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STRUCTURAL INVESTIGATIONS ON THE LIPOPOLYSACCHARIDE ISOLATED FROM *Vibrio cholera, Inaba 569 B*

ASISH K. SEN, AMAL K. MUKHERJEE*,

Department of Macromolecules, Indian Association for the Cultivation of Science, Calcutta-700032 (India)

BHAKTI GUHATHAKURTA, ARCHANA DUTTA, AND DIPTI SASMAL

Cholera Research Centre, 3 Kyd Street, Calcutta-700 016 (India)

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ABSTRACT

On hydrolysis, the purified lipopolysaccharide (LPS) isolated from *Vibrio cholera, Inaba 569 B*, yielded glucose, mannose, a heptose behaving like D-*glycero*-L-*manno*-heptose and one behaving like D-*glycero*-L-*gluco*-heptose, 2-amino-2-deoxyglucose, and glucuronic acid in the molar ratios of ~9:4:5:1:2:5. Studies on the LPS, the polysaccharide (PS), and carboxyl-reduced LPS showed that the PS has a branched structure, with (1→2)-linked mannopyranosyl and a heptopyranosyl, and (1→4)-linked glucopyranosyluronic and 2-amino-2-deoxyglucopyranosyl residues in the interior part of the molecule, and glucopyranosyl and heptopyranosyl residues as nonreducing end-groups.

INTRODUCTION

O-Antigens are the determining factors in serological specificities of Gram-negative bacteria; these are present in endotoxic lipopolysaccharides (LPS) located in the bacterial cell-walls. Detailed, structural investigations have been conducted on the O-antigenic determinants of some Gram-negative bacteria[1–3]. As far as *Vibrio cholera* is concerned, no such detailed work has been reported, although a number of publications[4–9] reported preliminary studies on the LPS constituents. As the serological specificities of different serotypes of *V. cholera* are determined by the LPS of these bacteria, we initiated structural and immunological studies on the LPS of *V. cholera*. In our earlier publication[10], we reported the structural pattern of the polysaccharide isolated from *V. cholera, NAG 384* strain. We now describe the results of our investigations on the LPS isolated from *V. cholera, Inaba 569 B*.

RESULTS AND DISCUSSION

Cells obtained from 18-h growth of *V. cholera, Inaba 569 B*, on nutrient agar

*To whom correspondence should be addressed.

in roll bottles were digested with 1:1 (w/w) water–phenol for 45 min at 65°. The aqueous layer from this digest contained the LPS; it was fractionated with cetyltrimethylammonium bromide, and isolated as described by Pant and Shrivastava[11]. The LPS, $[\alpha]^{23}_{589.5}$ +15°, was free from protein and nucleic acids, and contained ash 1.75%, nitrogen 2.48%, and phosphate 1.38%. In the i.r. spectrum (KBr), a small peak in the region of 1735 cm^{-1} indicated the presence of an *O*-acyl group. The LPS was found to be essentially homogeneous, as it gave a single precipitin line in the Ouchterlony gel-diffusion test[12a,b] with antisera raised against the whole organism in rabbit; however, a very small trace of impurity (a spot towards the cathode) was found along with the main spot when it was subjected to high-voltage electrophoresis at different gradients with different buffers. The LPS was therefore further purified by passing it through a column of Sephadex G-100, using ammonium hydrogencarbonate solution for elution. The purified LPS was obtained as a single peak in 75% yield, and it had $[\alpha]^{23}_{589.5}$ +14°, and contained nitrogen 2.1%, and phosphate 1.33%. This material also gave a single precipitin line in the Ouchterlony gel-diffusion test, and behaved in the same way as the original LPS when subjected to high-voltage electrophoresis.

