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The structure of the keratan sulphate chains attached to fibromodulin isolated from bovine tracheal cartilage: oligosaccharides generated by keratanase II digestion

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The repeat region and chain caps of the N-linked keratan sulphates attached to bovine tracheal cartilage fibromodulin were fragmented by digestion with keratanase II, and the oligosaccharides generated were isolated by strong anion-exchange chromatography. Each of these oligosaccharides has been examined by both HPAE chromatography and high field ^{1}H-NMR spectroscopy.

All of the capping oligosaccharides isolated terminated with α(2-3)-linked *N*-acetyl-neuraminic acid, and neither α(2-6)-linked *N*-acetyl-neuraminic acid chain terminators, nor fucose α(1-3)-linked to *N*-acetylglucosamine were found. The keratan sulphate chains were short, with average lengths of five to seven disaccharides, and the level of galactose sulphation varied along the length of the chain.

The repeat region and chain cap were confirmed as having the following general structure:

$$\begin{matrix} & (6S) & & 6S & & (6S) & & 6S \\ & / & & / & & / & & / \end{matrix}$$
$$\mathbf{NeuAc\alpha^{2}\text{-}^{3}Gal\beta^{1}\text{-}^{4}(GlcNAc\beta^{1}\text{-}^{3}Gal\beta^{1}\text{-}^{4})_{4\text{-}6}GlcNAc}$$

This study has identified a novel structure in fibromodulin, namely a cap containing a sulphated galactose adjacent to a non-reducing terminal *N*-acetyl-neuraminic acid. We have also confirmed that the general structure of the repeat units and chain caps of N-linked keratan sulphate attached to fibromodulin isolated from bovine tracheal cartilage, is similar to that of O-linked keratan sulphate chains attached to aggrecan from non-articular cartilage. However, there are important differences in chain lengths and sulphation patterns.

Keywords: fibromodulin, small proteoglycan, keratan sulphate, glycosaminoglycan, sulphation, keratanase II.

Abbreviations: KS, keratan sulphate; ELISA, enzyme linked immunosorbent assay; Gal, β-D-galactose; GlcNAc, *N*-acetylglucosamine (2-acetamido-β-D-glucose); GlcNAc-ol, *N*-acetylglucosaminitol; NeuAc, *N*-acetyl-neuraminic acid; 6S/(6S), *O*-ester sulphate group on C6 present/sometimes present; NMR, nuclear magnetic resonance; HPAE, high pH anion-exchange; PED, pulsed electrochemical detection.

Introduction

Fibromodulin is a small, leucine-rich, proteoglycan [1, 2], which has been identified in several connective tissues, including articular and tracheal cartilage, in which it was found to be abundant [1]. It is glycosylated by up to four N-linked keratan sulphate chains [3], and has a region rich in sulphated tyrosine residues [4].

A potential role for fibromodulin has been found in its ability to inhibit the formation of collagen fibrils *in vitro* [5, 6]. It has also been demonstrated to bind to both collagen types I and II [6, 7], and immuno-cytochemical

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localisation studies have found that it is localised mainly in the gap region, axially within the D-period of collagen fibrils [8]. These workers also found that, in articular cartilage, levels of fibromodulin were higher in the interterritorial matrix than adjacent to cells, and were higher at the cartilage surface than at deeper levels.

The keratan sulphate chains in fibromodulin are known to be N-linked [3], however, the only previous study of their structure [9] does not address that of the linkage region nor the precise biantennary nature. By contrast, two models have been proposed for the structure of the N-linked keratan sulphate chains from cornea. Both are biantennary, but in the first model, derived from studies of pig cornea [10], the keratan sulphate chain has one extended antenna and one short stub. In the second model, derived from studies of monkey cornea [11], both antennae are extended.

Keratanase II, an endo-β-*N*-acetylglucosaminidase, has been found to cleave at the β(1-3)-glycosidic bond of a 6-*O*-sulphated *N*-acetylglucosamine in keratan sulphates [12]. Di- and tetra-saccharides are generated from the sulphated poly-*N*-acetyllactosamine repeat region, and in the case of the *N*-acetyl-neuraminic acid-containing oligosaccharides pentasaccharides are recovered [13].

In this study fibromodulin has been isolated from bovine tracheal cartilage, and the entire proteoglycan has been subjected to degradation by keratanase II. In consequence, the oligosaccharides isolated derive from the cap and repeat structures and do not yield information about the linkage region or biantennary nature of the KS chains. The oligosaccharides generated have been isolated using strong anion exchange chromatography, and examined by HPAE chromatography and high field ^{1}H-NMR spectroscopy.

