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Note

Structural characterization of the *O*-antigenic polysaccharide from the lipopolysaccharide of *Vibrio cholerae O*37

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The chemical structure of the *O*-antigenic polysaccharide isolated from the lipopolysaccharide of *Vibrio cholerae O*37 by mild acid hydrolysis was elucidated. The *O*-antigenic polysaccharide is found to consist of D-glucose, *N*-acetyl-D-Quinovosamine and small amount of 4-*O*-methyl-*N*-acetyl-D-quinovosamine. The structure of the *O*-antigen is established by using sugar and methylation analyses, Smith degradation studies and by using GLC, GC-MS, FAB-MS, one dimensional ^{1}H and ^{13}C NMR spectroscopy and two dimensional NMR spectroscopy including COSY, TOCSY, HSQC, experiments.

Keywords: Lipopolysaccharide, *O*-antigen, polysaccharide, cholera, *Vibrio cholerae O*37.

The species *Vibrio cholerae* is classified into several serogroups[1] on the basis of their *O*-antigenic polysaccharides. Until 1992, only *V. cholerae* O1 was considered to be responsible for the disease cholera in human. Other non O1 strains were considered to be responsible for the sporadic cases of gastroenteritis with much less severity. However, emergence of *V. cholerae* O139, a non O1 strain having epidemic and pandemic potential[2] has drawn much attention of the scientists. Besides *V. cholerae* O139, some of the non O1 and non O139 serogroups were also found to be associated with cholera like disease[3,4]. In particular, *V. cholerae* O37 was associated with an outbreak[5,6] in Sudan in 1968. Since the cell surface lipopolysaccharides play an important role in protective immunity as well as in virulence, we have undertaken structural studies of the lipopolysaccharide isolated from *V. cholerae* O37. Structures of several *O*-antigenic polysaccharides from different serogroups of *V. cholerae* such as O1, O2, O3, O5, O6[7] O8, O9, O10, O21, O22, O76, O139, O140, O144 and O155 have already been reported. Here in the structural studies of the *O*-antigenic polysaccharide from *V. cholerae* O37 is reported.

V. cholerae O37, provided by Dr. G. B. Nair, was grown in liquid tryptone-yeast extract medium. The cells were washed and dried with acetone (1 g/L culture medium). LPS was extracted from the dry bacterial cells by 90% hot phenol-water method[8] to give crude lipopolysaccharide (0.19 g/g dry cell). Repeated ultracentrifugation (thrice) of the crude LPS at 120000 g for 4 hr gave the pure LPS (0.06 g/g dry cell). The crude LPS was also purified by treatment with DNase, RNase and protease[9] (0.04 g/g dry cell). The purity in both the cases were same as judged by GLC analysis but the yield in the later case was low due to loss during experimental procedure.

Sugar analysis of LPS by GLC and GLC-MS as alditol acetate[10] after acid hydrolysis with 2*N* trifluroacetic acid showed the presence of D-glucose (42%), *N*-acetyl-D-quinovosamine (35%) and small amounts of 4-*O*-methyl-*N*-acetyl-D-quinovosamine (5%), *N*-acetyl-D-glucosamine (6%), L-glycero-D-manno-heptose (12%). The LPS also contained fructose[11] (2.1%), 2-keto-3-deoxy-manno-octonic acid[12] (2.0%) and very small amount of phosphate[13] (0.6%).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)[14,15] of several LPS samples was carried out (**Figure 1**). LPS isolated from *V. cholerae* O37 (lane b) showed slower moving bands, indicating that it is of low molecular weight and semi-rough type (SR) in nature having molecular heterogeneity. The LPSs of *Salmonella montevideo* SH94 (lane e) and *E. coli* O8 (lane a) were used as standard smooth and rough type of LPS respectively.

