Glycosylation differences between pig gastric mucin populations: a comparative study of the neutral oligosaccharides using mass spectrometry

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Five mucin populations were isolated from the cardiac region, corpus and antrum of pig gastric mucosa. The released neutral oligosaccharides were permethylated and analysed using high-temperature gas chromatography–mass spectrometry (GC–MS) as well as matrix-assisted laser-desorption mass spectrometry (MALDI-MS). Thirty different oligosaccharides with up to six monosaccharide residues were characterized using both techniques, but the presence of an additional 49 structures was suggested on the basis of their molecular mass by MALDI-MS. Oligosaccharides based on core-1 (Galβ1-3GalNAcα1-) and core-2 [Galβ1-3(GlcNAcβ1-6)GalNAcα1-] structures were widely distributed, whereas core-3 structures (GlcNAcβ1-3GalNAcα1-) were present only in mucins from the cardiac region and corpus, and core-4 structures [GlcNAcβ1-3(GlcNAcβ1-6)GalNAcα1-] were present exclusively in mucins from the cardiac region. Furthermore the oligosaccharides from one of the mucins from the corpus were significantly longer than those from the other populations. The results illustrate vast structural diversity, but the relative abundances show only a few dominating structures, suggesting that many oligosaccharides may be quite rare in pig gastric mucins. Well-defined mucin populations with distinctly different glycosylation can thus be identified in pig stomach, suggesting that glycosylation of the large secreted mucins from this tissue is not a random event.

INTRODUCTION

In the stomach, the mucus gel protects the gastric epithelial cells from the harsh conditions in the gastric lumen [1]. The mucus glycoproteins (mucins), providing the polymer matrix of mucus, are extended linear oligomeric macromolecules that typically contain of the order of 80 % carbohydrate [2]. Gastric mucins originate from several cell types in both the surface epithelium and the glands. In normal human gastric mucosa, the *MUC5AC* and *MUC6* genes are expressed in the surface epithelium and glands respectively, and the corresponding mucins have been detected using antibodies directed against the mucin apoproteins [3–5]. Histochemical studies suggest that the various mucin-secreting cells produce structurally different mucins [6,7], and in the preceding paper we have shown that pig gastric mucins from different regions can be fractionated into several distinctly different populations [8].

The carbohydrates found in mucins occur mainly as O-linked oligosaccharides attached via GalNAc to serine and threonine residues in the apoprotein. Together with proline, these amino acids are enriched in one or several domains where they often appear as tandemly repeated motifs [9]. Glycosylation, in particular the initial addition of the GalNAc, favours an extended conformation of these domains [10]. In the oligomeric mucins, cysteine residues in the regions flanking these highly glycosylated domains are involved in joining the mucin subunits together, as suggested by structural and biosynthetic studies as well as by similarities to the von Willebrand factor [2,9,11].

Mucin oligosaccharides from pig gastric mucosa have been studied extensively [12–15], but so far little is known about the distribution of the various glycans in the different regions of the stomach. However, analysis of the monosaccharide composition indicates that the glycosylation of pig gastric mucins can vary between the different regions [16]. In the preceding paper it was shown that the degree of sulphation varies between the regions as well as between mucin populations from the same part of the stomach [8]. In the present investigation, the neutral oligosaccharides of mucins from the cardiac region, corpus and antrum were analysed in more detail by gas chromatography–mass spectrometry (GC–MS) and matrix-assisted laser-desorption mass spectrometry (MALDI-MS). The results show extensive differences in the glycosylation of the various mucin populations both within and between different regions.

MATERIALS AND METHODS

Materials

Methyl iodide was from Fluka, DEAE-Sephadex A-25 from Pharmacia, AG50W-X8 from Bio-Rad and solid NaOH from EKA (Bohus, Sweden). Water used for permethylation was double-glass-distilled and kept over chloroform. All other chemicals were from Merck.

Isolation of mucins

Mucins from the surface mucosa of the cardiac region, corpus and antrum of pig stomach were isolated using isopycnic density-gradient centrifugation as described [8]. Mucins from corpus and antrum were separated into high-density (corpus-HD and antrum-HD) and low-density (corpus-LD and antrum-LD) populations whereas those from the cardiac region were pooled as a single fraction (cardia).

Analysis of blood-group activities

Aliquots (100 μl) of fractions from density-gradient centrifugations were coated on to microtitre plates [poly(vinyl chloride); Falcon 3913] overnight in a humidified chamber at room temperature. Plates were washed with 0.15 M NaCl/5 mM sodium phosphate buffer, pH 7.4, containing 0.05 % Tween 20 (washing buffer) and then blocked for 1 h with washing buffer

Abbreviations used: MALDI, matrix-assisted laser-desorption/ionization; HexNAcol, *N*-acetylhexosaminitol; DHB, 2,5-dihydroxybenzoic acid.

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containing 1 % BSA. The wells were then incubated with an anti-(blood group A) monoclonal antibody (clone A003; Biotest Ag) diluted 1:100 in blocking solution. Reactivity was detected using an alkaline phosphatase-conjugated anti-mouse antibody with *p*-nitrophenyl phosphate (2 mg/ml in 5 mM $MgCl_2$/1.0 M diethanolamine buffer, pH 9.8) as a substrate. A_{405} was recorded after 1 h. The lectin-binding assay was performed in essentially the same way. After blocking, wells were incubated with biotinylated *Ulex europaeus I* lectin (Vector, Burlingame, CA, U.S.A.) at a concentration of 2 μg/ml in buffer consisting of 0.15 M NaCl/0.1 mM $CaCl_2$/50 mM Tris/HCl buffer, pH 7.6, containing 0.05 % Tween 20 and 1 % BSA. Bound lectin was detected with alkaline phosphatase-conjugated streptavidin.

