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OLIGOSACCHARIDES PRODUCED BY PARTIAL ACETOLYSIS OF XANTHAN GUM

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ABSTRACT

Partial acetolysis of the extracellular polysaccharide from *Xanthomonas campestris* gives the constituent monosaccharides, cellobiose, and three mannose-containing neutral oligosaccharides thought to be derived from the branch points of the polysaccharide The structural analyses of these oligosaccharides provide further evidence for the presence of β-D-glucopyranosyl and α-D-mannopyranosyl linkages in the polymer

INTRODUCTION

The structure originally proposed[1] for the industrially useful, extracellular polysaccharide (xanthan gum) elaborated by the bacterium *Xanthomonas campestris* has recently been revised[2] [3] An important new feature was the discovery that the main chain consists of 4-*O*-β-D-glucopyranose residues with side chains attached to alternate sugar residues Our results, which are in full accord with the revised structure, provide evidence for the α configuration of the internal D-mannopyranosyl linkages of the side chains We also report on the characterisation of five fragments of the polymer, which can be prepared and detected relatively easily and offer a means of comparing the structures of gums from various sources (such as mutants of *X campestris* or other xanthomonad species)

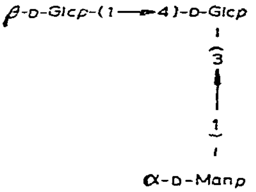
RESULTS AND DISCUSSION

Xanthan gum was partially acetolysed, and the crude deacetylated product was fractionated on ion-exchange resins[4] into acidic and neutral components The neutral fraction comprised the major proportion of the product and was relatively rich in oligosaccharides (p c) This observation was surprising in view of the considerable resistance of glycuronosyl bonds to aqueous acid, which usually leads to the isolation of mainly acidic oligosaccharides upon hydrolysis of uronic acid-containing polysaccharides Thus, of a series of aldo-oligouronides obtained from xanthan gum by partial hydrolysis with acid, cellobiose (**4**) was the only neutral oligomer identified[3]

The acidic fraction of the partial acetolysate contained glucuronic acid and another compound, which was presumed to be 2-*O*-(β-D-glucopyranosyluronic acid)-D-mannose (**1**) previously isolated from the gum[3] When the chromatographically homogeneous, latter product, obtained by using high-voltage paper electrophoresis, was hydrolysed, approximately equal proportions of glucuronic acid and mannose were formed (p c)

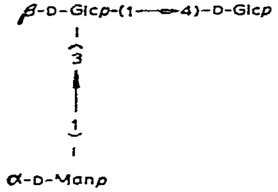
$\beta\text{-D-GlcA}p\text{-}(1\rightarrow 2)\text{-D-Man}p$

1



$$\begin{array}{ll}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-} & \text{D-Glc}p\\ & \quad 3\\ & \quad\uparrow\\ & \quad 1\\ & \alpha\text{-D-Man}p\end{array}$$

2



$$\begin{array}{l}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-D-Glc}p\\ \quad 3\\ \quad\uparrow\\ \quad 1\\ \alpha\text{-D-Man}p\end{array}$$

3

$\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-D-Glc}p$

4

$\alpha\text{-D-Man}p\text{-}(1\rightarrow 3)\text{-D-Glc}p$

5

Four oligosaccharides (**2**–**5**) were isolated from the deacetylated neutral fraction of the partial acetolysate by cellulose column and preparative paper chromatography

Fragments **2** and **3** had mobilities (p c) indicative of trisaccharides Specific colorimetric assays[5] for total carbohydrate and glucose showed the hydrolysates of **2** and **3** each to be composed mainly of glucose (**2**, 55%, **3**, 66%) When **2** and **3** were each treated with sodium borohydride and the products then analysed, a substantial decrease in the glucose yields was observed (**2**→45%, **3**→50%), thereby demonstrating the presence of a glucose reducing-residue in each saccharide G l c of the alditol acetates derived from the components of **2** and **3** confirmed that each trisaccharide was composed of glucose and mannose in the ratio 2 1

