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Rapid characterization of mucin oligosaccharides from rat small intestine with gas chromatography-mass spectrometry

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Mucin glycopeptides were isolated from rat small intestinal mucosa after reduction/alkylation, trypsin digestion and gel chromatography. The oligosaccharides were released by using alkaline-$NaBH_4$, separated into neutral and acidic species and permethylated. The derivatized mixtures were analysed with fast atom bombardment mass spectrometry and gas chromatography-mass spectrometry using thin film columns. Permethylated neutral oligosaccharides with up to seven sugars could be chromatographed and detected with mass spectrometry. The complex mixture revealed was partly due to the linkage GalNAc being substituted at both position 3 and 6. The approach will be very useful when analysing small amounts of mucins and mucin fragments.

Oligosaccharide; Mucin glycopeptide; Gas chromatography; Mass spectrometry; (Small intestine, Rat)

1. INTRODUCTION

The mucus gel of the gastrointestinal tract has its major role in lubrication and protection of the underlaying epithelial surface [1]. Mucins, the gel-forming component of mucus, are very large glycoproteins with a high (70–90%) content of carbohydrate [2]. Several investigations have been devoted to oligosaccharides obtained from different mucin preparations [3,4]. The most successful approach has been to purify the released oligosaccharides with HPLC and analyse the components with proton-NMR spectroscopy [5]. This approach is time consuming and large amounts of material are needed. As part of a long-term project aimed at comparing the oligosaccharides between different mucins we have now improved the conditions for gas chromatography-mass spectrometry (GC/MS) to allow quick analysis of small amounts of relatively large oligosaccharides. Furthermore, by selecting inbred strains of rats as the source of mucins a detailed comparison with the structurally related glycan part of glycosphingolipids will be possible [6].

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Abbreviations: Hex (or H), hexose; HexNAc (or HN), *N*-acetylhexosamine; Fuc (or F), fucose; HexNAcol (or HNol), *N*-acetylhexosaminitol

2. MATERIALS AND METHODS

2.1. *Preparation of glycopeptides*

Inbred Sprague-Dawley white rats were used [7]. The mucosa of the small intestine was scraped off and kept frozen until subjected to reduction with dithiothreitol (10 mM) in 6 M guanidine hydrochloride/5 mM Na_2EDTA/10 mM Tris-HCl, pH 8.0. The sample was alkylated with iodoacetamide, dialysed against 0.05 M sodium phosphate (pH 7.0, including 5 M $MgCl_2$), digested with DNase I (Sigma, type IV) and RNase A (Sigma, type I-A), dialysed against 0.1 M Tris-

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HCl (pH 8.0), treated with trypsin (Sigma, type XIII), dialysed against 4 M guanidine hydrochloride, and chromatographed on a column of Sephacryl S-200. The glycopeptides eluted in the void volume were chromatographed on Sephacryl S-500, where two major peaks were resolved. The first one (2.5 mg) was treated with 2.5 ml of 0.05 M KOH containing 1 M $NaBH_4$ for 45 h at 45°C [8]. The released oligosaccharides were passed through an AG50W × 8 column (BioRad), separated into neutral and acidic components on DE-23 (Whatman), and permethylated by a simple procedure [9].

2.2. *Gas chromatography–mass spectrometry*

GC/MS was performed on a Carlo Erba 4160 gas chromatograph directly interfaced to a VG ZAB-HF mass spectrometer equipped with a VG-11 250 data system.

2.2.1. Chromatographic conditions

A fused silica column (10 m × 0.25 mm i.d.) coated with 0.05 μm of cross-linked SE-54 was used. The column was D4-deactivated at 380°C, statically coated and cross-linked according to Blomberg et al. [10]. Helium was used as carrier gas with a head pressure of 0.15 bar and a linear gas velocity of 0.9 m/s at 87°C. Injections (1 μl of 200 μl) were made at 87°C in the on-column mode with ethyl acetate as solvent. The temperature program was increased 10°C/min to 370°C and held there for 5 min.