Lipid was removed from the purified LPS by treatment under nitrogen with 0.1M acetic acid for 2 h in a boiling-water bath, cooling, and extracting the lipid with diethyl ether. A solution of the resulting polysaccharide (PS) was passed through a column of Sephadex G-100, and the eluate containing the PS was lyophilized. The PS, obtained in 79% yield, had $[\alpha]^{23}_{589.5}$ +6°, and contained only traces of nitrogen (0.19%) and phosphate. The results of preliminary experiments on various fractions are summarized in Table I. The purified PS was also homogeneous by the Ouchter-

TABLE I

RESULTS OF PRELIMINARY ANALYSIS OF FRACTIONS OF *Vibrio cholera* LIPOPOLYSACCHARIDE

	Crude LPS	*Purified LPS*	*PS*	*Lipid*
Moisture (%)	2.3			
Ash (%)	1.75			
$[\alpha]^{23}_{589.5}$ (degrees)	+15	+14	+6	
Nitrogen[a] (%)	2.48	2.1	0.19	
Phosphate[b] (%)	1.38	1.33	0.12	
Total carbohydrate(%)[c]	32.2	30.1	34	0.9
Carbohydrate (%)[d]	21.5	23.3	28.1	0.7
	10.5	10.7	13.1	
Uronic acid (%)[e, f, g]		4.6	5.4	
		5.7	6.9	

[a]Estimated by the Dumas method. [b]Estimated by the Ames method[13a]. [c]Estimated by the orcinol method[12c]. [d]Estimated by g.l.c., using D-galactose as the internal standard, but excluding amino sugars which do not contribute to the orcinol test. [e]Estimated by the carbozole–sulfuric acid method[14]. [f]Estimated from the difference in the percentage of glucose in the reduced and non-reduced material (by g.l.c., using D-galactose as the internal standard). [g]Estimated by the carbazole–sulfuric acid method on the carboxyl-reduced materials.

TABLE II

RESULTS OF ESTIMATION OF SUGARS IN DIFFERENT FRACTIONS OF *V. cholera* LIPOPOLYSACCHARIDE

Sugars detected[a]	*Hydrolyzed with 0.5M H_2SO_4 for 18 h (with D-galactose as internal standard)*			
	Crude LPS	*Pure LPS*	*Carboxyl-reduced LPS*	*PS*
Mannose (%)	3.3	3.8	3.7	4.7
Glucose (%)	8.1	9.0	13.6	10.8
Major heptose (%)[b]	4.8	5.1	5.3	6.2
Minor heptose (%)[c]	0.7	0.8	0.9	1.0
2-Amino-2-deoxyglucose (%)	1.7	2.1	2.2	2.6
Glucuronic acid (%)[d]		4.6		5.4

[a]Estimated by g.l.c. as alditol acetates[15]. [b]Behaves like D-*glycero*-L-*manno*-heptose. [c]Behaves like D-*glycero*-L-*gluco*-heptose. [d]Estimated from the difference in the percentage of glucose in the hydrolyzates of carboxyl-reduced LPS and pure LPS (by g.l.c.). Average value of three estimations are given. All results are corrected for moisture.

lony gel-diffusion test, but still showed a trace of a spot towards the cathode (along with the main spot), as in the case of the original and the purified LPS. Attempts to isolate and identify this very minor component failed, because of the small amount of the substance available.

The LPS, PS, and carboxyl-reduced lipopolysaccharide (CR-LPS) were hydrolyzed with 0.5M sulfuric acid for 18 h in a boiling-water bath. The acidic, neutral, and basic sugars were identified and estimated by employing appropriate techniques. The results of these experiments are summarized in Table II. Total carbohydrate was also estimated by the orcinol method[12c]. From these results, a few important points were found that merit special discussion. The core structure of the LPS of *V. cholera, Inaba 569 B*, differs from those of other Gram-negative bacteria, as the LPS from *V. cholera, Inaba 569 B*, does not contain any galactose or 3-deoxy-D-*manno*-octulosonic acid (KDO), which are commonly found in other Gram-negative bacteria, *e.g., Salmonella typhimurium*[1]. Secondly, the carbohydrate content of this serotype of *V. cholera* is much less than that of *S. typhimurium*, and contains a large proportion of unknown organic constituents. Similar observations have been made by Hisatsune *et al.*[9]. Another interesting point is that, for the LPS or PS, the total amount of carbohydrate indicated by the orcinol method is more than that found by g.l.c. analysis (using D-galactose as the internal standard) for the same sample of LPS or PS. "Lipid" showed almost same the value of carbohydrate by the orcinol method and by g.l.c. analysis, so it seems possible that the large proportion of unknown organic matter present in the LPS contributes to some extent to the orcinol test.

