ACTA CHEMICA SCANDINAVICA 25 (1971) 2622–2628

Studies on the Polysaccharides of Lichens

I. The Structure of a Water-soluble Polysaccharide in *Stereocaulon paschale* (L.) Fr.

ERIK HAUAN and OVE KJØLBERG

Department of Chemistry, University of Oslo, Blindern, Oslo 3, Norway

A water-soluble polysaccharide has been isolated from the lichen *Stereocaulon paschale*. It has been found to be of a α-glucan type with a degree of polymerization (DP) ~140 glucose units and with a slight branding (CL ~38). The results indicate that the polysaccharide is built up mainly by 1,4-glucosidic linkages, and with 1,3-linkages as branch points. Furthermore, 1,3-linkages must also occur in the linear chain, approximately one per four 1,4-linkages, and also at, or fairly near, all the nonreducing ends.

The information available on the polysaccharides in lichens is mainly restricted to the water-soluble polysaccharides in *Cetraria islandica* (Iceland moss). Meyer and Gürtler[1] described the "cellulose" type polysaccharide, lichenin, in Iceland moss as a linear glucan, containing β-1,3- and β-1,4-linkages in proportion 3:7, and an α-glucosidic polysaccharide called isolichenin, which, besides glucose, also was found to contain small amounts of galactose and mannose.[2] Later works[3,4] have, however, shown that the isolichenin was a linear "starch" type polysaccharide, containing glucose as the only monomer, and with α-1,3- and α-1,4-linkages in the relative proportions *ca.* 3:2. Isolichenin was found to stain with iodine, giving a greenish-blue colour with low "blue value" (B.V. 0.005 – 0.01) and to be partly hydrolyzed by α- and β-amylase.[3] Lichenin has also been found in some Indian lichens, *e.g. Usnea longissima* Aeh.,[6] while *Roccella montagnei* has been described to be a lichen which contains isolichenin.[6,7] In *Umbelicaria pustulata* (L.) Hoffm., a water-soluble polysaccharide (pustulan) has been found,[8] which is built up as a linear β-1,6-linked glucose polymer.

So far, the hemicelluloses in lichens have been very sparingly investigated. Only an alkali-soluble polysaccharide isolated from *Cladonia alpestris* (L.) Rabenh. (reindeer moss) has been reported to be analysed in some detail.[9] This polysaccharide was found to be a highly branched polymer, containing a back-bone of glucose and mannose, and with mainly galactose in the terminal positions.

Reindeer moss and related species, *e.g.* *C. rangiferina* (L.) Web. and *C. arbuscula* Rabenh. have been found to contain more than 90 % of carbohydrates.[10] The lichens therefore act as an excellent food source for the Norwegian reindeer. The scope of this and further work in this series is therefore to investigate more in detail the different types of polysaccharides in the most common species of lichens found in the Norwegian mountains.

Stereocaulon paschale is a lichen closely related to reindeer moss. The first report on the carbohydrates in this species [11] concluded that the lichen contained mannose and galactose, while no glucose could be detected. Later work [12] has, however, shown that glucose occurs in fairly large amounts also in this moss. As so little work has been done on the carbohydrates in this common lichen, the first communication in this series describes the structural investigation of a water-soluble polysaccharide in *Stereocaulon paschale.*

After removing fatty material and lichen acids, the lichen was extracted with boiling water. As no precipitation occurred, neither on cooling nor by repeated freezing and thawing even after concentration to a smaller volume, it was concluded that the extract did not contain lichenin. By addition of ethanol (to 60 %), a precipitate was obtained (fraction A), counting for 6.8 % of dry weight. The lichen was thereafter extracted with 2 % of sodium hydroxide which by precipitation with ethanol gave fraction B (8.5 %), and then with 10 % of sodium hydroxide, giving fraction C, containing 3.8 % of polysaccharide. After these extraction procedures, the lichen was found still to contain carbohydrates.

By acid hydrolysis, the fraction A was found to contain mainly glucose, but also galactose, mannose, and xylose was found in the hydrolysate. Fractions B and C were found, by acid hydrolysis, to contain at least six different monosaccharide units, of which galactose, glucose, mannose, rhamnose, and xylose were identified. After repeated precipitation of fraction A with ethanol (four times), the fraction was found to have lost all the xylose. The polysaccharide containing mannose did not precipitate as a copper complex by addition of Fehling's solution. However, by chromatography on a DEAE-cellulose column, using a stepwise elution with phosphate buffer pH 6.6 (0.01, 0.05, and 0.1 M), three fractions (A1, A2, and A3) were obtained (Fig. 1).

