CHEMICAL AND IMMUNOCHEMICAL INVESTIGATION OF THE LIPOPOLYSACCHARIDES OF *Vibrio alginolyticus*

E. L. Nazarenko, R.P. Gorshkova,
T. I. Burtseva, and Yu. S. Ovodov

UDC 577.144.5:579.843.1

A chemical and immunochemical study of the LPSs of the microorganisms *Vibrio alginolyticus* (strains 1385-80, 945-80, and 2076-80) has been made. A difference in the monosaccharide composition of the LPSs of the strains studied has been shown. It had been established that the LPSs of strains 945-80 and 2076-80 possess a high serological specificity, while the LPS of strain 1385-80 differ serologically from them. A suggestion is made of the role of different monosaccharides in the formation of the immunochemical determinants of the LPSs of the strains studied.

The microorganism *Vibrio alginolyticus* belongs to the group of so-called halophilic vibrios. This microorganism is facultatively pathogenic, in contrast to the species *Vibrio parahaemolyticus* related to it, which causes severe food poisoning. A paper by Japanese authors [1] gives a comparative characterization of the monosaccharide compositions of the lipopolysaccharides (LPSs) of *V. alginolyticus* and *V. parahaemolyticus* with the aim of creating a chemotaxonomic classification of these two species of microorganisms.

The present work was devoted to a chemical and immunochemical investigation of the LPSs of three strains of *V. alginolyticus* (1385-80, 945-80, and 2967080), obtained from Japan. The cultivation of the microorganisms was carried out in seawater containing peptone and nutrient broth at room temperature (pH 7.5-7.8). The suspension of the microbes was centrifuged, and the precipitate was washed with acetone and was dried. A ground powder of the microorganisms was extracted three times with chloroform to eliminate free fatty acids.

The LPSs were isolated from the washed biomass by Westphal's method of phenol–water extraction [2] and were freed from nucleic acids by three ultracentrifugations at 105,000g. The analytical figures given in Table 1 show that the LPSs of the strains studied contained a considerable amount of protein, nucleic acid, and ash impurities. The standard procedures used for isolating and purifying the LPSs probably require some improvement in this case. The high level of uronic acids in the LPSs (10-16%) shows the acidic nature of the polysaccharides that they contain.

By mild acetic acid hydrolysis, the LPSs were fractionated into lipid A and a polysaccharide hapten. No 2-keto-3-deoxyoctonic acid (KDO) (a unique monosaccharide forming the bond between lipid A and the polysaccharide in the LPS molecule of the majority of Gram-negative bacteria) was detected. It has been shown previously [3] that the LPSs of the majority of representatives of the genus *Vibrio* studied do not include KDO.

As a result of the acid hydrolysis of lipid A, followed by analysis of the aqueous phase of the hydrolysate by paper and gas–liquid chromatography, it was shown that the only monosaccharide present in the carbohydrate components of lipid A was glucosamine. The presence of N-acetyl groups in the LPSs (see Table 1) showed that the glucosamine was acetylated at the amino group.

To determine their monosaccharide compositions, the LPSs and the polysaccharide haptens obtained as the result of acetic acid hydrolysis were subjected to acid hydrolysis followed by identification of the monosaccharides by paper chromatography, high-voltage paper electrophoresis and gas–liquid chromatography of the corresponding polyol acetates [4] and aldonitrile acetates [5] using authentic samples of monosaccharides and their derivatives. In a

Pacific Ocean Institute of Bioorganic Chemistry of the Far-Eastern Scientific Center of the USSR Academy of Sciences, Vladivostok. Translated from Khimiya Priorodnykh Soedinenii, No. 5, pp. 652-657, September-October, 1987. Original article submitted January 21, 1987; revision submitted May 25, 1987.