The hexuronic acid was identified as glucuronic acid by paper chromatography, and also by reducing the acid to glucose. The major heptose was identical in behavior to D-*glycero*-L-*manno*-heptose in g.l.c. using column *a* and by paper chromatography

in[16] solvent *E*, the authentic compound being obtained by synthesis. The minor heptose had R_G 2.54 (column *a*), and behaved like D-*glycero*-L-*gluco*-heptose. The proportion of glucuronic acid in the LPS was estimated to be 10.7% by the carbazole–sulfuric acid method[14], but, after reduction of the LPS twice with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC) plus sodium borohydride[17], the product (CR-LPS) still showed 5.7% of uronic acid by the carbazole–sulfuric acid method, and, after hydrolysis, showed a 4.6% increase in the percentage of glucose as estimated by g.l.c. However, this sample did not show any spot for uronic acid on paper chromatograms in solvents *A* and *B*. After repeated CMC–borohydride reduction of the CR-LPS, it showed the same proportion of uronic acid by the carbazole–sulfuric acid method although it could not be found by p.c.; this is due to the fact that heptoses respond, and contribute to some extent, to the value obtained by the carbazole–sulfuric acid method of estimation of uronic acid. This was confirmed by estimating a known mixture of glucuronic acid and heptose by the carbazole–sulfuric acid method. The unknown organic materials in the LPS or PS may also contribute to this behavior.

The LPS and CR-LPS were methylated by the Hakomori procedure[18,19] and then by Purdie's method[20]. The products were formylated with 90% formic acid, and then hydrolyzed with 0.5M sulfuric acid, to yield partially methylated sugars. The partially methylated amino sugars were separated from the partially methylated neutral sugars in each sample by means of a cation-exchange resin. The partially methylated neutral and amino sugars were then converted into their alditol acetates[21,22]. The peaks were identified and estimated by use of g.l.c.–m.s. and g.l.c. The results are summarized in Table III.

TABLE III

METHYL ETHERS OF SUGARS FROM THE HYDROLYZATES OF METHYLATED LPS (A), METHYLATED CARBOXYL-REDUCED LPS (B), AND METHYLATED AMINO SUGAR (C)

Sugars[a]	*T*[b]	*Approximate mole-%*[c]			*Mode of linkage*
		A	*B*	*C*	
2,3,4,6-Glc	1.00	45	34		Glc*p*-(1→
3,4,6-Man	1.95	17	13		→2)-Man*p*-(1→
2,3,4,6,7-Hep	2.45	24	18		Hep*p*-(1→
2,3,6-Glc	2.5	—	16		→4)-Glc*p*A-(1→
3,4,6,7-Hep	4.49	4	8		→2)-Hep*p*-(1→
3,4,7-Hep	8.66	6	6		→2,6)-Hep*p*-(1→
X[d]	10.9	4	4		
3,6-GlcNMe	1.71[e]			10	→4)-Glc*p*NAc-(1→

[a]2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, etc. [b]Retention times of the corresponding alditol acetates, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an ECNSS-M column at 165°. [c]Mole percent in total carbohydrate. Average values from three determinations. [d]2- or 6-*O*-methylheptose, or 2,6- or 2,7-di-*O*-methylheptose. [e]Retention time on a column of 3% of ECNSS-M at 190°, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

It may be seen that the nonreducing ends of the molecule are occupied by glucopyranosyl and heptopyranosyl residues, as, in the hydrolyzates of the methylated LPS and CR-LPS, 2,3,4,6-tetra-*O*-methylglucose and 2,3,4,6,7-penta-*O*-methylheptose were characterized. Although no partially methylated heptoses were available for comparison, three peaks, corresponding to 2,3,4,6,7-penta-*O*-methylheptose, 3,4,6,7-tetra-*O*-methylheptose, and 3,4,7-tri-*O*-methylheptose, were identified by g.l.c.–m.s.

