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SHORT COMMUNICATION

PRESENCE AND LOCATION OF AN O-ACETYL GROUP IN O4-ANTIGEN OF *VIBRIO PARAHAEMOLYTICUS*

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A polysaccharide moiety (PS) released from O4-LPS of *Vibrio parahaemolyticus* was purified. The constituent sugars of the purified PS were glucose, galactose, arabinose, fucose, heptose, N-acetyl galactosamine and an unknown N-acetyl amino sugar. The unknown amino sugar was deduced to be a terminal deoxy amino sugar from its NMR spectrum.

PNMR spectroscopy at 200 MHz, periodate oxidation and treatment with α-L-fucosidase of the purified PS sample before and after alcoholic sodium methoxide-treatment suggested that PS contained an O-acetyl and α-linked non-reducing terminal L-fucose and that the O-acetyl group was located at the C-2 or C-4 position of the non-reducing terminal fucose.

The contribution of the O-acetyl group located in L-fucose to the O4-antigenic determinant seemed to be small, judging from immunochemical experiments.

Several chemical studies on the chemotype of the lipopolysaccharide (LPS) of *Vibrio parahaemolyticus* have been reported (Deneke and Colwell, 1973; Hisatsune et al., 1980; Ishibashi et al., 1979; Miyano et al., 1980; Shimouchi, 1972; Terada and Yokodo, 1972; Torii et al., 1969). The chemical composition of the LPS has been especially extensively analyzed, but little is still known about its structure and antigenic determinant.

We recently became interested in the structure and antigenic determinant of O4-LPS, and we attempted to use 200 MHz proton nuclear magnetic resonance spectroscopy (PNMR) to obtain information on the structure of its polysaccharide moiety that is related to its serological type. Super conducting magnet high field NMR spectroscopy should provide information on the structure of the carbohydrate, because the very high sensitivity and first-order spin coupling pattern obtained with this instrument make it possible to measure the spectra of very small amount of carbohydrate sample and simplify spectral assignments. Another merit of using this instrument for carbohydrate analysis is that a well-resolved spectrum can be recorded in D_2O solution, whereas with a usual instrument (60 MHz or 100 MHz), O-acetylation of the carbohydrate is required to obtain a well-re-

solved spectrum. As a result, there have been many reports on the high field NMR spectra of carbohydrates in D_2O. This paper reports the presence and location of an O-acetyl group in the polysaccharide moiety of O4-LPS determined by 200 MHz PNMR spectroscopy, periodate oxidation and α-L-fucosidase action.

A crude sample of O4-LPS was extracted from boiled cells of the O4-pilot strain by the phenol/water procedure of Westphal et al. (1952), and purified by the P.C.P. method (Galanos et al., 1969) to remove α-glucan. The polysaccharide moiety (PS) was separated from Lipid A (LA) by treating the purified O4-LPS with 0.1 *M* acetic acid at 100 C for 11 h. During this treatment the LA became insoluble. The mixture was then centrifuged at 10,000 *g* for 30 min, and the supernatant was lyophilized. The product (crude PS) was purified on a Bio-gel P-10 column (1.5× 140 cm) eluted with H_2O. Fractions of the effluent were tested by the phenol/sulfuric acid method (Dubois et al., 1951). As shown in Fig. 1, two distinct peaks were obtained. The material (PS) in the first peak did not precipitate with anti-O4 serum, but showed O4 specificity in the quantitative precipitation inhibition assay. The second peak mainly contained monosaccharides and was not inhibitory. PS was lyophilized for further experiments. The yield of PS from purified LPS by this procedure was 48%.

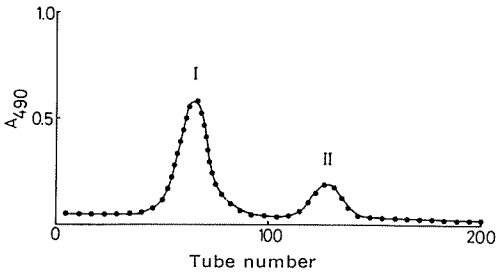

FIGURE 1. Gel chromatographic pattern of crude PS on Bio-gel P-10. Chromatographic conditions are described in the text.

PS was hydrolyzed in 2 *N* trifluoroacetic acid at 100 C for 16 h, and then its neutral sugar components were analyzed by gas-liquid chromatography (instrument, Shimazu GC-4BF; 3% ECNSS-M on Chromosorb Q; carrier gas, N_2; column temperature, 190 C) as the alditol acetate derivative and its amino sugar components were determined in an amino acid analyzer (Hitachi KLA-5). The results of sugar analysis are summerized in Table 1. Since no glucosamine was detected in PS, it was supposed to be present only in the Lipid A moiety. An unknown amino sugar was detected as a constituent sugar of PS. This amino sugar was a terminal deoxy-amino sugar judging by 200 MHz PNMR spectroscopy, but studies on its complete

TABLE 1. *Monosaccharide Compositions of Various Fractions (as molar ratios to fucose)*

	Glc	Gal	Fuc	Ara	Hep[a]	GlcN	GalN	XN[b]
O4-LPS	2.2	0.1	1.0	1.3	1.2	1.3	0.7	0.3
Lipid A	—	—	—	—	—	1.0	—	—
Crude PS	3.2	0.2	1.0	0.9	1.7	—	0.7	0.3
PS (Peak I[c])	2.7	0.7	1.0	1.1	2.4	—	2.0	1.0
Peak II[c]	1.3	—	1.0	1.1	—	—	—	—

[a] Hep was reported as L-glycero-D-mannoheptose by Hisatsune et al. (1980).