0009-3130/87/2305-0541$12.50

TABLE 1. Analytical Figures for the LPSs of *V. alginolyticus*

Strain of the microorganism	Composition, %									
	yield of LPS*	monosaccharides	protein	nucleic acids	heptoses	uronic acids	N-AG	phosphorus	ash	lipid
1385-80	1,3	22,7	15,6	3,2	1,9	10,0	0,7	2,3	9,1	34,9
945-80	0,9	35,9	7,4	3,9	2,4	6,6	0,9	2,3	7,7	35,9
2076-80	0,5	29,0	7,8	6,0	1,9	16,8	0,7	1,9	6,6	35,3

*On the weight of the dry bacterial cells.

hydrolysate of the LPS of strain 2076-80 we identified residues of galactose, glucose, L-glycero-D-mannoheptose, glucosamine, galactosamine, and glucoronic acid. The LPS of strain 945-80 lacked galactosamine residues, and that of strain 1385-80 lacked galactose residues.

Gel chromatography of the polysaccharide haptens on Sephadexes G-50 and G-100 did not succeed in fractionating them into polysaccharides of the O-specific chains and the oligosaccharide of the core. It is possible that in the process of partial hydrolysis of the LPSs no cleaveage of the bond between them took place, or they had similar molecular weights.

The monosaccharide compositions of the LPSs and the polysaccharides are given in Table 2. In all cases, glucuronic acid was identified by paper chromatography and high-voltage paper electrophoresis in comparison with authentic samples of oronic acids. The acidic nature of the polysaccharides obtained was confirmed by ion-exchange chromatography on DEAE-cellulose. It follows from Table 2 that the LPSs of all three strains of *V. alginolyticus* that were studied differed in their monosaccharide compositions. The polysaccharides had the same monosaccharide composition as the corresponding LPSs with the exception of the glucosamine present in lipid A.

To establish the serological affinity of the LPSs of the strains of *V. alginolyticus* that were studied we used the precipitation reaction (PR) and the indirect hemagglutination reaction (IHAR) [6]. Table 3 gives the titers of the antisera to erythrocytes sensitized by the LPSs and shows cross-reactions between the O-antigenic LOSs and the antisera to the microorganisms in the precipitation reaction.

In Ouchterlony double diffusion in agar [7], the LPSs reacted specifically with the homologous antisera obtained to the corresponding strains of microorganism, giving a single precipitation band. Furthermore, the LPS of stain 2076-80 reacted with the serum obtained to strain 945-80. These results agree well with those of the IHAR (Table 3), showing a serological closeness of the LPSs of the strain 945-80 and the antiserum to strain 2076-80 can be explained by the low titer of the serum (1:256).

In the inhibition of passive hemolysis reaction [8], three immune systems of antisera to the corresponding strains of *V. alginolyticus* were used. The results, which are shown in Table 4, indicate that strains 945-80 and 2076-80 of the microorganism *V. alginolyticus* possess close serological specificities, while strain 1385-80 of the microorganism is serologically different from them, and these results are confirmed by those of the IHAR and of immunodiffusion in agar.

To reveal the groupings taking part in the formation of the immunochemical determinants, we used, in addition, the inhibition of passive hemolysis reaction with individual structural components of the LPSs as inhibitors (see Table 4). These results indicate that glucose and glucosamine residues participate in the formation of the immunochemical determinants of the LPS of strain 1385-80 of the microorganism *V. alginolyticus*, and galactose and glucuronic acid residues in the LPSs of the 945-80 and 2076-80 and the serological differences of strain 1385-80 from them, which is connected with the monosaccharide composition of the polysaccharide moieties of the LPSs.

As the result of a chemical and immunochemical reaction investigation of the LPSs of *V. alginolyticus*, differences have been found in their chemical compositions and serological specificities. On the basis of this result it may be assumed that within the limit of the given species several serogroups exist which differ in the monosaccharide compositions of their O-antigenic LPSs, and this is also confirmed by the work of Japanese authors [1].

TABLE 2. Monosaccharide Compositions of the LPSs and the Polysaccharides (NPSs), mol.%*

Preparation	Gal	Glc	*L-D*-Hep	$GlcNH_2$**	$GalNH_2$**	GlcUA***
LPS 1385-80	—	12,0	10,7	11,7	6,7	10,0
LPS 945-80	15,6	10,4	9,9	10,9	—	6,6
LPS 2076-80	11,5	9,7	7,8	6,6	4,3	16,8
PS 1385-80	—	34,6	24,2	—	4,8	11,8
PS 945-80	29,4	20,5	14,6	—	—	17,9
PS 2076-80	15,6	29,8	16,3	—	17,5	11,9

*The molar percentages were calculated on the amount of monosaccharides determined by the phenol—sulfuric method.
**The amount of amino sugars was determined with the aid of an amino acid analyzer.
***The amount of glucuronic acid was determined analytically.

