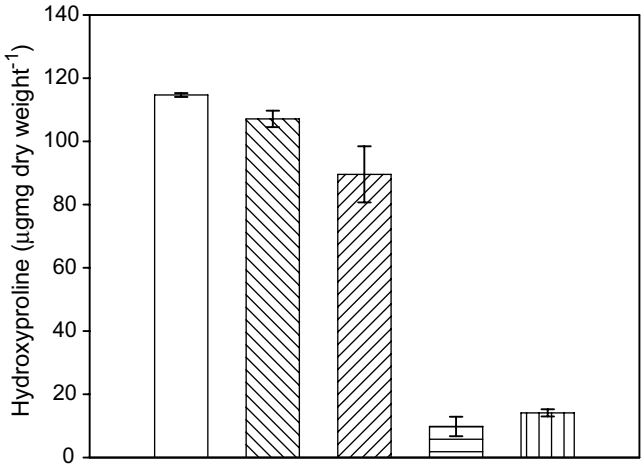

Fig. 4. The amount of hydroxyproline (µg/mg dry weight) released by collagenase after 1 week incubation at 37 °C from hide powder previously incubated for 24 h with either no added substrate (open), sphagnan from chlorite-treated leaves (negative diagonals), sphagnan from acetone/methanol-treated leaves (positive diagonals), chestnut-extract (horizontal lines) and mimosa-extract (vertical lines).

104–108°C versus 98–104 °C for various controls was observed as an endothermal peak (Fig. 4 in Børsheim et al., 2001), and cited as evidence that sphagnan is a tannin. This physical parameter, however, falls outside current definitions of what comprises tanning (Covington, 2001), and therefore cannot be used as evidence for such a process. Interestingly, sphagnan extracted from acetone/methanol-treated leaves did, after one wetting and drying cycle, increase the hydrothermal stability of the hide powder to a similar degree to that observed for the hydrolysable chestnut polyphenol extract (Table 2). In contrast, after four wetting and drying cycles, the increase in hydrothermal stability of this sphagnan-treated hide powder was lost. Many common plant-derived vegetable tannages are polyphenols which fall into a defined molecular weight range of 500–3000 Da (Covington, 1997). Whereas chestnut-extract is a low molecular weight sugar derivative based on glucose, sphagnan extracted from acetone/methanol-treated leaves is a complex carbohydrate polymer with an average molecular weight of 2.7×10^4 Da. As already mentioned it also contains some covalently linked aromatic compounds, as observed in ^{1}H NMR at a chemical shift of 6.5–7.0 ppm, whose fine structure is not known (Fig. 2c and d). It is possible that these aromatic compounds interact with collagen via hydrogen bonding in a similar way as polyphenols, but fail to tan the hide powder in these experiments either because they are too scarce, or the high molecular weight polymer is unable to penetrate sufficiently into the collagen. In the traditional practice of tanning with vegetable extracts, the hide was placed in pits and treated with the tannin for a 'year and a day' (Covington, 1997) to ensure sufficient penetration into the hide. In accordance with hydrothermal measurements, hide powder collagen treated with sphagnans for 24 h were readily degraded by collagenase (Fig. 4). Whether longer or repeated exposure to sphagnan would have reduced its degradation by collagenase and increased its hydrothermal stability has so far not been tested. However, given the results presented here, and the fact that no other plant-derived polyanionic polysaccharides are shown to be tannins, it seems unlikely that the carbohydrate residues in sphagnan will prove to be good tannins.

Specimens of tanned skin samples taken from three different bog bodies and analysed by pyrolysis GC–MS (Stankiewicz, Hutchins, Thomson, Briggs & Evershed) did not reveal the presence of any rhamnose- or hexose-derived components indicative of the presence of sphagnan, and which have previously been detected in subfossil *Sphagnum* leaves (van Smeerdijk & Boon, 1987). In fact, the only *Sphagnum*-derived compound identified in the bog body skin samples was 4-isopropenylphenol, which originated from sphagnum acid (a phenolic component unique to *Sphagnum*) tightly bound to collagen amino acid residues (Stankiewicz, Hutchins, Thomson, Briggs, & Evershed, 1997).

It seems that pectin-like polysaccharides released from *Sphagnum* moss or its holocellulose by acid hydrolysis, either in the lab or naturally in the environment, forms associations with proteins as polyelectrolyte complexes. If such complexes make any contribution towards the preservation of the protein it is probably not because carbohydrate directly participates in the process of tanning.

Acknowledgements

Linda Fonnes (Institute of Marine Research, Bergen) and Ann-Sissel Ulset (NTNU) are thanked for technical assistance. Professor A.D. Covington at the British School of Leather Technology, University College Northampton, UK, is thanked for his advice. Sybille Sondermann, Otto-Dille GMBH, Germany is thanked for the free gift of vegetable tannin. Espen Granum is thanked for proof-reading the manuscript. This work was financed by the Research Council of Norway (Grant no. 146974/130).

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