The purified LPS (100 mg) was hydrolyzed with 2% acetic acid at 100°C for 7 hr (optimum condition) to cleave core and lipid-A portions. In general mild acid hydrolysis[16] (1.5% acetic acid, 100°C for 1.5 hr) completely cleaves LPS into *O*-antigenic polysaccharide (OPS), core oligosaccharide (COS) and lipid-A, but use of higher acid concentration, temperature and hydrolysis time are also reported[17]. In the

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case of LPS from *V. cholerae* O37, although lipid-A was completely released, cleavage of the linkage between COS and OPS was incomplete under mild hydrolysis condition. Longer time of hydrolysis or use of stronger acid resulted in random cleavage of the OPS or COS. After hydrolysis at optimal condition, Lipid-A was separated from the hydrolyzate by centrifugation, washed with water and lyophilized (50 mg). The aqueous portion containing the degraded LPS was then fractionated by gel permeation chromatography using Sephadex G-25 and water as eluent (30 mg). The OPS was still heterogeneous as it contained some uncleaved COS revealed by sugar analysis. Attempt to separate these by gel permeation chromatography using various matrixes, HPLC (size exclusion column) or by ion-exchange chromatography was not successful.

Sugar analysis of the OPS as alditol acetate[10] showed presence of D-glucose, *N*-acetyl-D-quinovosamine, 4-*O*-methyl-*N*-acetyl-D-quinovosamine and L-glycero-D-manno-heptose in the ratio of 10:10:1:1. The absolute configuration of D-glucose and D-quinovosamine were determined by GLC of the acetylated (+)-2-butyl glycosides[18]. Both were found to have D-configuration. The L-glycero-D-manno-heptose was confirmed by direct comparison with a standard compound. The fatty acid in lipid-A was analyzed by GLC and GC-MS as their methyl esters. Presence of 3-OH:C12, C14, 3-OH:C14, C16 and small amount of C18 indicated that the nature of fatty acids in *V. cholerae* species is conserved in *V. cholerae* O37.

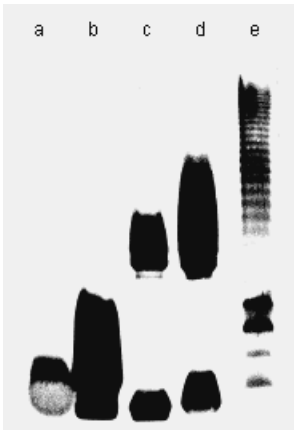

Figure1 — Silver stained SDS-PAGE patterns of lipopolysaccharides; lane (a) *E. coli* O8; lane (b) *V. cholerae* O37; lane (c) *V. cholerae* O31; (d) *V. cholerae* O139 and lane (e) *Salmonella montevideo* SH94

The methylation analysis[19] of the OPS revealed that the OPS contained 4-substituted glucose, terminal quinovosamine, 3-substituted quinovosamine and 3,4-disubstituted quinovosamine. Small amount of terminal glucose and 3,4,6-trisubstituted heptose detected as minor product, is likely to be from the uncleaved core oligosaccharide.

The FAB-MS[20] analysis of the methylated OPS showed two possible sequences of monosaccharides (I) and (II) (**Figure 2**) which are very similar and is due to the presence of heterogeneity in O-antigenic polysaccharide preparation. It is possible that terminal quinovosamine in (II) is 4-*O*-methylated in the native OPS, which could not be confirmed in this study and needs further investigation.

The ^{1}H NMR was carried out using 500 MHz machine at 70°C and acetone as internal standard (**Figure 3**). Because of the heterogeneity of the OPS preparation the spectra was complicated but some major signals could be identified including signals in the anomeric region.

The major peaks in the anomeric region, tentatively identified, are δ 5.31, 5.21 (2s, α-D-QuinNAc 1→), 5.06 (s, →4 α-D-Glc 1→), 4.93, 4.90 (2s, →3 α-D-QuinNAc 1→), 4.78 (s, →3,4 α-D-QuinNAc 1→),