It was difficult to decide the location of heptose residues at the various positions in the molecule. One point, however, seems to be clear; the heptose behaving like D-*glycero*-L-*manno*-heptose is located at the non-reducing end, as a large proportion of 2,3,4,6,7-penta-*O*-methylheptose is present in the hydrolyzates of the methylated LPS and CR-LPS. One, or both, of the heptose residues is present in the interior of the molecule. One of them is present as a (1→2)-linked residue, and the other is branched; and at the branch point, the heptose is linked through O-1, O-2, and O-6. Characterization of 3,4,6-tri-*O*-methylmannose indicated that the mannopyranosyl groups are exclusively present in the interior of the molecule as (1→2)-linked residues. The interior part of the molecule also contains the glucuronic acid as (1→4)-linked residues, as only the hydrolyzate of the methylated CR-LPS contained a new sugar, which was characterized as 2,3,6-tri-*O*-methylglucose. The 2-amino-2-deoxyglucose residue is also present in the interior part as (1→4)-linked residues, as it yielded 2-deoxy-3,6-di-*O*-methyl-2-(methylamino)-glucose in the hydrolyzates of the methylated samples.

The hydrolyzates of all of these methylated samples contained a partially methylated sugar (R_{TMG} 10.9, column *a*) that could not be characterized. From the signals in g.l.c.–m.s., the sugar could be a 2-*O*-methylhexose, a 2- or 6-*O*-methylheptose, or a 2,6- or 2,7-di-*O*-methylheptose (*m/e* 117, 70%), but, from the retention time in g.l.c., it does not seem to be a 2-*O*-methylhexose. In any case, the molecule has a multiple branching at this point.

It was also found that the relative amount of the 3,4,6,7-tetra-*O*-methylheptose was higher in the hydrolyzate of the CR-LPS only; this indicates that the release of this heptose derivative is higher when the glucuronic acid has been reduced. It is therefore possible that the glucuronic acid is linked to this heptose at O-2.

The low, positive, specific rotation of the PS, as well as of the LPS, indicates that both anomeric configurations are present in the molecule.

EXPERIMENTAL

General methods. — Optical rotations were measured with a Perkin–Elmer model 241 MC spectropolarimeter at 23 ±1° and 589.5 nm for solutions in water. Infrared spectra were recorded with a Beckman IR-20A instrument having cesium bromide optics. Ultraviolet and visible spectra were recorded with a Carl Zeiss VSU2-P and a Hilger spectrophotometer, respectively. For g.l.c., a Hewlett–Packard 5730 A gas chromatograph with flame–ionization detector was used. Resolutions were

performed on glass columns (1.83 m × 6 mm) containing (*a*) 3% of ECNSS-M on Gas Chrom Q (100–120 mesh) at 190° (for alditol acetates of sugars), and at 165° (for partially methylated alditol acetates), (*b*) 5% of OV-225 on Sil Rub (80–100 mesh) at 190° and (*c*) 1% of OV-225 on Gas Chrom Q (80–100 mesh) at 160° (for partially methylated sugars), and (*d*) 3% of POLY A-103 on Gas Chrom Q (100–120 mesh) at 200° (for alditol acetates of amino sugars). Paper chromatography was performed on Whatman No. 1 paper, with the following solvent systems (v/v): (*A*) 8:2:1 ethyl acetate–pyridine–water, (*B*) 5:5:1:3 ethyl acetate–pyridine–acetic acid–water, (*C*) 10:1:2 1-butanol–ethanol–water, (*D*) 4:1:1 1-butanol–ethanol–water, and (*E*) 6:4:3 1-butanol–pyridine–water. The sugars were detected with (*a*) alkaline silver nitrate, (*b*) 2% ninhydrin in acetone at 110°, and (*c*) 3% *p*-anisidine hydrochloride in ethanol at 120°. High-voltage electrophoresis was conducted in a Shandon model L 24 High-voltage electrophoresis apparatus. Evaporations were performed under diminished pressure at bath temperatures not exceeding 40°. Small volumes of aqueous solutions were lyophilized.