2.2.2. Mass spectrometric conditions

Interface and source temperature, 365°C; electron energy, 70 eV; trap current, 500 μA; acceleration voltage, 8 kV; mass range scanned, *m/z* 1600–160; scan speed, 2 s/decade; total cycle time, 3.5 s and resolution, 1400.

3. RESULTS

The high molecular mass mucin glycopeptides corresponding to the oligosaccharide 'clusters' of the macromolecules were prepared from the rat small intestine and separated into two major components with gel chromatography. The larger and more abundant one was subjected to alkaline-$NaBH_4$ treatment and the released oligosaccharides separated into neutral and acidic species. Fast atom bombardment mass spectrometry in the positive mode of the permethylated sample showed the presence of a complex mixture of neutral oligosaccharides with the largest M + 1 ion at *m/z* 1554 corresponding to a heptasaccharide. This mixture was analysed with GC/MS and the total ion chromatogram is shown in fig.1 together with interpretations.

Detailed characterization of the peaks requires interpretation of their mass spectra. Fig.2 shows the mass spectrum (scan 390) of a heptasaccharide alditol recorded at 370°C with a molecular mass of 1553.8 Da. A Fuc–Hex–HexNAc sequence is deduced from the oxonium ions at *m/z* 189, *m/z* 361 (393 – 32), *m/z* 638, and 606 (638 – 32). The fragment ion at *m/z* 1409 is M – 145 which was found characteristic for oligosaccharide alditols with a terminal Fuc–Hex sequence. Ions containing the alditol moiety at *m/z* 1145 and 1349 confirm the symmetrical nature of this compound. Mass spectra of partially methylated alditol acetates show that there is a preferred cleavage between two adjacent methoxylated carbon atoms [11]. This is also observed in mass spectra of C-3 and C-6 substituted permethylated oligosaccharide alditols [12,13] where cleavage between the methoxylated carbon atoms C-4 and C-5 of the alditol chain with charge retention at the carbon C-4 containing moiety gives a strong fragment at *m/z* 841 indicating substitution of sugar residues at C-3. Inductive cleavage at the C-3 oxygen with charge retention on the alditol moiety gives a fragment indicative of substitution at C-6 (*m/z* 899).

The mass spectra of two isomeric tetrasaccharide alditols with molecular masses of 1175.6 Da are shown in fig.3a and b (scans 257 and 292). The Fuc–Hex–HexNAc sequence is recognized in both spectra at *m/z* 189, *m/z* 393, 361 (393 – 32) and *m/z* 638, 606 (638 – 32) as well as the terminal HexNAc at *m/z* 260 and 228 (260 – 32). The strong fragment ion at *m/z* 521 in fig.3a is due to inductive cleavage at the C-3 linkage position giving the [HexNAcol–6-*O*-HexNAc]$^+$ fragment. The corresponding ion in fig.3b is at *m/z* 899 representing the [HexNAcol–6-*O*-HexNAc–Hex–Fuc]$^+$ fragment. Ions indicating substitution at C-3 are found at *m/z* 841 and at *m/z* 463, respectively. Fragment ions at *m/z* 1031 (M – 145) show the presence of a terminal Fuc–Hex sequence.