[b] XN was partially identified as a terminal deoxy amino sugar. The molar value was estimated from the color produced by galactosamine.

[c] Two peaks (I and II) in Fig. 1.

Abbreviations: Glc, glucose; Gal, galactose; Fuc, fucose; Ara, arabinose; Hep, heptose; GlcN, glucosamine; GalN, galactosamine; XN, the unknown amino sugar.

structure are still in progress.

Proton nuclear magnetic resonance spectra were recorded in a Fourier transform (FT) mode at 200 MHz (FT NMR spectrometer equipped with a superconducting magnet, Varian XL 200). The sample at a concentration of 10 mg/0.4 ml in D_2O was placed in a 5.0 mm NMR tube and measurements were made at 20 C. Sodium 2, 2-dimethyl-2-silapentane-5-sulfonate was used as a reference standard ($\delta=0.00$). The Fourier transform parameters used for recording the spectrum are indicated in each spectrum. For confirmation of the presence of an O-acetyl group in PS, alkaline methoxide treatment in methanol was undertaken to remove the O-acetyl group selectively. PS (10 mg) was suspended in 5 ml of methanol and mixed with 1 ml of 1 *N* sodium methoxide-methanol solution for 20 h at room temperature. Insoluble material was completely dissolved by adding H_2O, and then sodium ion was removed with cation exchange resin (Amberlite IR-120, H form) and the solution was lyophilized. A sample was then dissolved in D_2O (0.4 ml) and subjected to PNMR spectroscopy. The PNMR spectra of intact PS and methoxide-treated PS (MPS) are shown in Fig. 2. Because of the complex structure of PS, its full spectrum was not well resolved and was difficult to assign. However, the spectrum in the region from $\delta=1.00$ to $\delta=2.10$ was so fine and distinct, as shown in Fig. 2, that its assignment was possible. Two doublets ($\delta=1.20$, J=6.0 Hz and $\delta=1.31$, J=4.8 Hz) were assigned to the proton of C-CH_3, two singlets ($\delta=1.91$ and $\delta=2.04$) to that of N-$COCH_3$, and one singlet ($\delta=2.09$) to that of O-$COCH_3$. Furthermore, the singnal of O-$COCH_3$ was confirmed by comparing the spectrum of intact PS with that of MPS, whose O-$COCH_3$ group was selectively removed. MPS showed only one signal ($\delta=$ 2.09) less than PS. The presence of C-CH_3 and N-$COCH_3$ suggested that PS had a terminal deoxy sugar and amino sugar. One of the terminal deoxy sugars corresponds to L-fucose, and another should correspond to the unknown amino sugar mentioned above. Moreover, one of the amino sugars corresponds to D-galactosamine and another to the unknown amino sugar, judging from the constituent sugars of PS shown in Table 1. From a preliminary experiment on its PNMR spectroscopy, the unknown amino sugar was concluded to be a terminal deoxy amino sugar.

Fortunately, we could observe the signal of O-$COCH_3$, indicating the presence of an O-acetyl group in PS. It is usually difficult to detect an O-acetyl group, in an intact condition, because it is destroyed during chemical analysis. However, use of a 200 MHz PNMR spectrometer made it possible to detect the group by resolving the signal of O-$COCH_3$ from that of N-$COCH_3$ and by enhancing the sensitivity of detection.

For determination of the sugar with an

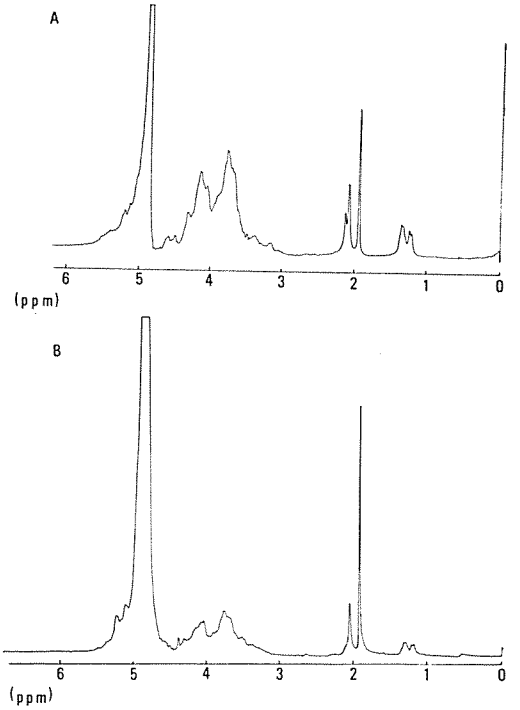

FIGURE 2. NMR spectra of PS and MPS at 200 MHz. (A) spectrum of PS and (B) spectrum of MPS (sodium methoxide treated PS) by the FT mode.

acetylated hydroxyl group, periodate oxidation and enzymatic hydrolysis were applied to PS and MPS. Periodate oxidation was carried out under the conditions reported by Torii et al. (1976). Sugar analysis of the periodate oxidation products showed that all the constituent sugars were destroyed, except galactosamine and the unknown amino sugar. This suggested that all the constituent neutral sugars involve a diol function, and that galactosamine and the unknown amino sugar residue are neither in a diol state nor locate at the non-reducing end of PS.

