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Determination of the Structure of Sulfated Tetra- and Pentasaccharides Obtained by Alkaline Borohydride Degradation of Hen Ovomucin. A Fast Atom Bombardment-Mass Spectrometric and ^{1}H-NMR Spectroscopic Study

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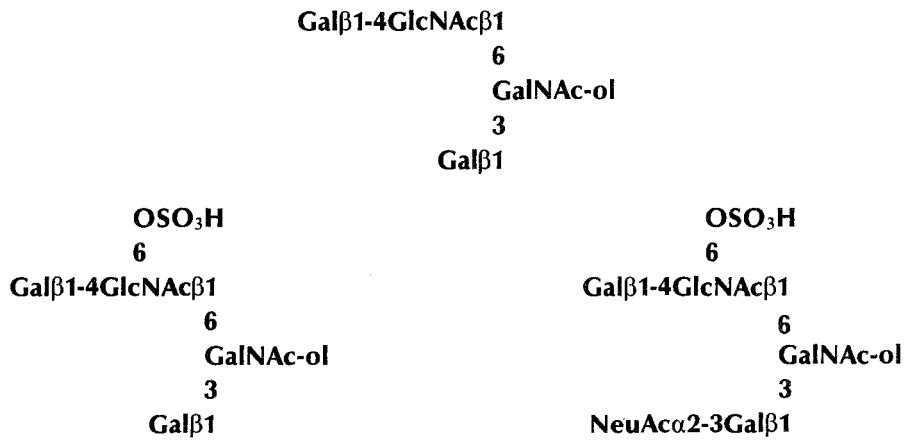
Alkaline borohydride reductive cleavage of hen ovomucin resulted in the release of a series of neutral and acidic oligosaccharide-alditols. ^{1}H-NMR spectroscopy in combination with fast ion bombardment-mass spectrometry in negative ion mode were used for investigation of the structures of three oligosaccharide-alditols. The following structures were established:



Abbreviations: NeuAc, *N*-acetyl-D-neuraminic acid; Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; GalNAc-ol, *N*-acetyl-D-galactosaminitol; NMR, nuclear magnetic resonance; FAB-MS, fast atom bombardment-mass spectrometry.

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Ovomucin, first described by Eicholz [1], is obtained as a precipitate on dilution of egg white with water. The presence of ester sulfate groups was reported by Astudillo *et al.* [2] and the glycan structure NeuAcα2-3Galβ1-3[6-O-SO_3]GalNAc-Ser(Thr) has been recently described by Kato *et al.* [3]. Although ovomucin is not a homogeneous glycoprotein, it represents a rich source of sulfated O-glycans, the structures of which have not yet been extensively analysed. Here, we report on the isolation and structural characterisation of three oligosaccharide-alditols obtained by β-elimination of ovomucin.

Materials and Methods

Isolation of Oligosaccharides

Ovomucin was isolated according to the method of Brooks and Hale [4]. O-Linked oligosaccharides were released from ovomucin (5 g) by alkaline borohydride treatment in 1 M sodium borohydride, 0.05 M sodium hydroxide at 37°C for 16 h [5]. The solution was applied to a column of charcoal-Celite (5 × 30 cm) [6]. The column was washed with 2 l water, in order to remove salts, and then eluted with 5 l of 50% ethanol. The eluate was concentrated to a small volume and applied to a column of Bio-Gel P-4 (150 × 3 cm, Bio-Rad, Richmond, CA, USA), using water as eluent. The purity of the oligosaccharide-alditols was checked by TLC on Silica-gel plates (Merck, Darmstadt, W. Germany) using *n*-butanol/ethanol/acetic acid/pyridine/water, 10/100/3/10/30 by vol. Oligosaccharides were located by spraying the plates with an orcinol reagent (0.2% orcinol in 80% aqueous H_2SO_4) and heating at 105°C for 10 min.

Analytical Methods

Sugar analysis was carried out by GLC of trifluoroacetylated derivatives of methyl glycosides formed by methanolysis in 0.5 M hydrochloric acid in methanol at 80°C for 24 h [7]. The nature of the sialic acid was determined according to the method of Schauer [8]. Phosphate and sulfate content were estimated as described by Beaufay *et al.* [9] and Silvestry *et al.* [10], respectively.

Fast-atom Bombardment-Mass Spectrometry

FAB-MS of native oligosaccharide-alditols was performed using a Kratos MS-50 mass spectrometer. The samples (2-5 μg) were applied to the target in aqueous solution; glycerol was used as matrix. The target was bombarded with xenon atoms having a kinetic energy equivalent to 9 KeV. The spectra were recorded, in negative-ion mode at 7 kV acceleration voltage, in a mass-controlled linear scan at a resolution of 300 ppm.