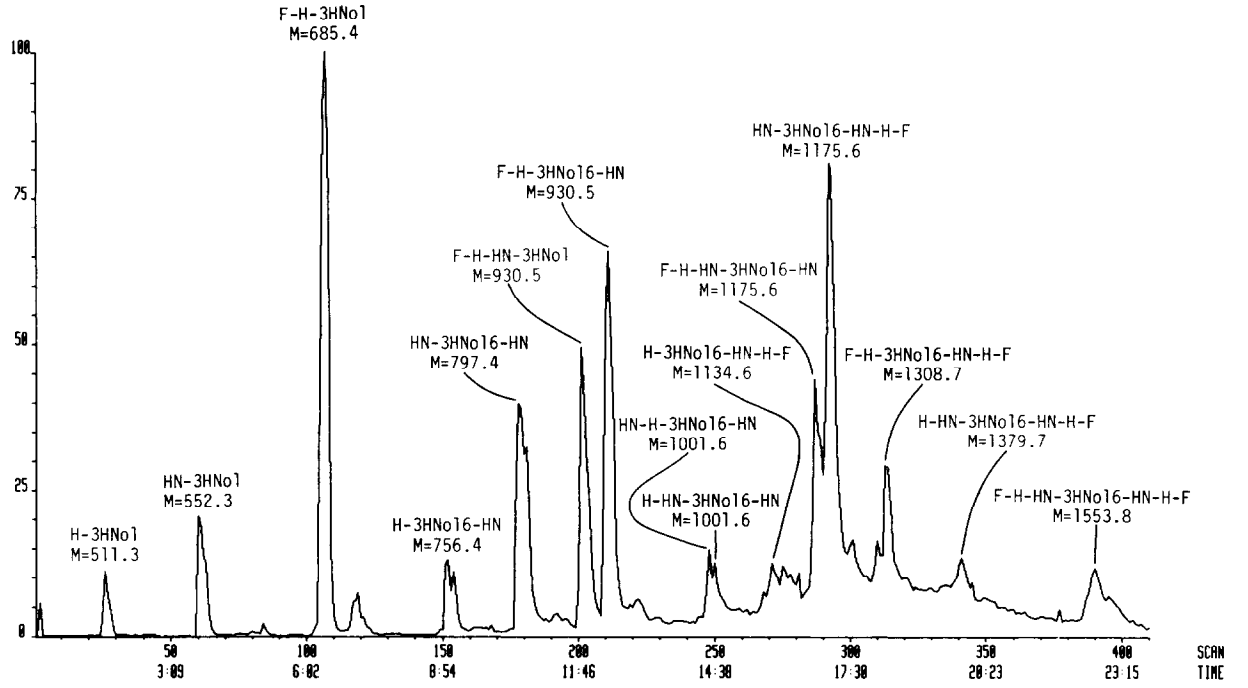

Fig.1. Total ion chromatogram of permethylated neutral oligosaccharide alditols released from rat mucin glycopeptides together with the interpreted components. The peak for *N*-acetylhexosaminitol is not shown. Abbreviations: H, hexose; HN, *N*-acetylhexosamine; HNol, *N*-acetylhexosaminitol; F, fucose. Conditions for the analysis are given in section 2.