The partially methylated alditol acetates derived from the glycitol of **2** gave two peaks (ratio ~1 2) on g l c (OV 225), the larger of which contained unresolved 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-hexitols (T_{TMG} 1 0), as the mass spectrum was indistinguishable from that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (TMG) The spectrum [*m*/*e* 277 (44%), 185 (43), 175 (21), 143 (71), 129 (37), 115 (10), 110 (10), 101 (4), 89 (57), 87 (50), 75 (16), 59 (36), 45 (33), and 43 (100)] of the minor, more volatile component (T_{TMG} 0 81) contained a prominent ion at *m*/*e* 89, as expected[6] for a 3,4-di-*O*-acetyl-1,2,5,6-tetra-*O*-methylhexitol, resulting from fission between C-2 and C-3 or C-4 and C-5 which occurs when the vicinal methoxyl groups are terminal

When **2** was reduced and then trimethylsilylated and the product (T_{RAF} 1 52) was subjected to g l c –m s , the absence of ions at *m*/*e* 307 (from primary cleavage of the C-3 and C-4 bond of the alditol moiety) and 525 [from primary cleavage of a pentakis(*O*-trimethylsilyl)alditol fragment] indicated[7] branching in the parent oligosaccharide When reduction of **2** was performed with NaB^2H_4 prior to silylation, the intensity of the ion at *m/e* 206 in the mass spectrum was enhanced, and this is consistent with disubstitution (at O-3 and O-4) of the reducing glucose residue

The detection (p c) of cellobiose in the partial, acid hydrolysate of **2**, together with the foregoing evidence, shows its structure to be *O*-D-mannopyranosyl-(1→3)-[*O*-β-D-glucopyranosyl-(1→4)]-D-glucose

The mass spectrum of the per(trimethylsilyl) ether of the glycitol of **3** (T_{RAF} 1 52) contained ions at *m/e* 451 and 525 arising by cleavage of the hexose and alditol residues, respectively The series of ions at *m/e* 103, 205, and 307 (409 absent) suggested substitution at O-4 of the reducing glucose moiety, and this conclusion was supported by the increased intensities of the ions at *m/e* 104, 206, 308, and 526 when NaB^2H_4 was used for the preparation of the glycitol

When the glycitol of **3** was subjected to methylation analysis, three partially methylated alditol acetates were obtained (g l c , ECNSS-M, 173°), the two slower-eluting compounds had T_{TMG} values of 1 00 and 1 93 (*cf* T_{TMG} 1 00 and 1 80 on OV 225 at 200°) Comparison with literature data[6] allowed these peaks to be assigned to the derivative arising from a terminal non-reducing hexose and either 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-mannitol or 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, respectively Since α-D-mannosidase converted **3** into cellobiose and mannose, the least-volatile, partially methylated alditol acetate must be the glucitol derivative Thus, **3** is *O*-α-D-mannopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→4)-D-glucose

Identification of **4** as cellobiose was based upon co-chromatography of the compound with an authentic sample of the disaccharide

Fragment **5** was assigned the structure *O*-α-D-mannopyranosyl-(1→3)-D-glucose upon the following evidence The total carbohydrate to glucose ratio of a hydrolysate of **5** was 1 94 1, but reduction with $NaBH_4$ prior to hydrolysis destroyed all the glucose, indicating **5** to be a disaccharide with a glucose reducing-residue Analysis (g l c) of the alditol acetates of the components of **5** showed equal proportions of mannose and glucose to be present in **5** The mass spectrum of the per(trimethylsilyl) ether of the glycitol of **5** (T_{SUC} 1 50) contained, *inter alia*, ions at *m/e* 103, 205, 307, and 451 Substitution at O-3 of the glucose moiety in **5** was shown by lead tetra-acetate oxidation[8] followed by hydrolysis, which gave (p c , g l c of the alditol acetate) arabinose

The conversion of **5** into glucose and mannose by α-D-mannosidase established the presence of an α-D linkage Although **2** was largely unaffected by α-D-mannosidase, the formation of traces of cellobiose and mannose suggested that an unusually resistant α-D-mannopyranosyl linkage was present The proximity of the glucosyl substituent on O-4 of the disubstituted glucose moiety probably hinders attack by the enzyme Similarly, **2** was not attacked by β-D-glucosidase

Presumably, the fragments **1–5** originate as shown in Fig 1 With the exception of the terminal mannose residue, all of the sugars in the repeating unit are present in at least one of the oligomers Thus, any major structural variations of the polysaccharide, which may result from its production under differing conditions, should be detectable qualitatively by p c of the oligomers formed on partial acetolysis

