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Structural Studies on the O-Specific Side-Chains of the Cell-Wall Lipopolysaccharide from *Pasteurella pseudotuberculosis* Group II B

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The structure of the O-specific side-chains of the cell-wall lipopolysaccharide from *Pasteurella pseudotuberculosis* group II B has been investigated, using methylation analysis and partial hydrolysis. As a result of these studies a structure for the oligosaccharide repeating unit (I) is proposed. Serological cross reactions between this group and *Salmonella* group B have been observed, and lipopolysaccharides from the two groups have a structural feature in common, a 3-*O*-α-abequosyl-α-D-mannose residue.

Lipopolysaccharides (LPS) from *Pasteurella pseudotuberculosis* group II, which have been investigated by Davies,[1] contain 3,6-dideoxy-D-*xylo*-hexose (abequose) residues, also found in *Salmonella* group B LPS. Sereological cross reactions between these groups have been observed.[1] The structure of the O-specific side-chains of the Salmonella group B LPS have been extensively studied,[2] but little is known on the structure of *Pasteurella* LPS. *P. pseudotuberculosis* group II has recently been divided into two subgroups, II A (5,6) and II B (5,7).[3] In the present communication structural studies on the LPS from group II B are reported.

RESULTS

The LPS was isolated from lyophilized cells of *P. pseudotuberculosis* group II B by extraction with phenol–water.[4] The high percentage of lipid in this material made it unsuitable for further study, thus an essentially lipid-free polysaccharide (PS) was prepared from it by treatment with aqueous acetic acid,[5] followed by partition between aqueous ethanol and hexane.

Acid hydrolysis of the PS and analysis of the resulting sugars, as their alditol acetates,[6] revealed the presence of abequose, fucose, mannose, galactose,

glucose, and heptose in the relative percentages 25 : 20 : 22 : trace : 7 : 26. The presence of 2-acetamido-2-deoxy-D-glucose was also demonstrated. The identities of the sugars were confirmed by MS of their alditol acetates.[7] Abequose has been unambiguously identified but it was not determined whether the other sugars belong to the D- or L-series. It is assumed that they have the configurations commonly observed, that is D-configuration for the hexoses and L-configuration for the fucose. Two heptitol acetates, with the same retention times on GLC as those derived from D-*glycero*-D-*manno*heptose (7 %) and L-*glycero*-D-*manno*heptose (19 %) were observed.

The PS was methylated by the Hakomori [8] procedure, the fully methylated PS hydrolysed and the mixture of partially methylated sugars analysed, as their alditol acetates by GLC-MS.[9] The results are summarised in Table 1, column A.

Table 1. Methyl ethers obtained from the hydrolysates of the fully methylated PS (A) and the partially hydrolysed, fully methylated PS (B).

Sugar[a]	T[b]	Mol %[c]	
		A	B
2,4-Abe	0.32	5.7	8.2
2,3,4,6-Man	1.00	8.6	19.2
2,4-Fuc	1.12	20.0	20.0
2,3,4,6,7-Hep	1.68	12.6	5.3
3,4,6-Man	1.95	7.2	16.2
3,4,6-G	1.98		
2,4,6-Man	2.09	8.4	0
4,6-Man	3.29	8.8	0
2,3,6,7-Hep	5.6	5.9	–
2,3,4,6-Hep	5.9	3.2	–
2,4,6-Hep	12	4.4	–

[a] 2,4-Abe = 2,4-di-*O*-methyl-abequose, *etc.*

[b] Retention time of the corresponding alditol acetate on the ECNSS-M column, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

[c] As considerable amounts of the volatile 2,4-di-*O*-methyl-abequose and derivatives were lost during the analysis, the molar percentages are given relative to that of 2,4-di-*O*-methyl-L-fucose, which is assumed to represent all the L-fucose in the original PS.

The identifications of the substitution patterns of the different alditols from their MS were unambiguous and require no comments. D-Glucose or D-mannose configuration could be assigned to some derivatives from their T-values. The tetra-*O*-methyl-derivatives, however, are not separated on the ECNSS-M column used. GLC, using an OV 225 column, however, demonstrated that only the D-mannose derivative was present. The 3,4,6-tri-*O*-methyl derivatives of D-glucose and D-mannose are not separated. For reasons discussed below, a considerable part of this peak should derive from the D-mannose derivative. In the absence of reference materials, we have no information on the configuration of the different heptose derivatives.

Part of the PS was subjected to a mild hydrolysis, by which essentially all the abequosidic linkages should be cleaved. The material was recovered and subjected to a methylation analysis (Table 1, column B). The 2,4-di-*O*-methyl-abequose found in this analysis certainly derives from free abequose, isolated together with the oligomeric material after the hydrolysis.

Part of the PS was subjected to a somewhat stronger hydrolysis, and a disaccharide was isolated from the hydrolysate. This on hydrolysis yielded equimolecular proportions of D-mannose and L-fucose. The hydrolysis was preceded by reduction with borodeuteride and followed by reduction with borohydride. GLC-MS of the alditol mixture, after acetylation, revealed that the L-fucitol but not the D-mannitol contained deuterium, and thus that L-fucose is the reducing sugar residue of the disaccharide. The disaccharide, $[\alpha]_{578} \approx 0°$, was indistinguishable on paper chromatography or paper electrophoresis from 3-*O*-α-D-mannopyranosyl-L-fucose, previously obtained on graded hydrolysis of fungal polysaccharides.[10]

The hydrolysis of the polysaccharide was also followed polarimetrically (Fig. 1). The initial decrease in rotation indicates that the most readily hydro-

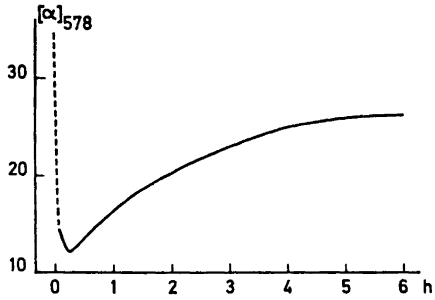


Fig. 1. Optical rotation *versus* time on acid hydrolysis of the *P. pseudotuberculosis* II B PS.

lysed linkages, the abequosidic, have the α-configuration. The subsequent increase indicates that the second most readily hydrolysed linkages, the L-fucopyranosidic, have the α-configuration.