400 MHz ^{1}H-NMR Spectroscopy

Oligosaccharide-alditols were repeatedly exchanged in ^2H_2O (99.96 atom % ^{2}H, Aldrich, Milwaukee, WI, USA) with intermediate lyophilisation and analyzed with a Bruker AM-400 WB spectrometer operating at 400 MHz in the Fourier transform mode at a probe temperature of 300°K. Chemical shifts are given relative to sodium-4,4,dimethyl-4-silapentanesulfonate but were actually measured indirectly to acetone in ^2H_2O (δ 2.225 ppm).

Table 1. Carbohydrate composition of native ovomucin and oligosaccharide-alditols OL-1, OL-2 and OL-3 obtained by alkaline borohydride degradation of the glycoprotein.

Monosaccharides	Carbohydrate content			
	Ovomucin[a]	OL-1[b]	OL-2[b]	OL-3[b]
Gal	9.4	1.9	2.0	1.95
Man	1.5	—	—	—
GlcNAc	9.0	0.95	0.96	0.97
GalNAc	5.0	—	—	—
GalNAc-o1	—	1.0	1.0	1.0
NeuAc	8.4	—	—	1.1
Total	33.3			

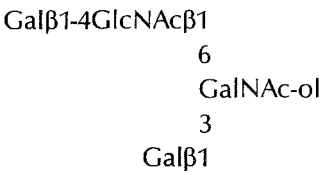
[a] % total weight.
[b] Molar ratios were calculated relative to one GalNAc-ol residue.

Results

The carbohydrate composition of native ovomucin is given in Table 1. Sialic acid was identified as *N*-acetylneuraminic acid, after mild hydrolysis. The phosphate assays gave negative results, while the presence of sulfate was clearly demonstrated with the sodium rhodizonate-$BaCl_2$ reagent.

The elution curve of the oligosaccharide-alditols on a Bio-Gel P-4 column is given in Fig. 1. The fractions were pooled as indicated. The material eluted in the first 650 ml was a heterogeneous mixture of oligosaccharides that was not further studied. Oligosaccharide-alditols OL-1, OL-2 and OL-3 were repeatedly submitted to Bio-Gel P-4 fractionation until they were pure by TLC criteria. 10, 22 and 45 mg of OL-1, OL-2 and OL-3, respectively, were finally obtained in pure state. The molecular weights of oligosaccharide-alditols were measured by FAB-mass spectrometry in the negative-ion mode (Fig. 2).

OL-1 was found to be a tetrasaccharide of mol wt 750, consisting of galactose, *N*-acetylglucosamine and *N*-acetylgalactosaminitol residues in the molar ratio 2:1:1 (Table 1). The ^1H chemical shifts of the structural reporter protons (Table 2) were identical to those of the same compound previously isolated from porcine blood-group H substance [11]. Therefore, the structure of OL-1 is



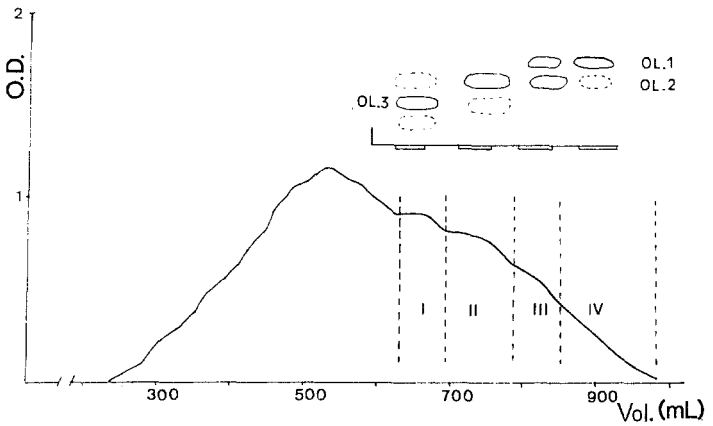


Figure 1. Bio-Gel P-4 fractionation of the products of alkaline borohydride degradation of hen ovomucin. Detection of neutral carbohydrate by the phenol-sulfuric acid test. Above the fraction numbers, a TLC analysis is shown. The material of higher mol wt (elution volume: 250-650 ml) was not resolved by TLC analysis.