Preparation of oligosaccharides

Oligosaccharides were isolated from a single aliquot of each pooled mucin population as described previously [8]. In short, the glycans were released by alkaline borohydride treatment in 0.05 M KOH/1.0 M $NaBH_4$ for 45 h at 45 °C and the neutral oligosaccharides were isolated using ion-exchange chromatography on a DEAE-Sephadex A-25 column. Aliquots of the glycans were permethylated with methyl iodide in a slurry of NaOH in DMSO as described [17], with some modifications [18]. Neutral permethylated oligosaccharides from corpus-HD were purified on a column (7 mm × 500 mm) of Sephadex LH-20 eluted with methanol (0.25 ml/min). Fractions (0.5 ml) were collected and 5–20 combined. The same approach was used to separate short oligosaccharides (less than seven residues, fractions 15–20) from longer ones (more than seven residues, fractions 5–14) that could not be analysed by GC–MS and that interfered with the GC separation of the short ones.

Time-of-flight MALDI-MS of permethylated oligosaccharides

Spectra were recorded on a VG TofSpec-E (VG-Analytical, Manchester, UK) in the positive reflection mode at 22.5 kV accelerating voltage. The mass spectrometer was equipped with a nitrogen laser operating at 337 nm, and external calibration was achieved with a mixture of permethylated polyfructanes using the isotopic average mass. Mass spectra were obtained by accumulating 40–150 laser shots, and the native spectra smoothed to give a resolution of approx. 500 (full width at half maximum). Samples for MALDI-MS were prepared by adding 2 μl of permethylated oligosaccharides diluted in ethyl acetate (100–400 μl) to 6 μl of 17 mM LiCl/33 mM 2,5-dihydroxybenzoic acid (DHB) in water/acetonitrile (1:1, v/v). Then 1 μl of this mixture was applied to the stainless-steel target and allowed to dry at room temperature before the target was introduced into the spectrometer. The accuracy of the mass measurement was better than 0.05 %.

GC–MS of permethylated oligosaccharides

The permethylated oligosaccharides were analysed by high-temperature GC–MS essentially as described previously [19]. Samples were diluted in ethyl acetate (100–400 μl) and 0.5–1 μl was injected on a fused-silica capillary column (11 m × 0.25 mm internal diameter) coated with 0.03 μm of cross-linked PS264. On-column injections were performed at 70 °C (1 min) followed by a temperature program (10 °C/min up to 390 °C). GC–MS was performed on a Hewlett–Packard 5890 series II gas chromatograph coupled to a JEOL SX-102 mass spectrometer (JEOL, Tokyo, Japan). The gas chromatograph was operated in constant-flow mode with He as carrier gas (linear gas velocity 75 cm/s at 70 °C). The interface was kept at 380 °C and the ion source at 370 °C. The mass spectrometer was scanned in the range m/z 100–1600 with a total cycle time of 1.8 s, electron energy 70 eV, trap current 300 μA, acceleration voltage + 10 kV, resolution 1400 ($m/\Delta m$, 10 % valley) and pressure in the ion source region 5×10^{-4} Pa.

RESULTS

Mucins originating from the surface mucosa of different regions (cardia, corpus and antrum) of pig stomach were fractionated by isopycnic density-gradient centrifugation [8], and pooled as indicated in Figure 1. The five fractions are referred to as cardia, corpus-HD, corpus-LD, antrum-HD and antrum-LD. Each mucin fraction was subjected to reductive β-elimination, and the released oligosaccharides were separated on a DEAE-Sephadex column into neutral (not retarded), sialylated (esterifiable, eluted after addition of DMSO/MeI) and sulphated (eluted with high-salt buffer) oligosaccharides. The monosaccharide composition [8] revealed that the acidity of the various mucin fractions,

