Determination of the structure of a novel acidic oligosaccharide with blood-group activity isolated from bovine submaxillary-gland mucin

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In this study we investigated the structure of an acidic fucose-containing pentasaccharide released from bovine submaxillary-gland mucin by alkaline-borohydride treatment. The structure, determined by a combination of one-dimensional and two-dimensional ^1H -n.m.r. spectroscopy at 270 MHz and methylation analysis involving g.l.c.-m.s., was as follows:

This pentasaccharide is a novel structure and is the first report of a blood-group-H type 2 determinant on a submaxillary-gland mucin.

INTRODUCTION

Previous investigations of the oligosaccharides released by alkaline-borohydride treatment of bovine submaxillary-gland mucin [1,2] have shown the presence of three acidic oligosaccharides: NeuAc (or NeuGc) $\alpha(2\rightarrow6)$ GalNAcol, Gal $\beta(1\rightarrow3)$ [NeuAc $\alpha(2\rightarrow6)$]-GalNAcol and GlcNAc $\beta(1\rightarrow3)$ [NeuAc $\alpha(2\rightarrow6)$]-GalNAcol. Tsuji & Osawa [2] have reported the structure of a neutral fucose-containing oligosaccharide in bovine submaxillary-gland mucin. In the present study we have extended the work of these investigators and report here the occurrence of a novel acidic fucose-containing oligosaccharide in bovine submaxillary-gland mucin. The structure was determined by an analysis of the one-dimensional ^1H -n.m.r. spectrum at 270 MHz in conjunction with two-dimensional correlation experiments (COSY and RELAYED-COSY). Methylation analysis results were in agreement with this structure. A preliminary report on this study has appeared [3].

MATERIALS AND METHODS

Mucin was isolated from bovine submaxillary glands by the procedure of Tettamanti & Pigman [4], with modifications by Hill *et al.* [5]. Reduced oligosaccharides were obtained from the 65–75%-(v/v)-ethanol precipitate by β -elimination according to published procedures [6,7] and separated into neutral and acidic fractions on a Dowex AG1-X2 column [6]. The acidic fraction was further fractionated by gel filtration as previously described [8] and assayed for sialic acid [9].

Oligosaccharides were purified by preparative h.p.l.c. on a Milton Roy CM4000 liquid chromatograph equipped with a Rheodyne 7125 injection valve and an SM4000 programmable wavelength detector operating

at 195 nm (Galway). Chromatograms were recorded with a LDC/Milton Roy CI-10 integrator. Chromatography was performed on a column (4 mm $\times$ 250 mm) of Spherisorb S5 NH₂ (5 μm particle size; Phase Separations, Queensferry, Clwyd, U.K.). The mobile phase consisted of a mixture of acetonitrile (h.p.l.c. grade) and deionized distilled water containing 15 mM-potassium phosphate, pH 5.2. The chromatogram was developed isocratically for 30 min with acetonitrile/15 mM-potassium phosphate (pH 5.2) (4:1, v/v), after which a linear gradient was applied to decrease the acetonitrile concentration at a rate of 0.5 %/min [10]. The flow rate was 2 ml/min.

Sialic acid identification, determination of carbohydrate composition and methylation analysis were performed essentially as described previously [8]. The products of methylation analysis were identified by using a Hewlett-Packard 5993 gas chromatograph/quadrupole mass spectrometer/computer combination (Amsterdam) equipped with an ultra-performance capillary column (12.5 m $\times$ 0.2 mm internal diam.) containing cross-linked methylsilicone. Helium was used as a carrier gas at a pressure of 50 kPa. The initial column temperature was 55 $^{\circ}\text{C}$, rising 25 $^{\circ}\text{C}/\text{min}$ to 130 $^{\circ}\text{C}$, then 4 $^{\circ}\text{C}/\text{min}$ to 214 $^{\circ}\text{C}$ and finally 8 $^{\circ}\text{C}/\text{min}$ to 230 $^{\circ}\text{C}$. Mass spectra were recorded at 70 eV at an ion-source temperature of 200 $^{\circ}\text{C}$ and a pressure of 1.33 kPa.

For ^1H -n.m.r. spectroscopic analysis the pentasaccharide-alditol was treated thrice with 0.4 ml of $^2\text{H}_2\text{O}$ (99.8 atom % ^2H ; Nuclear Magnetic Resonance, High Wycombe, Bucks., U.K.) at room temperature, with intermediate freeze-drying. The 270 MHz ^1H -n.m.r. spectroscopy was performed on a JEOL GX-270 spectrometer (Galway), at a probe temperature of 293 K. For the one-dimensional experiment, 1000 transients were collected, with a spectral width of 3000 Hz and 32000 data points. Resolution enhancement was achieved by

Abbreviation used: COSY, correlation spectroscopy.