TABLE 3. Antigenic Affinities of the LPSs of *V. alginolyticus*

Antiserum	1385-80		945-80		2076-80	
	IHAR	PR	IHAR	PR	IHAR	PR
1385-80	1 : 2048	+	1 : 16	—	1 : 16	—
945-80	1 : 8	—	1 : 2048	+	1 : 512	+
2076-80	1 : 64	—	1 : 128	—	1 : 256	+

EXPERIMENTAL

Descending paper chromatography was performed on Filtrak FN-3 paper in the solvent butan-1-ol—pyridine—water (6:4:3). Electrophoresis was performed on Filtrak FN-15 paper in pyridine—acetic acid (2:4:986) buffer at 90 V/cm for 1.5 h. The monosaccharides were detected with the following reagents: reducing sugars with an alkaline solution of silver nitrate; amino sugars with 0.2% ninhydrin in acetone; the KDO with 2-thiobarbituric acid (TBA).

The GLC of the monosaccharide in the form of the corresponding polyol acetates and adononitrile acetates was carried out on a Pye Unicam 104 chromatograph with a flame-ionization detector using a column (150 × 4 cm) packed with 3% of QF-1 on Gas Chrom Q (100-120 mesh) in the temperature interval of 175-225°C with the temperature programmed at the rate of 5°/min.

The total amount of monosaccharides was determined by the phenol/sulfuric acid method [9], KDO with the aid of the modified TBA reaction [10], protein by Lowry's method in [11], uronic acid as in [13], acetyl groups as described in [14], ash by a gravimetric method, and heptoses by a modified Sinilova method [15]. The quantitative analysis of the amino sugars and hydrolysates of the LPSs was performed on an LKB Biocal 3201 amino acid analyzer using a column packed with the resin Jeol LC R2. Gel chromatography was carried out in pyridine—acetate buffer (pH 5.3) using columns containing Sephadex G-50 (2.3 × 85 cm) and G-100 (1.8 × 65 cm). For ion-exchange chromatography a column with DEAE-celluose (2.4 × 58 cm) in phosphate buffer (pH 8.8) with elution by the same buffer was used, and then with increasing concentration of NaOH (0.1-0.5 N).

Microorganisms. The microorganism *V. alginolyticus* (strains 1385—80, 945-80, and 2076-80) were obtained from Dr. Sakazaki, Japan. The production of the biomass was carried out in a medium consisting of nutrient fish broth and seawater in a ratio of 1:4 containing 1% of peptone, at room temperature (pH 7.5-7.8), with aeration for two days. The suspension of microbes was separated by centrifugation at 300 rpm and the precipitate was washed twice with acetone and was dried. The ground powder of microorganisms was extracted three times with chloroform to eliminate free lipids. The yield of biomass was 1-2 g per liter of medium.

Isolation of the the LPSs. The LPSs were isolated from the chloroform-washed powder of microbes by extraction with 45% aqueous phenol, were freed from nucleic acids by three ultracentrifugations at 105,000g, and were lyophilized. The yields of LPSs are given in Table 1.

Partial Hydrolysis of the LPSs. An LPS (500 mg) was hydrolyzed with 50 ml of 1% acetic acid at 100°C for 2 h. The precipitate of lipid A was separated off by centrifugation at 5000 rpm, dissolved in chloroform, and the solution was evaporated. The yields of lipid for the LPSs of strains 1385-80, 945-80, and 2076-80 were 165, 180, and 178 mg, respectively.