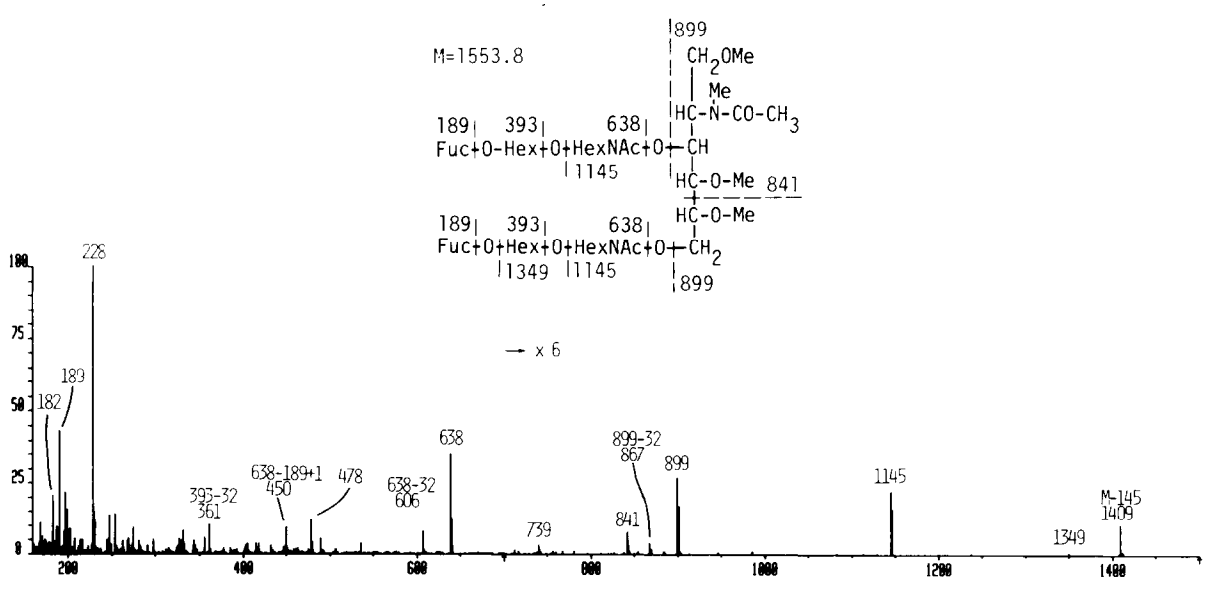

Fig.2. Mass spectrum of the component with scan number 390 of fig.1 and simplified interpretation formula.