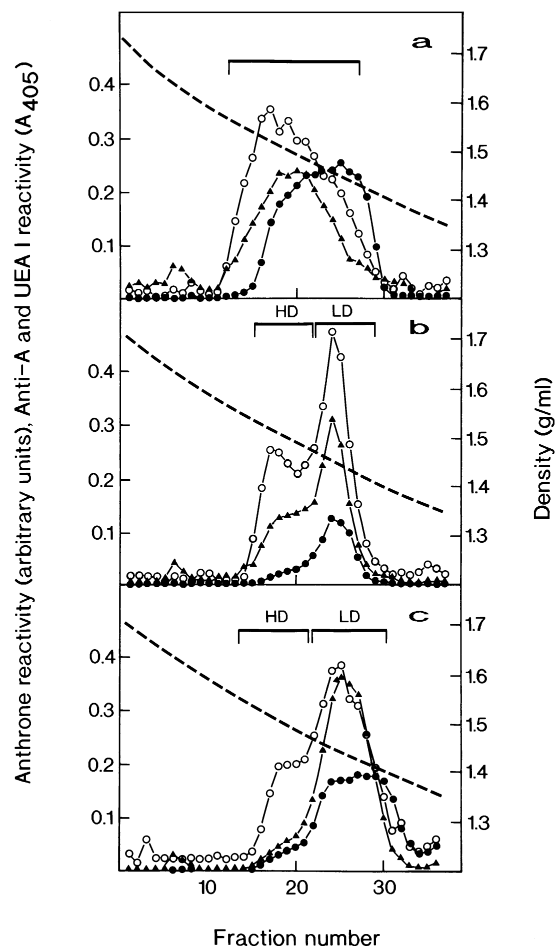

Figure 1 Analysis of blood-group antigens in pig gastric mucins

Mucins from the surface epithelium of (**a**) the cardiac region, (**b**) corpus and (**c**) antrum were subjected to isopycnic density-gradient centrifugation in CsCl/0.5 M guanidinium chloride as described in the preceding paper [8]. Fractions were collected from the bottom of the tubes and analysed for density (––––) and hexose/fucose by the anthrone method (▲) as described previously [8] as well as for blood-group-A reactivity (●) and reactivity with the *U. europaeus I* lectin (UEA I) (○) as described in the Materials and methods section. The bars indicate material corresponding to the cardiac mucins, corpus-HD, corpus-LD, antrum-HD and antrum-LD.

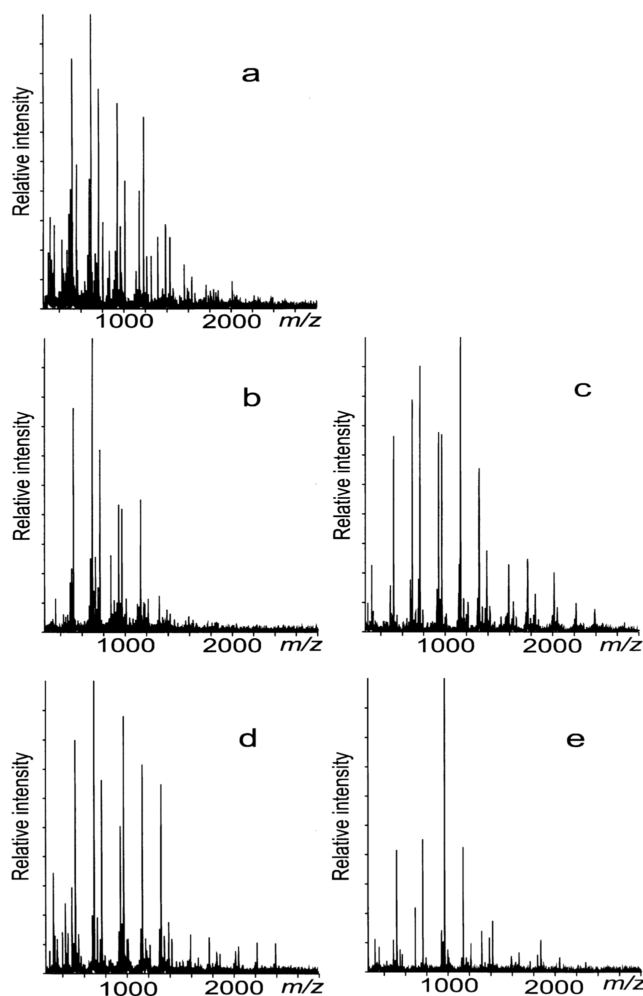

Figure 2 Mass spectra of the total permethylated neutral mucin oligosaccharides from pig gastric mucin populations obtained by MALDI-MS

The non-fractionated neutral oligosaccharides from (**a**) cardia, (**b**) corpus-HD, (**c**) corpus-LD, (**d**) antrum-HD and (**e**) antrum-LD were analysed by MALDI-MS. The oligosaccharides were detected as their $[M+\mathrm{Li}]^+$ ions after co-crystallization of the sample with LiCl in the DHB matrix. Most of the peaks represent molecular ions of one or several isomeric oligosaccharides as shown in Tables 1 and 2.

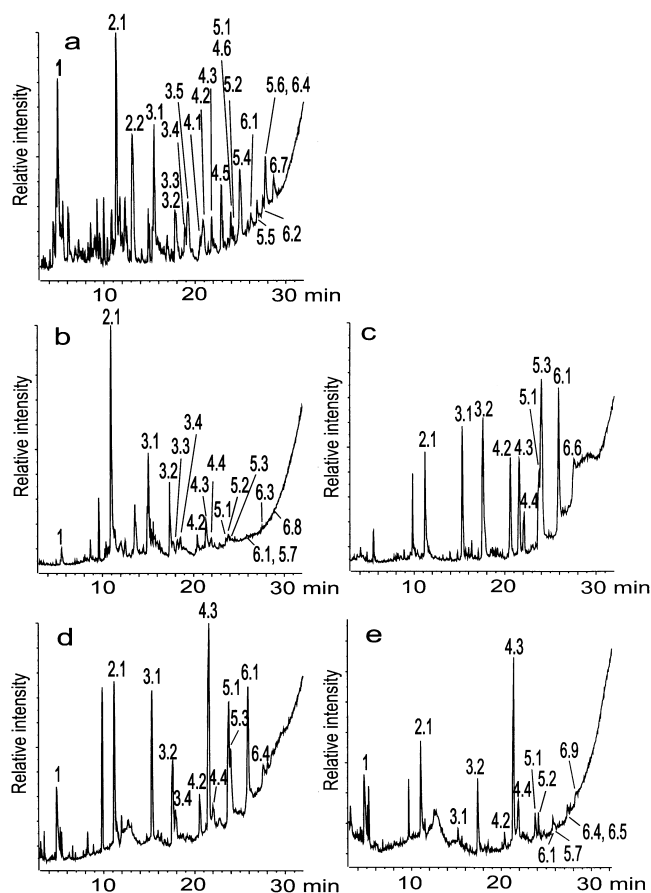

Figure 3 Total ion chromatogram from GC–MS of permethylated neutral mucin oligosaccharides from pig gastric mucin populations

