Carbohydrate Research, 38 (1974) 239–246

CHEMICAL STRUCTURE OF AN ACIDIC POLYSACCHARIDE PRODUCED BY *Serratia piscatorum**

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(Received December 31st, 1973; accepted in revised form, June 17th, 1974)

ABSTRACT

The acidic polysaccharide of *Serratia piscatorum* consists of L-rhamnopyranosyl, D-galactopyranosyl, and D-galactopyranosyluronic acid residues in the molar ratio of 2:1:1. Some of the D-galactopyranosyluronic acid residues are acetylated at O-2 or O-3, or both. Smith degradation and methylation analysis indicated that the L-rhamnopyranosyl, D-galactopyranosyl, and D-galactopyranosyluronic acid residues are substituted with glycosidic linkages at O-3, O-3, and O-4, respectively. Partial acid hydrolysis of the native polysaccharide gave four acidic oligosaccharides, each of which was isolated and characterized, suggesting the following tetrasaccharide repeating unit: →3)-L-Rha*p*-(1→4)-D-GalA*p*-(1→3)-L-Rha*p*-(1→3)-D-Gal*p*-(1→.

INTRODUCTION

In a previous study[1], we isolated a potent anti-inflammatory substance from a culture filtrate of *Serratia piscatorum*. It was a complex consisting of an acidic polysaccharide, as major moiety, and of such other moieties as hexosamines, protein, and lipid. Subsequently, the polysaccharide moiety was dissociated by ultrasonication and purified by gel filtration. The polysaccharide is homogeneous and composed[2,3] of L-rhamnose, D-galactose, and partially acetylated D-galacturonic acid residues in the molar ratio of 2:1:1. In the present study, we report the determination of the repeating unit of the polysaccharide chain by Smith degradation, methylation analysis, and fragmentation analysis.

RESULTS AND DISCUSSION

Periodate oxidation. — During the periodate oxidation of the native and deacylated polysaccharides, the consumption of oxidant and the production of formic acid were both very low (Table I). The resistance to periodate oxidation suggested that

*Biologically active polysaccharides produced by bacteria. Part IV.

most sugar residues in the polysaccharide are linked by (1→3)-glycosidic bonds. After one Smith degradation, analysis of the residual sugars (see Table II), indicated that the L-rhamnose and D-galactose residues in both polysaccharides remained unchanged after the degradation, while one half of the galacturonic acid residues in the native polysaccharide and all of the residues in the deacylated polysaccharide were degraded. These results suggest that both the L-rhamnose and the D-galactose residues are (1 → 3) linked, whereas the D-galacturonic acid residues are probably (1 → 2) or (1 → 4) linked.

TABLE I

PERIODATE OXIDATION OF NATIVE AND DEACYLATED POLYSACCHARIDES

Sample[a]	*Periodate consumed*[b]	*Formic acid produced*[b]
Native polysaccharide	0.2	0.09
Deacylated polysaccharide	0.3	0.1
Methyl α-D-glucopyranoside	2.05	1.03

[a]The sample (100 mg) was treated with 0.05M sodium periodate (100 ml) for 20 days at 5° in the dark. [b]Expressed in moles per glycosyl residue.

TABLE II

SUGAR COMPONENTS OF NATIVE AND DEACYLATED POLYSACCHARIDES AFTER PERIODATE OXIDATION[a]

Component	*Native polysaccharide*[b]	*Periodate-oxidized polysaccharide*	
		Native	*Deacylated*
L-Rhamnose	2.05	2.02	2.05
D-Galactose	1.0	1.0	1.0
D-Galacturonic acid	0.95	0.51	0

[a]Sugar components expressed as a molar ratio of the components. [b]As reference sample.

Methylation analysis. — To elucidate the glycosidic linkages of the polysaccharide, the deacylated polysaccharide was subjected to methylation analysis. Since the deacylated, acidic polysaccharide contains D-galacturonic acid residues, it was methylated at first by the Hakomori method[4], and the carboxyl groups were reduced with lithium aluminum hydride. An additional methylation gave the fully methylated, neutral polysaccharide.