Materials and methods

Materials

Sepharose CL-6B, Sephacryl S-300, Q-Sepharose, and a Mono Q 10/10 column were purchased from Pharmacia (Uppsala, Sweden), and a Nucleosil 5SB column was purchased from Technicol Ltd (Stockport, Cheshire, UK). Lithium perchlorate (A.C.S. grade) was from Aldrich Chemical Co. (Poole, Dorset, UK). Keratanase II was from ICN Biomedicals (High Wycombe, Bucks, UK). Bovine (tracheal) cartilage powder, guanidine hydrochloride (practical grade), Tween 20, phosphate buffered saline (PBS) tablets, 3,3′,5,5′-tetramethylbenzidine dihydrochloride and peroxidase-conjugated goat anti-rabbit IgG were from Sigma Chemical Co. (Poole, Dorset, UK). Sodium hydroxide (A.R. 46/48%) was from Fisons Scientific Equipment (Loughborough, Leics., UK). Sodium acetate was >99.5% Microselect grade from Fluka (Gillingham, Dorset, UK). The antibody to the protein core of fibromodulin used in this study was a kind gift of Dr A.H.K. Plaas (Shriners Hospital, Tampa, FL, USA). All other chemicals and reagents were of analytical grade.

Isolation of fibromodulin from bovine tracheal cartilage powder

Fibromodulin was isolated from bovine tracheal cartilage powder by ethanol precipitation as previously described [9]. Bovine tracheal cartilage powder (100 g) was suspended in 4 M guanidine hydrochloride plus protease inhibitor cocktail (50 mM sodium acetate, 100 mM 6-aminocaproic acid, 10 mM EDTA, 5 mM benzamidine hydrochloride, pH 6.8). Insoluble material was removed by filtration and soluble material precipitated at two and four volumes of ethanol. The fibromodulin rich four volume precipitate was subjected to ion exchange chromatography on a Q-Sepharose column (12 cm × 2 cm) which had previously been equilibrated with 6 M urea/0.15 M NaCl/50 mM Tris/HCl, pH 7.3. Following loading, the column was washed with 20 ml of buffer, and bound material eluted with a linear gradient of 0.15 M–1 M NaCl in 6 M urea/50 mM Tris/HCl, pH 7.3. The absorbance of the eluate was monitored at 280 nm, and fibromodulin was assayed by an ELISA.

Fibromodulin-containing fractions were pooled, and subjected to size exclusion chromatography on a Sepharose CL-6B column (152 cm × 3.2 cm) run in 4 M guanidine hydrochloride/50 mM Tris/HCl, pH 7.3, at 16 ml h^{-1}, fractions being collected over 30 min. The absorbance of the eluate was monitored at 280 nm, and fibromodulin-containing peaks, as determined by an ELISA, were pooled and dialysed against 0.2 M NaCl overnight and then extensively against water before being recovered by lyophilization.

Two fibromodulin preparations, I and II, were isolated using this protocol. Fibromodulin preparation II has also been used in a previous examination of the keratanase susceptibility of fibromodulin [9].

Isolation of fibromodulin from fresh bovine trachea

Fibromodulin was isolated from fresh bovine tracheal cartilage essentially as previously described for bovine articular cartilage [14]. Diced bovine trachea from 6–8-year-old animals were extracted into 4 M guanidine hydrochloride with a protease inhibitor cocktail. The extract was taken to 0.4 M guanidine hydrochloride by dialysis against the protease inhibitor cocktail, and solid CsCl added to achieve a density of 1.48 g ml^{-1}. Following density gradient centrifugation at 37 000 r.p.m. (100 000 × **g**) in a Sorval T-865 Ti rotor for 48 h at 4 °C, the A3 and A4 fractions ($\rho < 1.53$ g ml^{-1}) were pooled and exchanged into 6 M urea/0.15 M NaCl/50 mM Tris/HCl, pH 7.3.

This was subjected to ion exchange chromatography on a Mono-Q HR 10/10 column as described above for the

Q-Sepharose column. The absorbance of the eluate was monitored at 280 nm and the fibromodulin content monitored by an ELISA using an antibody to its protein core.