Permethylated neutral oligosaccharides from (**a**) cardia, (**b**) corpus-HD, (**c**) corpus-LD, (**d**) antrum-HD and (**e**) antrum-LD were analysed by GC–MS. The analyses were performed on either the total fraction of neutral oligosaccharides (**a**, **b**, **d**, **e**) or on a fraction of 'small' oligosaccharides isolated by gel chromatography as described in the Materials and methods section (**c**). The numbers in the total ion chromatograms represent individual oligosaccharide structures as shown in Table 1.

assessed as the elution profile obtained by anion-exchange chromatography, correlated well with the degree of sulphation, and only small amounts of sialylated species were found in all mucins. The cardia, corpus-HD and antrum-HD mucins contained approx. 40 % of neutral oligosaccharides and 60 % of the sulphated ones. In contrast, the low-density components (corpus-LD and antrum-LD) consisted of 80 % neutral oligosaccharides and only about 20 % sulphated glycans. The mean number of monosaccharide residues per oligosaccharide chain was estimated to be 9–14 for the neutral ones with the highest number of corpus-LD. The monosaccharides detected were GalNAcol, GalNAc, GlcNAc, Fuc, Gal and Man. At least a portion of the unreduced GalNAc is explained by the presence of blood-group-A antigens [GalNAcα1-3(Fucα1-2)Galβ1-] detected by the reactivity with an anti-(blood group A) monoclonal antibody (Figure 1). A higher blood group A activity was found in the cardiac region, compared with the other regions, when related to the reactivity with the *U. europaeus I* lectin (recognizing fucose and blood group H) and the amount of hexose/fucose. Most of the blood-group-A reactivity of the mucins from corpus and antrum was found in the low-density fractions. In contrast, the reactivity with the *U. europaeus I* lectin was also pronounced in the high-density fractions from these regions.

The Man detected in some of the oligosaccharide fractions on monosaccharide composition analysis suggested the presence of N-linked glycans in the mucins. Since N-linked oligosaccharides are not usually released by the alkaline conditions used here to release the O-linked ones, the detected Man probably represented unreleased N-linked oligosaccharides of glycopeptides remaining after the cation-exchange step.

The total neutral oligosaccharide fractions from the five mucin populations were permethylated and analysed by MALDI-MS (Figure 2). Most of the ions could be related to $[\mathrm{M}+\mathrm{Li}]^+$ ions of the theoretical permethylated oligosaccharide structures, and no obvious signs of remaining glycopeptides with N-linked glycans were seen. The spectra illustrate a remarkable structural diversity; however, the true heterogeneity is likely to be even larger since a substantial number of the molecular ions may represent oligosaccharide species with isomeric structures.

The neutral oligosaccharides from the various mucin popula-

Table 1 Proposed structures of neutral oligosaccharides from pig gastric mucins obtained by GC–MS and MALDI-MS

Neutral oligosaccharides isolated from pig gastric mucins were permethylated and analysed using high-temperature GC–MS and MALDI-MS. The abundance of each oligosaccharide is expressed relative to the amount of the disaccharide Gal → 3GalNAcol by dividing the peak area in the GC profile by the cognate molecular mass. The sequences are based on mass spectra from GC–MS of permethylated released oligosaccharides, and the compositions were verified by the presence of $[M+\mathrm{Li}]^+$ ions detected by MALDI-MS. In accordance with the monosaccharide composition, hexoses were assumed to be Gal, deoxyhexoses to be Fuc linked 1 → 2 as in the H-determinant, *N*-acetylhexosaminitols to be GalNAcol, and *N*-acetylhexosamines to be GlcNAc except for the terminal GalNAc in the A-determinants. The fragment ion at *m/z* 182 in the mass spectra from GC–MS, arising from either a terminal GlcNAc or a GlcNAc substituted at C-4, was used to indicate the presence of type-2 chains when possible. Bold letters are used for the C-6 branch of the GalNAcol residue.