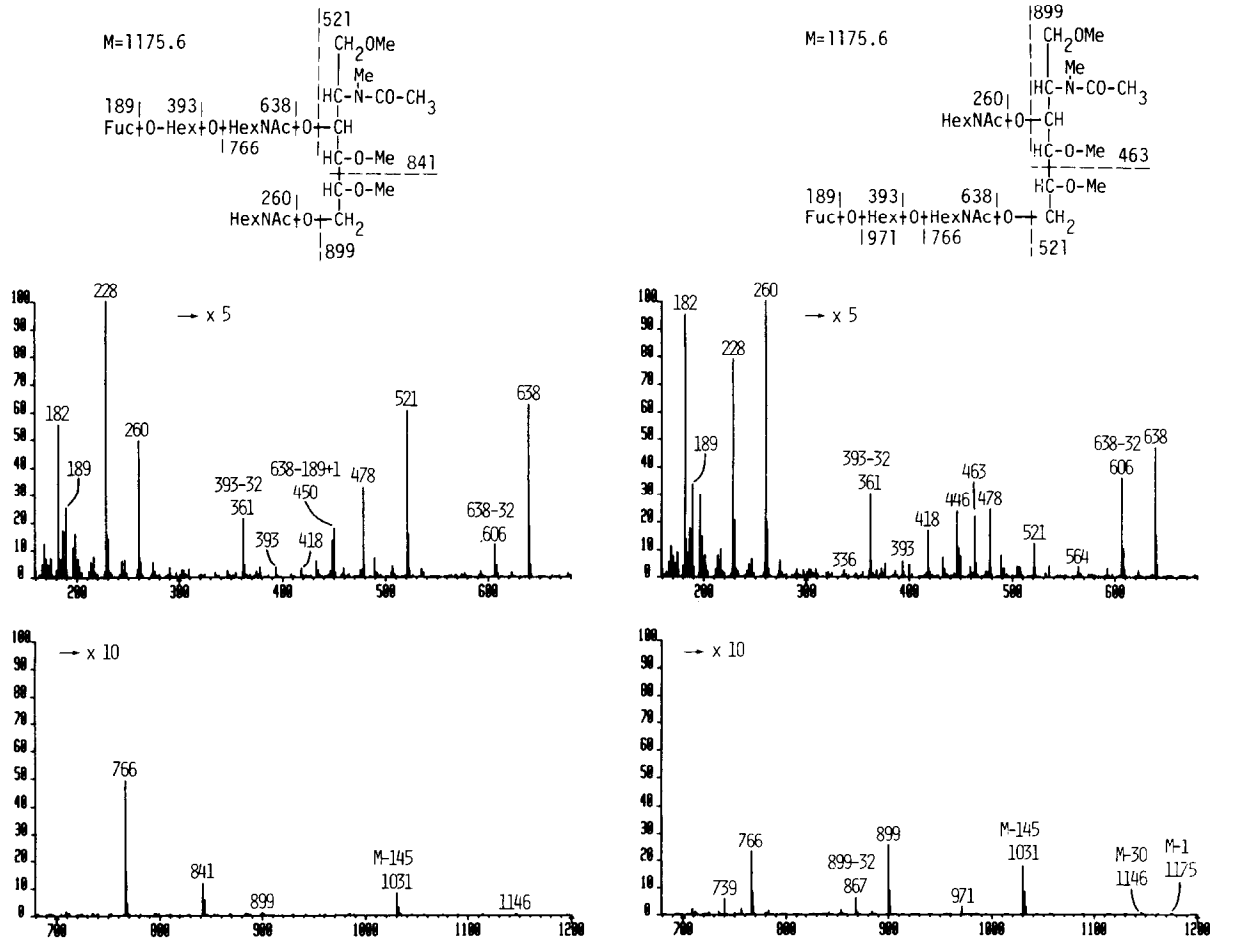

Fig.3. Mass spectra and interpretation formulas of the isomeric components with scan numbers 287 and 292 of fig.1, respectively.

The ion at *m/z* 182 is indicative of a terminal GlcNAc or an internal −4-GlcNAc, i.e. a type 2 chain [14]. All peaks containing terminal or internal HexNAc showed a relatively large *m/z* 182 (see for example figs 2 and 3) suggesting that these HexNAc units are *N*-acetylglucosamines and that the internal HexNAc on the branches is linked to C-4.

When the permethylated acidic fraction was analysed as described above, four peaks containing sialic acid were found, two with NeuAc and two with NeuGc. The carbohydrate sequence of these were NeuAc(NeuGc)–Hex–3-HexNAcol and NeuAc(NeuGc)–Hex–3-HexNAcol–6-HexNAc.

4. DISCUSSION

By improving the conditions for gas chromatography it has been possible to chromatograph larger permethylated oligosaccharides with up to 3 *N*-acetylhexosamines. In the present investigation we have identified 16 different neutral and 4 sialic acid containing oligosaccharides. The largest oligosaccharide found by fast atom bombardment of the permethylated oligosaccharide mixture contains 7 sugars and this component was also detected by GC/MS.

The oligosaccharides identified were based on certain core structures. The C-3 of the alditol could

be substituted by either a Hex or a HexNAc and further elongated. The C-6 of the alditol could be substituted with HexNAc when the C-3 had been substituted. Hexose was never bound directly to the C-6 of the alditol. The two branches could be elongated with up to 3 sugars giving a blood group H-type sequence. The four different ways the alditols were substituted are identical to the four major 'core' saccharides present in mucin oligosaccharides [15]. Some of the oligosaccharides are terminated by H-type sequences, a feature shared with the glycosphingolipids from the same rat strain [6]. However, the mucin oligosaccharides probably have internal type 2 chains (Galβ1→4GlcNAc) in contrast to the glycosphingolipids that are dominated by type 1 chains (Galβ1→3GlcNAc).

The GC/MS analyses are rapid, informative, the peaks well resolved and only small amounts of starting glycopeptides are needed. The approach can be used for neutral and sialic acid containing oligosaccharides, but the sulphate-containing ones must be analysed separately. The upper limit for the analysis is presently 7 sugars with 3 *N*-acetylhexosamines, but this can be improved by replacing the *N*-acetyl groups with *N*-trifluoroacetyl groups [16,17] and by using new high temperature stationary phases. The approach will be particularly useful in studies aimed at correlating structural changes in mucin oligosaccharides with alterations in mucin composition and properties in health and disease.

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