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STUDIES ON A NOVEL POLYSACCHARIDE GEL FROM THE FRUIT OF *Thaumatococcus daniellii* (BENTH)

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ABSTRACT

T. daniellii gel contains residues of L-arabinose, D-xylose, D-glucuronic acid, and 4-*O*-methyl-D-glucuronic acid in the ratios 1.00:7.20:1.91:0.66, together with nitrogen (~1%) and ash (3.1%). The ash-free gel contains 76% of pentose and 24% of uronic acid; 25% of the uronic acid occurs as the 4-*O*-methyl derivative. All of the uronic acid residues in the polysaccharide are susceptible to periodate oxidation. Methylation studies suggest that the uronic acids occur as terminal side-substituents to a xylan back-bone and that the polysaccharide is highly branched. Enzymolysis with β-D-glucuronidase liberates a substantial part of the uronic acid, but does not completely depolymerise the gel.

INTRODUCTION

The fruit of the West African shrub *Thaumatococcus daniellii* (Td) or Katemfe is known for its extremely sweet taste originating from sweet proteins[1–3] located in the aril at the base of each seed. The seeds are covered with a thin transparent gel having unusual physical and chemical properties; these include the ability to imbibe up to 500 times its own weight of water, thereby producing a resilient transparent gel of convoluted structure (Fig. 1) which is tasteless and very similar in appearance and texture to set gelatin. This apparently unique fruit constituent serves as a protection for the seed, initially splitting open the fruit pericarp during swelling and subsequently retaining moisture and nutrients for germination. Its composition (arabinose, xylose, and uronic acids) suggests close structural relationship to the gum exudates of the Rosaceae and the xylan hemicelluloses of angiosperm leaves. No such xylan has been described previously for related species or plant families of the same order, so that adequate assessment of the chemotaxonomic significance of this carbohydrate is not possible.

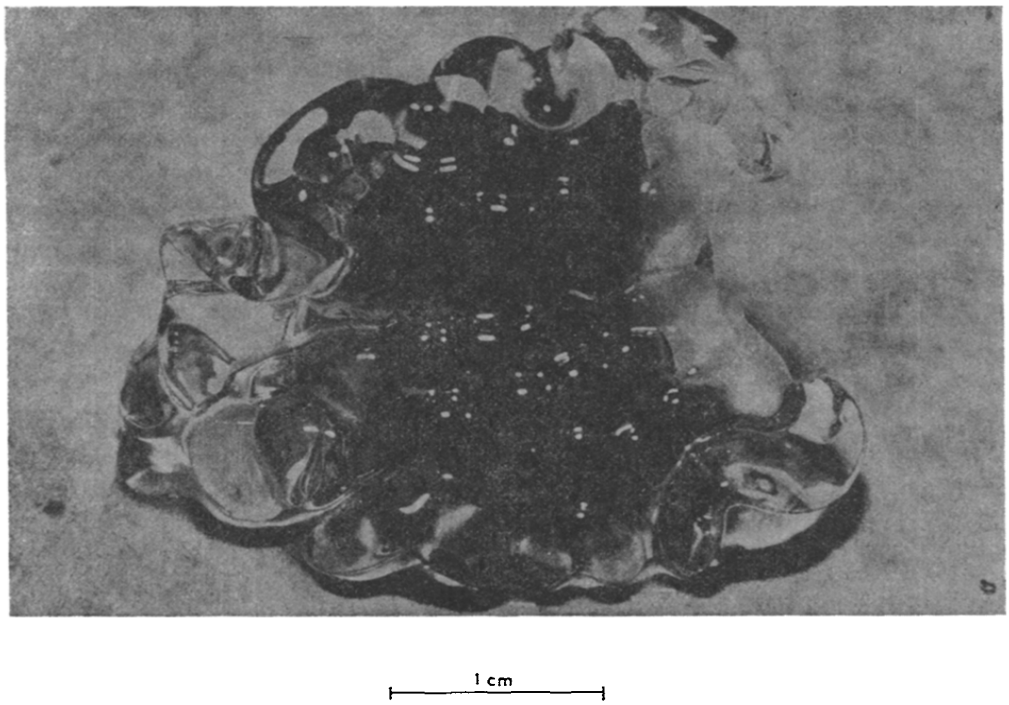

Fig. 1. A seed from *T. daniellii* soaked in water, showing the expanded polysaccharide gel.

