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STRUCTURAL STUDIES OF THE EXOCELLULAR POLYSACCHARIDES OF *Agrobacterium tumefaciens* AND *Agrobacterium radiobacter*

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ABSTRACT

The exocellular polysaccharides of *Agrobacterium tumefaciens* and *Agrobacterium radiobacter* have been investigated by the techniques of methylation and Smith degradation–periodate oxidation. The structure of the *A. tumefaciens* polysaccharide has been found to consist of hexasaccharide repeating-units having D-glucose as the sole sugar component, and pyruvic acid, *O*-succinyl, and *O*-acetyl in the molar ratios of ~5.8:1.03:0.91:0.87. *Agrobacterium radiobacter* polysaccharide was shown to contain D-glucose, D-galactose, pyruvic acid, *O*-succinyl, and *O*-acetyl in the molar ratios of ~3.1:2.2:1.2:1.1:1.02.

INTRODUCTION

Although each bacterial strain produces a unique D-glucan[1], a feature common to all glucans is the preponderance of (1→6) linkages. Some glucans are composed almost exclusively of (1→6) linkages, whereas others may contain as little as 50% of (1→6) linkages. As the non-(1→6) linkages may be (1→2), (1→3), or (1→4), a series of glucans may contain a variety of linkage types. Differences in solubility and rheological characteristics are apparently due to the proportions and types of linkages, and the way in which these are arranged in each glucan molecule. Most glucans are considered to have high molecular weights (in the millions); a potentially useful exception[2] was described by Hehre[3].

There are several reports on the exocellular polysaccharides of *Agrobacterium* species. Graham[4] noted that all strains of *A. radiobacter* and *A. tumefaciens* produce polysaccharides in which only glucose was detectable. Zevenhuizen[5,6] found that the exocellular polysaccharide of *A. tumefaciens* has glucose as the main component, and galactose, mannose, and glucuronic acid as minor components. He also noted that the polysaccharide have β-glycosidic linkages, with O-3, -4, -6- and O-4, -6-linked D-glucosyl residues. Tomonori *et al.*[7] studied the structure of the

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exocellular polysaccharide of *A. radiobacter*, and showed that it contains galactose, glucose, succinic acid, and pyruvic acid in the molar ratios of 1:7.2–7.3:1:0.85. Makoto *et al.*[8] examined the water-soluble, exocellular polysaccharides from all 9 strains of *Agrobacterium*, and found that they contain succinic, pyruvic, and acetic acid as organic constituents. Acetic and succinic acid are probably attached as esters, whereas pyruvic acid is linked in an alkali-stable form (possibly an acetal).

By using n.m.r. spectra, Harada *et al.*[9] confirmed that the exocellular polysaccharides of *Agrobacterium* strains contain succinyl and pyruvic acetal groups in the molar ratio of 1:0.45. Gorin *et al.*[10] studied the exocellular β-D-glucans from *A. tumefaciens* and *A. radiobacter* by ^{13}C-n.m.r. spectroscopy, and showed that the D-glucan of *A. tumefaciens* consists of (1→2)-linked β-D-glucopyranosyl residues and minor proportions of 2,3- and 2,6-di-*O*-substituted β-D-glucopyranosyl residues. The glucan of *A. radiobacter* was found to contain 7% of galactose, and 4-*O*- and 4,6-di-*O*-substituted D-glucopyranosyl residues.

EXPERIMENTAL

General methods. — Solutions were evaporated under diminished pressure at low temperature, unless specified otherwise. All products were dried *in vacuo* over calcium chloride. Fractionation of hydrolyzates was performed by descending p.c. at room temperature, using the non-aqueous phases of (*A*) 5:4:1 water–1-butanol–ethanol, (*B*) 4:1:5 1-butanol–acetic acid–water, and (*C*) 2:7:5 pyridine–ethyl acetate–water. Specific rotations were measured at 23 ±1° and 589 nm. Total carbohydrates were determined by the phenol–sulfuric acid[11] method. Gas–liquid chromatography (g.l.c.) was performed with a Varian 1700 chromatograph equipped with a flame-ionization detector, and fitted with columns (1–2 m) of 5% of NPGS, 3% of ECNSS-3M, and 3% of OV-225 on Chromosorb W (80–100 mesh).

