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Heterogeneity in Oligosaccharides from the *O*-Polysaccharide Chain of the Lipopolysaccharide from *Salmonella Typhi* 253Ty Determined by Fast Atom Bombardment Mass Spectrometry

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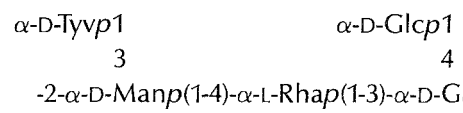
Oligosaccharides from the *O*-polysaccharide chain of the *Salmonella typhi* 253Ty lipopolysaccharide (LPS) were prepared from delipidated LPS by digestion with bacteriophage P22. The oligosaccharides were separated by gel chromatography into fractions representing monomers to polymers of the basic tetrasaccharide repeating unit. Fractions containing the dimer and the trimer were analysed by fast atom bombardment-mass spectrometry as methylated alditols. The mass spectra clearly showed heterogeneity in terms of D-glucose substitution of the D-galactose residue in the tetrasaccharide repeating unit: dimers with none, one, or two D-glucosyl branches and trimers with none, one, two, or three D-glucosyl branches were found. This suggests a random D-glucosylation of the *O*-polysaccharide chain of *S. typhi* 253Ty.

Earlier studies of the lipopolysaccharide from *Salmonella typhi* strain IS59 revealed an *O*-antigenic polysaccharide chain built of the following repeating pentasaccharide [1].



The α(1-4)-linked D-glucose branch is an addition to the basic tetrasaccharide repeating unit in *Salmonellae* of serogroups A,B,D and E [2, 3], and specifies the *O*-antigen 12_2 specificity. It is subject to what is called form variation, which means that the D-glucose substituent is either expressed or not, and consequently variable degress of substitution have been reported [4, 5]. When expressed, it has been debated whether individual

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O-polysaccharide chains are completely glucosylated and others not, or if there is a random glucosylation of the *O*-chains. We report here on the heterogeneity of glucosylation in the *O*-polysaccharide chain from *S. typhi* strain 253Ty by fast atom bombardment mass spectrometry of oligosaccharide fractions obtained by phage P22 hydrolysis of the lipopolysaccharide.

Experimental

Bacterial Strain

Salmonella typhi strain 253Ty was obtained from Prof. B.A.D. Stocker, Department of Medical Microbiology, Stanford University School of Medicine. The strain was derived from strain Ty2, by transducing from a *S. typhimurium* donor a purine gene blocked by Tn10, i.e. *purD1735*: Tn10 and by transducing an aromatic acid gene blocked by Tn10, i.e. *aroA544*: Tn10. After each step tetracycline-sensitive non-reverting, presumed deletion or deletion-inversion, mutants were isolated [6]. The strain has the *O*-antigenic formula $09,12_2$ and is Vi-antigen specific.

Preparation of the Oligosaccharides

Bacteria were grown in submerged culture, and the LPS was extracted by the hot phenol-water method from formaldehyde-killed bacteria [7, 8]. Isolated LPS was subjected to mild alkaline treatment (0.25 M NaOH, 37°C, 16 h) to cleave off the ester-linked fatty acids [9]. The partially delipidated LPS was then hydrolysed using the endorhamnosidase activity associated with bacteriophage P22 tail protein [10]. The phages were mixed in a ratio 10^9 plaque forming units/mg delipidated LPS in 5 mM ammonium carbonate buffer, pH 7.0, and incubated at 37°C within a dialysis tubing immersed in 10 times the volume of the same buffer. After 48 h the dialysis buffer was replaced and dialysis continued for an additional 48 h. The outer dialysates were pooled and concentrated to dryness under vacuum to remove the remaining ammonium carbonate.

Oligosaccharides were separated by gel chromatography on Bio-Gel P-2 and P-4 columns (Bio-Rad Labs, Richmond, CA, USA). The columns were eluted with water containing trichlorobutanol (0.05%) as antibacterial agent. The effluent was monitored with a differential refractometer R403, (Waters).

Analytical Methods

Fractions from the gel chromatography were assayed for total hexose using the phenol-sulfuric acid method [11].

Preparation of methylated NaB^2H_4-reduced oligosaccharide alditols was carried out as previously described [12]. The methylated products were isolated after fractionation using a Sep-Pak C18 cartridge [13] (Waters). A fraction of the methylated oligosaccharide was hydrolysed (2 M trifluoroacetic acid, 120°C, 2 h) and converted to partially methylated alditol acetates [14] which subsequently were separated on an SE 54 W.C.O.T. capillary column (25 m × 0.2 mm) fitted in a Hewlett-Packard 5890 gas chromatograph equipped with a Hewlett-Packard 5970 mass selective detector. All reagents used were of analytical grade. For FAB-MS (positive ion mode) a VG ZAB-SE instrument was used.

