ELSEVIER

Carbohydrate Research 268 (1995) 249–255

CARBOHYDRATE RESEARCH

Structural studies of O-specific polysaccharide chains of the lipopolysaccharide from *Yersinia enterocolitica* serovar O : 10

Raisa P. Gorshkova, Vladimir V. Isakov, Elena N. Kalmykova, Yury S. Ovodov *

Pacific Institute of Bioorganic Chemistry, Far East Branch of the Russian Academy of Sciences, 690022, Vladivostok, Russia

Received 25 April 1994; accepted 7 October 1994

Abstract

Lipopolysaccharide (LPS) was isolated from *Yersinia enterocolitica* serovars O : 10 and O : 10 KL and the structural pattern of O-specific sugar chains elucidated. The rhamnan and L-xylulose (L-*threo*-pent-2-ulose) as constituents of the O-specific polysaccharide were obtained by autohydrolysis of the LPS. The rhamnan was shown to be a linear, α-(1 → 3)-linked polysaccharide in the D configuration. L-Xylulose was purified using paper chromatography on a preparative scale and its structure was confirmed by ^{13}C NMR spectroscopy. Using sugar and methylation analysis and ^{13}C NMR spectroscopy of the LPS and the rhamnan, the structural features of the disaccharide repeating unit of the *Y. enterocolitica* O : 10 O-specific polysaccharide were elucidated as:

$$\begin{array}{l} \rightarrow 3)\text{-}\alpha\text{-D-Rha}p\text{-}(1 \rightarrow \\ \qquad\quad 2 \\ \qquad\quad \uparrow \\ \qquad\quad 2 \\ \beta\text{-L-Xul}f \end{array}$$

Keywords: *Yersinia enterocolitica*; Lipopolysaccharide; O-Specific polysaccharide; D-Rhamnan; L-Xylulose (L-*threo*-Pent-2-ulose)

1. Introduction

Structural studies of O-specific polysaccharides of *Y. enterocolitica* LPSs isolated from the various serovars of the microorganism have been carried out earlier [1]. The

* Corresponding author.

SSDI 0008-6215(94)00339-4

present paper describes the elucidation of structural features of the O-specific polysaccharide of LPSs from *Y. enterocolitica serovars* O:10 and O:10 KL.

2. Results and discussion

LPSs were isolated from the microorganism *Y. enterocolitica* serovars O:10 and O:10 KL, using extraction with hot aqueous phenol in accordance with Westphal's procedure [2]. The samples of LPS obtained were found to be closely related. Thus, identical mixtures of sugars were obtained on complete hydrolysis of the LPSs with 0.5 M H_2SO_4, and 0.5 M trifluoroacetic acid. The sugars were separated and identified by paper chromatography (PC) and by gas–liquid chromatography (GLC) of the corresponding alditol acetates. The following sugars were determined in the hydrolysates: rhamnose, glucose, galactose, 3-deoxy-*manno*-octulosonic acid (Kdo), D- and L-*glycero*-D-*manno*-heptose, glucosamine, and galactosamine. Because of the similarity of the LPS samples obtained, the LPS from *Y. enterocoltica* serovar O:10 was used for further investigations.

Mild acid hydrolysis of the LPS with dilute acetic acid afforded a rhamnnan and xylulose (R_{Rha} 0.98) which gave rise to xylitol and lyxitol (arabinitol) after reduction followed by identification using GLC of the corresponding alditol acetates. These data demonstrated that xylulose residues were constituents of the sugar chains of LPS. Xylulose was isolated on a preparative scale using a partial hydrolysis of LPS with 0.01 M HCl followed by preparative PC of the mixture obtained. The specific rotation, $[\alpha]^{20}_{578}$ + 30° (H_2O), of the purified xylulose indicated the L configuration of this sugar [3]. Identification of L-xylulose was confirmed by ^{13}C NMR spectroscopy. Signals in the spectrum were assigned (Table 1) in accord with reference data for an authentic sample of synthetic D-xylulose [4]. The spectral data indicated the presence of three forms in the aqueous solution of L-xylulose as follows: the keto-form, and α- and β-furanoses with the β-furanose form as the major component.

Thus, L-xylulose (L-*threo*-pent-2-ulose) is shown to be a constituent of a native microbial LPS for the first time. D-Xylulose residues have previously been discovered in the sugar chains of LPSs from *Pseudomonas diminuta* [5] and *Y. enterocolitica* O:5 and O:5.27 [1,6].