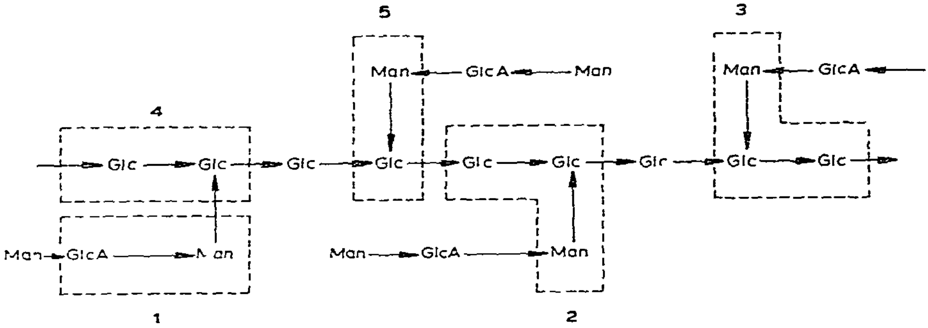


Fig 1 Schematic representation of the structure of xanthan gum and the origin of oligosaccharides 1–5

EXPERIMENTAL

Materials and methods — P c was performed on Whatman No 1 paper with *A* 1-butanol–pyridine–water (6 4 3) or *B* ethyl acetate–pyridine–water (8 2 1)[9] Washed Whatman 3MM paper was used for preparative p c, and detection was effected with alkaline silver nitrate[10] G l c was performed on a Pye 104 chromatograph with glass columns (150 × 0 3 cm) packed with *A*, ECNSS-M, *B*, OV 225, or *C*, SE 52 (column, 100 × 0 3 cm) Alditol acetates were prepared as described by Sawardeker *et al* [11], and per(trimethylsilyl) ethers by the two-step procedure of Ohashi and Yamakawa[12] Relative retention values for di- and tri-saccharide derivatives are given relative to that of the per(trimethylsilyl) ethers of sucrose (T_{SUC}) or raffinose (T_{RAF}), respectively, on column *C* at 210° or 250° A Pye 104 chromatograph linked to an A E I MS-12 mass spectrometer fitted with an A E I DS-30 data system was used for g l c –m s

Paper electrophoresis was conducted on Whatman 3MM paper in 0 2M pyridine acetate buffer (pH 4 5) at 85 V/cm, with an apparatus previously described[13]

Total carbohydrate assays were effected by the phenol–sulphuric acid method[5], and D-glucose was determined by the D-glucose oxidase (EC 1 1 3 4) method with reagents supplied by Boehringer–Mannheim G m b H, Mannheim, Germany α-D-Glucosidase (EC 3 2 1 20), β-D-glucosidase (EC 3 2 1 21), and α-D-mannosidase (EC 3 2 1 24) were obtained from the Sigma Chemical Co, St Louis, Mo, U S A

Glycitols were prepared by reduction of oligosaccharides with $NaBH_4$, and the products were methylated by the Hakomori procedure as described by Hellerqvist *et al* [14] Methylated products were hydrolysed[15] with 90% formic acid at 100° for 4 h and, after dilution with water (5 vol), for a further 2 h The hydrolysates were reduced ($NaBH_4$), and the products acetylated with pyridine–acetic anhydride (1 1) at 100° for 20 min prior to g l c (columns *A* and *B*) Retention times (T_{TMG}) are given relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol[6]

Enzymic hydrolyses — A solution of oligosaccharide (100–500 μg/ml) in 0 05M pyridine acetate (pH 4 5) was treated with α-D-mannosidase (50 μl of suspension, 1 8 units or 0 1 mg of enzyme) overnight at 20° with toluene (50 μl) as a bactericide The mixture was deionised with Amberlite IR-120 (H^+) and IRA-410 (HCO_3^-) resins, and the concentrated solution examined by p c

The oligosaccharides were treated with the glucosidases (~0 2 μmol/20 μg of enzyme) at 30° for 20 h, and the solutions were concentrated to dryness Without further processing, the residues were analysed by p c

Purification of xanthan gum — A solution of the gum (5 g), recovered from cultures of *X campestris* NRRL B-1459 by precipitation with propan-2-ol, in water (1 6 l) was centrifuged (36,000 *g*, 1 h) The centrifugate was made up to 2 litres with HEPES buffer (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid) to a final concentration of 0 1M (pH 7), and saturated, ethanolic mercuric chloride (1 ml) was added as bactericide The solution was incubated with trypsin (15 mg) at 30° for 48 h and then dialysed against deionised water at 4° for 15 days The purified polysaccharide (N, 0 5%), recovered by freeze-drying the dialysate, contained glucose and mannose (1 1, as shown by g l c of the alditol acetates) and glucuronic acid, acetate, and pyruvate (colorimetric assays) in the proportions previously reported[1]