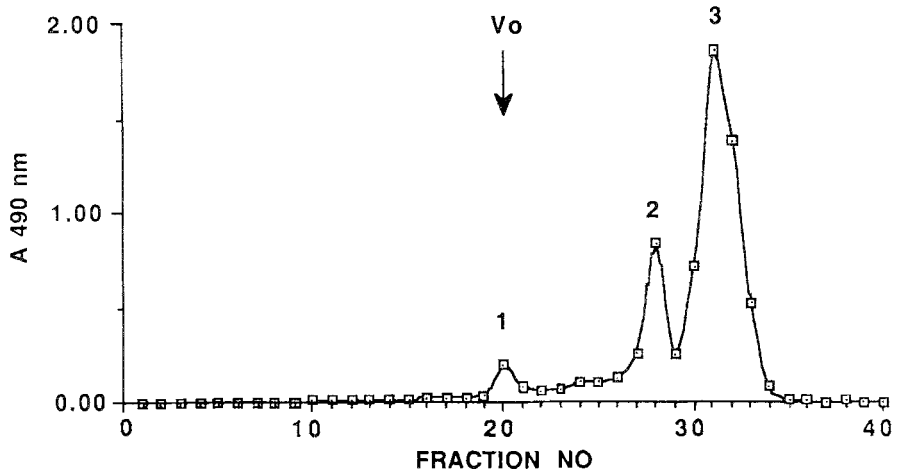

Fig. 1. Elution profile of oligosaccharides isolated from *Salmonella typhi* 253Ty on a Bio-Gel P-2 column (2.6 × 90 cm). V_o indicates the void volume. Peak 2 is the trimer, i.e. three repeating units, and Peak 3 is the dimer, i.e. two repeating units.

Samples were dissolved in thioglycerol (1-thio-2,3-propanediol) before being loaded on the stainless-steel target, which was bombarded with xenon atoms with a kinetic energy of 8 keV.

Results and Discussion

Oligosaccharides from *Salmonella typhi* 253Ty were obtained by digestion with endo-rhamnosidase from bacteriophage P22 and separated first on a Bio-Gel P-4 and then on a Bio-Gel P-2 column (Fig. 1). The material representing the dimer and the trimer of the repeating unit (peaks 2 and 3) were reduced (NaB^2H_4), methylated and analysed by FAB-MS. Part of the methylated dimer was analysed by GLC-MS after hydrolysis, reduction ($NaBH_4$) and acetylation. The major D-galactose derivatives represented 4- and 3,4-substituted residues. Traces of non-reducing terminal and 3-substituted D-galactose indicated heterogeneity in terms of glucosylation. All other derivatives were in agreement with the structure of the repeating unit. Further information about the heterogeneity of the dimer was obtained by FAB-MS of the rest of the methylated material. In order to determine ions representing the molecular weights (determined from the protonated molecular ions, $[M+1]^+$) the sample was re-run after addition of sodium iodide. This results in shifts of the $[M+1]^+$ ions to $[M+23]^+$, discriminating them from fragment ions for which no shifts are observed. The FAB spectrum obtained after addition of sodium iodide is shown in Fig. 2. The ions, m/z 1539, 1743 and 1947, could be assigned to be $[M+23]^+$ ions, showing the presence of at least three different species. The pseudomolecular ion of m/z 1947 is consistent with the molecular weight of the dimer of the pentasaccharide repeating unit containing two D-glucose branches (Fig. 3, structure A). The ions m/z 1743 and 1539 are respectively 204 and 408 mass units (m.u.) less than m/z 1947, showing species with one and two hexoses less than the dimer of the repeating unit, respectively.

