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CHARACTERIZATION OF AN EXTRACELLULAR POLYSACCHARIDE FROM A *XANTHOMONAS* SPECIES

TOSHIRO YADOMAE, HARUKI YAMADA, TOSHIO MIYAZAKI*

Department of Microbial Chemistry, Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachiōji, Tokyo 192-03 (Japan)

TOSHIHIRO ŌMORI, AND TETSUJI HIROTA

Technical Research Institute, Snow Brand Milk Products Co., Ltd., 1–2 Minamidai 1-chome Kawagoe, Saitama Prefecture 350 (Japan)

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ABSTRACT

An extracellular polysaccharide containing glucose, mannose, D-*manno*-octulosonic acid (KDO), an unidentified component (X), and acetyl groups in the molar ratio of 1.3:3.8:1.6:1.1:2.9, was obtained from the incubated medium of a *Xanthomonas* species. The extracellular polysaccharide contained traces of phosphate and nitrogen but no lipid. Mild hydrolysis with 0.025M sulfuric acid released all of the KDO in the polysaccharide and a KDO-free product was obtained, which on hydrolysis with 0.05M sulfuric acid, gave mainly an oligosaccharide containing glucose, mannose, and X in molar ratio of 1:1:1. The reducing end-group of this oligosaccharide was X, and other hexose residues were linked (1 → 4). Compound X seems to be a 6-deoxyhexose that differs from fucose and rhamnose.

INTRODUCTION

A strain of *Xanthomonas* species was isolated from soil during a search for microorganisms that produce a viscous material used as a paste or stabilizer for emulsification in the food industry. This microorganism secretes into the culture medium a viscous material that is a polysaccharide consisting of glucose, mannose, D-*manno*-octulosonic acid (KDO), and an unidentified component (X).

It is known that some strains of *Xanthomonas* produce very viscous, extracellular polysaccharides[1]. These polysaccharides generally contain of D-glucose, D-mannose, and D-glucuronic acid. However, the extracellular polysaccharides of *Xanthomonas stewartii*[2] and *Xanthomonas S19*[3] also contain galactose as well. Lindberg *et al.*[4], and Melton *et al.*[5] proved that the extracellular polysaccharide of *Xanthomonas campestris* is composed of D-glucose, D-glucuronic acid, and D-mannose

*To whom inquiries should be addressed.

combined with acetate or pyruvate groups. Furthermore, they proved the structure of a pentasaccharide that seemed to be a repeating unit of the extracellular polysaccharide.

Volk[6] has reported that the cell-wall lipopolysaccharides of certain *Xanthomonas* species contained KDO, uronic acid, phosphate, mannose, glucose, rhamnose, and fucose (or xylose), but no heptose nor galactose.

The extracellular polysaccharide, isolated here, contains glucose, mannose, KDO, and a sugar (X), but it differs from the other extracellular polysaccharides in that it contains no uronic acid. It resembles the cell-wall lipopolysaccharide in containing KDO, but it is not such a lipopolysaccharide as it contains no lipid, uronic acid, rhamnose, or fucose (or xylose). Such an extracellular polysaccharide has not previously been reported.

Is this compound a precursor or an intermetabolite of a lipopolysaccharide? We are interested in the relation between the extracellular polysaccharide and the lipopolysaccharide. In this paper, the isolation and partial characterization of the extracellular polysaccharide are discussed.

EXPERIMENTAL

Materials and methods. — Organisms were identified according to Bergey's manual of determinative bacteriology. Crystalline ammonium KDO was a gift from Prof. N. Kasai (Showa University, Tokyo).