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Fig. 1. H.p.l.c. separation of oligosaccharides: isolation of two oligosaccharides constituting peak A1 of the Bio-Gel P-4 fractionation of the acidic oligosaccharide-alditols released from bovine submaxillary-gland mucin by β -elimination

The arrows indicate the elution positions of (1) NeuAc α (2 $\rightarrow$ 3)Gal β (1 $\rightarrow$ 3)GalNAcol and (2) NeuAc α (2 $\rightarrow$ 3)Gal β (1 $\rightarrow$ 3)[NeuAc α (2 $\rightarrow$ 6)]GalNAcol.

applying a Gaussian weighting function to the time-domain data and Fourier-transforming. Double quantum-filtered COSY and 1-RELAYED-COSY spectra were measured with 256×512 data matrices and a spectral width of 1500 Hz. The time-domain data were multiplied by a $\sin^2$ function in both dimensions before Fourier transformation. The contour display of spectra is presented in the absolute-value mode. The mixing time for the 1-RELAYED-COSY spectrum was 32.5 ms, calculated on the basis of $J_{1,2} = 7.7$ Hz for the β -N-acetylglucosamine residue. Chemical shifts are expressed in p.p.m. downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulphonate but were actually measured by reference to internal acetone ($\delta = 2.225$ p.p.m.).

RESULTS

Isolation of oligosaccharide

Approx. 90% of the total mucin-bound sialic acid was precipitated in the 65–75%-(v/v)-ethanol fraction. From 4 g of this mucin 2.5 g of oligosaccharides was released by β -elimination. The acidic fraction from ion-exchange chromatography (approx. 1 g), when applied to a Bio-Gel P-4 column, gave seven peaks. Only peak A1, which was eluted at a volume corresponding to approximately nine hexose units, yielding approx. 3.5 mg of oligosaccharides, was further investigated by h.p.l.c. Two components, A1a and A1b, were identified by h.p.l.c. (Fig. 1). The major component, A1a, was isolated by preparative h.p.l.c. and desalted, giving approx. 1.5 mg of oligosaccharide.

Fig. 2. H.p.l.c. identification of sialic acid: separation of products of mild acid hydrolysis of oligosaccharide A1a and identification of the sialic acid residue

The arrows indicate the elution positions of (1) NeuAc, (2) NeuGc, (3) Fuca(1 $\rightarrow$ 2)Gal β (1 $\rightarrow$ 3)GalNAcol and (4) GalNAca(1 $\rightarrow$ 3)[Fuca(1 $\rightarrow$ 2)]Gal β (1 $\rightarrow$ 3)GalNAcol.

Composition of oligosaccharide

Analysis of oligosaccharide A1a after mild acid hydrolysis, by the use of h.p.l.c., showed it to be composed of *N*-acetylneuraminic acid and an oligosaccharide that was eluted slightly earlier than standard GalNAca(1 $\rightarrow$ 3)[Fuca(1 $\rightarrow$ 2)]Gal β (1 $\rightarrow$ 3)GalNAcol (Fig. 2). The sialic acid species was also identified by the use of g.l.c.-m.s. [11], giving the same result. The oligosaccharide was shown to be composed of equimolar amounts of sialic acid, fucose, galactose, *N*-acetylglucosamine and *N*-acetylgalactosaminitol by the use of g.l.c.

Methylation analysis

The molar ratios of the partially methylated monosaccharides obtained on methanolysis and acetylation of the permethylated oligosaccharide-alditol are given in Table 1. From the substitution pattern of *N*-acetylgalactosaminitol it can be deduced that it is substituted at both positions 3 and 6. Similarly, galactose and *N*-acetylglucosamine residues are substituted at positions 2 and 4 respectively, and the fucose and *N*-acetylneuraminic acid residues are unsubstituted.

Table 1. Molar proportions of monosaccharide methyl ethers present in the methanolysate of the permethylated reduced oligosaccharide

Abbreviations: Me-2,3,4-Me₃Fuc etc., methyl 2,3,4-tri-*O*-methylfucoside etc. Molar proportions are given relative to Me-3,4,6-Me₃Gal.