No.	Sequences of neutral oligosaccharides	Relative abundance (mol)				
		Cardia	Corpus-HD	Corpus-LD	Antrum-HD	Antrum-LD
1	GalNAcol	3.2	< 0.1		1.1	2.2
2.1	Gal → 3GalNAcol	1.0	1.0	1.0	1.0	1.0
2.2	GlcNAc → 3GalNAcol	0.8				
3.1	Fuc → 2Gal → 3GalNAcol	0.7	0.3	1.0	0.8	0.1
3.2	Gal → 3(**GlcNAc → 6**)GalNAcol	0.3	0.2	1.4	0.5	0.6
3.3	Gal → 4GlcNAc → 3GalNAcol	< 0.1	0.1			
3.4	GlcNAc → Gal → 3GalNAcol	0.2	< 0.1		0.2	
3.5	GlcNAc → 3(**GlcNAc → 6**)GalNAcol	0.5				
4.1	Fuc → 2Gal → 4GlcNAc → 3GalNAcol	0.1				
4.2	Fuc → 2Gal → 3(**GlcNAc → 6**)GalNAcol	0.3	< 0.1	0.7	0.2	0.1
4.3	Gal → 3(**Gal → 4GlcNAc → 6**)GalNAcol	0.1	0.1	0.6	0.8	1.5
4.4	Gal → 4GlcNAc → Gal → 3GalNAcol		< 0.1	0.2	< 0.1	0.4
4.5	GlcNAc → 3(**Gal → GlcNAc → 6**)GalNAcol	0.2				
4.6	GlcNAc → Gal → GlcNAc → 3GalNAcol	< 0.1				
5.1	Gal → 3(**Fuc → 2Gal → 4GlcNAc → 6**)GalNAcol	< 0.1	< 0.1	0.6	0.5	< 0.1
5.2	Fuc → 2Gal → 3(**Gal → 4GlcNAc → 6**)GalNAcol	< 0.1	< 0.1			< 0.1
5.3	Fuc → 2Gal → 4GlcNAc → Gal → 3GalNAcol		< 0.1	1.0	0.3	
5.4	GalNAc → (Fuc → 2)Gal → 4GlcNAc → 3GalNAcol	0.3				
5.5	Gal → 3(**GlcNAc → Gal → GlcNAc → 6**)GalNAcol	< 0.1				
5.6	GlcNAc → 3(**GlcNAc → Gal → GlcNAc → 6**)GalNAcol	< 0.1				
5.7	Gal → GlcNAc → (GlcNAc →)Gal → 3HexAcol		< 0.1			< 0.1
6.1	Fuc → 2Gal → 3(**Fuc → 2Gal → 4GlcNAc → 6**)GalNAcol	< 0.1	< 0.1	0.5	0.4	< 0.1
6.2.	Fuc → 2Gal → 4(3)GlcNAc → 3(**Gal → 4(3)GlcNAc → 6**)GalNAcol	< 0.1				
6.3	Fuc → 2Gal → GlcNAc → 3(**GlcNAc → Gal → 6**)GalNAcol		< 0.1			
6.4	Gal → 3(**GalNAc → (Fuc → 2)Gal → 4GlcNAc → 6**)GalNAcol	< 0.1			< 0.1	< 0.1
6.5	Fuc → 2Gal → GlcNAc → (GlcNAc →)Gal → 3GalNAcol					< 0.1
6.6	GalNAc → (Fuc → 2)Gal → 4GlcNAc → Gal → 3GalNAcol			< 0.1		
6.7	GlcNAc → 3(**GalNAc → (Fuc → 2)Gal → GlcNAc → 6**)GalNAcol	< 0.1				
6.8	Fuc → 2Gal → 4(3)GlcNAc → Gal → 4(3)GlcNAc → 3GalNAcol		< 0.1			
6.9	Gal → 4(3)GlcNAc → (Gal → 4(3)GlcNAc →)Gal → 3GalNAcol					< 0.1

tions were also analysed by high-temperature GC–MS. Since structures larger than approximately seven residues are not volatile enough for this technique, and larger oligosaccharides often interfere with the chromatography of the smaller ones, oligosaccharides from corpus-LD were first fractionated into large and small oligosaccharides by gel chromatography. Total ion chromatograms from GC–MS of the five different populations are shown in Figure 3, and the combined results from the analyses of the mass spectra obtained by GC–MS and MALDI-MS are presented in Table 1. Additional structures implicated only by the molecular ions detected by MALDI-MS are listed in Table 2.

Neutral oligosaccharides from cardia

The total ion chromatogram obtained by GC–MS of the mixture of the permethylated oligosaccharides showed a very complex pattern (Figure 3a). The mass spectra were dominated by B_i oxonium ions, inductive Z_i ions from cleavages of glycosidic bonds and α-cleavages between C-4 and C-5 of the permethylated GalNAcol residues [20]. In Figure 4, the features of these fragmentations are exemplified by the mass spectrum of a blood-group-A-type hexasaccharide (designated 6.7) having an $M-145$ at m/z 1276. The series of B-type fragment ions at m/z 260, m/z 189, m/z 638 and m/z 883 were used to deduce the C-6 branch of the GalNAcol to be a tetrasaccharide moiety terminating in an A-determinant, and the C-3 branch was deduced to be a single HexNAc confirmed by the fragment ion m/z 463 from the α-cleavage between C-4 and C-5. The presence of the Z-type fragment ions at m/z 521, m/z 766 and m/z 1145 corroborates the proposed structure.

A total of 22 structures were identified in cardia by GC–MS ranging from a single GalNAcol to hexasaccharides (Table 1). The glycans contain core-1 (Galβ1-3GalNAcα1-), core-2 [Galβ1-3(GlcNAcβ1-6)GalNAcα1-], core-3 (GlcNAcβ1-3GalNAcα1-) as well as core-4 [GlcNAcβ1-3(GlcNAcβ1-6)GalNAcα1-] sequences, and the mucins from the cardiac region were the only ones proved to contain core-4 structures. The large number of different oligosaccharide structures in these mucins is probably a consequence of the ability to add GlcNAc in a β1-linkage to both the C-3 and C-6 branches of the proximal GalNAc. This substitution opens up additional glycosylation pathways apparently not available in the mucins from the other regions of pig stomach (see below).

Table 2 Proposed composition of neutral oligosaccharides from pig gastric mucin obtained by MALDI-MS

Permethylated neutral oligosaccharides were co-crystallized with a mixture of DHB and lithium chloride on the MALDI target. Ions were assigned (X) as their lithium adducts when present within 0.05% of the calculated value.