Evaporations were performed under diminished pressure at $\leqslant 40°$ (bath). Total hexose was determined by the method of Dubois[7], and quantitative estimations of phosphate[8], protein[9], and nitrogen were performed by the methods of Fiske and SubbaRow, of Lowry *et al.*[9], and by elemental analysis, respectively. KDO was estimated by the method of Aminoff[10]. Periodate consumption was determined by the method of Avigad[11] and formic acid was estimated by the method of Whistler[12]. The method of Hakomori[13] was used for methylation. Paper chromatography was performed on Toyo filter paper, No. 50, at room temperature by the ascending method with the following solvent systems; (*A*) 10:4:3 (v/v) ethyl acetate–pyridine–water, (*B*) 5:5:1:3 (v/v) ethyl acetate–pyridine–acetic acid–water, (*C*) 1-butanol saturated with water, (*D*) 1:4:1 (v/v) acetic acid–1-butanol–water, (*E*) 8:1:1 (v/v) butanone–acetic acid–water, (*F*) 3:1:3 (v/v) ethyl acetate–acetic acid–water, and (*G*) 5:3:2 (v/v) 1-butanol–pyridine–0.05M hydrochloric acid. The indicator sprays were alkaline silver nitrate[14], *p*-anisidine hydrochloride[15], and ninhydrin[16]. KDO was detected on paper chromatograms by the method of Warren[17]. Gas–liquid chromatography (g.l.c.) was conducted with a Shimazu GC-6A instrument, equipped with columns (0.3 × 200 cm) packed with 3% ECNSS-M* and mixture of 17% FFAP†–2% phosphoric acid. G.l.c.–m.s. was performed on a Hitachi RMU-7L mass

*Cyanoethylsilicone polymer, Gasukuro Kogyo Ltd., Tokyo.
†Carbowax 20M terminated with 2-nitroterephthalic acid, Gasukuro Kogyo Ltd., Tokyo.

spectrometer at 70 eV ionization voltage with a metal column (0.3 × 150 cm) of 3% of OV-225 on Chromosorb W. Optical rotations were determined with a Jasco Dip SL polarimeter at 22°. A Hitachi model 282 ultracentrifuge was used for ultracentrifugal analysis, and a Hitachi model 215 spectrometer for i.r. spectra. A JNM-4H-100 instrument was used for proton magnetic resonance (p.m.r.) spectra and sodium 2,2,3,3, -tetradeuterio-4,4-dimethyl-4-silopentanoate and tetramethylsilane were used as internal references.

Isolation of the extracellular polysaccharide. — The culture medium contained 6% of D-glucose, 0.5% of peptone, 0.5% of yeast extract (Oriental Yeast Ind. Ltd., Japan), 0.1% of dipotassium hydrogenphosphate, and 0.1% of magnesium sulfate. Culture of a strain of *Xanthomonas* from the soil was performed at 30° until the culture medium became viscous. After 72 h, the culture broth (1500 ml) was separated from organisms by centrifugation at 15,000 r.p.m., the supernatant was concentrated, and 3 volumes of acetone were added. The resultant precipitate (3.3 g) was dissolved in 500 ml of distilled water and the solution was adjusted to pH 7.8 with sodium hydrogencarbonate. To the solution was added 83 mg of Pronase (Kaken Kagaku Co. Ltd.) and the mixture was incubated for 24 h at 37°. The solution was dialyzed in a Visking tubing against distilled water for 48 h. The solution in the tube was concentrated to 150 ml and deproteinized by the Sevag procedure[18]. Ethanol (3 volumes) was then added to the treated solution and a precipitate (1.5 g) was obtained. An aqueous solution (1%) of the precipitate was prepared, and ethanol was added to it until a concentration of 70% ethanol was reached. The precipitate (0.6 g) that appeared was collected by centrifugation. This procedure was repeated once more by using the precipitate obtained by the first procedure and, furthermore, ethanol was added to the 1% solution of the precipitate obtained by the second procedure until a concentration of 80% ethanol was attained. The purified extracellular polysaccharide (48 mg) was thus obtained.

Estimation[19] of acetyl groups in the extracellular polysaccharide. — The polysaccharide (1 mg) was hydrolyzed with M hydrochloric acid (0.1 ml) in a sealed tube for 2 h at 100°. The hydrolyzate was analyzed by g.l.c. on a glass column with a mixture of 17% FFAP–2% phosphoric acid on Chromosorb W at 118°. Acetic acid was used as a standard.