Oligosaccharide-alditol	Per- <i>O</i> -acetylated derivative of ...	Molar proportions (mol/mol of oligosaccharide)				
		Me-2,3,4-Me ₃ Fuc	Me-3,4,6-Me ₃ Gal	Me-3,6-Me ₂ GlcNAcNMe	1,4,5-Me ₃ GalNAcNMe-ol	Me-4,7,8,9-Me ₄ NeuAcNMe
A1a		0.85	1.00	1.00	0.90	0.95

Fig. 3. Resolution-enhanced 270 MHz ^1H -n.m.r. spectrum of $\text{Fu}\alpha(1\rightarrow2)\text{Gal}\beta(1\rightarrow4)\text{GlcNAc}\beta(1\rightarrow3)[\text{NeuAc}\alpha(2\rightarrow6)]\text{GalNAcol}$

* denotes a non-protein non-carbohydrate contaminant.

^1H -n.m.r. spectroscopy

In order to determine the anomeric configurations of the linkages and to determine its primary structure, oligosaccharide A1a was investigated by ^1H -n.m.r. In the one-dimensional spectrum (Fig. 3), the *N*-acetylgalactosaminitol H-2 resonance is found at $\delta = 4.263$ p.p.m. (Table 2). Its typical triplet-like shape cannot be readily recognized, because the signal partly coincides with that of H-5 of the fucose residue. Nevertheless, the chemical shift is characteristic of *N*-acetylgalactosaminitol substituted by *N*-acetylglucosamine in $\beta(1\rightarrow3)$ -glycosidic linkage [12]. The β -configuration of the $(1\rightarrow3)$ -glycosidic linkage between *N*-acetylglucosamine and *N*-acetylgalactosaminitol is evident from the chemical shift of *N*-acetylglucosamine H-1 ($\delta = 4.613$ p.p.m.) in conjunction

with its $J_{1,2}$ value (7.7 Hz). The chemical shifts of *N*-acetylgalactosaminitol H-5 ($\delta = 4.210$ p.p.m.) and H-6' ($\delta = 3.490$ p.p.m.) reflect substitution at C-6 [12,13]. Such substitution at C-6 by *N*-acetylneuraminic acid in $\alpha(2\rightarrow6)$ -glycosidic linkage is evident from the chemical shifts of the *N*-acetylneuraminic acid structural-reporter groups, H-3_{ax} ($\delta = 1.699$ p.p.m.) and H-3_{eq} ($\delta = 2.734$ p.p.m.) [14,15]. The chemical shifts of the fucose structural-reporter groups, H-1 ($\delta = 5.313$ p.p.m.), H-5 ($\delta = 4.223$ p.p.m.) and H-6 ($\delta = 1.233$ p.p.m.), are characteristic of a fucosylated type 2 chain: $\text{Fu}\alpha(1\rightarrow2)\text{Gal}\beta(1\rightarrow4)\text{GlcNAc}\beta(1\rightarrow-)$ [16]. The β -configuration of the linkage between galactose and *N*-acetylglucosamine is evident from the chemical shift of galactose H-1 ($\delta = 4.534$ p.p.m.) and its $J_{1,2}$ value (8 Hz).

Table 2. ^1H -n.m.r. chemical-shift data for *N*-acetylneuraminic acid-containing pentasaccharide-alditol obtained from bovine submaxillary-gland mucin

Reporter group	Residue	Chemical shift	Reporter group (p.p.m.)	Residue	Chemical shift (p.p.m.)
H-2	GalNAcol	4.263	H-1	GlcNAc	4.613
H-3		3.984	H-2		3.77–3.8
H-5		4.210	H-3		3.7–3.8
H-6		3.80–3.81	H-6		4.010
H-6'		3.490	NAc		2.080
NAc		2.034			
H-3 _{ax}	NeuAc	1.699	H-1	Fuc	5.313
H-3 _{eq}		2.734	H-2		3.77–3.8
NAc		2.034	H-5		4.223
			H-6		1.233
			H-1	Gal	4.534
			H-2		3.64–3.65
			H-3		3.87–3.88

Fig. 4. ^1H -n.m.r. absolute-value RELAYED-COSY spectrum (0.5–5.5 p.p.m.) of $\text{Fuc}\alpha(1\rightarrow2)\text{Gal}\beta(1\rightarrow4)\text{GlcNAc}\beta(1\rightarrow3)[\text{NeuAc}\alpha(2\rightarrow6)]\text{GalNAcol}$ obtained at 270 MHz

Key: F_1 and F_2 frequencies of some of the multiplets are labelled by using an abbreviated form iH_j , where i denotes the residue and j indicates the position within the residue. Residues are numbered as follows: $\text{Fuc}^5\alpha(1\rightarrow2)\text{Gal}^4\beta(1\rightarrow4)\text{GlcNAc}^3\beta(1\rightarrow3)[\text{NeuAc}^1\alpha(2\rightarrow6)]\text{GalNAcol}^2$.