Partial acetolysis of xanthan gum — The purified polysaccharide (1 g) was dried *in vacuo* over P_2O_5 for 17 h, and then shaken with a mixture (30 ml) of acetic acid, acetic anhydride, and sulphuric acid (18 25 2 5)[16] at 20° for 48 h in a sealed tube The resulting, clear-brown solution was poured into ice-cold water (30 ml) and extracted with chloroform (3 × 100 ml) The combined extracts were washed with aqueous $NaHCO_3$, dried (Na_2SO_4), and concentrated to yield a pale-yellow oil which was deacetylated in dry, 0 04M methanolic sodium methoxide (500 ml) at 4° overnight Water (~50 ml) was added dropwise until the suspension clarified, and the solution was then passed through IR-120 (H^+) resin (20 ml) Concentration of the eluant and aqueous washings yielded a pale-yellow syrup (~1 g), which was separated into a neutral and an acidic component on IRA-410 (AcO^-) resin[4]

Isolation of oligosaccharides **2–4** — A slurry of microcrystalline cellulose (Merck) in acetone was packed into a column (70 × 4 cm), and irrigated in a stepwise manner[17] so that the solvent was changed to 1-butanol–pyridine–water (6 4 1 5) The neutral product described above (~400 mg) was adsorbed onto cellulose[17], and this was then applied to the column Elution with the foregoing solvent system for 72 h gave fractions (300 × 15 ml) which were monitored on paper with alkaline silver nitrate Fractions giving a positive response were further examined by p c (solvent *A*) Appropriate fractions were combined, adjusted to pH 6 5 with acetic acid, and concentrated to dryness under reduced pressure

Each oligomer was purified by preparative p c to give **2** (R_{GLC} 0 37, 7 3 mg), **3** (R_{GLC} 0 46, 3 3 mg), **4** (R_{GLC} 0 67, 1 02 mg), and **5** (R_{GLC} 0 76, 2 6 mg)

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REFERENCES

1 J H Sloneker and A Jeanes, *Can J Chem*, 40 (1962) 2066–2071, I R Siddiqui, *Carbohydr Res*, 4 (1967) 284–291

2 P E Jansson, L Kenne, and B Lindberg, *Carbohydr Res*, 45 (1975) 275–282

3 L D Melton, L Mindt, D A Rees, and G R Sanderson, *Carbohydr Res*, 46 (1976) 245–257

4 J D Blake and G N Richards, *Carbohydr Res*, 14 (1970) 375–287

5 M Dubois K A Gilles J K Hamilton P A Rebers and F Smith, *Anal Chem*, 28 (1956) 350–356

6 H Bjorndal C G Hellerqvist, B Lindberg, and S Svennson, *Angew Chem Int Ed Engl*, 9 (1970) 610–619, J Lonngren and A Pilotti, *Acta Chem Scand*, 25 (1971) 1144–1145

7 J Karkkainen, *Carbohydr Res*, 11 (1969) 247–256

8 A S Perlin, *Anal Chem*, 27 (1965) 396–399

9 L D Ough, *Anal Chem*, 34 (1962) 660–664

10 W E Trevelyan, D P Procter, and J S Harrison, *Nature (London)*, 166 (1950) 444–445

11 J S Sawardeker, J H Sloneker, and A Jeanes, *Anal Chem*, 37 (1965) 1602–1604

12 M Ohashi and T Yamakawa, *J Lipid Res*, 14 (1973) 698–700

13 D Gross *J Chromatogr*, 5 (1961) 194–206

14 C G Hellerqvist, B Lindberg, S Svennson, T Holme, and A A Lindberg, *Carbohydr Res* 8 (1968) 43–55

15 E Percival, *Carbohydr Res*, 7 (1968) 272–283, 17 (1971) 121–126

16 K Morgan and A N O'Neill, *Can J Chem*, 37 (1959) 1201–1209

17 R L Whistler and J N BeMiller, *Methods Carbohydr Chem*, 1 (1962) 47–50