The micro-organisms studied. — The bacteria used in this study were kindly identified by the staff of the National Collection of Industrial Bacteria at Torry Research Station, Scotland, as two strains of *Agrobacterium*. Throughout this article, they are referred to as MSI_1 and MSI_2. The former strain was isolated from garden soil in Alexandria, whereas the latter was from a salt marsh situated in the Burg El-Arab area of Alexandria. Pathogenicity tests (unpublished results) confirmed the suggestion of *A. tumefaciens* as the source for isolate MSI_1, and of *A. radiobacter* for isolate MSI_2. The bacteria were maintained in a medium containing, per liter, D-glucose, 20 g; yeast extract (Difco), 2 g; agar (Difco), 15 g; peptone (Difco), 10 g; and micro-elements 0.5 mL. Following inoculation, the cultures were stored at room temperature, transfers of inocula being made every 45 days.

Cultures were obtained by growing at pH 7 in Roux bottles on a medium containing, per liter, D-glucose, 20 g; peptone, 10 g; yeast extract, 2 g; and micro-elements, 0.5 mL, by incubating for 10 days at 30°.

Extraction of the polysaccharides. — Cell-suspension cultures of the two isolates MSI_1 and MSI_2 were grown as described, and centrifuged; the clear, supernat-

ant liquor was poured into ethyl alcohol (6 vol.), and kept overnight in a refrigerator. The precipitated polysaccharides were obtained by centrifugation, dialyzed, and dried.

Purification of the isolated polysaccharides. — Each polysaccharide was dissolved in 10mM Tris · HCl (pH 8), and the solution applied to a column (2 × 50 cm) of DEAE-cellulose equilibrated with the same buffer. The column was eluted with the same buffer with a linear gradient of NaCl (0–1M, 1 L). The fractions were analyzed for carbohydrates by the phenol–sulfuric acid method[11]. The carbohydrate fractions were collected, dialyzed, and dried. The residue was dissolved in 10mM Tris · HCl (pH 8), and applied to a column (2 × 50 cm) of Sepharose 4B, equilibrated with the same buffer and precalibated by using 2 mL of 0.20% (w/v) Blue Dextran 2000. The column was eluted with the same buffer but containing 20mM NaCl. The fractions were monitored for carbohydrates[11], and the carbohydrate fractions were collected, dialyzed, and freeze-dried.

Sugar analysis of the polysaccharides. — The polysaccharides were hydrolyzed with 0.5M sulfuric acid for 12 h, and the SO_4^{2-} ions were removed as $BaSO_4$. The monosaccharides were reduced with $NaBH_4$, the resulting alditols were acetylated with Ac_2O–pyridine, and the alditol acetates were characterized, and quantitatively estimated[12], by g.l.c.

Methylation of the polysaccharides. — Each polysaccharide (50 mg) was twice methylated by the Hakomori method[13], and the methylation products were hydrolyzed with 2M trifluoroacetic acid for 18 h at 100°. The hydrolyzates were chromatographed on paper (solvents *A* and *B*), and their *O*-acetyl-*O*-methylalditols were estimated by g.l.c. A portion of the methylated materials was hydrolyzed with 90% formic acid for 30 min at 95°, to remove the pyruvic group, followed by methylation under the Purdie conditions[14] for 2 days.