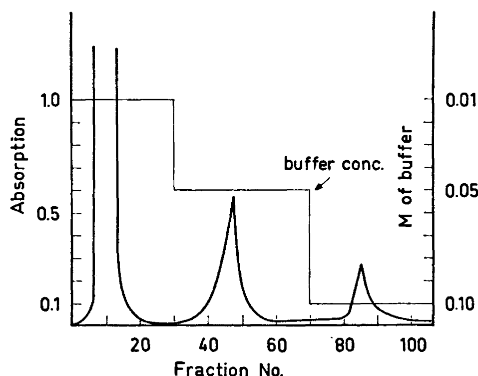

Fig. 1. Chromatography of the polysaccharide fraction A on DEAE-cellulose.

Of these, the main fraction (A1) was found, by acid hydrolysis, only to contain glucose, while the two other fractions both contained glucose, galactose, and mannose. As so small amounts of fractions A2 and A3 were obtained, only fraction A1 has so far been investigated in detail.

By methylation and hydrolysis of fraction A1, 2,3,4,6-tetra-*O*-methyl glucose (*ca.* 3 %), 2,3,6-tri-*O*-methyl glucose (63 %), 2,4,6-tri-*O*-methyl glucose (25 %) besides a small amount of 2,3,-di-*O*-methyl glucose, and at least one other, presumably dimethyl-substituted glucose, were found, as determined by gas chromatography after reduction and silylation of the hydrolysate. The fraction A1 had $[\alpha]_D^{20}+233°$, which indicated α-glucosidic linkages, and according to Jeanes *et al.*,[13] this high rotation should account for 30 – 40 % 1,3-linkages. Determination of the DP by end group assay of the reducing end gave a value equal to 140. Because of the low reducing power, this value cannot be taken as more than approximate. However, molecular weight determination by the gel filtration method, using Sephadex G 75, gave an approximate M.W. 24 000. This molecular weight may not be absolutely correct, as it is obtained by using two linear 1,6-linked dextrans of known M.W. as standards. Nevertheless, this M.W. is in fairly good agreement with the DP (M.W. 22 400). By periodate oxidation using sodium periodate, a consumption of 0.69 mol of periodate per 162 g of polysaccharide was found, indicating approximately 30 % of 1,3-linkages. End group assay by periodate oxidation gave formation of 0.026 mequiv. of formic acid from 110 mg polysaccharide. Using the usual formula for chain length calculation

$$(\overline{\mathrm{CL}}) = \frac{\text{mg polysaccharide}}{\text{mequiv. HCOOH} \times 162}$$

(giving $\overline{\mathrm{CL}} = 26$) will not be correct, as the formula neglects the formation of formic acid from the reducing end. As no overoxidation occurs under the conditions used, the reducing end must be oxidized as a hemiacetal, forming a formyl ester. The reducing unit bound in the 4-position will then give 2 mol of formic acid, while when bound in the 3-position, only 1 mol of formic acid will be produced. Assuming 30 % of 1,3-linkages and an approximate equal distribution of the 1,3- and the 1,4-linkages throughout the molecule relative to their amount, the equation

$$\overline{\mathrm{CL}} = \frac{\text{mg polysaccharide}}{162 \times (\text{mequiv. HCOOH}) - \dfrac{1.7 \times \text{mg polysaccharide}}{162 \times \mathrm{DP}}}$$

would be more correct.

Using this formula, the formation of formic acid should give a $\overline{\mathrm{CL}} = 38$ equivalent to approximately three branch points per molecule. By a Smith degradation of the oxidized product, erythritol, glucose, glucosyl erythritol and a trace of glycerol were detected by paper chromatography. No nigerose could be detected.

Partial acid hydrolysis to an apparent conversion into glucose to about 32 and 68 % was carried out. According to the Kuhn formula,[15] the latter should give maximum conversion to disaccharides. In the 68 % of hydrolysate,

maltose and nigerose were detected, as shown by their chromatographic and electrophoretic mobilities, but no isomaltose could be detected. The colour intensities of the spots indicated more maltose than nigerose, but this was not checked by quantitative determination. In the 32 % of hydrolysate, small amounts of the same disaccharides were detected, together with two spots, which both by their chromatographic and ionophoretic mobilities presumably were maltotriose and maltotetraose. Another "trisaccharide" with higher ionophoretic mobility (M_G 0.63 in borate buffer and 1.2 in germanate buffer) was also obtained. This should indicate a trisaccharide with a 1,3-linkage to the reducing end. This trisaccharide is expected not to be nigerotriose, as no nigerose could be detected after the Smith degradation. It seems therefore reasonable to assume that the trisaccharide is either a mixture of – or one of the two trisaccharides 3-*O*-α-D-maltosyl-glucose and 3,4-di-*O*-(α-D-glucopyranosyl)-glucose. So far, a too small amount has been obtained of the trisaccharide for further investigation. The polysaccharide did not stain blue with iodine, and no increase in reducing power could be detected by incubation with β-amylase.