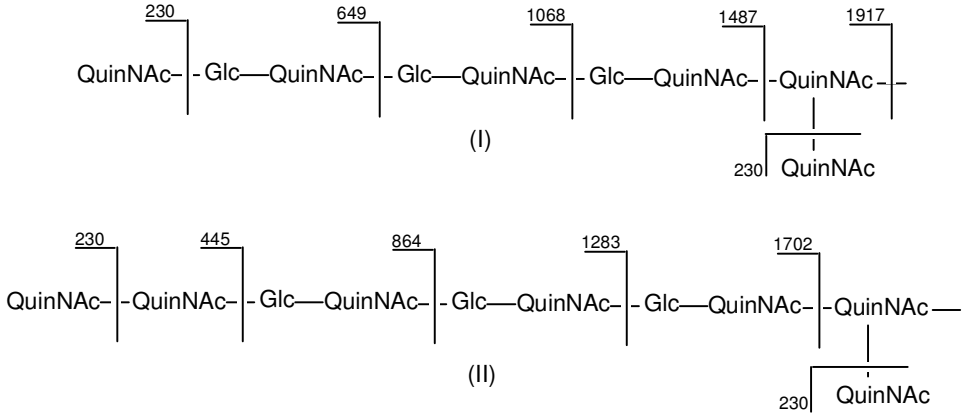

Figure 2 — FAB-MS fragmentation patterns of the methylated *O*-antigenic polysaccharide of *Vibrio cholerae* O37

and 4.58 (d, J = ~7Hz, β-D-Glc 1→). The peak for β-D-glucose probably comes from the attached COS. Except the peak for β-D-glucose (δ 4.56, d, J = ~7Hz) all other peaks appeared as broad singlet indicating α-anomeric linkage and all are in pyranose form. Other characteristic signals at δ1.27 (m, 6-deoxy CH_3 of QuinNAc), 1.38 (m, 6-deoxy CH_3 of QuinNAc), 1.97 (*O*-COCH_3), 2.03-1.95 (m, *N*-COCH_3 of QuinNAc) and 3.03-4.15 for ring protons, they are in good agreement with sugar analysis and methylation data. The small *O*-acetyl signal appears to be insignificant and location of *O*-acetyl group could not be assigned.

^{13}C NMR spectra of the OPS was recorded in a 75 MHz NMR machine (**Figure 4**). The characteristic signals tentatively identified are δ 175.73, 175.62, 175.0, 174.22 and 174.15 (5s, -*C*OCH$_3$ signals, QuinNAc) anomeric signals 101.74 (β-D-Glc 1→), 100.31 (→4 α-D-Glc 1→), 99.25 and 98.26 (→3 α-D-QuinNAc 1→), 98.04 (→3,4 α-D-QuinNAc 1→), 96.04, 94.81 (α-D-QuinNAc 1→) were observed. The other characteristic signals are 18.11, 17.83, 17.28, 17.19 and 17.15 (5s, 6-deoxy *C*H$_3$ of QuinNAc), 23.28, 23.07, 22.87, 22.70 and 22.63 (5s, *N*-CO*C*H$_3$ of QuinNAc). The other ring carbon signals appeared between 55.0 and 80.0 could not be assigned. In addition to the major peaks as mentioned above, there were minor signals in the spectrum due to the presence of uncleaved core oligosaccharide attached with the OPS as mention earlier.

For the assignment of proton and carbon signals of the OPS, COSY, TOCSY and HSQC experiments were performed. Due to complexity of the spectra only anomeric signals could be assigned.

Periodate oxidation of the OPS showed the presence of tetritol, *N*-acetyl quinovosamine, glucose and heptose in approximately 3:6:1:1 ratio. The

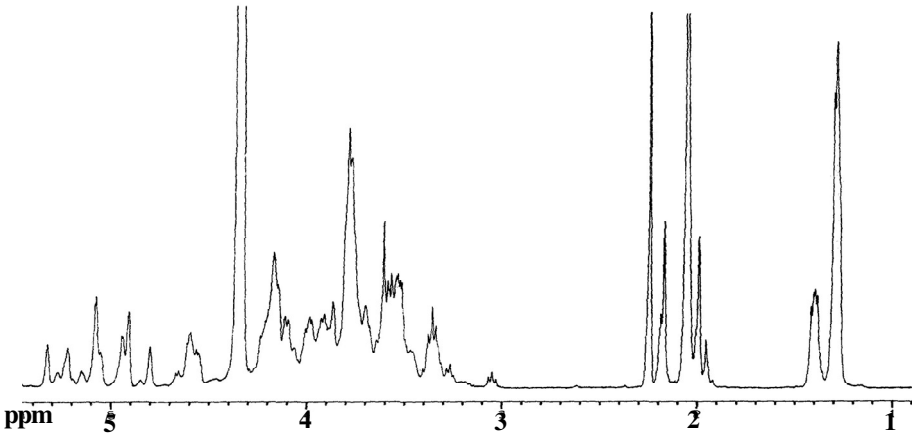

Figure 3 — ^{1}H NMR spectrum (70°C, 500 MHz, D_2O) of *O*-antigenic polysaccharide of *Vibrio cholerae* O37 with acetone as internal standard