TABLE 4. Results of the Inhibition of Passive Hemolysis Reaction, %

Inhibitor	1385-80 system			945-80 system			2076-80 system		
	amount of inhibitor, μg								
	1000	100	1	1000	100	1	1000	100	1
PS 1385-80	100	98	50	65	65	10	35	35	3
PS 945-80	40	40	12	90	89	40	70	70	5
PS 2076-80	50	50	15	80	80	20	85	83	15
Methyl α-D-galactopyranoside	20	0	0	60	5	0	49	0	0
Methyl α-D-glucopyranoside	35	0	0	30	0	0	30	0	0
Methyl β-D-glucopyranoside	95	85	50	12	0	0	22	0	0
Methyl 2-acetamido-2-deoxy-α-D-glucopyranoside	90	80	0	8	0	0	10	0	0
Glucuronic acid	45	10	0	85	7	0	90	0	0

The polysaccharide fractions were lyophilized, giving yields of 205, 174, and 165 mg, respectively. The fractions obtained were subjected to gel chromatography on Sephadex G-100. The polysaccharides eluted as a single peak. The yields were 183, 152, and 148 mg, respectively.

Complete Acid Hydrolysis. An LPS (10 mg) or a polysaccharide (3-5 mg) was hydrolyzed with 1 N H_2SO_4 (0.5 ml, 100°C, 3 h), and the reaction mixture was neutralized with $CaCO_3$ and was deionized with KU-2 cation-exchanger (H^+ form). The monosaccharides were identified by paper chromatography, high-voltage paper electrophoresis, and gas–liquid chromatography of the corresponding polyol acetates and aldonitrile acetates. For the quantitiative determination of aminosugars, weighed samples of the LPSs and of the polysaccharides (3-5 mg) were each hydrolyzed with 0.5 ml of 4 N HCl at 100°C for 4 h, and the hydrolysates were evaporated several times with ethanol and were analyzed on an amino acid analyzer. Lipid A (3-5 mg) was hydrolyzed with 0.5 ml of 0.5 N HCl for 3 h, the hydrolystate was washed with chloroform, the aqueous phase was evaporated to methanol, and the residue was analyzed by paper chromatography and gas–liquid chromatography of the corresponding polyol acetates.

Serological Methods. Antisera were obtained by the immunization of rabbits intravenously by seven injections of suspensions of $1 \cdot 10^9$ living cells of the corresponding strain in 1 ml every four days.

The indirect hemoagglutination [6] and inhibition of passive hemolysis [8] reactions for the sensitization of erythrocytes were performed with LPSs activated by treatment with 0.1 N NaOH at 90°C for 5 min.

The sensitization of the erythrocytes, in the passive hemolysis inhibition reaction was carried out as described previously [8].

CONCLUSIONS

A chemical and immunochemical study has been made of the O-antigenic LPSs of three strains of the microorganism *V. alginolyticus*, as a result of which differences have been revealed in their chemical compositions and serological specificities.

LITERATURE CITED

1. K. Hisatsune, A. Kiuye, and S. Kondo, Microbiol. Immonol., 25, 127 (1981).
2. O. Westphal, O. Lüderitz, and F. Bister, Z. Naturforsch., 76, 148 (1952).
3. K. Hisatsune, S. Kondo, T. Iguchi, M. Machida, S. Asou, S. Inaguma, and F. Yamamoto, Microbiol. Immunol., 26, 649 (1982).
4. J. S. Sawardeker, J. H. Sloneker, and A.R. Jeanes, Anal. Chem., 37, 1602 (1965).
5. V. M. Easterwood and B. J. L. Huff, Svensk. Paperstidn., 72, 768 (1969).
6. A. A. Hirata, S. J. Ronspies, J. S. Petruska, M. Hargie, J. R. Schenck, and W. T. Stall, J. Immunol. Methods, 13, 167 (1976).
7. Ö. Ouchterlony, Acta Pathol. Microbiol. Scand., 32, 148 (1952).
8. T. Kontrohr and K. Peterffy, J. Immunol. Methods, 13, 271 (1976).
9. M. Doubois, K. A. Gilles, J. K. Hamilton, R. A. Rebers, and F. Smith, Anal. Chem., 28, 350 (1956).

10. T. I. Burtseva, J. I. Glebko, and Yu. S. Ovodov, Anal. Biochem., 64, No. 1 (1975).
11. O. H. Lowry, N. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem., 193, 265 (1951).
12. L. Hortman, M. Cordoso Elias, and E. Esteves, Analyst, 105, 173 (1980).
13. L. P. Kosheleva and L. I. Glebko, Khim. Prir. Soedin., 500 (1977).
14. H. Trutnovsky, A. B. Sakla, and A. A. Taleb, Microchem. J., 20, 415 (1975).
15. T. I. Burtseva and Yu. S. Obodov, Khim. Prir. Soedin., 159 (1982).