From the results it is concluded that the polysaccharide is an α-glucan which in some respects reminds one of isolichenin, containing α-1,3- and α-1,4-glucosidic linkages. The ratio of 2,3,6- and 2,4,6-tri-*O*-methyl glucose indicates a molar proportion of 1,3- to 1,4-linkage of the order 1:2.5, *i.e.* less 1,3-linkages than in isolichenin. The amount of 2,3,4,6-tetra-*O*-methyl glucose, together with the formic acid produced by periodate oxidation, indicates approximately one branch point per 40 glucose units. These branch points are presumably 1,3-linkages. Some 1,3-linkages must also occur in the linear chains, approximately one per four 1,4-linkages, and every terminal unit or two to three glucose units from the nonreducing ends must also be 1,3-linkages, otherwise one might except some degradation with β-amylase. 1,4-Glucans with $\overline{CL} > 20$ have been found to stain blue with iodine. The most probable explanation why the present polysaccharide ($\overline{CL} = 38$) does not stain with iodine is that the 1,3-linkages do not allow the polysaccharide to coil up in a helix which is perfect enough for forming an iodine complex. This will also be in agreement with the fact that the linear polysaccharide nigeran, which is built up by approximately equal amounts of α-1,3- and α-1,4-glucosidic linkages, does not stain with iodine under blue value conditions.[16] With such an explanation, however, it is difficult to understand why isolichenin with D.P. ~42 glucose units, and with approximately equal amounts of 1,3- and 1,4-linkages, gives a blue colour with iodine. It might, however, be that the isolichenins investigated have been slightly contaminated with a starch-like substance. This possibility has also been discussed by Peat *et al.*[4] as a probable explanation for the iodine staining and the action of α- and β-amylase on isolichenin. If this is the fact, one can conclude that the polysaccharide described here is closely related to the isolichenins found in other species of lichens.

EXPERIMENTAL

Paper chromatograms were run by the descending method, using Whatman No. 1 filter paper with one of the following system (v/v): A. Ethyl acetate:pyridine:water

10:4:3. B. Ethyl acetate:pyridine:water 5:2:7. C. Butanol:pyridine:water 6:4:3. D. ethyl acetate:acetic acid:formic acid:water 9:1.5:0.5:2.

As spray reagent served either aniline oxalate [17] or silver nitrate.[18] Quantitative determination of the carbohydrate in the different fractions was carried out with the anthrone method.[19] Gas chromatography was carried out on an Aerograph Autoprep A-700 with an Apiezon L (30 %) on Chromosorb W (60/80 mesh) column, 8.5 ft. × 0.25 in., keeping the temperature at 190 – 195°C, or on a Varian Aerograph 1200 (flame ionization detector) with a 3 % SE30 on chromosorb W (70/80 mesh) column, 5 ft. × 0.25 in., at 130°C. Paper electrophoresis was carried out as described by Lindberg and Swan [20] in 0.05 M sodium germanate buffer, pH 10.7, and in 0.05 M borate buffer, pH 10.6 and 1500 V (25 – 30 V/cm) for 90 min, using 2,3,4,6-tetra-*O*-methyl-D-glucose for determination of the electroendosmosis. Reducing power was determined by the Somogyi method.[21]

Isolation of crude water-soluble polysaccharide. The dry lichen (200 g), after being ground in a mill and extracted with (a) benzene and (b) methanol (Soxhlet) to remove waxy materials, was treated with a cold solution (1000 ml) of sodium carbonate (2 %) for 3 h. The residue, after filtration, was washed free from alkali and thereafter extracted with water (1000 ml) at 95 – 100°C for 3 h. Any lichenin present was removed, as described by Chanda *et al.*[8] A crude polysaccharide was precipitated by addition of ethanol (to 60 %), giving 1.364 g.

The polysaccharide was analyzed by complete acid hydrolysis (2 N sulphuric acid for 2.5 h) and chromatography of the hydrolysate, using solvents A and C as eluents.