Table 1
^{13}C NMR data for L-xylulose, rhamnan, and LPS from *Y. enterocolitica* serovar O:10

Compound	Chemical shifts (ppm)					
	C-1	C-2	C-3	C-4	C-5	C-6
α-L-*threo*-Pent-2-ulofuranose	63.2	105.3	81.5	76.6	72.5	
β-L-*threo*-Pent-2-ulofuranose	64.08	103.6	77.3	75.8	70.6	
L-*threo*-Pent-2-ulose (keto form)	66.6	213.5	76.0	72.5	62.7	
Rhamnan → 3)-α-D-Rha *p*-(1 →	103.3	71.3	79.5	72.6	70.6	18.0
LPS	101.6	75.4	76.7	73.2	70.9	17.0
β-L-Xul *f*-(2 →	62.0	106.2	78.7	75.4	71.5	

Table 2
Quantitation of sugars in the hydrolysates of LPSs from *Y. enterocolitica* O:10 and O:10 KL

Serovars	Molar percent of sugars						
	Rha	Xylulose as		Glc	Gal	D-D-Hep	L-D-Hep
		Lyx-ol	Xyl-ol				
O:10	32	12	15	20	10	3	8
O:10 KL	29	11	13	22	12	3	10

A gradual hydrolysis of LPS from *Y. enterocolitica* O:10 and O:10 KL with 0.01 M HCl followed by 0.5 M trifluoroacetic acid allowed the quantitative determination of sugars by GLC of the corresponding alditol acetates (Table 2).

The polysaccharide fraction obtained on mild hydrolysis of LPS with dilute acetic acid was subjected to further fractionation by molecular-sieve chromatography on Sephadex G-50 followed by purification of the material obtained using rechromatography on Sephadex G-25. However, an impurity of the core oligosaccharide could not be completely removed. The major polysaccharide fraction obtained was shown to consist of the following sugar residues: D-rhamnose, D-galactose, D-glucose, D- and L-*glycero*-D-*manno*-heptose.

Autohydrolysis of LPS for 4 h afforded Lipid A as a precipitate and a mixture of xylulose and Kdo together with a polysaccharide fraction. The latter was subjected to further fractionation using gel filtration on Sephadex G-50. As a result, three polysaccharide fractions were isolated: a rhamnan; a fraction composed of residues of rhamnose, xylulose, galactose, glucose, and L- and D-*glycero*-D-*manno*-heptose (12:9:26:33:6:14); and a core oligosaccharide. The rhamnan obtained was subjected to complete hydrolysis to furnish D-rhamnose, $[\alpha]_{578}^{20} - 6°$ (H_2O). In addition, methyl α-rhamnopyranoside, obtained by treatment with methanolic HCl, was found to show $[\alpha]_{578}^{20} + 59°$ (H_2O), thus confirming the D configuration [7]. The α-D configuration of the rhamnosidic bonds in the rhamnan, $[\alpha]_{578}^{20} + 79°$ (H_2O), was determined using calculations of specific rotation values by Klyne's rules [8] for an α-D-rhamnan, $[\alpha]_{578}^{20} + 81.6°$ and for a β-D-rhamnan, $[\alpha]_{578}^{20} - 81.6°$.

The rhamnan was permethylated using Hakomori's procedure [9] followed by a complete methanolysis of the permethylated material obtained. Using combined GLC-MS, methyl 2,4-di-*O*-methyl-α-D-rhamnopyranoside was identified as the major component of the methanolysate, demonstrating $(1 \rightarrow 3)$ linkages between the D-rhamnopyranosyl residues in the rhamnan.

A single signal for the 6-methyl group (18.0 ppm) of the rhamnose residues was observed in the ^{13}C NMR spectrum of the rhamnan (Table 1). In addition, a signal at 103.3 ppm was assigned to the anomeric carbon, while a signal of a non-anomeric carbon linked by a glycosidic bond was observed at 79.5 ppm. A C-5 signal (70.6 ppm) is characteristic of an α anomer of rhamnopyranose. In all, six signals were observed in the ^{13}C NMR spectrum of the rhamnan (Table 1). A comparison of the chemical shifts of the rhamnan with reference data [10] demonstrated that the polysaccharide is composed of $(1 \rightarrow 3)$-linked α-D-rhamnopyranosyl residues as the regular repeating sugar unit.