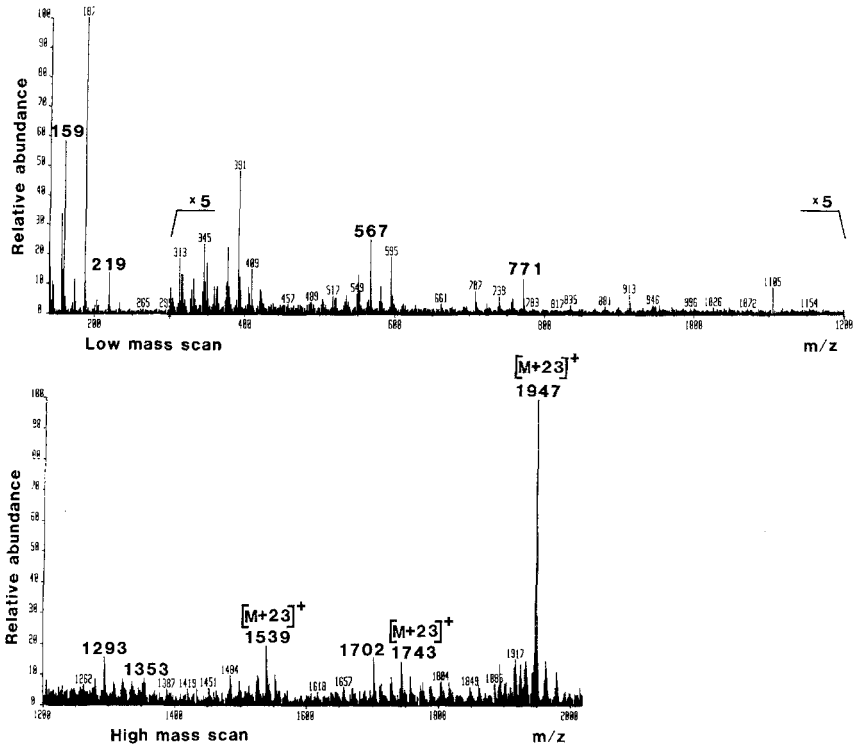

Fig. 2. FAB-MS spectrum of the dimer as its methylated oligosaccharide alditol (for structures see Fig. 3).

In order to identify the different species in the mixture, the primary sequence ions are of some help. The sequence ions from the non-reducing terminal are formed by cleavage of the glycosidic bonds as indicated in Fig. 3. By adding 204 mass units for a hexosyl residue and 174 for a deoxyhexosyl residue the monosaccharide sequence is determined. For m/z values above 1000 the contribution from the non-integral part of the atomic masses exceeds 0.5 m.u., resulting in mass peaks one unit higher than expected using integral mass numbers. Two different non-reducing terminals are observed by the ions m/z 159 and m/z 219 representing dideoxyhexosyl and hexosyl residues, respectively. Abundant primary fragments are formed by cleavage of the glycosidic bonds of disubstituted residues, determining the position of glucosylation. The set of sequence ions m/z 771, 1353 and 1702 and an $[M+23]^+$ ion of m/z 1947 are compatible with structure A (Fig. 3.).

Structure B containing two hexoses less than structure A is identified by the ions m/z 567, 1293 together with an $[M+23]^+$ ion of m/z 1539. As previously mentioned a third $[M+23]^+$ ion of m/z 1743 was present showing one hexose more than in structure B. Two structures C and D (Fig. 3) with different glucosylation patterns are possible. The position of the extra D-glucose cannot be determined from the spectrum since diagnostic

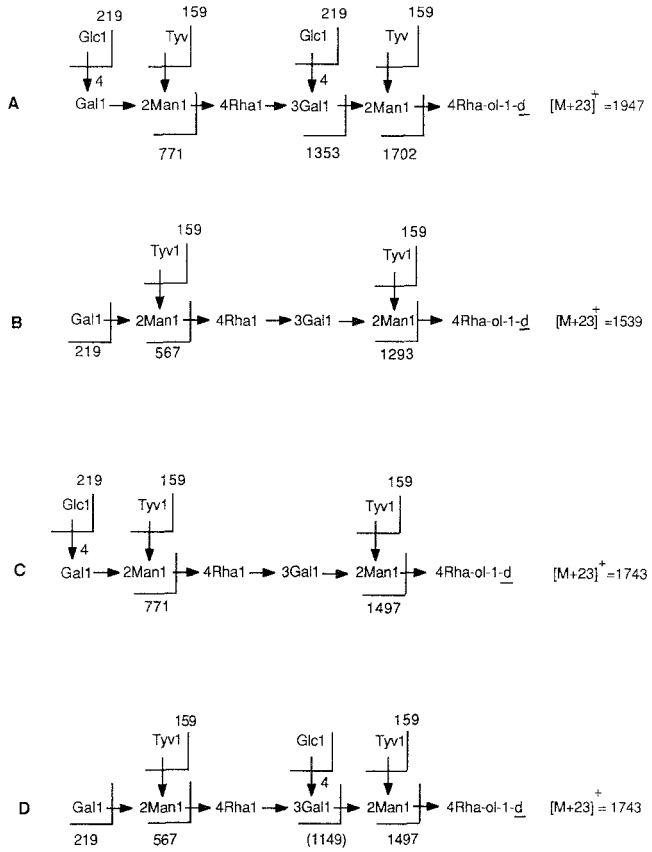

Fig. 3. Major fragments derived from the NaB^2H_4-reduced and methylated dimer.