This chemical shift is also indicative of substitution at H-2 of $\text{Gal}\beta(1\rightarrow4)$ by $\text{Fuc}\alpha(1\rightarrow2)$, as opposed to terminal $\text{Gal}\beta(1\rightarrow4)$ ($\delta = 4.45\text{--}4.47$ p.p.m.) [12]. The (1→4)-glycosidic linkage between galactose and *N*-acetylglucosamine has been confirmed by the assignment of H-3 of *N*-acetylglucosamine ($\delta = 3.7\text{--}3.8$ p.p.m.) from the 1-RELAYED-COSY spectrum (Fig. 4). This value is indicative of a (1→4)-glycosidic linkage as opposed to a value of approx. 3.9 p.p.m. for a (1→3)-glycosidic linkage [17]. In addition, the chemical shift of H-6 of *N*-acetylglucosamine ($\delta = 4.010$ p.p.m.) is in agreement with galactose in $\beta(1\rightarrow4)$ -glycosidic linkage as opposed to $\beta(1\rightarrow3)$ -glycosidic linkage [13]. On the basis of these data, the structure of oligosaccharide A1a is proposed as:

DISCUSSION

We report here the structure of a novel acidic pentasaccharide. Although the occurrence of oligosaccharides in bovine submaxillary-gland mucin containing sialic acid and fucose had been proposed in 1970 by Bertolini & Pigman [1], this is the first report of the structure of an acidic oligosaccharide from bovine submaxillary-gland mucin containing fucose. This is also the first time that the blood-group-H type 2 determinant [fucose linked $\alpha(1\rightarrow2)$ to galactose on type 2 $\text{Gal}\beta(1\rightarrow4)\text{GlcNAc}$ chains] has been found in submaxillary-gland mucin, although it has been found on ovarian-cyst mucin [18,19], human bronchial mucin [20], pig trachea mucin [21], sheep gastric mucin [22] and pig stomach mucin [16]. Previously, the blood-group-H type 3 determinant [fucose linked $\alpha(1\rightarrow2)$ to $\text{Gal}\beta(1\rightarrow3)\text{GalNAc}$] has been found in bovine [2], pig [23] and sheep [24] submaxillary-gland mucins.

On the basis of the 'one enzyme—one linkage' concept of oligosaccharide biosynthesis [25], five different glycosyltransferases are necessary for the biosynthesis of this pentasaccharide. The fucosyltransferase is probably the blood-group-H β -galactoside $\alpha(1\rightarrow2)$ -fucosyltransferase that has been characterized from pig submaxillary glands [26] and has been shown to act, although at a lower rate, on type 2 chains. In analogy with the biosynthesis of the trisaccharide $\text{Gal}\beta(1\rightarrow3)[\text{NeuAc}\alpha(2\rightarrow6)]\text{GalNAcol}$ [27,28] it is likely that the *N*-acetylglucosamine residue is transferred to *N*-acetylgalactosamine before the *N*-acetylneuraminic acid residue.

A $\beta(1\rightarrow3)$ -*N*-acetylglucosaminyltransferase acting on $\text{GalNAc}\alpha\text{-R}$ structures has been reported in pig and rat colon mucosa [29]. The *N*-acetylgalactosaminide $\alpha(2\rightarrow6)$ -sialyltransferase from pig submaxillary glands [30] has been shown to have a strict requirement for protein at the aglycone but has a loose specificity with regard to the oligosaccharide structure [31]. The specificity of both enzymes is compatible with the suggested order of attachment of *N*-acetylglucosamine and *N*-acetylneuraminic acid, and probably very similar enzyme activities are present in bovine submaxillary gland.

Further extension of the carbohydrate structure with a $\beta 4$ -galactosyl and a $\alpha 2$ -fucosyl residue may occur after sialic acid has been attached. It has been shown that sialylation does not block the formation of the blood-group-A determinant by α -fucosylation and α -*N*-acetylgalactosaminylation on pig submaxillary mucin [28]. Similarly, sialylation might not impair further glycosylation of the oligosaccharides on the bovine mucin.

Both *N*-acetylneuraminic acid- and *N*-glycolylneuraminic acid-containing oligosaccharides have been reported in bovine submaxillary mucin [2,3]. The sialic acid species in oligosaccharide A1a has been identified as *N*-acetylneuraminic acid rather than *N*-glycolylneuraminic acid by using h.p.l.c., and this has been confirmed by g.l.c.-m.s. The sugar sequence has been determined by analysis of the ^1H -n.m.r. spectrum at 270 MHz. Evidence for the blood-group-H type 2 chain is confirmed by analysis of the 1-RELAYED-COSY spectrum, and the methylation analysis data are in agreement.

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