For oligosaccharide-alditol OL-2, containing galactose, *N*-acetylglucosamine and *N*-acetylgalactosaminitol in the molar ratio 2:1:1, the pseudo-molecular ion (M-H) was found at m/z 829 (Fig. 2B), indicating the presence of a sulfate residue. The NMR analysis (Fig. 3B and Table 2) shows a significant alteration of the GlcNAc H-6 and H-6′ chemical shift ($\Delta\delta$ +0.421 and 0.521 respectively) when compared to those of OL-1, suggesting the sulfate residue to be present at C-6 of $GlcNAc^6$. Other significant shift alterations are also observed in the $GlcNAc^6$ H-1 ($\Delta\delta$ +0.023), Gal^4 H-2 ($\Delta\delta$ +0.017) and Gal^4 H-1 ($\Delta\delta$ +0.069), while the other chemical shifts of structural reporter protons are not modified ($\Delta\delta < 0.010$). Therefore, the structure of OL-2 is

$$\begin{array}{l} \qquad\qquad\quad OSO_3H \\ \qquad\qquad\quad\ 6 \\ Gal\beta1\text{-}4GlcNAc\beta1 \\ \qquad\qquad\qquad\ 6 \\ \qquad\qquad\qquad GalNAc\text{-}ol \\ \qquad\qquad\qquad\ 3 \\ \qquad\qquad\quad Gal\beta1 \end{array}$$

Oligosaccharide-alditol OL-3 was found to be an extension of OL-2 by an additional *N*-acetylneuraminic acid residue, as shown by the FAB-MS analysis (Fig. 2C) which indicated for the pseudo-molecular ion (M-H) the m/z value of 1120. Fragment ions observed at m/z 1040, 958, 878, 829 and 667 are of the $[(Sn+H)\text{-}H]^-$ even-electron fragment ion series [12]. The NMR analysis (Table 2 and Fig. 3C) enables establishment of the α(2-3)-linkage of *N*-acetylneuraminic acid to Gal^3, as confirmed by the *N*-acetylneuraminic acid H-3_{ax} and H-3_{eq} chemical shift values at δ 1.801 and 2.774, respectively; and the effect