STRUCTURAL INVESTIGATION OF THE LIPOPOLYSACCHARIDE OF *Yersinia enterocolitica* SEROVAR 0:8

S. V. Tomshich, R. P. Gorshkova, and Yu. S. Ovodov

UDC 579.842.23+577.114.5+543.544.45+51

The lipopolysaccharide of *Yersinia enterocolitica* serovar 0:8 (strain 161) isolated from the microbial mass by aqueous–phenol extraction contains residues of L-fucose-6-deoxy-D-gulose, D-mannose, D-galactose, D-glucose, D- and L-glycero-D-mannoheptoses, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, and 2-keto-3-deoxyoctonic acid (KDO). The polysaccharide was obtained by mild acid hydrolysis of the lipopolysaccharide followed by gel filtration on Sephadex G-50. On the basis of the results of monosaccharide analysis, methylation, Smith degradation, and partial hydrolysis the following structure is suggested for the repeating unit of the O-specific polysaccharide of the LPS of *Yersinia enterocolitica*, serovar 0:8:

$$\begin{array}{l} \rightarrow 4\text{-}D\text{-Man}_p\,1 \rightarrow 3\text{-}D\text{-Gal}_p\,1 \rightarrow 3\text{-}\alpha\text{-}D\text{-GalNAc}_p\,1 \rightarrow \\ \quad\;\; 3 \qquad\quad 2 \\ \quad\;\; \uparrow \qquad\quad \uparrow \\ \quad\;\; 1 \qquad\quad 1 \\ \text{6d-}D\text{-Gul}_p \qquad L\text{-Fuc}_p \end{array}$$

In recent years, the microorganism *Yersinia enterocolitica* has been isolated more and more frequently from patients with the symptoms of the intestinal disease called yesriniosis, and also from animals, birds, and the environment. The bulk of publications [1-3] on infections caused by *Y. enterocolitica* relates to its biochemical and serological characterization; the O-anigens of this microorganism have been little-studied structurally [4-6].

The lipopolysaccharides (LPSs) were isolated from a dry acetone powder of the microbial mass of *Y. enterocolitica*, serovar 0:8 (strain 161), by aqueous phenol extraction according to Westphal [7]. The monosaccharide composition of the LPSs was determined (after preliminary hydrolysis with 0.5 N CF_3COOH or 2 N HCl) with the aid of paper chromatography, gas–liquid chromatography (GLC), and chromato-mass spectrometry (GLC-MS) of the corresponding polyol acetates [8]. The following monosaccharides were identified: L-fucose, 6-deoxy-D-gulose, D-mannose, D-galactose, D-glucose, D- and L-glycero-D-mannoheptoses, D-glucosamine, D-galactosamine, and 2-keto-3-deoxyoctonic acid (KDO). The ratio of the monosaccharides in the LPS determined by the GLC of the corresponding polyol acetates was as follows: L-Fuc:6d-D-Gul:D-Man: D-Gal:D-Glc:Hep_{1+2} ∿ 1:1.5:1:1:2:1:3, respectively, the D- and L-glycero-D-mannoheptose residues being in a ratio of 1:3.

To isolate the O-specific polysaccharide, the LPSs were subjected to weak acid hydrolysis, and the lipid A that then precipitated was separated off by centrifugation. In gel filtration on Sephadex G-50, the polysaccharide moiety issued mainly as a single peak (without considering a glucan, amounting to 3-5% of the main fraction). Analysis with the aid of GLC of the monosaccharide composition of the polysaccharide isolated showed that it contained amino sugars (D-GlcN and D-GalN) and all the monosaccharides of the initial LPSs with the exception

Pacific Ocean Institute of Bioorganic Chemistry of the Far-Eastern Scientific Center of the USSR Academy of Sciences, Vladivostok. Translated from Khimiya Prirodnykh Soedenenii, No. 5, pp. 657-664, September-October, 1987. Original article submitted February 10, 1987.

0009-3130/87/2305-0545$12.50