The methylated and carboxyl-reduced, and the fully methylated neutral polysaccharides were hydrolyzed, and the resulting methyl sugars were converted into the corresponding alditol acetates, which were analyzed by gas chromatography–mass spectrometry according to Björndal *et al.*[5]. The gas chromatograms, shown in Fig. 1, revealed that components A, B, and C were derived from the methylated, carboxyl-reduced polysaccharide, and components A, B, and D from the fully methylated neutral polysaccharide, respectively. The mass spectrum of component A showed major fragments at *m/e* 117, 131, and 233, and it was identical with that of the alditol

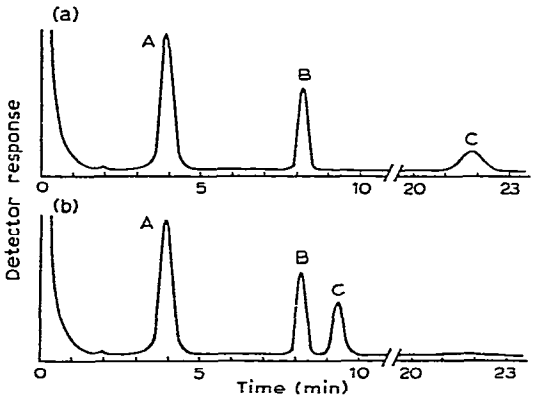

Fig. 1. Gas-chromatographic separations of the methylated sugars, as their alditol acetates, obtained after permethylation and hydrolysis of the polysaccharide. Conditions: 3% ECNSS-M on Gas Chrom Q (3 mm × 2 m), 170°, 40 ml He/min. (a) Methylated and carboxyl-reduced polysaccharide. (b) Methylated, carboxyl-reduced, and remethylated polysaccharide.

acetate derived from authentic 2,4-di-*O*-methyl-L-rhamnose*. The spectrum of component B showed major fragments at *m/e* 101, 117, 129, 161, 233, and 305, and it was in good agreement with that of the alditol acetate derived from authentic 2,4,6-tri-*O*-methyl-D-galactose*. Component C gave a mass spectrum with major fragments at *m/e* 101, 117, 127, 161, 261, and 305 that indicated a 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylhexitol[6], and component D a spectrum with major fragments at *m/e* 101, 113, 117, 161, 233, and 305, which is a fragment pattern typical for a 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol[7]. Since the remethylation of the hydroxymethyl group of the methylated, carboxyl-reduced polysaccharide replaced component C with component D (as shown in Fig. 1) and the hydroxymethyl group originated from the carboxyl group of the D-galacturonic acid residues in the original polysaccharide, the methyl sugars corresponding to components C and D were identified as 2,3-di-*O*-methyl-D-galactose and 2,3,6-tri-*O*-methyl-D-galactose, respectively.

Other di-*O*-methyl-D-galactoses or mono-*O*-methyl-L-rhamnoses, which may have originated from the branching points, could not be detected. The results of the methylation (Table III) are in good agreement with the structure suggested by the Smith degradation. In addition, the molar ratio of L-rhamnose, D-galactose, and D-galacuronic acid residues (2:1:0.7–0.8) estimated from the results of the methylation is consistent with that obtained from the sugar analysis of the acid hydrolyzate of the unmethylated polysaccharide[3].

Fragmentation analysis. — The acidic polysaccharide was partially hydrolyzed with acid, and the isolation of oligosaccharides from the hydrolyzate gave four acidic oligosaccharides, designated as S-1, S-2, S-3, and S-4. No neutral oligosaccharide was detected in the hydrolyzate.

*Kindly supplied by Dr. A. Misaki.

TABLE III

METHYL ETHERS OF SUGARS OBTAINED BY PERMETHYLATION OF THE POLYSACCHARIDE

Methylated sugar	*Peak*	T[a]	*Molar ratio*		*Structural feature indicated*
			Me-I[b]	*Me*-II[c]	
2,4-Di-*O*-methyl-L-rhamnose	A	1.00	2.0	2.1	→3)-L-Rha*p*-(1→
2,4,6-Tri-*O*-methyl-D-galactose	B	2.08	1.0	1.0	→3)-D-Gal*p*-(1→
2,3,6-Tri-*O*-methyl-D-galactose	D	2.40	—	0.8	→4)-D-GalA*p*-(1→
2,3-Di-*O*-methyl-D-galactose	C	5.57	0.7	—	→4)-D-GalA*p*-(1→

[a]Retention times of the corresponding alditol acetates relative to that of 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-L-rhamnitol. [b]Methylated and carboxyl-reduced polysaccharide. [c]Methylated, carboxyl-reduced, and remethylated polysaccharide.