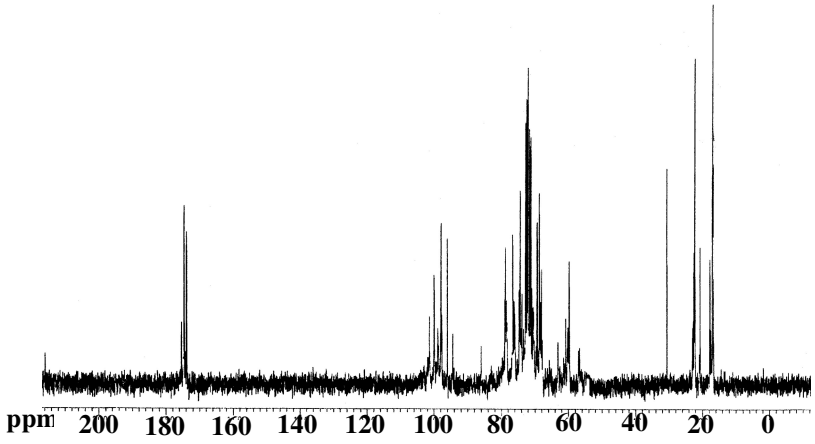

Figure 4 — ^{13}C NMR spectrum (RT, 75 MHz, D_2O) of *O*-antigenic polysaccharide of *Vibrio cholerae* O37 with acetone as internal standard

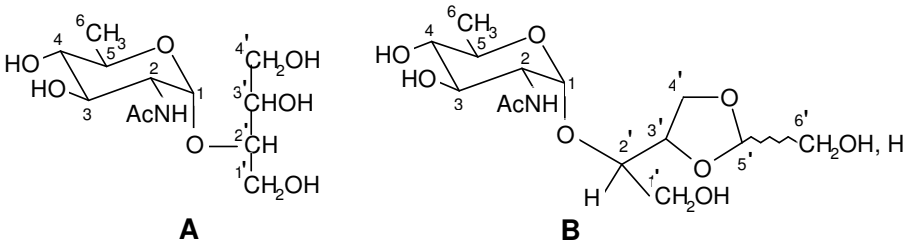

Figure 5 — Structure of the two oligosaccharides obtained from the Smith degraded OPS

tetritol was generated from the 4-substituted glucose moiety present in the main chain. The 3-linked or 3,4-linked QuinNAc were survived.

Smith degradation gave mixture of two oligosaccharides as revealed from methylation, NMR, FAB-MS and ES-MS/MS analysis. These oligosaccharides could not be separated by any means. Methylation analysis using GLC-MS showed the presence of 2-*O*-acetyl-1,3,4-tri-*O*-methyl-tetritol, 2,3,4-tri-*O*-acetyl-1-*O*-methyl tetritol and 1,5-di-*O*-acetyl 3,4-*O*-methyl-2-*N*-methylacetamido quinovositol. The presence of two tetritol units was only possible due to the presence of the two oligosaccharides A and B (**Figure 5**). In FAB-MS, two molecular ion peaks at 416(A), 444(B) and one secondary ion 230 ($QuinNAc^+$) were observed. In ES-MS/MS of the same methylated sample, MS/MS of the peaks *m/z* 416 and 444 gave oxonium ions, 187 (164+23) and 215 (192+23) respectively, which confirmed the structure of oligosaccharides (A) and (B) (**Figure 5**). The compound B was formed *via* a intramolecular rearrangement[21] of the Smith degraded product under mild acid condition. The compound (A) was a normal Smith degraded product.