RESULTS AND DISCUSSION

Gel was extracted, from fruit of *Thaumatococcus daniellii* (Benth) collected in Western Nigeria, into dilute alkali and purified by precipitation with acidified ethanol, followed sequentially by deproteinisation, dialysis, and filtration. Lyophilisation then gave a white, fluffy product. A single fraction was obtained by elution of the material from columns of DEAE-cellulose, DEAE-Sephadex, or Sephadex G-200, suggesting a single homogeneous constituent. The physical and chemical properties of this material are summarised in Table I.

Elemental analysis indicates that the gel exists as calcium (1.40% w/w Ca^{2+} to gel) potassium (0.94%), sodium (0.47%), and magnesium (0.30%) salts. Following dialysis against distilled water, some calcium (0.45%), potassium (0.11%), sodium (0.03%), and magnesium (0.007%) remain, which can be removed by prolonged dialysis first against water containing EDTA and then against deionised water. Since the gel swells in the absence of these ions, they may not be necessary for hydration. The free-acid form of the polysaccharide dissolves readily in water to give a solution of pH 2.6–3.1, whereas the sodium salt disperses in water with difficulty to give a solution of pH ~6.2.

I.r. bands at 1735 and 1639 cm^{-1} for the arabinoglucuronoxylan have been assigned to un-ionised and ionised free-carboxyl groups, respectively, suggesting the

TABLE I

ANALYTICAL DATA FOR THE GEL FROM KATEMFE FRUIT

Xylose (%)	66.9[a], 63[b]
Glucuronic acid (%)	17.7[a], 17[b]
4-*O*-Methylglucuronic acid (%)	6.1[a], 7[b]
Arabibose (%)	9.3[a], 13[b], 11[c]
Protein (%)	6.7[d], 6.0[e]
Mol. wt. of reduced polysaccharide	56,000
Equiv. wt. of solubilised polysaccharide	666
Ash (%), original gel	3.1
after dialysis	0.6
$[\alpha]_D^{20}$ (*c* 0.43, M sodium hydroxide)	−26
Moisture content, freeze-dried gel (%)	13.0

[a]As alditol acetates. [b]Chromoscan analysis. [c]As trimethylsilyl derivative. [d]From amino-acid analysis. [e]From direct determination of nitrogen.

presence of hexuronic acids[4,5]. Native gel, lyophilised and further dried over P_2O_5 *in vacuo*, gave very strong absorptions at 1650 and 1410 cm^{-1}, indicating[6,7] that the polysaccharide occurs naturally in the salt form. No absorption bands were detected at 2580–2500 (S–H), 1250–1230 (S=O), or 820 cm^{-1}, suggesting the absence of sulphate groups. A weak absorption at 890 cm^{-1} suggested a preponderance of β-D linkages[4], which was confirmed by digestion with a β-D-glucuronidase that liberated some uronic acid residues, and further substantiated by a negative specific rotation.

Acid hydrolysis (5mM H_2SO_4, 2 h, 90°) of the gel yielded arabinose (p.c.), suggesting that the arabinose residues are present in the acid-labile, furanoid form. The polysaccharide could not be completely hydrolysed using either 0.5M or M acid, and p.c. of hydrolysates revealed xylose, glucuronic acid, and an aldobiouronic acid. Hydrolysis (90% HCOOH–M H_2SO_4) of the aldobiouronic acid gave[8,9] xylose and glucuronic acid, the identities of which were confirmed by mass spectrometry of methylated derivatives. The mass spectrum of the methylated aldobiouronic acid contained peaks at *m/e* 424 (4.2%), which confirms[25] the presence of a disaccharide, and 233 (8.5), 201 (79.4), 169 (12.2), 191 (23.0), 173 (5.9), 141 (8.0), 101 (100), 88 (38.4), and 75 (25.4) attributable[25,26] to the methylated glucuronic acid. The ratios of abundancies of the ions *m/e* 201/233 and 101/88 are >1, confirming that glucuronic acid and xylose are linked together in the polysaccharide.

When the gel was treated with 37% aqueous propylene oxide[11] and a water-soluble carbodiimide[10], and the product was reduced with sodium borohydride, the acid-resistant glycosiduronic acid linkage was replaced by a glycosidic linkage, and acid hydrolysis then yielded arabinose (11%), xylose (65%), glucose, and 4-*O*-methylglucose; no oligosaccharides were detected. Glucose and 4-*O*-methylglucose were present in the ratio 100:29.

