Carbohydrate Research, 124 (1983) C18–C21
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Preliminary communication

Residues of D-*lyxo*-5-hexosulopyranuronic acid in *Sphagnum* holocellulose, and their role in cross-linking

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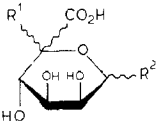
(Received August 8th, 1983; accepted for publication, September 30th, 1983)

The presence of only one anomeric centre in all the known, naturally occurring sugars has hitherto implied that glycan chains can be cross-linked in Nature only through glycosyluronic ester linkages or some non-carbohydrate moiety. We now report the presence of ~10% of residues of the title compound (**1**) in the holocellulose of *Sphagnum* moss, and evidence indicating their involvement in cross-linking between cellulosic, hemicellulosic, and pectin-like chains.



1 $R^1 = R^2 = OH$
2 $R^1 = OH, R^2 = H$

The unusual properties of the chlorite-holocellulose from *Sphagnum quinquefarium* (Braithw.) Warnst. have been reported briefly[1]. Provided that the living moss was freshly harvested, and delignified and extracted immediately, aqueous 24% potassium hydroxide removed little hemicellulose. The holocellulose was insoluble in aqueous cuprammonium hydroxide, and did not stain with iodine in the presence of concentrated, aqueous zinc chloride. It was also very resistant to cellulase. It had an ion-exchange capacity of ~1.4 mequiv./g, and contained only 38% of D-glucose residues. The other sugar components were D-galacturonic acid (21%), L-rhamnose and L-fucose (6%), D-xylose and L-arabinose (14%), D-galactose (3%), and D-mannose (6%). These figures added up to only 88% and, despite careful efforts to correct for any destruction of the identified sugars occurring during acid hydrolysis, a higher total could not be reached. The same problem was encountered in an earlier study by Theander[2].

A further indication of the presence of an unidentified monomer, which decomposes under ordinary conditions of acid hydrolysis, was obtained when the holocellulose, in its free-acid form, was allowed to undergo autohydrolysis in distilled water at 98°. Whereas

0008-6215/83/$03.00

all glycuronans turn brown when hydrolysed, the rate at which the holocellulose did so under these mild conditions was extraordinary.

Simultaneously with the browning, a soluble glycuronoglycan (**A**) was liberated into solution, which contained residues of D-galacturonic acid (25%), L-rhamnose (19%), pentoses (6%), D-glucose (7%), D-galactose (10%), and D-mannose (4%). These figures added up to only 71%, but there was also ~20% of an acid-insoluble, brown polymer in the hydrolysate.

Sphagnum is easily delignified because of its high porosity, so the presence of aromatic material was unlikely. Consistent with this, **A** showed no aromatic u.v. absorption and gave no colour with aqueous ferric chloride or diazotised 4-nitroaniline. It reacted strongly with the Folin–Denis reagent for phenols (alkaline phosphotungstate–phosphomolybdate)[3], but so also did L-*threo*-ascorbic acid (vitamin C). It also decolorised aqueous 2,6-dichlorophenol–indophenol, suggesting that the unidentified monomer either contained, or readily isomerised to, a reductone type of structure.

After prolonged autohydrolysis (or storage of the dry holocellulose, in its free-acid form, for 2 years at room temperature, followed by extraction with water), the fibrous residue (yield, ~40%) behaved more like a typical holocellulose. Extraction with aqueous 24% potassium hydroxide yielded an apparently typical "α-cellulose" (**B**, ~20% of the original holocellulose) and a hemicellulose (**C**) which, after purification *via* an insoluble copper complex[4], contained residues of D-glucose (33%), D-xylose (29%), D-mannose (23%), D-galactose (9%), and L-fucose (4%). A fraction similar to **C** has been isolated from peat by Black *et al.*[5].

It seemed likely that, in the original holocellulose, the unidentified monomer was involved in cross-linking between the separate parts, **A**, **B**, and **C**. Since the linkages were stable to alkali but very sensitive to acid, they were probably acetals, ketals, or enolic ethers. Confirmation of the presence of reactive ketone groups was readily obtained by warming the holocellulose, or **A**, with aqueous phenylhydrazine. By decomposing the orange-coloured complexes with conc. hydrochloric acid, and measuring the liberated phenylhydrazine hydrochloride by its absorption maximum at 275 nm, it was shown that the material unaccounted for in the sugar analyses contained one ketone group for a unit weight of ~200 daltons.