On the basis of fragmentation analysis, periodate oxidation, and methylation, we propose that the native polysaccharide is an unbranched, acidic polysaccharide consisting of the following tetrasaccharide repeating-unit, with a D-galacturonic acid residue partially acetylated at O-2 or O-3, or at both positions: →3)-L-Rha*p*-(1→4)-D-GalA*p*-(1→3)-L-Rha*p*-(1→3)-D-Gal*p*-(1→. The presence of this tetrasaccharide repeating-unit in the polysaccharide molecule is also supported by the observation that the molecular weight of the unit (690) was in good agreement with the neutralization equivalent[2] (710) of the polysaccharide. A molecular weight[3] of 2×10^4 indicates a polysaccharide chain consisting of about 30 tetrasaccharide repeating-units. The occurrence of (1→3)-linked L-rhamnose or (1→3)-linked D-galactose residues was reported for several microbial polysaccharides, *e.g.*, polysaccharides of *Bacillus polymyxa var. lactoviscosus*[8], *Serratia marcescens*[9], *Escherichia coli*[10] type 42, and *Klebsiella*[11] type 47. Among these polysaccharides, *Klebsiella* type 47 capsular polysaccharide, composed of L-rhamnose, D-galactose, and D-glucuronic acid residues in the molar ratio of 1.7:1.0:0.7, appears to be related closely to the present *S. piscatorum* polysaccharide. Both polysaccharides gave, on partial acid hydrolysis, the similar two oligosaccharide fragments, *i.e.*, 3-*O*-(D-hexopyranosyluronic acid)-L-rhamnose and *O*-(D-hexopyranosyluronic acid)-(1→3)-*O*-L-rhamnopyranosyl-(1→3)-D-galactose. However, *S. piscatorum* polysaccharide differs from *Klebsiella* type 47 polysaccharide, which contains D-glucuronic acid residues, branched side chains, but no *O*-acetyl groups.

It is of biological interest that the presence of *O*-acetyl groups linked to D-galacturonic acid residues in the *S. piscatorum* polysaccharide is essential for the manifestation of adjuvant activity, as described previously[3]. The relationship between the adjuvant activity and the fine structure of this polysaccharide is being further investigated.

EXPERIMENTAL

General. — The total carbohydrate content was determined by the phenol–sulfuric acid method[12], reducing sugar content by the method of Somogyi–Nelson[13],

and hexuronic acid content by the carbazole–sulfuric acid method[14]. Paper chromatography was usually performed by an ascending method on Toyo No. 51A or Whatman 3MM filter paper with the following solvent systems (v/v): (A) 6:4:3 1-butanol–pyridine–water, and (B) 5:5:1:3 pyridine–ethyl acetate–acetic acid–water. Reducing sugars were detected on chromatograms by spraying with the alkaline silver nitrate or the *p*-anisidine hydrochloride reagent. Electrophoresis was performed on Toyo No. 51A or Whatman 3MM filter paper at 40 V/cm in 5:2:43 pyridine–acetic acid–water (pH 5.3). Sugars and oligosaccharides were detected on paper electrophoregrams by spraying alkaline silver nitrate. G.l.c. was performed with a Hitachi K-53 gas chromatograph, fitted with a flame-ionization detector and with a stainless-steel column (3 mm × 2 m) packed with (A) 5% SE-30 on Chromosorb W, (B) 3% silicone OV-1 on Chromosorb W-AW-DMCS, or (C) 3% ECNSS-M on Gas Chrom Q[15]. The methyl ethers of sugars were analyzed by gas chromatography–mass spectrometry, the alditol acetates[5] being prepared from the corresponding methylated sugars by reduction with sodium borohydride and subsequent acetylation with acetic anhydride–pyridine. The solution of the mixture of alditol acetates in chloroform was injected into the ECNSS-M column (3 mm × 2 m) of a Perkin–Elmer 800 gas chromatograph connected with a Hitachi RMU-6D mass spectrometer. The gas–liquid chromatography was performed at 170° and the mass spectra were recorded at an ionization potential of 70 eV and at a temperature of the ionic source of 200°.

Preparation and purification of the polysaccharide. — *Serratia piscatorum* IFO 12527 was grown for 4 days at 28° under aeration and agitation in a 50-liter fermentor containing a sucrose medium (30 liters), which was composed of 5% sucrose, 0.1% ammonium sulfate, 0.2% dipotassium hydrogen phosphate, 0.15% potassium dihydrogen phosphate, 0.02% magnesium sulfate heptahydrate, 0.1% yeast extract, and 0.5% calcium carbonate, pH 7.0. The culture broth was centrifuged to remove bacterial cells and other insoluble materials. To the supernatant was added acetone (90 liters) to give a precipitate of crude polysaccharide that was collected by centrifugation, washed with acetone, and then dried in a current of air (yield, 108 g).