The presence of large proportions of uronic acid in the gel precluded methylation analysis. However, acid hydrolysis of the methylated, reduced polysaccharide

and conversion of the products into alditol acetates gave, *inter alia*, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, which confirmed that the uronic acids are present as side substituents.

Periodate oxidation of the acidic polysaccharide for 72 h destroyed all the uronic acids; about two thirds of the xylose residues and most of the arabinose residues resisted oxidation. P.c. of the products from total, acid hydrolysis of the oxidised polysaccharide revealed the presence of xylose, arabinose (ratio 3.7:1), and a minor, coloured (pink–red) compound. Thus, a substantial proportion of the xylose residues and most of the arabinose residues do not contain vicinal-diol groups. After borohydride reduction of the oxidised polysaccharide, followed by mild hydrolysis with acid (Smith degradation), a second periodate oxidation gave xylose and arabinose in the ratio 1.09:1, suggesting that a greater proportion of the xylose residues had been oxidised and that some arabinose residues may be linked in the xylan backbone. The periodate-oxidation data and a consideration of the molar ratios eliminate the possibility that xylose and glucuronic acid occur as a regular repeating-unit.

The possibility of a linear structure is supported by the fairly strong film which the polysaccharide forms on drying, and the threadlike pattern it forms on precipitation. The polysaccharide did not reduce Fehling's solution, suggesting a large size with few (if any) sugar residues having reducing end-groups in the exterior chains.

These initial studies suggest that the gel elaborated by *T. daniellii* is a polysaccharide having a xylan backbone, to which are attached at positions 2 or 3, as side chains, glucuronic acid and its 4-*O*-methyl derivative.

EXPERIMENTAL

General methods. — All evaporations were performed under diminished pressure at 40–45°. Whatman 1 and 3MM papers were used for descending p.c. with *A*, ethyl acetate–acetic acid–formic acid–water (18:3:1:4); *B*, 1-butanol–pyridine–water (6:4:3); *C*, 1-butanol–benzene–pyridine–water (5:1:3:3); *D*, 1-butanol–ethanol–water (5:1:4); *E*, phenol–water (3:1); and *F*, ethyl acetate–pyridine–water (4:1:1, top layer); and detection with aniline hydrogen phthalate[13] or *p*-anisidine hydrochloride[13]. The optical rotation was recorded with a Perkin–Elmer 141 polarimeter. I.r. spectra (KBr discs) were recorded with a Perkin–Elmer Model 457 spectrophotometer. Mass spectra (70 eV) were obtained with an A.E.I. MS-12 spectrometer that had a direct-inlet system (source temp., 200°) and was coupled to a Pye 104 gas chromatograph fitted with flame-ionisation detectors and (*a*) coiled glass columns (60 × 0.25 in.) of 3% of ECNSS-M on Gas Chrom Q (80–100 mesh), (*b*) columns (72 × 0.25 in.) of 3% of SE-30C on Gas Chrom Q (80–100 mesh), or (*c*) columns (72 × 0.25 in.) of 3% of SE-52 on Chromosorb N (85–100 mesh, acid-washed). Trimethylsilyl ethers were prepared as described by Sweeley *et al.*[14], alditol acetates by the procedure of Rosik *et al.*[15], and partially methylated alditol acetates by the method of Björndal *et al.*[16]. Results were quantified by using an Autolab 6300 Digital integrator. Regeneration of exchangers and resins and packing of columns were as recommended by the

manufacturer. Colorimetric assays[8,9,17] were used to monitor the elution of sugars in paper and column chromatography.

Isolation and purification of the polysaccharide. — The fruits were opened by hand, and the seeds were separated from the aril and integument with a scalpel. The clean seeds, coated with the gel, were then stirred into dilute alkali (0.1 or 0.2M NaOH, or 10% Na_2CO_3) for ~1 h. After solubilisation, the green–yellow extracts were neutralized by cautious addition of dilute hydrochloric acid and filtered through glass wool. The clear filtrate was dialysed against distilled water, and passed through a column of Amberlite IR-120(H^+) resin, and the crude polysaccharide was recovered by lyophilisation. Alternatively, the crude aqueous extract was centrifuged and the polysaccharide was precipitated in acidified ethanol (~5 vol.). A solution of the precipitated polysaccharide in distilled water was filtered and lyophilised to give a white, fluffy product. Deproteinisation, when necessary, was effected by the method of Staub[18].