Preparation of lipopolysaccharide. — *V. cholera, Inaba 569 B*, was isolated at the Haffkine Institute, Bombay. Eighteen-hour growths of this strain on nutrient agar in roll bottles at 37° were harvested in 0.85% cold saline, and filtered through muslin cloth. The filtrate was passed through a Sharples supercentrifuge, and the sediment was washed repeatedly with cold saline. The washed cells were digested with 1:1 (w/w) water–phenol for 45 min in a water bath at 65°. The mixture was kept overnight in a refrigerator (5°), and the aqueous layer, obtained after centrifugation, was fractionated with cetyltrimethylammonium bromide, and further purified as described by Pant and Shrivastava[11]. The neutral LPS fraction was used in all of the experiments. The LPS preparations were respectively confirmed to be free from protein, nucleic acid, and degraded LPS by the biuret test, ultraviolet absorption at ~260 nm, and the Ouchterlony gel-diffusion test. The LPS showed $[\alpha]_{589.5}^{23}$ +15° (*c* 1, water), nitrogen 2.48%, phosphate 1.38%, and ash 1.75%. In the i.r. spectrum (KBr), a small peak in the region of 1735 cm^{-1} indicated the presence of some *O*-acyl group.

Purification of the lipopolysaccharide (LPS). — A solution of LPS (50 mg) in 0.05M ammonium hydrogencarbonate solution (5 mL) was applied to a column (100 × 2.2 cm) of Sephadex G-100. The column was eluted with 0.05M ammonium hydrogencarbonate, and the effluents were collected in 3-mL portions. The carbohydrate content of each fraction was determined by the phenol–sulfuric acid method. The eluate showed only one major peak. The LPS was recovered in 75% yield by lyophilization; $[\alpha]_{589.5}^{23}$ +14° (*c* 1, water), nitrogen 2.1%, and phosphate 1.33%.

Removal of lipid. — A 1% solution of LPS in 0.1M acetic acid was heated for 2 h on a boiling-water bath, cooled, and repeatedly extracted with diethyl ether. The extract was then washed with water, and the washings were added to the aqueous solution. The aqueous solutions were combined, dialyzed against distilled water, and concentrated to ~2 mL. The derived PS was purified by passing through a column (60 × 2.2 cm) of Sephadex G-100. The sole product, eluted as a single peak,

was lyophilized. The PS, obtained in 79% yield, showed $[\alpha]_{589.5}^{23}$ +6° (*c* 1, water), and contained only traces of nitrogen (0.19%) and phosphate.

High-voltage electrophoresis. — High-voltage electrophoresis was performed in (*a*) phosphate buffer (pH 7.5, 0.02M, 35 V/cm, 1 h), (*b*) borate buffer (pH 9.5, 0.02M, 25 V/cm, 1 h), and (*c*) acetate buffer (pH 4.7, 0.05M, 25 V/cm, 1 h). In *a*, *b*, and *c*, both LPS and purified LPS remained stationary, although a very faint spot, 2 cm towards the cathode, could be detected. However, the PS moved 4, 9, and 22 cm towards the anode in *a*, *b*, and *c*, respectively, with a very faint spot, 2 cm towards the cathode, in all of them. Benzidine–periodate was used as the spray reagent.

Preparation of antiserum. — Antiserum was produced by immunizing rabbits (two for each group) intravenously with a suspension of bacteria (0.25 mL) containing ~1,400 × 10^6 organisms per mL. Increasing doses were given twice a week for three weeks. The animals were bled one week after the last injection, and the serum was stored at —20°. Gel-diffusion studies were performed with this serum.

Ouchterlony gel-diffusion test[12a,b]. — Original LPS, purified LPS, and PS, in different concentrations in physiological saline, were added to the gel-diffusion plates. In all instances, only a single precipitin-line was observed.