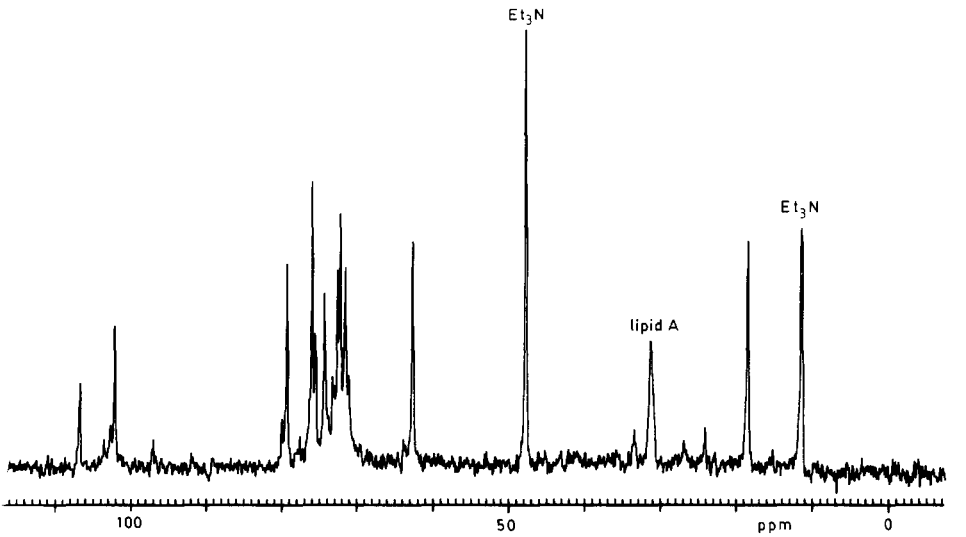

Fig. 1. ^{13}C NMR spectrum of the LPS from *Y. enterocolitica* serovar O:10.

Thus, the rhamnan appeared to represent a part of the O-specific polysaccharide of the LPS. In order to elucidate the complete structure of the repeating unit of the O-specific polysaccharide, the LPS obtained was subjected to an additional purification using gel filtration on Sepharose 4B. The purified LPS isolated was found to contain the monosaccharide residues rhamnose, xylulose, galactose, glucose, and D- and L-*glycero*-D-*manno*-heptose in the proportions 45:37:4:10:1:3. The purified LPS was permethylated [9] followed by mild hydrolysis of the resulting product. Combined GLC–MS of the methylated sugars as the corresponding alditol acetates allowed identification of 4-*O*-methylrhamnitol and two derivatives of 1,3,4-tri-*O*-methylpentitols. These data demonstrated that the O-specific polysaccharide has a backbone composed of (1 → 3)-linked α-D-rhamnopyranose residues and side chains consisting of terminal residues of L-xylulofuranose attached to the C-2 position of rhamnose residues.

The results of chemical analysis were confirmed by ^{13}C NMR spectral data (Fig. 1, Table 1).

Two signals, at 106.2 (singlet) and 101.6 ppm (doublet, $J_{C\text{-}1,H\text{-}1}$ 175.6 Hz), were observed in the region of anomeric C-atoms. In addition, the characteristic signals of the methyl group of the rhamnopyranose residue and of the hydroxymethylene group of xylulofuranose were observed at 17.8 and 62 ppm, respectively. A comparison of the ^{13}C NMR spectra of the LPS and the rhamnan demonstrated that the C-2, C-1, and C-3 signals of the rhamnopyranose residue in the spectrum of LPS are shifted by +4.1, −1.7, and −1.1 ppm relative to their positions in the spectrum of the rhamnan. At the same time, a signal at 106.2 ppm similar to the signal (105.8 ppm) of the C-2 atom of methyl β-D-*threo*-pent-2-ulofuranoside indicated the β-furanose form of the xylulose residues. Therefore, β-xylulofuranose residues are attached at C-2 of the α-rhamnopyranose residues. An assignment of ^{13}C-signals in the spectrum of the LPS is given in

Table 1, using the spectral data of rhamnan and xylulose and taking into account the effects of glycosylation [11].