No.	Composition of oligosaccharides	$[M+\text{Li}]^+$(Da)*	Cardia	Corpus-HD	Corpus-LD	Antrum-HD	Antrum-LD
6.10	Hex$_3$, HexNAc$_2$, HexNAcol	1416.5		X	X	X	X†
6.11	Hex$_2$, HexNAc$_3$, HexNAcol	1458.6	X	X			
7.1	Fuc$_3$, Hex$_2$, HexNAc, HexNAcol	1490.6		X			
7.2	Fuc$_2$, Hex$_3$, HexNAc, HexNAcol	1520.7	X		X		
7.3	Fuc$_2$, Hex$_2$, HexNAc$_2$, HexNAcol	1561.7	X	X			
7.4	Fuc, Hex$_3$, HexNAc$_2$, HexNAcol	1591.7	X	X	X	X	X
7.5	Fuc, Hex$_2$, HexNAc$_3$, HexNAcol	1632.8	X		X		X
7.6	Hex$_3$, HexNAc$_3$, HexNAcol	1662.8	X			X	X
8.1	Fuc$_2$, Hex$_3$, HexNAc$_2$, HexNAcol	1765.9	X		X	X	X
8.2	Fuc$_2$, Hex$_2$, HexNAc$_3$, HexNAcol	1807.0	X		X		
8.3	Fuc, Hex$_3$, HexNAc$_3$, HexNAcol	1837.0	X		X	X	X
8.4	Hex$_4$, HexNAc$_3$, HexNAcol	1867.0				X	X
9.1	Fuc$_2$, Hex$_3$, HexNAc$_3$, HexNAcol	2011.2	X		X	X	
9.2	Fuc, Hex$_4$, HexNAc$_3$, HexNAcol	2041.1			X	X	X
9.3	Fuc, Hex$_3$, HexNAc$_4$, HexNAcol	2082.3			X		
10.1	Fuc$_2$, Hex$_4$, HexNAc$_3$, HexNAcol	2215.4	X		X	X	X
10.2	Fuc$_2$, Hex$_3$, HexNAc$_4$, HexNAcol	2256.5	X		X		
10.3	Fuc, Hex$_4$, HexNAc$_4$, HexNAcol	2286.5			X		X
11.1	Fuc$_3$, Hex$_4$, HexNAc$_3$, HexNAcol	2389.6	X		X	X	
11.2	Fuc$_2$, Hex$_4$, HexNAc$_4$, HexNAcol	2460.7			X		
11.3	Fuc, Hex$_5$, HexNAc$_4$, HexNAcol	2490.7			X		
11.4	Fuc$_2$, Hex$_3$, HexNAc$_5$, HexNAcol	2501.8			X		
12.1	Fuc$_4$, Hex$_4$, HexNAc$_3$, HexNAcol	2563.8			X		
12.2	Fuc$_3$, Hex$_4$, HexNAc$_4$, hexNAcol	2634.9			X		
12.3	Fuc$_2$, Hex$_5$, HexNAc$_4$, HexNAcol	2664.9			X		
12.4	Fuc$_2$, Hex$_4$, HexNAc$_5$, HexNAcol	2704.4			X		
12.5	Fuc, Hex$_5$, HexNAc$_5$, HexNAcol	2734.4			X		
13.1	Fuc$_3$, Hex$_5$, HexNAc$_4$, HexNAcol	2839.1			X		
13.2	Fuc$_3$, Hex$_4$, HexNAc$_5$, HexNAcol	2880.2			X		
13.3	Fuc$_2$, Hex$_5$, HexNAc$_5$, HexNAcol	2910.2			X		
14.1	Fuc$_4$, Hex$_5$, HexNAc$_4$, HexNAcol	3013.3			X		
14.2	Fuc$_3$, Hex$_5$, HexNAc$_5$, HexNAcol	3084.4			X		
14.3	Fuc$_2$, Hex$_6$, HexNAc$_5$, HexNAcol	3114.4			X		
14.4	Fuc$_2$, Hex$_5$, HexNAc$_6$, HexNAcol	3155.5			X		
14.5	Fuc, Hex$_6$, HexNAc$_6$, HexNAcol	3185.5			X		
15.1	Fuc$_3$, Hex$_6$, HexNAc$_5$, HexNAcol	3288.6			X		
15.2	Fuc$_3$, Hex$_5$, HexNAc$_6$, HexNAcol	3329.7			X		
15.3	Fuc$_2$, Hex$_6$, HexNAc$_6$, HexNAcol	3359.7			X		
16.1	Fuc$_4$, Hex$_6$, HexNAc$_5$, HexNAcol	3460.8			X		
16.2	Fuc$_3$, Hex$_6$, HexNAc$_6$, HexNAcol	3533.9			X		
16.3	Fuc$_2$, Hex$_7$, HexNAc$_6$, HexNAcol	3563.9			X		
16.4	Fuc$_2$, Hex$_6$, HexNAc$_7$, HexNAcol	3605.0			X		
16.5	Fuc, Hex$_7$, HexNAc$_7$, HexNAcol	3635.0			X		
17.1	Fuc$_4$, Hex$_6$, HexNAc$_6$, HexNAcol	3708.1			X		
17.2	Fuc$_3$, Hex$_7$, HexNAc$_6$, HexNAcol	3738.1			X		
17.3	Fuc$_3$, Hex$_6$, HexNAc$_7$, HexNAcol	3779.2			X		
17.4	Fuc$_2$, Hex$_7$, HexNAc$_7$, HexNAcol	3809.2			X		
18.1	Fuc$_4$, Hex$_7$, HexNAc$_6$, HexNAcol	3912.3			X		
18.2	Fuc$_3$, Hex$_7$, HexNAc$_7$, HexNAcol	3983.4			X		

* Calculated isotopic average masses for the permethylated structure.