Detection of KDO in the extracellular polysaccharide. — The extracellular polysaccharide (10 mg) was hydrolyzed with 0.05M sulfuric acid for 1 h at 80°. The hydrolyzate was neutralized with barium carbonate and filtered. The filtrate was passed through a column of Dowex-50 (H^+, 100–200 mesh) and subsequently through a column (0.8 × 5 cm) of Dowex-1 (CO_3^{2-}, 100–200 mesh). After washing the column of Dowex-1 with distilled water (50 ml), it was eluted with 20 ml of 0.5M sodium hydrogencarbonate. The hydrogencarbonate eluate was treated with an excess of Dowex-50 (H^+). The acidified hydrogencarbonate eluate was made neutral with ammonium hydroxide. The neutral solution was concentrated and then examined by paper chromatography and by a ceric sulfate degradation[20]. Paper chromatography was carried out with solvent systems *E*, *F*, and *G* with crystalline ammonium KDO.

Ceric sulfate degradations of the sample and authentic KDO were performed according to the procedure of Ghalambor[20].

Stepwise acid hydrolysis of the extracellular polysaccharide. — The extracellular polysaccharide (20 mg) was heated with 0.025M sulfuric acid (4 ml) for 30 min at 80° and, after dialysis, the external solution (600 ml, containing the dialyzable fragments) was neutralized with barium carbonate. After filtration, the filtrate was concentrated (DF-1, Scheme 1). The content of total sugar and KDO, other components, and the presence of oligosaccharides in DF-1 were examined. The solution (containing non-dialyzable material) inside the tube was evaporated (NDF-1, Scheme 1) and the residue heated again with 0.05M sulfuric acid (2.5 ml) for 5 h at 100°. The dialyzable fraction (DF-2) from the hydrolyzate was collected and examined by the same procedures as already described.

Subsequently, the solution remaining in the tube was evaporated and treated with 0.25M sulfuric acid (0.4 ml) for 5 h at 100°. The dialyzable fraction (DF-3) was examined as before. Next, the final, nondialyzable fraction was hydrolyzed with 0.5M sulfuric acid (0.2 ml) for 5 h at 100° and the constituents of the hydrolyzate were examined. In another experiment, the extracellular polysaccharide was hydrolyzed with 2% acetic acid (pH 2.5) for 45 min at 100°, and the hydrolyzate was dialyzed against distilled water with a Visking tube. The solution external to the tube was evaporated. The dried sample was reconstituted with water and redried. This step was repeated once more with methanol. The solution remaining in the tube was lyophilized. The dialyzable and non-dialyzable fractions thus obtained were examined by p.m.r. spectroscopy.

Preparation of de-KDO-polysaccharide (de-KDO-PS). — The extracellular polysaccharide was hydrolyzed with 0.025M sulfuric acid for 45 min at 80°. The mixture was dialyzed against distilled water for 2 days, and the solution remaining in the tube was lyophilized.

Methylation of de-KDO-PS. — De-KDO-PS (10 mg) was dissolved in methyl sulfoxide (1 ml), 0.02M methylsulfinyl carbanion in methyl sulfoxide (10 ml) was added, and the mixture was kept for 18 h with stirring at room temperature. Methyl iodide (2 ml) was added with cooling. These procedures were performed under nitrogen. The fully methylated product was heated with 90% formic acid for 10 h at 100°. Formic acid was distilled off and the residue hydrolyzed with 0.5M sulfuric acid for 5 h. The hydrolyzate was analyzed (as the corresponding alditol acetate derivatives) by g.l.c. and g.l.c.–m.s., with columns of 3% OV-225 and 3% ECNSS-M.

Isolation and characterization of oligosaccharides. — De-KDO-PS was hydrolyzed with 0.05M sulfuric acid for 5 h at 100°. The hydrolyzate was made neutral with barium carbonate and filtered. The filtrate was dialyzed with a Visking tube against 200 ml of distilled water, and dialysis was repeated twice. The dialyzate was concentrated, and the concentrate was resolved (papergram, solvent system *A*). Two oligosaccharides were obtained, in addition to monosaccharides, as components from the extracellular polysaccharide. The principal one was termed the major oligosaccharide (M-OS); this was hydrolyzed and the hydrolyzate examined by g.l.c. of the alditol

acetate derivatives. The oligosaccharide was reduced with sodium borohydride and then hydrolyzed with 0.5M sulfuric acid. The hydrolyzate was acetylated with pyridine–acetic anhydride and examined by g.l.c. The oligosaccharide was also methylated[13] and subjected to methylation analysis.