Periodate oxidation. — Each polysaccharide (1 g) was oxidized in 0.05M sodium metaperiodate (100 mL), with stirring in the dark. Samples (10 mL) were taken at different times for spectrophotometric determination (223 nm) of periodate consumption, and for potentiometric determination of the formic acid produced[15]. The excess of periodate was reduced with ethylene glycol, the mixture dialyzed, and the product reduced with $NaBH_4$, dialyzed, and freeze-dried. Smith degradation[15] of the polyol (50 mg) was conducted with 0.5M trifluoroacetic acid (25 mL) for 48 h at 23°, and the solution was evaporated to dryness. The residue was separated on Whatman No. 1 paper (solvent *C*), and the chromatograms were sprayed with benzidine–sodium metaperiodate. A portion of the polyol was subjected to Smith degradation with 0.1M trifluoroacetic acid for 1 h on a steam bath, followed by dialysis, and freeze-drying, to give respectively, MSI_1 (P-1) and MSI_2 (P-1). Methylation of MSI_1 (P-1) and MSI_2 (P-1) was performed by the Hakomori procedure, followed by treatment with the Purdie reagents. Methylated MSI_1 (P-1) and MSI_2 (P-1) were hydrolyzed with 2M trifluoroacetic acid for 18 h at 100°. The methylated sugars were examined by paper chromatography (solvents *A* and *B*), and their alditol acetates were analyzed by g.l.c.

Compounds MSI_1 (P-1) and MSI_2 (P-1) were subjected to a second periodate oxidation, with 0.05M sodium metaperiodate for 5 days at 5° in the dark. Sugar analysis of these second polyols was performed by reduction, hydrolysis, and examination of the hydrolyzates on Whatman No. 1 paper (solvent C). Smith hydrolysis of the polyols MSI_1 (P-1) and MSI_2 (P-1) with 0.1M trifluoroacetic acid for 1 h on a steam bath, followed by dialysis and freeze-drying, gave polysaccharides MSI_1 (P-2) and MSI_2 (P-2). Hakomori methylation of MSI_2 (P-2) and MSI_2 (P-2), followed by treatment with the Purdie reagents, gave methylated MSI_1 (P-2) and MSI_2 (P-2); these were hydrolyzed with 2M trifluoroacetic acid for 18 h at 100°. The methylated sugars were separated on Whatman No. 1 paper (solvents *A* and *B*), and their alditol acetates were analyzed by g.l.c.

Identification and determination of the acidic residues. — After acid hydrolysis of the polysaccharides at 100°, pyruvic acid was determined colorimetrically by the (2,4-dinitrophenyl)hydrazone method[16]. Identification of pyruvic acid was achieved by p.c. comparison of the (2,4-dinitrophenyl)hydrazone with an authentic sample[16].

The hydroxamic acid method[17] as adapted by Knutson *et al.*[18] was used to measure the *O*-acetyl content. A freshly prepared, 1:1 mixture of 9.5% sodium hydroxide and 2.25% hydroxylamine hydrochloride was added to the polysaccharide solution, and allowed to react for 5 min at room temperature. The mixture was made neutral with methanolic hydrogen chloride, and evaporated to dryness. Hydroxamates were extracted with ethanol, and the extract was concentrated to low volume, in order to precipitate the excess of salt, and decrease interference on chromatograms. The ethanolic solution was spotted on paper, and chromatographed (solvent *B*)[19], the spots being developed with ferric chloride spray[20]. The acetyl group was verified by t.l.c. of the free acid. A solution of the polysaccharide in M NaOH was kept for 30 min at room temperature, and the base neutralized to pH 7. The polysaccharide was precipitated with ethanol, and removed by centrifugation. The supernatant solution was evaporated to dryness, acidified with HCl, extracted with ether, and the extract treated with 10% Na_2CO_3, acidified, and distilled. The residue and the distillate were extracted with ether, made alkaline with NH_4OH, and the aqueous layer tested by t.l.c. on silica gel G, developed[21] with 25:3:4 ethanol–water–25% NH_4OH, and sprayed with Bromocresol Green

Succinic acid was determined by formation of the dihydronaphthazarine derivative [22], after hydrolysis of the polysaccharides.