† In GC–MS the structure 6.9 [Hex → HexNAc → Hex → 3(Hex → HexNAc → 6)HexNAcol] was detected in antrum-LD but not in the other fractions. However, this may not be the only isomer with the same composition as 6.10.

Neutral oligosaccharides from the corpus

In the corpus, two distinct mucin populations with different density were found. The corpus-HD population contain predominantly short neutral oligosaccharides (less than eight monosaccharides) as detected by MALDI-MS whereas corpus-LD contain the largest oligosaccharides (up to 18 residues) detected with MALDI-MS (Figure 2 and Table 2). Owing to the presence of these longer oligosaccharides, attempts to analyse the total fraction of the neutral oligosaccharides from corpus-LD by GC–MS failed, and the permethylated oligosaccharides were therefore separated into 'large' and 'small' species by gel-

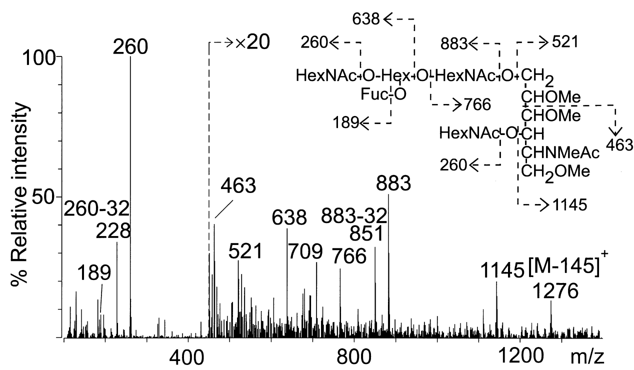

Figure 4 Mass spectrum of a blood-group-A-type hexasaccharide from GC–MS of permethylated pig gastric mucin oligosaccharides from cardia

Mass spectrum and interpretation of the fragmentation of the component 6.7 with a core-4 structure having a terminal blood-group-A determinant on the C-6 branch. The mass spectrum is the average of 14 scans recorded at 28.7 min and at 347 °C of the GC–MS chromatogram in Figure 3.

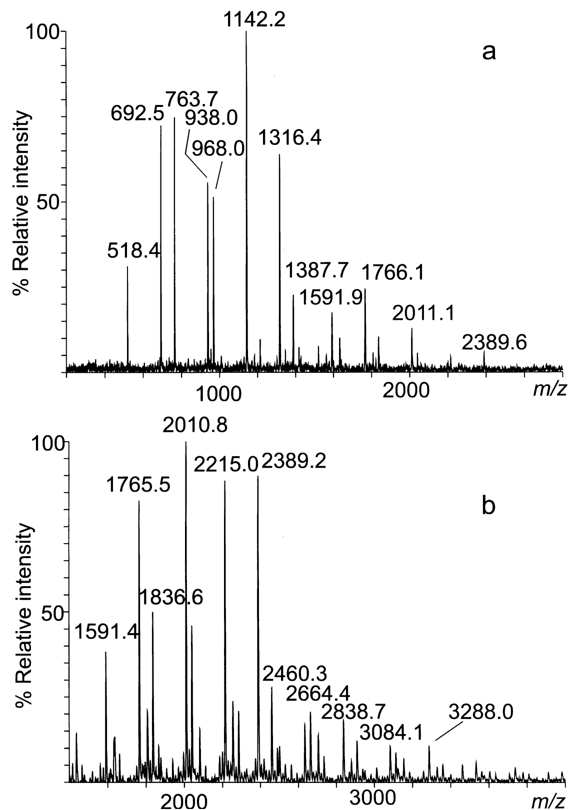

Figure 5 MALDI-MS of (a) the 'smaller' and (b) the 'larger' permethylated pig gastric mucin oligosaccharides from corpus-LD

The fractions were obtained by gel chromatography on LH-20 Sephadex of permethylated mucin oligosaccharides from the corpus-LD. Ions detected in the spectra reflect the composition of the $[M+\mathrm{Li}]^+$ ions of the permethylated oligosaccharides described in Table 1 and 2.

filtration chromatography. MALDI-MS of the small ones showed only oligosaccharides with up to eleven residues, whereas the size of the large ones ranged between seven and eighteen (Figure 5). The small oligosaccharides were also analysed by GC–MS (Figure 3c). Comparison of the MALDI-MS spectrum of the total fraction of neutral oligosaccharides from corpus-LD (Figure 2c) with that obtained for the glycans fractionated by gel chromatography (Figure 5) indicates that there was a suppression of the ions for the high-molecular-mass species when the total fraction was analysed by MALDI-MS. This finding initiated the separation of the permethylated oligosaccharides from cardia, corpus-HD, antrum-HD and antrum-LD into 'small' and 'large' oligosaccharides. However, the subsequent analysis of the 'large' oligosaccharides by MALDI-MS did not show any additional $[M+\mathrm{Li}]^+$ ions, implying that the corpus-LD mucins indeed contain longer oligosaccharides than the other pig gastric mucin populations.