DISCUSSION

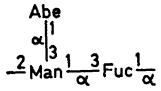
Assuming that the O-specific side chains are composed of oligosaccharide repeating units, the simplest unit, from the sugar analysis, should contain one residue each of abequose, D-mannose and L-fucose. The other sugars, D-glucose and heptose, probably derive from the core. The percentage of abequose in the sugar analysis is higher than expected under this assumption, as part of this sugar is destroyed during the hydrolysis.[9] From the methylation analysis, all three sugar residues in the repeating unit are pyranosidic.

The disappearance of 4,6-di- and 2,4,6-tri-*O*-methyl-D-mannose after the mild hydrolyses indicates that the abequose residue is attached to D-mannose in the 3 position. The low percentage of 2,3,4,6-tetra-*O*-methyl-D-mannose in the methylation analysis of the original PS could be a result of the mild hydrol-

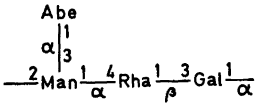
ysis during the preparation of lipid free PS from LPS. A comparable amount of 3,4,6-tri-*O*-methyl-D-mannose should be formed and most of the 3,4,6-tri-*O*-methyl-hexose should consequently consist of the D-mannose derivative. According to experience, the major part of the 3,6-dideoxy-hexapyranosidic linkages should not be cleaved during this treatment.

The determination of the anomeric nature of the abequosidic and L-fucopyranosidic linkages has already been discussed, and from the accumulated evidence, structure I is proposed for the oligosaccharide repeating unit.

The 2,4,6-tri-*O*-methyl-D-mannose must derive from the terminal repeating unit and as this sugar and 4,6-di-*O*-methyl-D-mannose are found in comparable quantities, the O-specific side-chains contain at average only two repeating units. This is also in agreement with the high percentage of the heptose, which most probably derives from the core.



I



II

The agreement between the sugar analysis and the methylation analysis is not absolute. The proportion between D-mannose and L-fucose residues is considerably higher in the latter analysis and most of the D-glucose is not accounted for. These discrepancies cannot be explained, and the proposed structure should therefore be regarded as tentative.

The presence of terminal, chain and branch residues of heptose in the core is indicated. The structural significance of these results is, however, uncertain, and studies on the core structure should preferably be performed with LPS from R-mutants.

The repeating unit of the *Salmonella* group B LPS (II) also contains a terminal α-abequosidic residue, linked to the 3-position of an α-D-mannopyranose residue, which is further substituted in the 2-position. As serological cross reactions between the two groups have been observed, a common structural element in them is not unexpected.

EXPERIMENTAL

Isolation of LPS. Pasteurella pseudotuberculosis strain 1779 of serotype IIB, obtained through the courtesy of Dr. W. Knapp, was cultivated in a medium containing 3 % NZ Amine, Type A (Sheffield), 1 % D-xylose, and the salt component contained in the medium described by Higuchi and Smith.[12] Following aeration at 26°C on a shaker (400 ml of medium per 2 l flask), the cells were harvested in the early stationary phase ($\approx 10^{10}$ cells per ml), washed in 0.033 M $KHPO_4^-$ (pH 7.0), resuspended in distilled water, and lyophilized. LPS was then prepared from the dried organisms by extraction with hot phenol : water, separation of the aqueous layer in the cold, dialysis, and differential centrifugation. Final yields of purified LPS approximated 1 % of the initial dry weight.

Preparation of lipid-free PS. A solution of LPS (47 mg) in 0.5 % aqueous acetic acid (10 ml) was kept at 100° for 1.5 h, cooled and concentrated to 2 ml. Ethanol (8 ml) was added and the solution extracted with hexane (10 ml). The PS (24 mg) was recovered from

the ethanol-water phase. Part of the material was hydrolysed with 0.25 M sulphuric acid for 12 h at 100°, neutralized and concentrated. The sugars in the hydrolysate were converted into alditol acetates and analysed by GLC-MS.

Methylation analyses. Methylation analyses of the PS (2 mg) was performed as previously described.[9]

Partial hydrolysis studies. Part of the PS (3.9 mg) was dissolved in 0.25 M sulphuric acid (1 ml), transferred into a 10 cm polarimeter tube, kept at 80°, and the optical rotation was determined at intervals. The result is given in Fig 1. The resulting hydrolysate after 5 h was neutralized, concentrated and fractionated by chromatography on Whatman 3 MM paper (ethyl acetate – acetic acid – water, 3 : 1 : 1). The main component (1 mg) which on hydrolysis yielded D-mannose and L-fucose (1 : 1), had $[\alpha]_{578}^{22} \approx 0^0$. It was indistinguishable from an authentic sample of 3-*O*-α-D-mannopyranosyl-L-fucose [10] on paper chromatography or paper electrophoresis in germanate buffer.[11]

PS (3.9 mg) was hydrolysed for 15 min under the conditions described above, the solution neutralized, concentrated and the residue dried. This material was subjected to methylation analysis.

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