RESULTS

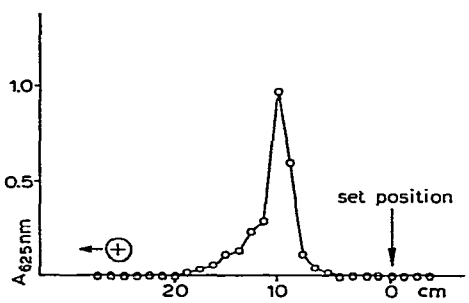
Homogeneity of the extracellular polysaccharide. — Zone electrophoresis was performed on Pevicon C-870 (polyvinyl resin, M & S Instruments, Inc., Japan) support, pre-equilibrated with 1% borax. A block (1.5 × 3 × 40 cm) was prepared and a solution of the sample in 1% borax was placed 10 cm from the cathode and allowed to migrate at a constant current of 3mA/cm^2 for 13 h. After migration, the Pevicon block was cut into 40 segments. Each segment was extracted with 10 ml of distilled water and the carbohydrate content was determined by the anthrone reagent[21]. A single peak was detected (Fig. 1).

Sepharose 4B (Pharmacia Fine Chemicals) was washed with water and equilibrated with 0.2M sodium chloride. The extracellular polysaccharide (3 mg), dissolved in 0.2M sodium chloride (0.5 ml), was applied to the column (1.5 × 90 cm) of Sepharose and the column was eluted with 0.2M sodium chloride at flow rate of 10 ml/h. Fractions (2 ml) were collected, and an aliquot of each fraction was assayed for carbohydrate by the anthrone reagent. A single peak was detected (Fig. 2).

Sedimentation analysis of the extracellular polysaccharide was conducted with a Hitachi model 282 ultracentrifuge at 60,000 revs min^{-1} in 0.2M sodium chloride. Routine, analytical assays were performed at 25°. The extracellular polysaccharide showed a single, symmetrical peak.

Properties of the extracellular polysaccharide. — The polysaccharide, $[\alpha]_D$ −28° (*c* 1.0, water), contained 0.33% nitrogen, 1.0% protein (with bovine serum albumin as standard), and 0.05% phosphorus.

The intrinsic viscosity (4.4 dl/g) was estimated by using a Cannon viscosity microanalyzer at six different concentrations (0.05, 0.1, 0.2, 0.3, 0.4, and 0.5%, in



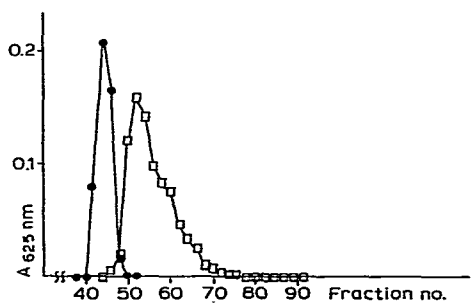


Fig. 1. Zone electrophoresis of the extracellular polysaccharide (detection by anthrone).

Fig. 2. Elution pattern by gel filtration of the extracellular polysaccharide on Sepharose 4B; —●—●—, Blue Dextran; —□—□—, extracellular polysaccharide (detected by the anthrone reagent).

phosphate buffer, pH 7.0) at 37°. The total sugar content was 59.3% (as mannose) by the procedure of Dubois *et al.*[7]. The i.r. spectrum showed absorptions at 1720 and 1620 cm^{-1} (ester and carboxylate groups). The p.m.r. spectrum of the polysaccharide showed signals at 1.25 and 2.14 p.p.m. (methyl and acetyl groups). The acetyl-group content, determined by the method of Nagai *et al.*[19], was 9.3%. Glucose, mannose, compound-X, and galactose, in the molar ratio of 1.3:3.8:1.1:0.07 were detected by hydrolysis with 0.5M sulfuric acid. No uronic acid, pyruvic acid, heptose, or amino sugars were detected. The polysaccharide was hydrolyzed with 0.5M sulfuric acid and the hydrolyzate extracted with chloroform, but no material was detected in the extract.