The core types found in the two mucin populations from corpus include both core-1 (Galβ1-3GalNAcα1-) and core-2 [Galβ1-3(GlcNAcβ1-6)GalNAcα1-] structures (Table 1). Although the same dominating oligosaccharide species were present in both mucin populations, the sequence GlcNAc-3GalNAcol was only detected in corpus-HD, showing the presence of a core-3 pathway (GlcNAcβ1-3GalNAcα1-) in these mucins. Apart from the cardiac region, this is the only indication of a structure containing GlcNAc linked directly to C-3 of the GalNAcol residue. The lower blood-group-A reactivity of corpus-HD relative to that with *U. europaeus I* lectin, as compared with corpus-LD (Figure 1), was also reflected in the GC–MS analysis, where no blood-group-A-type sequences were found in corpus-HD (Table 1). The presence of a branching Gal residue also distinguished corpus-HD from corpus-LD mucins. This feature is represented by component 5.7 [Gal-GlcNAc-(GlcNAc-)Gal-3GalNAcol] which is only present in corpus-HD. This type of branching has been described previously in porcine stomach [13,14].

Neutral oligosaccharides from the antrum

The different appearances of the total chromatograms of the permethylated neutral oligosaccharides from antrum-HD (Figure 3d) and antrum-LD (Figure 3e) were mainly due to a lower fucosylation of the antrum-LD mucins. Consequently, a simpler spectrum was observed with MALDI-MS (Figures 2d and 2e).

The vast majority of the neutral oligosaccharides from the mucins from the antrum contained the sequences Gal-3GalNAcol or Gal-3(GlcNAc-6)GalNAcol, indicating the presence of core-1 and core-2 sequences (Table 1). The largest oligosaccharides detected were species containing eleven (antrum-HD) and ten (antrum-LD) residues (Table 2), and the molecular-mass distributions were similar to that of the cardia mucins, although not as complex. The glycosylation of the mucins from antrum-LD exhibits a pathway with Gal as a branching point (structures 5.7, 6.2 and 6.9, Table 1) similar to corpus-HD.

DISCUSSION

MALDI-MS and high-temperature GC–MS were demonstrated to be powerful tools for the investigation of mucin oligosaccharides. Five mucin populations from the cardiac region, corpus and antrum from pig gastric mucosa were shown to be glycosylated differently both quantitatively and qualitatively. A comparison of the results for the monosaccharide composition of these mucin populations [8] and those obtained by GC (Table 1) shows the mean oligosaccharide size deduced from the monosaccharide composition (approximately ten residues) to be higher than that obtained by using GC and MALDI-MS. However, it is our experience that monosaccharide composition analysis performed as described [21] usually overestimates the mean oligosaccharide length, probably because of the presence of glycopeptides with unreleased oligosaccharides.

The amount of unsubstituted GalNAcol varied between the five mucin populations, with only low levels found in the two mucin fractions from corpus. The presence of significant amounts of unsubstituted GalNAcol has been noted before in rat intestinal mucins [22] and is probably not due to chemical degradation. The fact that unsubstituted GalNAc could be detected in mucins from the cardia, but not from the other regions, by anti-Tn antibodies (H. Nordman, unpublished work) indicates that the epitopes may be shielded by larger oligosaccharides.

The core structures (core-1, core-2, core-3 and core-4) found here in the pig stomach are the same as those shown to be present in human gastric mucins [23]. However, to our knowledge this is the first indication of the presence of core-3 structures in pig gastric mucins. Core-2 and core-4 structures have also been isolated from the stomach of sheep [24]. The ability to add GlcNAc in β1-linkage to both C-3 and C-6 of the protein-bound GalNAc appears to be exclusive to the glycosylation pathway of the cardiac mucins. No glycosyltransferase adding GlcNAc to C-3 of GalNAc has been cloned and nothing is known about how this pathway is regulated in mucin-secreting cells. In contrast, the addition of GlcNAc in a β1-linkage to C-6 and the addition of Galβ1- to C-3 and/or C-6 are more general pathways in the glycosylation process of gastric mucins. Finally, the differences in the fucosylation of the two antrum populations could reflect an important mechanism for regulating glycosylation of mucins.

A striking difference between the mucin populations was that the oligosaccharides from corpus-LD were much longer than those from the other regions. Among the oligosaccharides from the corpus-LD mucins, there was no indication, on GC–MS analysis, of structures containing a branch point in an *N*-acetyl-lactosamine unit. This type of structure was instead found in the shorter oligosaccharides from corpus-HD and antrum-LD, suggesting that the larger oligosaccharides in corpus-LD were not the result of branching, but rather of an increased number of linearly arranged *N*-acetyl-lactosamine units. How the number of *N*-acetyl-lactosamine units added to a growing oligosaccharide chain is regulated is still not known.

Although the oligosaccharides from pig gastric mucins display vast structural diversity, the relative molar abundance shows that there are only a few 'dominating' species (Table 1). A possible main function of the shorter and less complex oligosaccharides is to give the highly glycosylated domains an extended conformation, whereas the longer and more complex glycans may impart more subtle properties to the macromolecules such as binding sites for the interaction with bacterial surface proteins. The identification of mucin populations with similar overall macromolecular behaviour, but with distinctly different glycosylation, offers the possibility of studying how the properties of mucins relate to oligosaccharide substitution. The powerful tools now available for determining oligosaccharide structure using small amounts of material, as well as for assessing their relative abundance, will be instrumental in this context and eventually provide an insight into how glycosylation affects the properties of mucins. Furthermore a knowledge of the cellular origin of mucins that can be isolated as well-defined populations and subjected to oligosaccharide and apoprotein analysis is needed to elucidate to what extent glycosylation is modulated by the apoprotein and by regulatory mechanisms at the glycosyltransferase level.

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