When the polymeric phenylhydrazones were heated with an excess of phenylhydrazine under the conditions of the Barry degradation[6], a yellow phenylosazone or phenylhydrazone was liberated which, after extraction into diethyl ether, showed absorption maxima at 203, 240, and 275 nm. It was very unstable, and polymerised to a brown tar on evaporation of the extract to dryness, but p.m.r. spectroscopy of a fresh $CDCl_3$ extract showed the presence of one phenylhydrazino unit for every four aliphatic protons.

Since it was likely that reduction of the ketone group would stabilise the monomer to acid, and hence permit its isolation by acid hydrolysis, holocellulose (10 g) was treated with aqueous 2% sodium borohydride (500 mL) overnight at room temperature. Surprisingly, this treatment solubilised ~10% of the holocellulose. The soluble part con-

tained some polymeric material, but consisted mainly of D-*arabino*-2-hexulosonic acid (2-keto-D-gluconic acid, **2**), and of oligosaccharides linked glycosidically to **2**. Identification of **2** was achieved by acid-catalysed decarboxylation, to yield (mainly) D-arabinose, and by comparison of its calcium salt with an authentic specimen.

The monomer was finally isolated in unmodified form by enclosing the free-acid form of the holocellulose (10 g) with water (300 mL) in a cellophane dialysis-casing, and heating it in distilled water (1.5 L) at 98°. At 12-h intervals, the dialysis casing was replaced by a new casing, and the dialysate by fresh distilled water. The liberation of carbon dioxide was observed in this experiment. The dialysates were neutralised with calcium carbonate, filtered, and concentrated. The solution from three successive dialysates was passed through a column of Dowex 1 (acetate form) resin, which was then washed well with water. After elution of D-galacturonic acid with 0.5M acetic acid, **1** was eluted with 0.2M calcium acetate, as a single peak of material reacting positively with phenol–sulphuric acid. Calcium ions were removed by addition of the calculated amount of the pyridine salt of oxalic acid, followed by filtration. The filtrate was cautiously concentrated *in vacuo* to dryness, with constant adjustment of the pH to ~6 with pyridine. After reconversion into its calcium salt, the residue yielded hygroscopic crystals from aqueous ethanol, having $[\alpha]_D^{22}$ +25° (equil.; *c* 3.25, water). At the time of writing, identification of the crystals rests upon their partial reduction to **2** with dilute, aqueous sodium borohydride.

To investigate the possible involvement of residues of **1** in cross-linking, a suspension of the holocellulose (10 g) in aqueous 20% sodium borohydride (500 mL) was kept at room temperature for 48 h, after which it gave no reaction with phenylhydrazine. It was then heated in water, in its free-acid form, at 98° for 24 h. The liberated glycuronoglycan, corresponding to **A**, was isolated and dissolved in aqueous 2% sodium borohydride. After 24 h, the dialysable portion was recovered, and shown to contain **2**. It is therefore likely that a minor proportion of the residues of **1** in the holocellulose are linked glycosidically at both anomeric centres (positions 1 and 5). Since glycosidic linkages formed at position 5 would be similar to those formed by *N*-acetylneuraminic acid, their extreme sensitivity to acid hydrolysis is readily understood.

The possibility that **1** was an artefact, formed during chlorite-bleaching of the moss, was ruled out by the finding that the unbleached, acetone-extracted moss also yielded **2** upon treatment with dilute, aqueous sodium borohydride. It was additionally demonstrated that citrus pectin developed no significant reactivity towards phenylhydrazine when treated with chlorite under the conditions used to delignify the moss.

In a survey of holocelluloses from lower plants, the criteria of reactivity with phenylhydrazine, the Folin–Denis reagent, and 2,6-dichlorophenol–indophenol were used as indicators of the likely presence of keto-acids related to **1**. Positive results were obtained with ten different mosses selected from five different Orders (Sphagnales, Polytrichales, Hypnobryales, Grimmiales, and Dicranales), a liverwort (Order Jungermanniales), three horsetails (*Equisetum arvense, E. pratense*, and *E. hyemale*), and lawn-grass clippings (a mixture of species). Since the grasses are Spermatophytes, it seems likely that residues of **1** are not exclusively associated with the holocelluloses of lower plants, except insofar as they

lack the thick, secondary cell-walls characteristic of woody plants, and hence contain a much higher proportion of primary cell-wall material. The Sphagnales, in particular, contain many large, empty cells (hyalin cells, or leucocysts), whose thin walls seem to lack a secondary layer[7]. Fruit pectins all gave negative results, possibly because they are intercellular in origin.

ACKNOWLEDGMENTS

The author thanks Kari-Anne Holsen for skilful technical assistance, Hans Grasdalen for help with the n.m.r. spectroscopy, Professor N. A. Sørensen for collecting mosses and for his interest, and Norges almenvitenskapelige forskningsråd for financial support.

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