The serological tests showed that LPSs obtained from serovars O:10 and O:10 KL gave rise to positive cross-reactions of precipitation with the homologous antisera and with antisera to the parent microbial cells. Precipitation tests were negative for antisera to other serovars of *Y. enterocolitica*. An inhibition with L-xylulose of the interaction of LPS with the homologous antisera was observed, indicating that the terminal residue of L-xylulose in the furanose form appears to be an immunodominant sugar of the O-specific polysaccharide of the LPS from *Y. enterocolitica* serovar O:10.

Thus, on the basis of the data obtained, the structural features of the O-specific sugar chains of the LPS from *Y. enterocolitica* serovar O:10 may be proposed as follows:

$$\begin{array}{l} \rightarrow 3)\text{-}\alpha\text{-D-Rha}p\text{-}(1 \rightarrow \\ \qquad\qquad\quad 2 \\ \qquad\qquad\quad \uparrow \\ \qquad\qquad\quad 2 \\ \qquad\quad \beta\text{-L-Xul}f \end{array}$$

This is the first description of β-L-xylulofuranose as the immunodominant sugar of an O-specific polysaccharide of an enterobacterial LPS.

3. Experimental

General.—The microorganisms *Yersinia enterocolitica* serovar O:10 (strain 500) and serovar O:10 KL (strain 551) were kindly provided by Professor H. Mollaret (Institute Pasteur, Paris, France). Microbial cells were grown and harvested, then treated with acetone as described earlier [1] to furnish a dried acetone powder of the bacterial cells.

PC, GLC, and GLC–MS were carried out as described earlier [1]. Gel chromatography was performed on a column of Sephadex G-50 (2.5 × 100 cm) or G-25 (2 × 60 cm) in aq 0.3% AcOH and on columns of Sepharose 4B (3 × 100 cm) in 0.01 M ammonia. Elution curves were obtained using a differential refractometer RIDK-101 (Czechoslovakia).

All solutions were evaporated *in vacuo* at 40°C followed by lyophilization.

Antisera to the parent microorganisms and to the LPS were obtained and serological assays were carried out as described earlier [1].

The ^{13}C NMR spectra were recorded using a Bruker WM-250 spectrometer for solutions in D_2O (internal standard MeOH, 50.15 ppm) at 60°C. Optical rotations for solutions in H_2O were determined with a Perkin–Elmer 141 M polarimeter at 20°C.

Isolation of LPS.—An acetone powder of dried microbial cells was treated with hot aqueous phenol by Westphal's procedure [2]. Nucleic acid impurities were removed by precipitation with aq 50% trichloroacetic acid (pH 2), and the aqueous solution obtained was subjected to ultracentrifugation to afford LPS (yield 1.5–2% of dry cells).

Complete hydrolysis of LPS.—The LPSs (10 mg each) obtained from *Y. enterocolitica* serovars O:10 and O:10 KL were subjected to complete hydrolysis as described earlier [1], to furnish the same mixtures of sugars identified using PC as follows: rhamnose, glucose, galactose, glucosamine, galactosamine, heptoses, and Kdo. GLC of

the corresponding alditol acetates showed the presence in the hydrolysate of the following LPS constituents: glycerol, rhamnose, glucose, galactose, D-*glycero*-D-*manno*-heptose, L-*glycero*-D-*manno*-heptose, glucosamine, and galactosamine.

Gradual acid hydrolysis.—The LPSs of *Y. enterocolitica* serovars O:10 and O:10 KL (5 mg each) were hydrolyzed with 0.01 M HCl for 1 h at 100°C (or with aq 1% AcOH for 1.5 h at 100°C), and the hydrolysates obtained were evaporated thrice with MeOH. The remaining materials were chromatographed on paper to give xylulose which was extracted with water. The residual paper was extracted with water and the mixture obtained was subjected to a treatment with 0.5 M trifluoroacetic acid followed by evaporation with MeOH. Additional amounts of xylulose as well as other sugars were obtained. The sugar fractions were combined and quantitition was carried out using GLC of the corresponding alditol acetates. The data obtained are listed in Table 2.

Autohydrolysis of LPS.—The LPS (350 mg) was dissolved in distilled water (40 mL) and the solution was refluxed for 4 h at 100°C. Lipid A precipitate was removed by a centrifugation at 17000 rpm/min for 1.5 h to yield 120 mg of the precipitate. The supernatant solution was evaporated to 5 mL and EtOH (25 mL) was added to the residual material, to yield the polysaccharide fraction (135 mg) as a precipitate and the monosaccharide mixture as an ethanolic extract (ca. 40 mg). The sugar mixture was separated by preparative PC to yield L-xylulose (15 mg) and Kdo (18 mg).