Sugar analysis. — The crude LPS, purified LPS, and PS were hydrolyzed with 0.5M sulfuric acid for 18 h, the acid neutralized with barium carbonate, and the suspension centrifuged. The neutral, basic, and acidic sugars were detected by paper chromatography. Solvents *A*, *C*, and *E*, and spray reagents *a* and *c*, were used for neutral sugars; solvents *A* and *B* and spray reagent *a* for acidic sugars; and solvent *A* and spray reagents *a* and *b* for amino sugars. Neutral sugars, as their alditol acetates[15], were respectively detected and estimated by g.l.c.–m.s. and g.l.c. (column *a*), using D-galactose as the internal standard. Hexosamine was estimated (with the same internal standard) in column *d*, and also by ninhydrin degradation[13b] followed by chromatography in solvent *D*, and g.l.c. in column *a*. Total phosphate was estimated by an ashing procedure[13a].

Uronic acid was estimated by the carbazole–sulfuric acid method[14] (with D-glucuronic acid as the standard), and also from the difference in the percentage of glucose in the hydrolyzates of CR-LPS and LPS, by g.l.c. (column *a*), with D-galactose as the internal standard.

Total carbohydrate present in the crude LPS, purified LPS, PS, and lipid was estimated by the orcinol method[12c].

Preparation of carboxyl-reduced LPS (CR-LPS)[17]. — LPS (15 mg) was reduced with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC). To a stirred solution of LPS (15 mg) in water (14 mL) was added CMC (0.38 g), and the pH was kept at ~4.75 by adding 0.01M hydrochloric acid dropwise. After 2 h, 2M aqueous sodium borohydride (25 mL) was added dropwise during 45 min, and the pH was kept at ~7 by simultaneous addition of 4M hydrochloric acid. After 1 h, the solution was dialyzed against distilled water for 24 h, and lyophilized. The product was reduced once again, as just described. The carboxyl-reduced LPS was obtained in 90% yield.

CR-LPS (2 mg) was hydrolyzed with 0.5M sulfuric acid for 18 h at 100°, and estimated as the alditol acetates (with D-galactose as the internal standard) by g.l.c. in column *a*.

Methylation analysis[18] *of LPS and CR-LPS.* — LPS (20 mg) and CR-LPS (5 mg) in dimethyl sulfoxide (20 mL and 5 mL, respectively) in two vials were treated with 2M methylsulfinyl sodium[19] (20 mL and 5 mL, respectively) under nitrogen. The solutions were stirred overnight at room temperature. Methyl iodide (20 mL and 5 mL, respectively) was then added dropwise, with external cooling, and the mixture was stirred for 2 h (when clear solutions were obtained). These were then dialyzed, first against running tap-water, and then against distilled water. The methylated samples obtained after lyophilization were remethylated by the Purdie method[20], to yield fully methylated LPS (21 mg) and CR-LPS (5 mg). The methylated samples were then purified by passing them through a column (1.5 × 30 cm) of Sephadex LH-20 in 1:1 chloroform–acetone.

The methylated samples were individually hydrolyzed with 90% formic acid for 2 h at 100°. The acid was removed by co-distillation with water, and the products were further hydrolyzed with 0.5M sulfuric acid for 18 h at 100°. The solutions were made neutral with barium carbonate, and then passed through a column (10 × 1.5 cm) of Dowex-50W X-8 (H^+) ion-exchange resin. The columns were thoroughly washed with water (500 mL) to remove all of the partially methylated neutral and acidic sugars. Each eluate was concentrated to ~1 mL, reduced with sodium borohydride, converted into the alditol acetates[21], and examined by g.l.c.–m.s.

The columns of Dowex-50W X-8 (H^+) resin were then successively eluted with 2M (25 mL), M (25 mL), and 0.5M (50 mL) hydrochloric acid, to isolate the partially methylated, basic sugars. The hydrochloric acid was partially removed by evaporation, and then completely by neutralization with silver carbonate. The silver salts were removed by filtration, the filtrate was concentrated to ~2 mL, and the contents reduced with sodium borohydride and converted into their alditol acetates[10,22], and analyzed by g.l.c.

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