The crude polysaccharide (11 g) was dissolved in water (2 liters) and the solution was centrifuged to remove the insoluble material. To the supernatant was added 10% cetyltrimethylammonium bromide (150 ml) to precipitate an acidic polysaccharide fraction, which was collected by centrifugation, washed with water (300 ml), dissolved in 2M sodium chloride (2 liters), and centrifuged. To the clear supernatant was added ethanol (6 liters) to give a precipitate that was collected by centrifugation, dissolved in 1% sodium chloride (500 ml), and fractionated by stepwise addition of ethanol. The precipitate obtained at 65–70% ethanol concentration was dissolved in water (500 ml), and the solution was passed through a column (2 × 30 cm) of Amberlite IR-120 (H^+) resin. The effluent was dialyzed against deionized water for 3 days and lyophilized to give a partially purified polysaccharide (~6 g).

A part (300 mg), dissolved in M sodium chloride (20 ml), was applied onto a column (5 × 90 cm) of Sepharose 2B and eluted with M sodium chloride at a flow rate of 1 ml/min into 20-ml portions. The fractions corresponding to the carbohydrate

peaks (tubes No. 25 to 40) were collected, combined, dialyzed, and rechromatographed. The polysaccharide fraction thus obtained was dialyzed, passed through a column (2 × 30 cm) of Amberlite IR-120 (H^+) resin, dialyzed again, and lyophilized (yield, 210 mg), $[\alpha]_D^{25}$ +120° (*c* 1.0, water).

The polysaccharide (800 mg) was dissolved in water (80 ml) and disrupted by sonication for 1 h at 10 kHz in the cold. The sonicate was fractionated on a Sepharose 4B column (5 × 90 cm), as just described, to give 580 mg, $s_{20,w}$ 1.6 S; $[\alpha]_D^{25}$ +120° (*c* 1.0, water); $[\eta]$ 0.7; neutralization equivalent, 710; carbohydrate content as D-glucose equivalent, 84%; *O*-acyl content, 5%; nitrogen content, 0.1%.

Deacylation. — A solution of the polysaccharide (100 mg) in 0.5M sodium hydroxide (10 ml) was heated for 1 h at 70°, passed through a column (1.3 × 30 cm) of Amberlite IR-120 (H^+) resin, dialyzed, and lyophilized (yield, 82 mg), $[\alpha]_D^{25}$ +117° (*c* 1.0, water); carbohydrate content as D-glucose equivalent, 95%.

Periodate oxidation. — The polysaccharide (100 mg) dissolved in water (50 ml) was oxidized with 0.1M sodium periodate (50 ml) for 20 days at 5° in the dark. Methyl α-D-glucopyranoside was oxidized as control. Periodate consumption was determined on aliquots by arsenite[16] and formic acid production by alkaline titration[17], after decomposition of residual periodate with 1,2-ethanediol. After 20-day oxidation, 1,2-ethanediol (10 ml) was added to destroy the excess periodate and the solution dialyzed.

Borohydride reduction of periodate-oxidized product. — To the dialyzed solution was added solid sodium borohydride (100 mg). The mixture was kept overnight at room temperature, dialyzed, and concentrated to 5 ml. The sugar components of the reduction product were analyzed by paper chromatography after hydrolysis with 0.5M sulfuric acid for 15 h at 100°.

Methylation. — The deacylated polysaccharide (2.0 g) was added to dimethyl sulfoxide (100 ml) and heated for 30 min at 60° under a stream of nitrogen. The solution was cooled to room temperature, freshly prepared methylsulfinyl carbanion[18] (20 ml) was added, and the reaction mixture was stirred for 20 h. To the resulting alkoxide solution, adjusted to 20°, was added dropwise methyl iodide (10 ml). The mixture was stirred for 5 h, dialyzed, and lyophilized to give an amorphous powder (1.5 g) that was subjected to a second methylation, as just described. The reaction mixture was dialyzed and extracted with chloroform (200 ml). The extract was dried and evaporated to dryness to give the fully methylated polysaccharide (500 mg). No free hydroxyl group was observed in the i.r. spectrum.

The methylated polysaccharide (500 mg), dissolved in ether (50 ml), was added to a suspension of lithium aluminum hydride (2 g) in ether (50 ml), and the mixture was stirred for 20 h. The excess of hydride was destroyed by dropwise addition of water (30 ml) under vigorous stirring. The ether solution was filtered to remove the insoluble material, which was extracted with chloroform (50 ml). The filtrate and chloroform extract were combined and evaporated to dryness to give the methylated, carboxyl-reduced polysaccharide (406 mg).

A sample of this polysaccharide (100 mg) was dissolved in methanol (2 ml),

and methyl iodide (10 ml) and silver oxide (2 g) were added. The reaction mixture was heated under reflux for 8 h, and after being cooled, it was filtered to remove the insoluble material, which was extracted with chloroform (50 ml). The filtrate and the chloroform extract were combined, dried (sodium sulfate), and evaporated to dryness. This methylation process was repeated twice to give the permethylated product of the methylated, carboxyl-reduced polysaccharide (65 mg).