RESULTS AND DISCUSSION

The polysaccharides of MSI_1 and MSI_2 were isolated, purified on a column of DEAE-cellulose, and the purified fractions applied to a column of Sephadex-4B, which revealed an average molecular weight of 6.5×10^5 for MSI_1 and 5.4×10^5 for MSI_2. Analyses of the isolated polysaccharides are shown in Table I

Acid hydrolysis of the polysaccharides followed by p.c. (solvents *A* and *B*)

TABLE I

ANALYSIS OF THE MSI_1 AND MSI_2 POLYSACCHARIDES

Composition	*MSI_2*	*MSI_2*
Yield (g/L of medium)	0 65	1.06
$[\alpha]_D^{23}$ (c 0.1 H_2O), degrees	−164	−122
Ash (%)	0.04	0.05
Crude protein (N × 6.25)	0.187	0.375
Total carbohydrate	81.72	79.53

TABLE II

SUGAR ANALYSIS[a] OF MSI_1 AND MSI_2 POLYSACCHARIDES, AND OF COMPOUNDS DERIVED THEREFROM

Sugars and alditols	*Relative proportions*					
	I_1	*I_2*	*II_1*	*II_2*	*III_1*	*III_2*
Glucose	5.8	3.1	4.9	1.9	4.1	1.9
Galactose	—	2.2	—	1.9	—	1
Erythritol	—	—	—	—	0.8	—
Threitol	—	—	—	—	—	+
Glycerol	—	—	+	+	—	—

[a] I_1 and I_2, native polysaccharides MSI_1 and MSI_2; II_1 and II_2, periodate oxidation, reduction, and total hydrolysis of MSI_1 and MSI_2; III_1 and III_2, compound MSI_1 (P-1) and MSI_2 (P-1), periodate-oxidized, reduced, and hydrolyzed.

revealed the presence of only glucose for the polysaccharide of MSI_1, and the presence of glucose and galactose for the polysaccharide of MSI_2. This result was confirmed by conversion of the sugars into their alditol acetates, followed by g.l.c. using D-mannitol hexaacetate as the internal standard. Quantitative analyses of the sugar components are listed in Table II (columns I_1 and I_2).

By mild, alkaline hydrolysis of the polysaccharides for 5 min with 9.5% NaOH in the presence of 2.25% hydroxylamine hydrochloride, the alkali-unstable, acidic groups were hydrolyzed, and transformed into their hydroximate derivatives. Examined by p.c., these showed a clear spot that migrated identically with authentic acetyl hydroximate. There were two other (faint) spots, corresponding to mono- and di-succinyl hydroximate. Verification of the acetyl group was achieved by t.l.c. of the ammonium salt of the free acid. Hydroxamic acid assay for the *O*-acetyl groups indicated a molar ratio of 0.87:1 for the MSI_1 polysaccharide and 1.02:1 for the MSI_2 polysaccharide.

The alkali-stable, pyruvic acetal group was liberated by acid hydrolysis; this was followed by conversion into the (2,4-dinitrophenyl)hydrazone, which, in p.c., migrated identically with an authentic sample of the hydrazone. Quantitative determination by the (2,4-dinitrophenyl)hydrazone assay indicated a molar ratio of 1.03:1 for the MSI_1 polysaccharide and 1.2:1 for the MSI_2 polysaccharide.

TABLE III

METHYLATION ANALYSIS OF MSI_1 AND MSI_2 POLYSACCHARIDES AND COMPOUNDS DERIVED THEREFROM

Methylated sugars[a] (as alditol acetates)	*Molar ratio*[b]							
	I_1	I_2	II_1	II_2	III_1	III_2	IV_1	IV_2
2,3,4,6-Glc	0.8	0.9	1	0.9	—	—	—	—
2,4,6-Glc	3.1	1.9	3.1	2.1	3.1	2.1	3.9	2.1
3,4,6-Glc	—	—	0.9	—	—	—	—	—
2,4-Glc	0.9	—	1.1	—	1	—	—	—
3-Glc	1	—	—	—	—	—	—	—
2,4,6-Gal	—	—	—	1	—	—	—	1.1
2,4-Gal	—	0.8	—	0.9	—	1.1	—	—
2,3-Gal	—	—	—	—	—	1	—	—
2-Gal	—	0.9	—	—	—	—	—	—

[a] 2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, etc. [b] Column 2: I_1 and I_2, original polysaccharides; II_1 and II_2, original polysaccharides, methylated, mildly hydrolyzed, remethylated, and hydrolyzed; III_1 and III_2, compounds MSI_1 (P-1) and MSI_2 (P-1); IV_1 and IV_2, compounds MSI (P-2) and MSI_2 (P-2).