The hydrolyzate obtained by use of 0.05M sulfuric acid gave a positive color reaction in the thiobarbituric acid assay of Aminoff[10], but a negative color reaction with the resorcinol reagent[22] and the indirect Ehrlich test[22]. The product that gave a positive color reaction in the thiobarbituric acid assay was isolated by the literature procedure, converted into the ammonium form, and then identified by comparison with authentic ammonium KDO by paper chromatography and ceric sulfate degradation[20]. In order to estimate the proportion of KDO in the extracellular polysaccharide, three different sets of hydrolytic conditions, 0.025M sulfuric acid at 80°, 0.05M sulfuric acid at 80°, and 2% acetic acid at 100°, were examined. The most effective was 0.025M sulfuric acid for 45 min at 80° and the proportion of KDO in the extracellular polysaccharide was found to be 20.2%.

Studies on compound X. — The extracellular polysaccharide (100 mg) was hydrolyzed with 0.5M sulfuric acid for 6 h at 100°. The hydrolyzate was resolved into its components by paper chromatography with solvent system *A*. The area corresponding to X on the chromatogram was excised and extracted with water. Compound X gave positive color-reactions with the Molisch reagent[23], anthrone reagent[21], phenol–sulfuric acid[7], and the acetone–hydrochloric acid reagent[24] characteristic for 6-deoxy sugars, but it gave a negative color reaction with the Kiliani reagent[24] for 2-deoxy sugars. The behavior of X in a reaction[24] for 3,6-dideoxyhexoses was indecisive. Elemental analysis showed C, 37.7 and H, 6.4%. Compound X had $[\alpha]_D$ −25.0 (*c* 1.0, water, after 30 min) → −24.5 (1 h) → −13.0° (24 h). The p.m.r. spectra of X and its alditol acetate are shown in Fig. 3. The spectrum of X

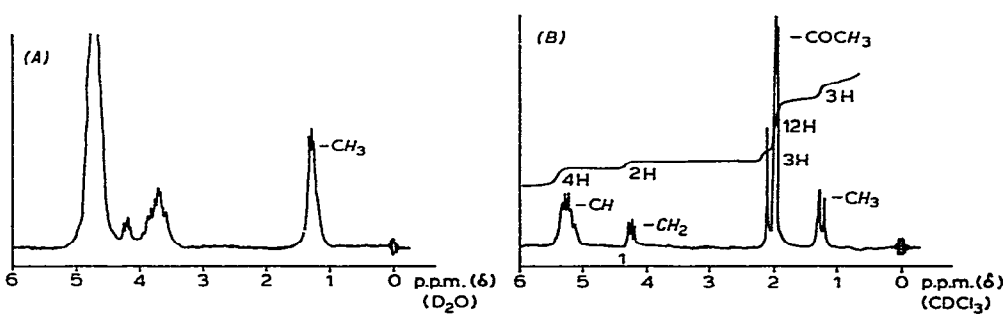
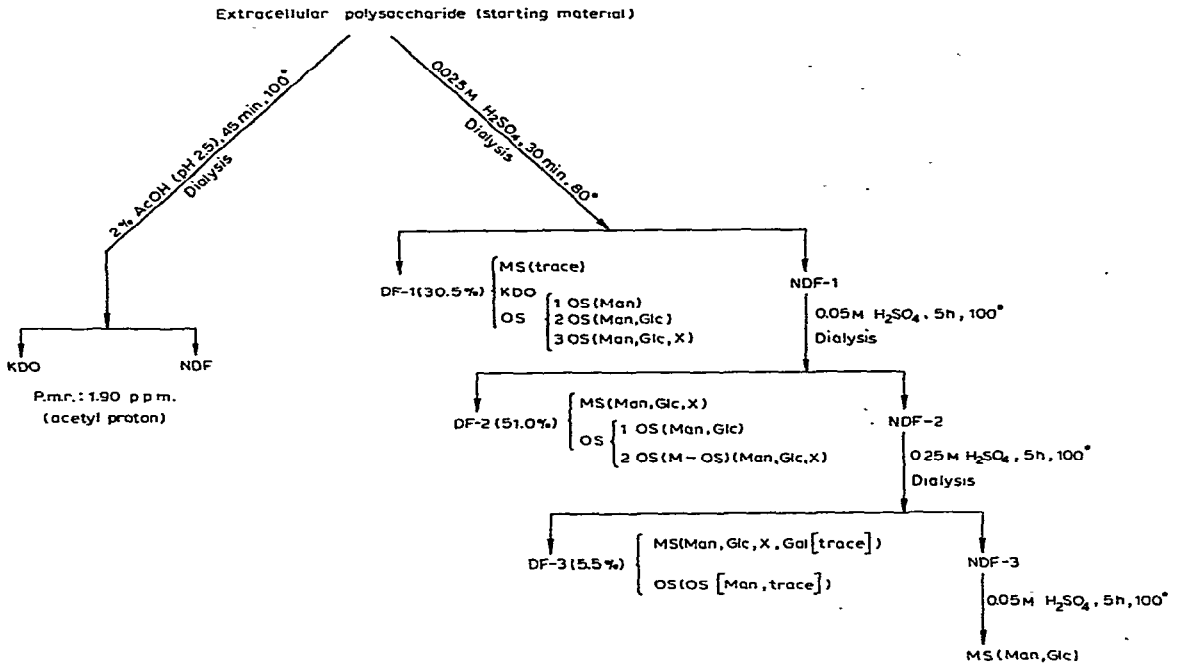