Purification of the polysaccharide. The polysaccharide (1.3 g) was dissolved in water (100 ml), filtered and precipitated by addition of ethanol (160 ml) five times. An attempt to obtain a precipitate by addition of Fehling's solution to a solution of the polysaccharide failed. The polysaccharide (300 mg) dissolved in water (20 ml) was then chromatographed on a DEAE-cellulose column (3 × 35 cm), using a stepwise elution with phosphate buffer pH 6.8 (i) 0.01 M (500 ml). (ii) 0.05 M (500 ml), and (iii) 0.1 M (500 ml); the effluent was collected in 13 ml fractions. Fractions 5 – 20, 35 – 60, and 80 – 100 were mixed (Fig. 1), dialyzed against running tap water, and freeze-dried, giving fraction A1:200 mg, A2:12 mg, and A3:18 mg. Molecular weight determination by the gel filtration method was carried out as described by Whitaker,[22] using Sephadex G 75 and a column 1.5 × 150 cm with water as eluent. The void volume V_0 was found to be 85 ml, using a rabbit muscle glycogen. For the determination of the molecular weight, the $V/V_0 = 1.88$ for the polysaccharide was compared with two dextrans of known molecular weights. Dextran 20 $M_w \sim 20\,000$ gave $V/V_0 = 1.92$ and dextran 40 $M_w \sim 40\,000$ was found to have $V/V_0 = 1.66$. Compared with these two dextrans, the polysaccharide should have $M_w \sim 24\,000$.

Methylation was carried out as described by Kuhn *et al.*,[23] giving a polysaccharide with no absorption in the region 3400 – 3600 cm^{-1} after three successive methylations. The methylated polysaccharide (150 mg) was hydrolyzed in sulphuric acid, as described by Bouveng and Lindberg,[24] and the hydrolysate neutralized with barium carbonate and filtered. To ensure that a complete hydrolysis had taken place, the solution was chromatographed on paper, using solvents C and D as eluents. The solution was reduced with sodium borohydride (100 mg), deionized using ion exchange resins (Amberlite IR-120 and IR-45), and evaporated to dryness.

Trimethylsilylation was carried out as described by Sweeley *et al.*[25] The dry hydrolysate (20 mg) was dissolved in pyridine (2 ml), and a mixture of hexamethyldisilazane (0.4 ml) and trimethylchlorosilane (0.2 ml) was added, and the solution was kept at room temperature for 8 h. The solution was centrifuged, evaporated to dryness, dissolved in chloroform (10 ml), centrifuged, and again evaporated to dryness. All the ammonium chloride was removed by repeating this procedure three times. The substance was then dissolved in acetone prior to the gas chromatography. A similar reduction and silylation procedure was carried out with the following reference substances: 2,3,4,6-tetra-*O*-methylglucose, 2,3,4-, 2,3,6-, and 2,4,6-tri-*O*-methylglucose, 2,3-di-*O*-methylglucose, and 2- and 3-*O*-methyl glucose.

Periodate oxidation for the determination of chain length was carried out with potassium periodate as described by Bell and Manners,[14] starting with 110 mg polysaccharide. The method as described by Perlin [26] for periodate oxidation, followed by determination of consumed periodate, was used, starting with 120 mg of polysaccharide, and the oxidation was carried out for 120 h. The consumed periodate was estimated by extrapolation of the linear part of the oxidation curve back to zero time. The two assays were combined,

excess of periodate was reduced with ethylene glycol, and the iodate removed by dialysis. After evaporation to a small volume (50 ml), sodium borohydride (0.2 g) was added. The solution was kept at room temperature for 48 h, and then neutralized to pH 7 with sulphuric acid, and deionized with ion exchange resins (Amberlite IR-45 and IR-120). After evaporation to dryness, the last traces of boric acid were removed by five times evaporation with methanol. One aliquot was used for partial acid hydrolysis which was carried out, using the method as described by Goldstein *et al.*[27] Another aliquot was treated with sulphuric acid (2 N) at 100° for 2 h. After deionization, the hydrolysates were chromatographed using solvent B as eluent, and the different spots obtained were identified by comparison with authentic species.

Partial acid hydrolysis of the polysaccharide was carried out as described by Wolfrom and Franks.[28] The polysaccharide (10 mg) was dissolved in sulphuric acid (0.1 N; 10 ml) and kept on boiling water until 32 and 68 % hydrolysis, determined as apparent conversion to glucose by its reducing power. The solutions were cooled to room temperature, neutralized with barium carbonate, filtered and evaporated to dryness *in vacuo*. The hydrolysates were chromatographed on paper, using solvents A and C, and were also analyzed by electrophoresis in germanate and borate buffer. Nigerose, maltose, maltotriose, and maltotetraose were identified by comparison of their mobilities with authentic samples.

The attempted β-amylolysis was carried out as described previously,[29] and the iodine staining as described by Archibald *et al.*[30]

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Received November 10, 1970.