In summary, the molar ratios of the different components were, for the MSI_1 polysaccharide glucose:*O*-succinyl:pyruvic acetal:*O*-acetyl = 5.8:0.91:1.03:0.87, and for the MSI_2 polysaccharide, glucose:galactose:*O*-succinyl:pyruvic acetal:*O*-acetyl = 3.1:2.2:1.1:1.2:1.02.

The highly negative values of the specific rotation, $[\alpha]_D$ $-164°$ for the MSI_1 polysaccharide, and $-122°$ for the MSI_2 polysaccharide, indicate a preponderance of β-D links in both polymers.

Complete methylation of the MSI_1 and MSI_2 polysaccharides was achieved by two Hakomori methylations, to afford a product that showed no hydroxyl absorption in its infrared spectrum. The methylated polysaccharide from MSI_1 was hydrolyzed, and the hydrolyzate was examined by p.c. (solvents *A* and *B*), and by g.l.c. of the alditol acetates (see Table III column I_1). Quantitative determination of the partially methylated sugars indicated the structure of the hexasaccharide repeating-units of the polymer. The identification of 2,4,6-tri-*O*-methylglucose in the molar ratio of 3.1:1 indicated that the polymer chain has (1→3) linkages. The 2,4-di-*O*-methylglucose indicated a branched point at C-3 or C-6. The presence of 3-*O*-methylglucose indicated O-2-, O-4-, and O-6-linked glucose residues. The isolation of 2,3,4,6-tetra-*O*-methylglucose indicated a (terminal) glucosyl group.

When the polysaccharide of MSI_1 was methylated, mildly hydrolyzed to remove the pyruvic acetal group, remethylated, and then hydrolyzed (see Table III, column II_1), the loss of 3-*O*-methylglucose, and the appearance of 3,4,6-tri-*O*-methylglucose with the same molar ratio, indicated that the 1-carboxyethylidene group is linked to O-4 and O-6 of a glucose residue. It also indicated that the repeating unit has only one branch point and one terminal glucosyl group.

The methylation product of the MSI_2 polysaccharide was hydrolyzed, and the hydrolyzates were examined by p.c. (solvents *A* and *B*), and g.l.c. (see Table III,

column I_2). Quantitative analysis of the partially methylated sugars indicated pentasaccharide repeating-units. The identification of 2,4,6-tri-*O*-methylglucose indicated O-3-linked glucose residues. The appearance of 2,4-di- and 2-*O*-methylgalactose indicated O-3, O-6- and O-3, O-4, O-6-linked galactose residues. Mild hydrolysis of the fully methylated MSI_2 polysaccharide was followed by remethylation, hydrolysis, and examination of the methylated sugars (see Table III, column II_2). The loss of 2-*O*-methylgalactose and the appearance of 2,4,6-tri-*O*-methylgalactose with the same molar ratio indicated that the 1-carboxymethylidene group is linked to O-4 and O-6 of a galactosyl residue; this also indicated that another galactosyl residue is the branch point and that the repeating unit has only one (terminal) glucosyl group.

Periodate oxidation. — On periodate oxidation, the MSI_1 polysaccharide consumed 2 mol of periodate per repeating unit. Reduction of the oxidized product was followed by hydrolysis, and the hydrolyzates were examined by p.c. (solvent *C*), and their alditol acetates were quantitized by g.l.c. (see Table II, column II_1). Quantitative analysis indicated the loss of one glucose unit. As only a terminal glucose unit of the polysaccharide was periodate-oxidizable, it was selectively removed by Smith degradation, to give polysaccharide MSI_1 (P-1), having a pentasaccharide repeating-unit.