Fractionation of the polysaccharide. — A portion of the crude polysaccharide (50 mg) was dissolved in distilled water (10 ml) and loaded on to a column (30 × 2.5 cm) of DEAE-Cellulose, which was then eluted successively with 0.2M Tris–HCl buffer (pH 5.9–6.0) and a 0.3M NaCl gradient in the buffer. Fractions (4 ml) were assayed for carbohydrates[17] and for protein[19]. The protein peak was eluted with the buffer, and the polysaccharide with the salt gradient. The polysaccharide preparation (1 mg) was further chromatographed on a column (60 × 1.5 cm) of Sephadex G-200 by elution with sodium acetate–acetic acid buffer (pH 4.0) containing 0.5M NaCl. Fractions (5 ml) were assayed for polysaccharide[20], giving a single band due to the arabinoglucuronoxylan at the void volume.

Properties of the polysaccharide. — The equivalent weight of the gel was determined by titration[15] using two different preparations. Protein-free polysaccharide was dried at 60° *in vacuo* for 24 h and cooled in a desiccator over P_2O_5. Three samples (70–120 mg) were separately soaked in 300-ml portions of distilled water, covered with toluene (20 ml), and stirred for 24 h. Titration with 0.1M NaOH followed for ~8 h, 0.2 ml of alkali being added each time until a steady pH of 7.6 was reached. Appropriate blank titrations were performed. Dried, scalpel-removed gel was washed repeatedly for 3 h with ethanol containing 1% of HCl. The gel was washed free of acid and recovered by lyophilisation. Titration with alkali to pH 7.6, as described earlier, gave comparable results.

Estimation of the molecular size of the carboxyl-reduced polysaccharide was effected by gel filtration on a column (60 × 1.5 cm) of Sepharose 4B by elution with sodium acetate buffer (pH 4.0) containing M NaCl at 10.50 ml/h; 4-ml fractions were collected and assayed for carbohydrate[17]. The column was calibrated with *Leuconostoc mesenteroides* dextrans T10 (mol. wt. 9,300), T20 (mol. wt. 22,300), T40 (mol. wt. 42,000), T110, T250, and T500 (Pharmacia Ltd.). Void volumes and inclusion volumes were determined by fractionating freshly prepared solutions of blue dextran and potassium chromate. The molecular weight was then estimated from a curve of K_{av} versus log mol. wt. Because of the expected differences in the unknown shape of

the modified polysaccharide and the Pharmacia (1→6)-α-D-linked dextrans used for calibration, the molecular weight reported is tentative. The molecular weight of the original polysaccharide could not be estimated on columns of Sephadex G-200 and 4B. Total exclusion on Sephadex G-200 may be due to repulsive forces between the carboxylic groups of the polysaccharide and the residual carboxylic groups on the Sephadex gel, or the shape and conformation of the polysaccharide in solution. Total inclusion on Sepharose 4B could be due to the structure of the polysaccharide which encourages extensive hydrogen bonding. The variations of the elution pattern of the polysaccharide were not due to changes of the properties of the gels, as the K_{av} values of the dextran fractions were not influenced by ionic strength.

Hydrolysis of the gel. – (*a*) *With 5mM sulphuric acid.* The freeze-dried product (100 mg) was hydrolysed under nitrogen at 100° for 2 h with 5mM H_2SO_4 (25 ml). The cooled solution was neutralised with barium carbonate, decationised over Amberlite IR-120(H^+) resin, and concentrated. The residue contained arabinose (p.c.).

(*b*) *With 0.5M sulphuric acid.* Purified polysaccharide (1 g) was hydrolysed under nitrogen at 100° for 20 h with 0.5M H_2SO_4 (100 ml). The cooled solution was neutralised with barium carbonate and decationised on AG 50W X4 resin. P.c. (solvent *D*) revealed xylose, arabinose, glucuronic acid, and an aldobiouronic acid. The hydrolysate was applied to a column (60 × 2.5 cm) of Sephadex A-25($HCOO^-$) resin. The neutral sugars were eluted with distilled water (2 l), and the acidic fraction with water–5% formic acid gradient that separated glucuronic acid and an aldobiouronic acid (125 mg), $[\alpha]_D$ +112° (*c* 1, water); ν_{max}^{KBr} 3400 (s, broad), 2900 (m), 1735 (s), and 1640 (w) cm^{-1}. P.c. (solvents *A* and *D*) of the neutral fraction showed only arabinose and xylose. This was confirmed by g.l.c. of the trimethylsilyl derivatives on a glass column (72 × 0.25 in.) of 3% of SE-52 (temperature programme 150→250° at 5°/min).