Fig. 3. The 100-MHz p.m.r. spectra (*A*) of compound X, and (*B*) its alditol acetate, recorded at 26°.



Abbreviations: MS = monosaccharide, OS = oligosaccharide, PS = polysaccharide, NDF = non-dialyzable fraction, DF = dialyzable fraction

Scheme 1. Stepwise acid hydrolysis of the extracellular polysaccharide. Figures in parentheses give the yield with respect to the starting material.

exhibited a signal at 1.25 p.p.m. (methyl protons), and that of its alditol acetate exhibited signals at δ 1.16–1.22 (methyl), 2.02–2.18 (acetyl), 4.16–4.32 (methylene), and 5.12–5.30 p.p.m. (methine). The ratio of the integrated values of these signals was approximately 3:15:2:4 (methyl:acetyl:methylene:methine). Compound X appeared to be a 6-deoxyhexose, but it was not identical with fucose or rhamnose by paper chromatography and g.l.c. The mass spectrum of its alditol acetate showed the same fragmentation pattern as that of fucose. Paper-chromatographic analysis of X, with solvent systems *C* and *D*, gave R_{Rhamnose} values[3] of 1.41 and 1.19.

Stepwise, acid hydrolysis of the extracellular polysaccharide. — The yield of dialyzable material (DF-1, Scheme 1) was 30.5% of the starting material and contained 64.7% of the total KDO and 11.4% of the total sugar. Paper chromatography showed trace of monosaccharide and three oligosaccharides in DF-1. One of the oligosaccharides consisted of mannose only, the next one contained mannose and glucose in 1:1 ratio, and the last one consisted of mannose, glucose and compound X in the molar ratio of 1:1:1. None of these oligosaccharides contained KDO. Further hydrolysis of the dialyzate (NDF-1, Scheme 1) with 0.05M sulfuric acid gave DF-2 (51.0% of the starting material) and NDF-2. The dialyzable material (DF-2) contained 0.4% of KDO and 74.0% sugars; the sugar constituents (papergram) were mannose, glucose, compound X, and two oligosaccharides. The oligosaccharides were isolated from the paper chromatogram and the components of each examined by g.l.c. One of them consisted of mannose and glucose, and the other one consisted

of mannose, glucose and compound X. Neither oligosaccharide contained KDO. Subsequent hydrolysis of NDF-2 with 0.25M sulfuric acid gave DF-3 (5.5% of starting material) and NDF-3.