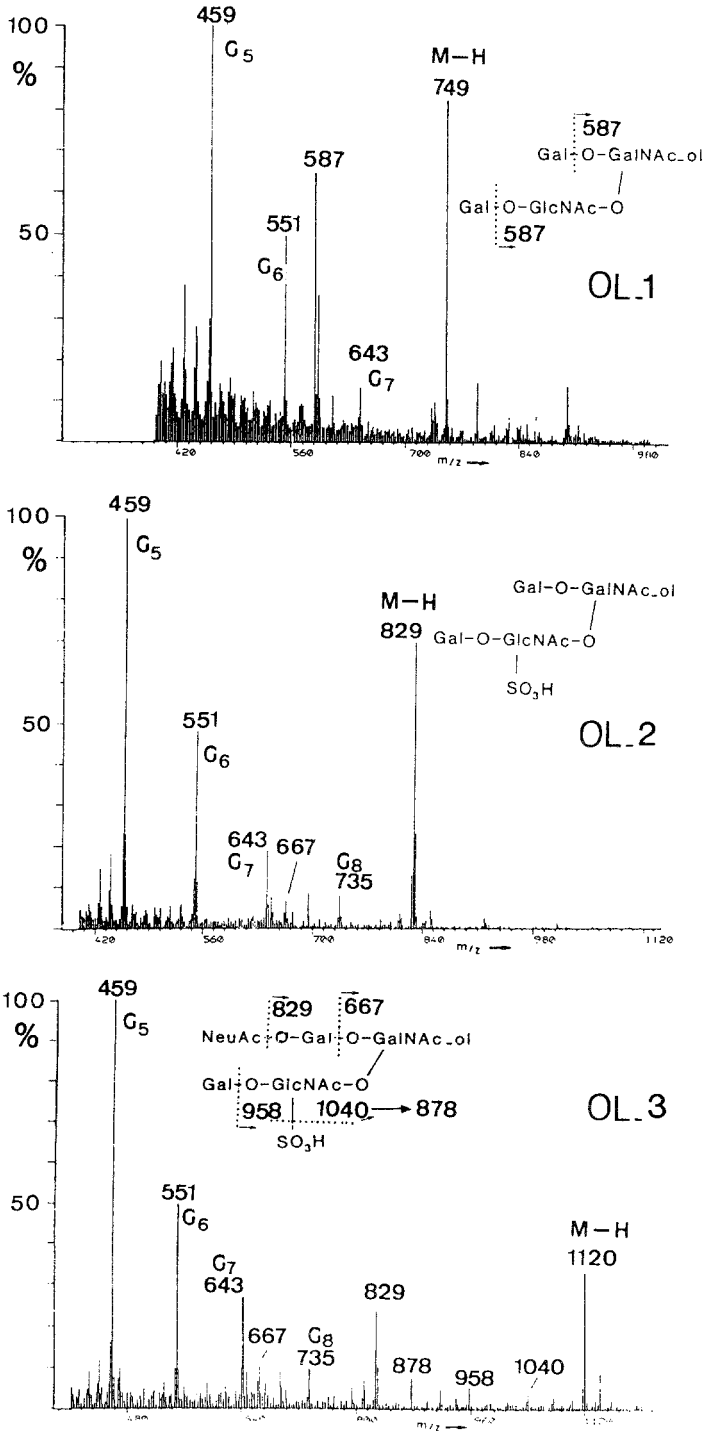


Figure 2. Negative ion FAB mass spectrum of compounds OL-1, OL-2 and OL-3. G_5 to G_8: polymers $(Glycerol)_5$ to $(Glycerol)_8$ from the glycerol matrix.

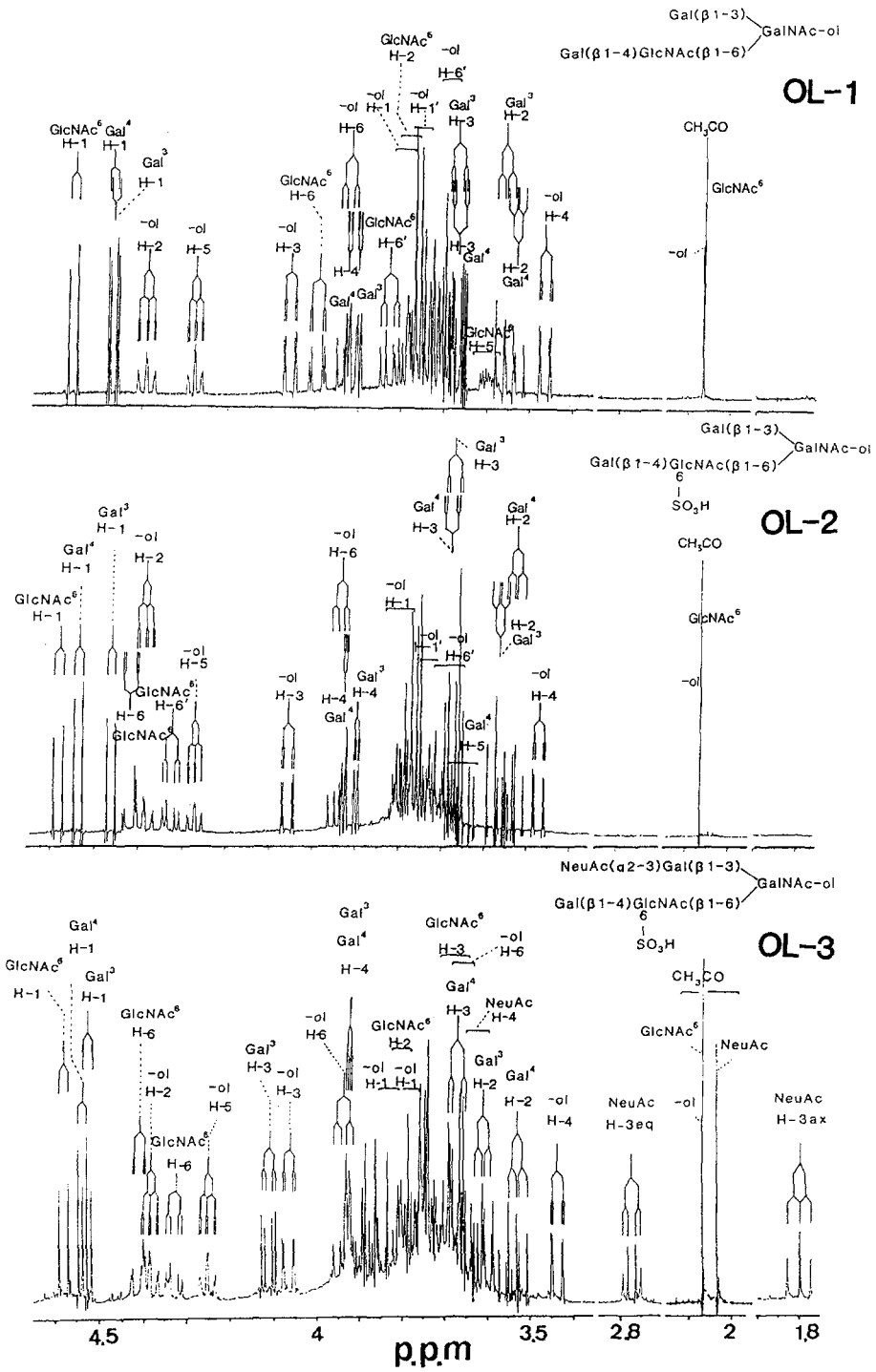


Figure 3. 400 MHz ^{1}H-NMR spectra of oligosaccharide fractions OL-1, OL-2 and OL-3.