The ^1H NMR spectrum of the mixture of Smith degraded products showed signals at δ1.79 (d, 3H, J = 6.0Hz, CH_3) and 2.56 (s, 3H, $COCH_3$) indicating presence of a 6-deoxy sugar and one *N*-acetyl group. The signal at δ 5.48 (d, 1H, J = 3.0Hz, H-1) and signal at 5.58 (d, 1H, J = 3.0Hz, H_B - 5′) were also observed.

The ^{13}C NMR and DEPT (90° and 135°) experiments of the same product showed signals at δ174.74 and 174.71 (>C=O of A and B), 103.14 (C_B - 5′), 98.10 and 97.85 (C_A - 1 and C_B -1), 80.13, 77.81, 76.45, 75.81, 75.84, 70.76, 70.79, 70.82, 68.53, 68.56 (C_A -3, C_A -4, C_A -5, C_A -2′, C_A -3′, C_B -3, C_B -4, C_B -5, C_B -2′, C_B -3′), 62.77, 61.94, 61.63, 61.32 (C_A-1′, C_A -4′, C_B -1′ and C_B -6′) and 66.16 (C_B -4′), 54.5 and 54.4 (C_A-2 and C_B -2), 22.34 and 22.23 ($COCH_3$ of A and B), 16.84 and 16.81 (C_A - 6 and C_B - 6).

On the basis of the experimental data, the following partial structure of the *O*-antigenic polysaccharide from the LPS of *V. cholerae* O37 is proposed. The linkages at the branched *N*-acetyl-D-quinovosamine could not be established. The OPS contains small amount of 4-*O*-methyl-*N*-acetyl-D-quinovosamine, the position of which could not be unambiguously established. It has been now established that the specificity of *V. cholerae* O1 Ogawa serotype is due to the presence of terminal 2-*O*-methyl-*N*-(3-deoxy-L -glycero-tetronyl)-D-perosamine[22]. It is likely that in case of the OPS of *V. cholerae* O37, the 4-*O*-methyl-*N*-acetyl-D-quinovosamine is present at the terminal position and needs further investigation.

Experimental Section

Cultivation of the bacteria, isolation of lipopolysaccharide and *O*-antigenic polysaccharide

Vibrio cholerae O37 was grown in liquid tryptone-yeast extract medium in a gyratory shaker at 37°C and 120 rpm for 16 hr. LPS was extracted from the dry bacterial cells by 90% hot phenol-water method[8] to give crude lipopolysaccharide. The LPS was purified by repeated ultracentrifugation at 40,000 rpm for 4 hr (three times) or by enzymes[9]. The crude LPS (100 mg) was dissolved in 0.1*N* tris-buffer (*p*H 4.2) containing 10 m*N* KCl, RNase (3.5 mg, Sigma, R-5503, 4 u/mg) and DNase (1 mg, Sigma, D-4138, 3870 u/mg) was added and kept at 38°C for 17 hr. Protease (1.5 mg, Sigma, P-6911, 3870 u/mg) was added to the solution and kept at 37°C overnight. The solution was centrifuged and the supernatant containing LPS was collected and lyophilized. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of the LPS along with other reference samples was carried out using Tris-glycine buffer (*p*H 8.3) containing 2% SDS in 15% polyacrylamide gel[14]. The bands were visualized by silver staining procedure[15]. The LPS in 2% aqueous HOAc was hydrolyzed at 100°C for 7 hr (optimal

condition). The precipitated Lipid-A was centrifuged and washed twice with water and dried. The supernatant containing the OPS was purified by gel permeation chromatography using Sephadex G-25 (fine, 100 cm × 1.6 cm) with water as eluent. The *O*-antigenic polysaccharide (OPS) was eluted after the void volume as a single peak.

Chemical analyses

For detection of fructose[11] LPS, (5 mg) was hydrolyzed with 0.5*M* H_2SO_4 (3 mL) in a sealed tube at 100°C for 2 hr. The acid was neutralized with barium carbonate and centrifuged. The clear solution was concentrated and used for the detection of fructose (2.1%) by HPLC using Ultropac TSK NH_2-60 (0.5μ) column and acetonitrile:water (70:30) as solvent system. A Waters RI detector was used for detection of sugars. An authentic sample of fructose was used for comparison. The total phosphate in LPS (0.6%) and OPS (0%) was determined by ashing procedure using ammonium molybdate in conc. H_2SO_4 and ascorbic acid. KDO was detected and estimation in LPS by thiobarbituric acid assay method[12].