The fraction DF-3 contained 8.4% of total sugars, but contained no KDO.The constituents of DF-3 were monosaccharides [mannose, glucose, compound X, galactose (trace)] and oligosaccharides (trace). Hydrolysis of the dialyzate NDF-3 with 0.5M sulfuric acid gave mannose and glucose.

In contrast, the dialyzable material obtained by hydrolysis with 2% acetic acid contained only KDO. After complete removal of acetic acid from the KDO fraction, the p.m.r. spectrum showed a signal at 1.90 p.p.m., (acetyl protons), but the spectrum of non-dialyzable material obtained by hydrolysis with 2% acetic acid showed no such signal for acetyl protons. These results are shown in Scheme 1.

Periodate oxidation of de-KDO-PS. — De-KDO-PS (2.5 mg) was oxidized in 5 ml of 5mM sodium periodate at room temperature, pH 5.5, in the dark. A blank determination without sample was processed similarly. The number of mol of periodate reduced per monosaccharide residue of de-KDO-PS, was as follows: 0.12 (1 h), 0.19 (3 h), 0.26 (6 h), 0.30 (10 h), 0.38 (25 h), 0.45 (48 h), 0.49 (72 h), 0.53 (96 h), and 0.57 (112 h); values for formic acid: 0.01 (3 h), 0.02 (9 h), 0.03 (72 h), and 0.08 (96 h).

Periodate oxidation, reduction, and hydrolysis of de-KDO-PS. — De-KDO-PS (5 mg) was oxidized with sodium periodate for 48 h under the same conditions as already described. After oxidation, ethylene glycol (0.1g) was added to decompose the excess of periodate and the mixture was dialyzed against distilled water. The solution retained in the tube was concentrated to low volume and stirred overnight with 5 mg of sodium borohydride. The excess of borohydride was decomposed with acetic acid and the mixture was dialyzed against distilled water. The solution retained in the tube was evaporated and hydrolyzed with 0.5M sulfuric acid. The hydrolyzate was neutralized with barium carbonate, filtered, and the filtrate concentrated to a low volume and examined by paper chromatography and g.l.c. Glycerol, erythritol, mannose, glucose and compound X were detected in the molar ratio of 2.1:0.7:1.5:0.9:1.1.

Methylation analysis of de-KDO-PS. — The peracetates of 2,3,4,6-tetra-*O*-methylhexitol, 2,3,6-tri-*O*-methylhexitol, 2,4,6-tri-*O*-methylhexitol, 3,4-di-*O*-methylhexitol, and the methylated and acetylated alditol of compound X were detected by g.l.c. and g.l.c.–m.s. (Fig. 4). These results indicate that de-KDO-PS contains (1 → 4)- and (1 → 3)-linked hexose, and branches corresponding to 3,4-di-*O*-methylhexitol.

Studies on the major oligosaccharide (M-OS). — This oligosaccharide consisted of mannose, glucose, and compound X in the molar ratio of 1:1:1. After borohydride reduction, its hydrolyzate gave neither mannitol nor glucitol. The methylation analysis showed the presence of the peracetates of 2,3,4,6-tetra-*O*-methylhexitol, 2,3,6-tri-*O*-methylhexitol, and an unidentified component which probably corresponded to the methylated and acetylated alditol of compound X.

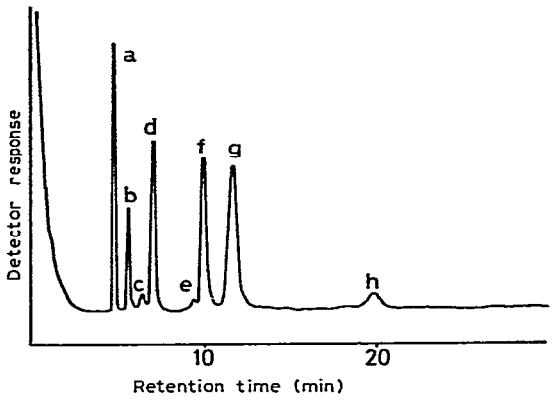


Fig. 4. G.l.c. trace of the hydrolyzate of fully methylated de-KDO-polysaccharide (as alditol acetate derivatives): a, methylated and acetylated alditol of compound X; b, 2,3,4,6-tetra-*O*-methylhexitol; c, unknown; d, unknown; e, unknown; f, 2,4,6-tri-*O*-methylhexitol; g, 2,3,6-tri-*O*-methylhexitol; h, 3,4-di-*O*-methylhexitol. Column, 3% ECNSS-M; temperature, 180°.