The actions of α-L-fucosidase on PS and MPS were examined, because L-fucose is reported usually to be located at the non-reducing end of polysaccharide and to be partially acetylated in some other LPS's. A commerical specimen of α-L-fucosidase obtained from *Charonia lampas* (Seikagaku Kogyo Ltd., Tokyo, Japan) was used without further purification. Samples were withdrawn at intervals during the enzymatic reaction of PS and MPS in phosphate buffer pH 4.0, containing 0.5 *N* NaCl at 25 C. The samples were developed by paper chromatography on Toyo Roshi No. 51A in pyridine: ethyl acetate: H_2O: acetic acid=5: 5: 3: 1 (v/v), and then the paper was sprayed with silver nitrate-sodium hydroxide reagent (Trevelyan et al., 1950). The paper chromatogram (Fig. 3) of the reaction mixture showed that L-fucose was released from MPS but not from PS, indicating that L-fucose is located at the non-reducing end and that some of hydroxyl groups of the L-fucose residue are acetylated. From the results of periodate oxidation, the hydroxyl group at position C-2 or C-4 of L-fucose was concluded to be acetylated, because the diol function of L-fucose became available only when the hydroxyl group at C-2 or C-4 was blocked.

Next, immunochemical experiments were made to determine the role of the O-acetyl group in the antigenic determinant. Anti-O4 serum was obtained from rabbits immunized with boiled cells of *V. parahaemolyticus* by the method of Barrows' (1946). A precipitation-

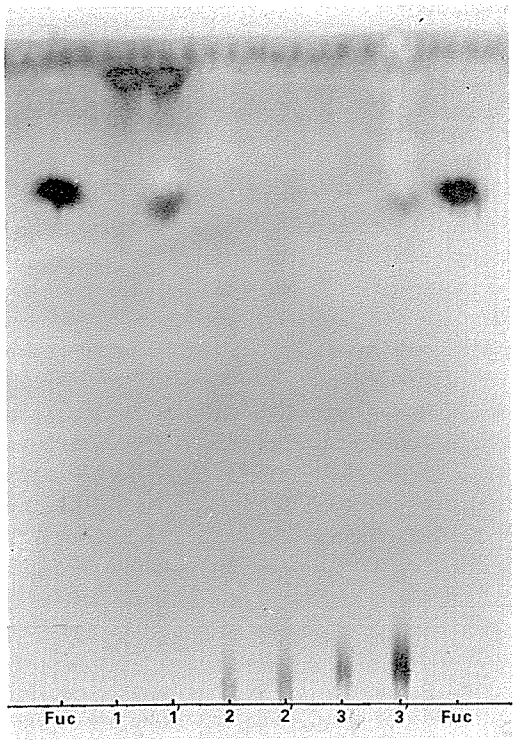

FIGURE 3. Paper chromatogram of the products of PS, MPS and phenyl α-L-fucoside on treatment with α-L-fucosidase.
1 : Phenyl α-L-fucoside
1′: Phenyl α-L-fucoside plus enzyme
2 : PS
2′: PS plus enzyme
3 : MPS
3′: MPS plus enzyme

inhibition experiment did not show a distinct difference between PS and MPS (Fig. 4), indicating, that the O-acetyl group has little function in the antigenic determinant. A previous paper (Torii and Igarashi, 1969) showed that on treatment with alkali solution the precipitin lines of all O-antigens changed, and this change was suggested to be produced by cleavage of an ester linkage and/or subsequent structural change of the antigenic molecule. From this and the present results, it seems likely that the contribution of the O-acetyl group in PS to binding to anti-O4

FIGURE 4. Inhibitions by PS and MPS of O4-anti O4 precipitation. 40 μl of O4-antiserum (85V) and 10 μg of O4-LPS were used. PS (O——O), MPS (●——●)

antibody is smaller than that of other determinant groups of PS, because no structural changes other than removal of the O-acetyl group in the antigenic molecule should occur under the present conditions, although in the previous experiment the more drastic conditions probably resulted in many structural changes in the antigenic molecule.

As concluded from the experiments described above, the O-acetyl group of L-fucose of O4-LPS was fortunately detected by 200 MHz NMR spectroscopy, the O-acetylation was shown to occur at the C-2 or C-4 position of the non-reducing terminal L-fucose, and the O-acetyl group did not seem to contribute to binding of PS with antibody. More sensitive and precise experiments are required to determine the role of the O-acetyl group in the antigenic specificity. Structural studies on the determinant group of PS are in progress.

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