The fatty acids in Lipid-A were methanolyzed (2*M* methanolic HCl, 16 hr, 100°C) and were detected as methyl esters[23] by GLC and GLC-MS. A fused silica HP-5 column (0.32 mm × 0.25 μm × 30 m) and a temperature programme 160°-2 min-3°C/min-220°-18 min was used for resolving peaks.

For sugar analysis, samples (1 mg) were hydrolyzed with 2N trifluoroacetic acid (0.5 mL) at 120°C in sealed tube for 2 hr. The liberated monosaccharides were converted to alditol acetate derivatives[10] and analyzed by GLC using an Agilent 6890 plus Gas chromatogram fitted with FID. For resolution, HP-5 (0.32 mm × 0.25 μm × 30 m) or SP-2380 (0.25 mm × 0.25 μm × 30 m) fused silica columns and temperature programme 150°C-5 min-2°C/min-200°C or 220°C-5 min-2°C/min-250°C-15 min respectively, were used. A Hewlett-Packard 3398A Chemstation was used for quantitation of peaks. For GLC-MS analysis, a Hewlett-Packard 5890 series II gas chromatograph tandemly linked to JEOL-AX500 mass spectrometer was used with electron impact ionization (EI) at 70 eV and ion source temperature at 200°C. Separation was performed on HP-1 (0.25 mm × 0.25 μm × 25 m) and HP-5 (0.25 mm × 0.32 μm × 30 m) capillary columns using temperature programme of 150°C-5 min-2°C/min-220°C. For determination of absolute configuration of the monosaccharides the OPS was heated with (+)-2-butanol (0.15 mL) in the presence of catalytic amount of anhydrous trifluroacetic acid at 100°C for 16 hr, acetylated with acetic anhydride in pyridine (100°C, 1 hr) and analyzed by GLC as mentioned above.

For methylation analysis, methylation was performed using Kerek's method[19] with dry DMSO/powdered NaOH/CH_3I in anhydrous condition. The product was purified using Sep-pack C18 cartridge. The methylated PS was first hydrolyzed with 85% formic acid at 100°C for 1.5hr and then with 2*N* trifluroacetic acid at 120°C for 2 hr, reduced and converted to partially methylated acetylated monosaccharides and analyzed by GLC and GC-MS as their partially methylated partially acetylated alditol acetates. For resolution a fused silica HP-5MS (0.25 mm × 0.25 μm × 30 m) column and a temperature programme 140°-5 min-2°C/min-200°-5 min was used.

FAB-MS was performed on methylated PS in positive mode on a JEOL JMS-AX505H mass spectrometer with glycerol:thioglycerol (1:3) mixture.

Smith degradation

A solution of the OPS (20 mg) and $NaIO_4$ (70 mg) in 0.1N sodium acetate buffer (*p*H 5.8, 5 mL) was kept in the dark at 4°C for 48 hr. Excess of periodate was reacted with ethylene glycol and the solution was dialyzed overnight against water and lyophilized. The product was reduced with excess of $NaBH_4$ at room temperature for 2 hr, acidified with acetic acid and desalted on Bio-Gel P-2 column using water as eluent. The product was hydrolyzed with 2% acetic acid (3 hr, 100°C) and the hydrolyzate was fractionated using Bio-Gel P-2 column, monitoring with an RI detector when only one peak was observed. The product was completely hydrolyzed with 2*N* trifluroacetic acid at 120°C for 2 hr and was analyzed by GLC as alditol acetate.

NMR spectroscopy

For NMR spectroscopy, sample was exchanged twice with 99.9% D_2O and dissolved in D_2O (99.9%) before use. ^1H and ^{13}C NMR spectra were recorded at room temperature or at 70°C using acetone as internal standard (2.225 for ^1H and 31.07 for ^{13}C) using Brucker 300, 500 or 600 MHz NMR machines as mentioned in appropriate places. COSY, TOCSY and HSQC spectra were run using standard pulse sequences.

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