observed on δ H-1 and H-3 of Gal^3 (Δδ +0.065 and +0.434, respectively) [13]. The alterations which affect the other structural reporter protons, such as GalNAc-ol H-4 (Δδ -0.034), $GlcNAc^6$ H-6 (Δδ -0.024), Gal^4 H-2 (Δδ -0.027) are of lower intensity. In comparison to compound B [13], the structural reporter skeleton protons of $GlcNAc^6$ in OL-3 show the same shift effects due to the attachment of sulfate at C-6 as those observed between compound A and OL-2: Δδ H-6 = +0.419; Δδ H-1 = +0.022 (Table 2). Therefore, the structure of compound OL-3 is

$$\begin{array}{l} \qquad\qquad\quad OSO_3H \\ \qquad\qquad\quad\ 6 \\ Gal\beta1\text{-}4GlcNAc\beta1 \\ \qquad\qquad\qquad\ 6 \\ \qquad\qquad\qquad GalNAc\text{-}ol \\ \qquad\qquad\qquad\ 3 \\ NeuAc\alpha2\text{-}3Gal\beta1 \end{array}$$

Discussion

The present study reports the FAB-MS and ^{1}H-NMR analysis of sulfated oligosaccharide-alditols released from a mucin by alkaline borohydride degradation. Similar reports concerning the polysulfated proteoglycans have been previously published [14-18]. As shown in the case of the neutral oligosaccharide-alditol OL-1 and the two monosulfated oligosaccharides OL-2 and OL-3, sulfation at C-6 of *N*-acetylglucosamine results in a downfield shift of 0.4 to 0.5 ppm for the H-6 and H-6' protons, while smaller differences in chemical shifts are observed for the other protons. Identical downfield shifts were observed for sulfated oligosaccharides released from keratan sulfate [18]. Therefore, our results confirm that FAB-MS and ^{1}H-NMR analysis are suitable methods to establish the primary structure of mucin-type sulfated O-glycans.

The main difficulties remain in fractionating complex mixtures of sialylated/sulfated oligosaccharides. More than a dozen larger oligosaccharides are presently under investigation, despite the failure of conventional procedure to furnish separations of these compounds. Methods elaborated with such an available material as ovomucin will be easily extended for the study of human sulfated mucin.

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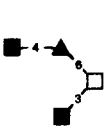
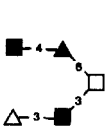
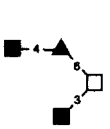
Table 2. ^{1}H Chemical shifts of structural-reporter-group protons of the constituent monosaccharides for oligosaccharide-alditols derived from hen ovomucin, together with those of reference compounds isolated from porcine blood-group H substance (A) [11] and cow colostrum K-casein (B) [13].

Residue	Reporter group	Chemical shift in				
		A	B	OL-1	OL-2	OL-3
						
GalNAc-ol	H-2	4.394	4.390	4.390	4.385	4.384
	H-3	4.060	4.072	4.060	4.058	4.066
	H-4	3.465	3.456	3.466	3.471	3.437
	H-5	4.282	4.272	4.280	4.271	4.252
	H-6	3.931	3.927	3.933	3.943	3.937
	NAc	2.067	2.066	2.066	2.065	2.066
GlcNAc^6	H-1	4.560	4.559	4.560	4.583	4.581
	H-6	3.998	3.993	3.998	4.419	4.912
	H-6′	—	—	3.828	4.349	4.373
	NAc	2.064	2.066	2.064	2.065	2.064
Gal^4	H-1	4.470	4.470	4.469	4.538	4.537
	H-2	—	—	3.538	3.555	3.528
	H-3	—	3.7	3.669	3.677	3.675
	H-4	3.925	3.931	3.925	3.927	3.925
Gal^3	H-1	4.465	4.534	4.464	4.462	4.527
	H-2	—	—	3.560	3.571	3.609
	H-3	—	4.116	3.674	3.677	4.111
	H-4	3.900	3.922	3.901	3.898	3.925
NeuAc^3	H-3 ax	—	1.801	—	—	1.801
	H-3 eq	—	2.774	—	—	2.774
	NAc	—	2.033	—	—	2.032

■ = Gal; ▲ = GlcNAc; △ = NeuAc; □ = GalNAc-ol; S = sulfate.

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