The polysaccharide fraction obtained was subjected to gel chromatography on Sephadex G-50. The following fractions were isolated: rhamnan (23 mg), $[\alpha]_{578}^{20}$ +79° (*c* 1.0, H_2O), as fraction I; the mixture of polysaccharides (57 mg) as fraction II; and the core oligosaccharide as fraction III (40 mg). Fraction II was completely hydrolyzed to furnish rhamnose, xylulose, galactose, glucose, D-*glycero*-D-*manno*-heptose, and L-*glycero*-D-*manno*-heptose in the ratios 15:9:26:33:6:14, respectively.

Mild acid hydrolysis.—The LPS (900 mg) was dissolved in aq 1% AcOH (90 mL) and the solution was refluxed for 1.5 h at 100°C. A precipitate of Lipid A was separated by a centrifugation at 15000 rpm for 30 min to yield 350 mg of the material. The supernatant solution was evaporated to 10 mL followed by precipitation with 5 vol of EtOH. A precipitate was separated to yield a polysaccharide fraction (270 mg). The residual ethanolic solution was evaporated to yield crude xylulose (200 mg). The material obtained was purified using PC on a preparative scale, to afford the purified xylulose (87 mg), $[\alpha]_{578}^{20}$ +30° (*c* 1.2, H_2O), R_{Rha} 0.98.

Complete hydrolysis of rhamnan.—Rhamnan (30 mg) was hydrolyzed with 0.5 M trifluoroacetic acid (1 mL) for 3 h at 100°C. The mixture obtained was thrice evaporated with MeOH to yield crude rhamnose which was purified by preparative PC to yield the purified rhamnose (20 mg), $[\alpha]_{578}^{20}$ − 6° (H_2O). A part of the rhamnose (10 mg) was converted into methyl α-D-rhamnopyranoside (8 mg), $[\alpha]_{578}^{20}$ +59°, by treatment with 0.1 M methanolic HCl (1 mL) for 1 h at 100°C in a sealed tube.

Methylation studies. — The LPS or rhamnan (5 mg each) was permethylated by Hakomori's procedure [9]. The permethylated LPS was hydrolyzed with 0.01 M HCl for 1 h at 100°C, the mixture obtained was thrice evaporated with MeOH, and the residual material was reduced with $NaBH_4$ followed by acetylation to yield partially methylated alditol acetates which were analyzed by GLC–MS.

The permethylated rhamnan was methanolyzed with methanolic 0.1% HCl to yield

partially methylated methyl glycosides which were analyzed by GLC–MS as the corresponding acetates.

References

[1] Yu.S. Ovodov, R.P. Gorshkova, S.V. Tomshich, N.A. Komandrova, V.A. Zubkov, E.N. Kalmykova, and V.V. Isakov, *J. Carbohydr. Chem.*, 11 (1992) 21–35.
[2] Q. Westphal and K. Jann, *Methods Carbohydr. Chem.*, 5 (1965) 83–89.
[3] T. Sticzay, C. Peciar, K. Babor, M. Fedoronko, and K. Linek, *Carbohydr. Res.*, 6 (1968) 418–422.
[4] S.J. Angyal, G.S. Bethell, D.E. Cowley, and V.A. Pickles, *Aust. J. Chem.*, 29 (1976) 1239–1247.
[5] S.G. Wilkinson, *Carbohydr. Res.*, 98 (1981) 247–252.
[6] M.B. Perry and L.L. MacLean, *Biochem. Cell. Biol.*, 65 (1987) 1–7.
[7] E. Fisher, M. Bergmann, and A. Kabe, *Ber.*, 53 (1920) 2362–2368.
[8] W. Klyne, *Biochem. J.*, 47 (1950) xli–xlii.
[9] S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
[10] Yu.A. Knirel, A.S. Shashkov, B.A. Dmitriev, N.K. Kochetkov, N.V. Kosyanchuk, and I.Ya. Zakharova, *Bioorg. Khim.*, 6 (1980) 1851–1859.
[11] G.M. Lipkind, A.S. Shashkov, Yu.A. Knirel, E.V. Vinogradov, and N.K. Kochetkov, *Carbohydr. Res.*, 175 (1988) 59–75.