DISCUSSION

A strain of *Xanthomonas* isolated from soil in Japan, secreted a viscous polysaccharide into the incubation medium. This extracellular polysaccharide, $[\alpha]_D$ −28°, $[\eta]$ 4.4 dl/g, consisted of glucose, mannose, *O*-acetyl-*manno*-octulosonic acid, and an unidentified deoxyhexose (compound X). Glucuronic acid and pyruvate were not detected.

As a KDO-containing polysaccharide, it appears similar to the cell-wall lipopolysaccharides of *Xanthomonas* species reported by Volk[6]. However, our extracellular polysaccharide contained no lipid and very little phosphate. Hydrolysis of the extracellular polysaccharide with 0.025M sulfuric acid gave a non-dialyzable polysaccharide (de-KDO-PS) containing no KDO, and showed a single spot on the paper electrophoregram. The KDO content of the extracellular polysaccharide was 20.2%. The extracellular polysaccharide was also hydrolyzed under conditions (2% acetic acid, pH 2.5, at 100°) that did not split *O*-acetyl linkages. The hydrolyzate was separated into dialyzable and non-dialyzable fractions. The dialyzable fraction contained only KDO, and its p.m.r. spectrum exhibited a signal at 1.90 p.p.m. arising from acetyl protons, but the non-dialyzable fraction showed no such signal, indicating that *O*-acetyl goups are linked to KDO in the extracellular polysaccharide.

When the nondialyzable fraction (NDF-1, Scheme 1) was hydrolyzed with 0.05M sulfuric acid, the major part of the hydrolyzate appeared in the dialyzable fraction (DF-2, Scheme 1), which contained monosaccharides and two oligosaccharides. Fractions that contained oligosaccharides were DF-1 and DF-2 (Scheme 1), and the components of oligosaccharides in DF-1 resembled those in DF-2. We presume that these oligosaccharides constitute repeating units of the extracellular

polysaccharide. The major of two oligosaccharides obtained from DF-2 was examined more detail than the others.

Mannitol and glucitol were not present in the product from reduction and subsequent hydrolysis of the major oligosaccharide. Methylation analysis revealed 2,3,4,6-tetra-*O*-methylhexose and 2,3,6-tri-*O*-methyl hexose, indicating that the major oligosaccharide has (1 → 4)-linked hexopyranose residues with compound X at the reducing end.

After reduction and subsequent acetylation of compound X, the p.m.r. spectrum showed signals corresponding to five acetyl groups, one methylene group, and four methine protons, by integration in comparison with the signal of a methyl group (Fig. 3B). Furthermore, compound X showed the mutarotation behavior and R_F value expected for 6-deoxy-L-talose[25]; the identification is tentative, but the compound is evidently a 6-deoxyhexose from the p.m.r. data.

Methylation analysis of de-KDO-PS indicates that it contains (1 → 3) and (1 → 4)-linked hexopyranose residues and forms branches at C-2 or C-6 of some hexose residues, but the position of compound X in de-KDO-PS could not be established.

Summarizing, the core part of the extracellular polysaccharide appears to be de-KDO-PS. *O*-Acetylated KDO is attached to the core as side-chains, because the extracellular polysaccharide releases KDO by hydrolysis with mild acid and gives the non-dialyzable de-KDO-PS.

The relation between this extracellular polysaccharide and cell-wall lipopolysaccharide cannot yet be discussed in detail because the structure of the cell-wall lipopolysaccharide has not been completely elucidated. The composition and structure of our extracellular polysaccharide differs from those of other extracellular polysaccharides reported by Fareed *et al.*[3] and Lindberg *et al.*[4], and of the cell-wall lipopolysaccharide reported by Volk[6].

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