Hydrolysis of methylated polysaccharides. — A sample of methylated polysaccharide (50 mg) was added to 85% formic acid (5 ml), and the solution was heated for 2 h at 100°. The resulting solution was evaporated to dryness, and the residue was added to 0.05M sulfuric acid (5 ml) and hydrolyzed for 15 h at 95°. The hydrolyzate was neutralized with barium carbonate, filtered, and concentrated to a syrup.

Partial hydrolysis with acid. — The polysaccharide (1 g) was hydrolyzed with 0.25M sulfuric acid (50 ml) for 1 h at 95°. The hydrolyzate was neutralized with barium carbonate, filtered, and the filtrate was dialyzed against deionized water (2 liters) for 20 h at 5°. The dialyzable fraction was concentrated to about 5 ml and applied onto a column (1.3 × 30 cm) of Dowex 1 (OH^-) resin. The column was washed with water (50 ml), and a mixture of acidic oligosaccharides was eluted with M formic acid (200 ml). The eluate was passed through a column (1.3 × 20 cm) of Amberlite IR-120 (H^+) resin and concentrated to a syrup (120 mg).

Fractionation and characterization of acidic oligosaccharides. — This syrup was dissolved in water (5 ml), and the solution was applied in 1-ml portions to a sheet (40 × 40 cm) of Whatman 3MM filter paper and chromatographed by multiple development (four times) with solvent A. The chromatography separated the mixture into four acidic oligosaccharide fractions, each of which was eluted from the chromatogram with water and evaporated to dryness. This procedure yielded S-1 (23 mg), S-2 (28 mg), S-3 (9 mg), and S-4 (6 mg). A small proportion of galacturonic acid and neutral sugars was also detected.

Oligosaccharide S-1: $[\alpha]_D^{25}$ +85° (*c* 0.5, water); M_{GalA} (electrophoretic mobility relative to that of D-galacturonic acid), 0.76. Acid hydrolysis gave equimolar amounts of D-galacturonic acid and L-rhamnose. Borohydride reduction, followed by hydrolysis, yielded L-rhamnitol, and periodate oxidation of the methyl glycoside of methyl ester degraded the D-galacturonic acid but not the L-rhamnose residue, indicating a D-galacturonosyl-L-rhamnose structure.

Oligosaccharide S-2: $[\alpha]_D^{25}$ +115° (*c* 0.5, water); M_{GalA} 0.60. Acid hydrolysis gave equimolar amounts of D-galacturonic acid, L-rhamnose, and D-galactose. The terminal reducing residue was D-galactose, as shown by borohydride reduction. Mild acid hydrolysis with 0.25M sulfuric acid for 20 min at 100° released almost equimolar amounts of S-1 and D-galactose. Periodate oxidation degraded both D-galacturonic acid and D-galactose residues but not the L-rhamnose residue, indicating a (D-galacosyluronic acid)-L-rhamnosyl-D-galactose structure.

Oligosaccharide S-3; M_{GalA} 0.51. Acid hydrolysis gave D-galacturonic acid, L-rhamnose, and D-galactose in the molar ratio of 1:2:1. The terminal reducing residu was shown to be L-rhamnose. Mild acid hydrolysis released a trace amount of

S-2 together with S-1, D-galacturonic acid, L-rhamnose, and D-galactose, indicating a (D-galactosyluronic acid)-L-rhamnosyl -D-galactosyl-L-rhamnose structure.

Oligosaccharide S-4; M_{GalA} 0.42. Acid hydrolysis gave D-galacturonic acid, L-rhamnose, and D-galactose in the molar ratio of 2:3:2. The terminal reducing residue was shown to be D-galactose. Partial acid hydrolysis gave a trace amount of S-2 together with S-1, D-galacturonic acid, L-rhamnose, and D-galactose, suggesting a (D-galactosyluronic acid)-L-rhamnosyl-D-galactosyl-L-rhamnosyl-(D-galactosyluronic acid)-L-rhamnosyl-D-galactose structure.

ACKNOWLEDGMENTS

We thank Prof. K. Ogata, Kyoto University and Dr. A. Misaki, Osaka University for their helpful advice and fruitful discussions, and Drs. S. Tatsuoka, R. Takeda, and E. Ohmura, Central Research Division of our company for their support and encouragement during the course of this study. Grateful acknowledgments are extended to Mr. T. Shima of the Chemical Research Laboratories, for his operation of the gas chromatography–mass spectrometry system and to Miss K. Nishiike for her technical assistance.

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