ions which should discriminate between structures C and D are absent (m/z 1149) or found in structures A and B (m/z 567 and 771). Furthermore the relative amounts of this component might be too low to be unambiguously identified. Comparison of the pseudomolecular ions gives a rough estimate of the relative amounts of the different species in the mixture. Structures C and D are each less than 10% of the total mixture and structure B is about 10-15%. Analysis of the trimer as methylated alditol by FAB-MS gave a spectrum shown in Fig. 4. Four $[M+23]^+$ ions, m/z 2266, 2470, 2674 and 2878, all 204 m.u. apart, showed that the trimer contained four species containing zero, one, two and three D-glucose residues, respectively. The FAB-MS spectrum gave, however, no further information concerning the positions of glucosylation of the trimer. Phage 22 cleaves the α-L-Rha*p*(1-3)-α-D-Gal*p*- linkage irrespective of whether the D-galactosyl is D-glucosylated or not [15]. We do not know, however, which linkage is preferably hydrolysed. The results show that in *S. typhi* strain 253Ty most of the tetrasaccharide repeating units are glucosylated. This glucosylation, which takes place after the repeating unit polymerization [4], is nevertheless incomplete and random. The unambigous demonstration of the structures C and D in the dimer, and of structures with one or two

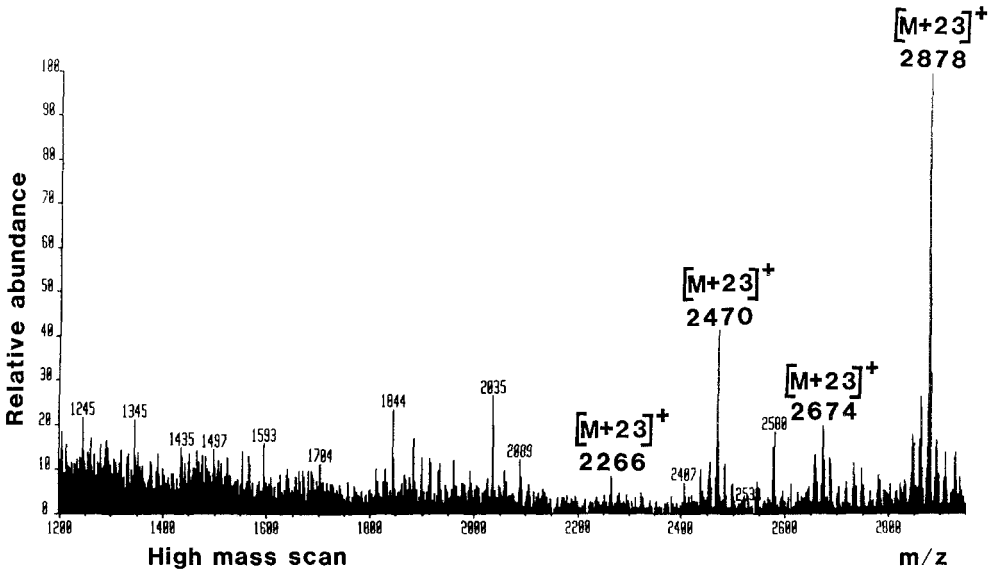

Fig. 4. FAB-MS spectrum of the trimer as its methylated oligosaccharide alditol. Only the high mass range is shown.

D-glucose residues in the trimer, conclusively shows that some of the *O*-polysaccharide chains of *S. typhi* 253Ty are randomly glucosylated. The use of phage P22 hydrolysed *O*-chains generating di- and trimers prevented an analysis of whether some of the chains are completely D-glucosylated, and others not. The studies of Nghiem on *S. zuerich O*-polysaccharide, which expressed *O*-antigen 1 specificity is a consequence of α(1-6)-linked D-glucose branches [16]. The use of polymerized concanavalin A for selective removal of *O*-chains with D-glucose branches was of considerable help in this study, although intra *O*-chain heterogeneity could not be demonstrated. Our results presented above clearly show that FAB-MS is useful for analysis of heterogeneity in oligosaccharides with known structures.

Acknowledgements

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