Methylation analysis of compound MSI_1 (P-1) yielded 2,4,6-tri-, 2,4,-di-, and 2,3-di-*O*-methylglucose (see Table III, column III_1). The loss of 3-*O*-methylglucose and the appearance of 2,3-di-*O*-methylglucose confirmed that the terminal glucosyl group in the original polysaccharide had been attached to O-2 of the glucose residue bearing the 1-carboxyethylidene group at O-4 and O-6.

On periodate oxidation compound MSI_1 (P-1) consumed one mol of periodate per mol. Reduction of the oxidation product, to give a new polyol, was followed by hydrolysis, and the sugars were analyzed (see Table II, column III_1). It was, again, only the terminal unit that was oxidized, and hence, the terminal glucosyl unit had been selectively removed by Smith degradation, to give polysaccharide MSI_1 (P-2), having tetrasaccharide repeating-units. Methylation analysis of MSI_1 (P-2) yielded 2,4,6-tri-*O*-methylglucose (see Table III, column IV_1). The loss of 2,4,-di-*O*-methylglucose and appearance of 2,4,6-tri-*O*-methylglucose with the same ratio established that the glucosyl residue which bore the 1-carboxyethylidene group had been attached to O-6 of a glucosyl residue in the polysaccharide chain. Polysaccharide MSI_1 (P-2) was found to be periodate-resistant, indicating glucosyl linkages at O-3.

After periodate oxidation of the MSI_2 polysaccharide, reduction yielded a polyalcohol; this was hydrolyzed, the hydrolyzate was examined by p.c. (solvent *C*), and the alditol acetates were quantitized by g.l.c. (see Table II, column II_2). As only the terminal glucose residue was oxidized, it was selectively removed by Smith degradation, to give MSI_2 (P-1) having a tetrasaccharide repeating-unit. Methylation analysis of compound MSI_2 (P-1) yielded 2,4,6-tri-*O*-methylglucose and 2,4- and 2,3-di-*O*-methylgalactose (see Table III, column III_2). The loss of 2-*O*-methyl-

galactose, and the appearance of 2,3-di-*O*-methylgalactose indicated that the terminal glucosyl group was linked to O-3 of a galactose residue. On periodate oxidation, compound MSI_2 (P-1) consumed one mol of periodate per mol. The product was reduced, the alditol hydrolyzed, and the hydrolyzate examined by p.c. (solvent *C*) and quantitized by g.l.c. (see Table II, column III_2). Again, a galactose unit was selectively oxidized, and it was removed by Smith degradation, to give MSI_2 (P-2), which was found to be periodate-resistant. Methylation analysis of compound MSI_2 (P-2) yielded 2,4,6-tri-*O*-methylglucose and 2,4,6-tri-*O*-methylgalactose, indicating a chain of glucosyl and galactosyl residues in the molar ratio of 2:1, joined by linkages at O-3. The loss of the 2,4-di-*O*-methylgalactose and the appearance of 2,4,6-tri-*O*-methylgalactose indicated that O-6 of a galactosyl residue is the point of branching.

Our findings confirm those of Zevenhuizen[5,6], who reported that *A. tumefaciens* exocellular polysaccharide is composed of glucose as the main component, and has β linkages with O-3, O-4, and O-6 linked D-glucose. Tomonori *et al.*[7] found that *A. radiobacter* exocellular polysaccharide is composed of galactose, glucose, succinic acid, and pyruvic acid in the molar ratios of 1:7.2–7.3:1:0.85, which differs from our finding of 2.2:3.1:1.1:1.2; also, they did not record the presence of the *O*-acetyl group. Harada *et al.*[9] confirmed the presence, in the exocellular polysaccharides of *Agrobacterium* strains, of succinyl and pyruvic groups in the molar ratio of 1:0.45, but we find 0.91:1.03 and 1.2:1.02 for MSI_1 and MSI_2, respectively.

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