Carboxyl-reduction of the polysaccharide. — Using the method of Taylor and Conrad[10], the polysaccharide was not completely reduced. The partially reduced polysaccharide (41.1 mg) was hydrolysed under nitrogen (M H_2SO_4, 4 h), and the products were examined by p.c., converted into the alditol acetates, and subjected to g.l.c. Protein-free polysaccharide (1 g) was esterified[11] with 37% propylene oxide during 20 days; fresh propylene oxide solution was added 7 times. The product was subsequently reduced with sodium borohydride (1.5 g) for 20 h, and recovered after purification by lyophilisation. The reduced polysaccharide gave a negative test with harmine[8].

Methylation analysis. — The carboxyl-reduced polysaccharide (60 mg) was methylated by the Hakomori method[21] to give a product which showed no i.r. absorption for the hydroxyl groups. The sulphinylcarbanion was generated by the method of Conrad[22], and the product of methylation was fractionated by the method of Anderson *et al.*[23] to give a product (35 mg). The methylated polysaccharide was hydrolysed, and constituent sugars were examined as permethylated alditol acetates by g.l.c. The aldobiouronic acid isolated on partial hydrolysis of the polysaccharide with acid was methylated as described above. The methylated product was purified by t.l.c.[24] and analysed[25] by mass spectroscopy.

Periodate oxidations. — The polysaccharide (200 mg) was oxidised in the dark with 15mM sodium metaperiodate (50 ml) for 7 days. Ethylene glycol (specially pure, 5 ml) was then added to reduce excess of periodate, the solution was dialysed for 48 h and then filtered, and the oxopolysaccharide was reduced with sodium borohydride[26]. The polyalcohol was hydrolysed (0.5M H_2SO_4, 18 h, 100°), and the purified hydrolysate was examined by p.c. (solvents *A* and *C*).

Two separate samples of the polysaccharide (50 mg) were oxidised as described above for 3 days, but including 1.25 ml of 1-propanol and M sodium perchlorate (10 ml). A reference solution contained polysaccharide, 1-propanol, and sodium perchlorate, but no periodate. The consumption of periodate was monitored spectrophotometrically[27]. The solutions were dialysed against several changes of distilled water for 48 h at 4° and then treated with boric acid (50mM, 25 ml) and potassium borohydride (250 mg) at ~0° for 30 h. Excess of hydride was decomposed with dilute acid, and salts were removed by dialysis. A sample of the oxidised-reduced polysaccharide was analysed for hexuronic acid by the harmine reagent[8] and for pentoses by p.c. (Chromoscan method). A second sample was hydrolysed with 0.1M H_2SO_4 at room temperature for 30 h. Sulphate ions were removed by the addition of barium carbonate, and barium ions were removed on a short column of Amberlite IR-120(H^+) resin. A second periodate oxidation was then carried out for 103 h under the conditions of the first oxidation, but in the absence of propanol or sodium perchlorate. Oxidation was terminated with ethylene glycol (5 ml), and the polysaccharide was purified as described above. The oxidised-reduced polysaccharide was treated with 0.6M H_2SO_4 under nitrogen at 100° for 22 h, and p.c. then revealed xylose and arabinose.

Action of β-D-glucuronidase on the gel. — Katemfe gel (110 mg) was dissolved in 27.5 ml of sodium acetate buffer (0.1M, pH 3.8). The crude enzyme [5 mg, β-D-glucuronidase from limpets (*Patella vulgata*), EC 3.2.1.31, Sigma] was added, and hydrolysis proceeded at 37° in a shaker for 40 h. Enzyme and polysaccharide were precipitated with ethanol, the mixture was centrifuged, and the concentrated supernatant was examined by p.c. (solvents *C*, *E*, and *F*), which revealed glucuronic acid and xylose, but no oligosaccharide. The liberation of xylose may be due to xylanases in the crude enzyme preparation.

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