Fibromodulin-containing fractions were pooled, exchanged into 4 M guanidine hydrochloride/50 mM Tris/HCl, pH 7.3, and subjected to size exclusion chromatography on a Sephacryl S-300 column, as described above for the Sepharose CL-6B, at 9 ml h^{-1}, fractions being collected over 20 min. The absorbance of the eluate was monitored at 280 nm and fibromodulin was assayed by an ELISA. Fibromodulin-containing fractions were pooled and dialysed overnight against 0.2 M NaCl and then extensively against water.

Two fibromodulin preparations, III and IV, were isolated using this protocol.

Analytical methods

Glycosaminoglycan concentrations were monitored on microtitre plates using a 1,9-dimethylmethylene blue assay [15]. Fibromodulin levels were determined with a microtitre plate ELISA using an antibody specific to the protein core of fibromodulin, as previously described [3, 9].

Keratanase II digestion

Purified fibromodulin preparations I (200 mg) and II (10 mg) from tracheal cartilage powder, and III (12 mg) and IV (15 mg) from fresh trachea, were subjected to exhaustive digestion by keratanase II, (0.002 U per mg of fibromodulin) in 10 mM sodium acetate, pH 6.8, for 24 h at 37 °C.

Isolation of oligosaccharides

The oligosaccharides generated by keratanase II digestion were isolated as previously described [9]. Following digestion they were reduced by addition of $NaBH_4$ to 1 M. After 3 h at room temperature, reduction was stopped by stepwise addition of ethanoic acid. The reduced oligosaccharides were isolated by size exclusion chromatography on a Sephadex G-50 (medium) column (84 cm × 1.5 cm) run in 0.15 M NaCl at 9 ml h^{-1}, fractions being collected over 20 min intervals. The oligosaccharides were pooled and desalted by chromatography on a Bio-Gel P-2 column (11 cm × 1 cm) run in water at 12 ml h^{-1}, then lyophilized.

Preparative strong anion exchange chromatography

The reduced oligosaccharides from fibromodulin preparation I were separated by ion exchange chromatography on a semi-preparative Nucleosil 5SB column (25 cm × 1 cm) using a Bio-Rad 700 HRLC titanium gradient system fitted with UV and conductivity detectors. Oligosaccharides were eluted with a linear gradient of 0–0.25 M lithium perchlorate at 2 ml min^{-1} over 200 min and absorbance of the eluate monitored at 206 nm. Fractions were pooled and desalted on a Bio-Gel P-2 column (11 cm × 1 cm) by elution with water at 12 ml h^{-1}, then lyophilized.

Analytical high pH anion exchange chromatography

The compositions of the reduced oligosaccharides from fibromodulin preparations I, II, III and IV were examined by HPAE chromatography on a Dionex chromatography system (Dionex, Camberley, Surrey, UK) as described previously [13]. They were separated on an IonPac AS4A-SC column (4mm × 250 mm), with a AG4A-AS guard column (4 mm × 50 mm), maintained at 30 °C and running at 1 ml min^{-1}. An isocratic period of 5% 1 M NaOH/95% water, was followed by a linear gradient of 0–95% 1.5 M sodium acetate and constant 5% 1 M NaOH over 50 min. The eluted oligosaccharides were monitored on-line by a pulsed electrochemical detector (PED) using integrated amperometry.

Previous work in this laboratory [13] has determined PED response factors for each oligosaccharide generated by keratanase II digestion of keratan sulphates. These values were used for the calculations of relative oligosaccharide abundance and chain length reported in Table 1. The average length of the antennae of keratan sulphate attached to fibromodulin from bovine tracheal cartilage has been estimated by calculating the ratio of capping oligosaccharides to repeat region oligosaccharides.

NMR spectroscopy

Samples, typically 0.2–1 mg, were buffered to pH 7 with phosphate, referenced with sodium 3-trimethylsilyl-[$^2H_4$]propionate (TSP) as internal standard and dissolved in 0.5 ml 99.96% ^2H_2O after micro-filtration, several previous exchanges with 99.8% ^2H_2O and one using 99.96% ^2H_2O. Proton NMR spectra were determined at 60 °C using a Bruker AM500 spectrometer with 5 mm probe. All chemical shifts are quoted relative to internal TSP at 0.0 ppm.

Spectra were reprocessed for presentation using the NMR1 (Version 1-4-1) software package supplied by New Methods Research Inc, Syracuse NY, USA.

Results

Isolation and characterisation of oligosaccharides

Four preparations of fibromodulin were studied: those numbered I and II were from commercially available bovine tracheal cartilage powder, while those numbered III and IV were derived from fresh bovine trachea.

The reduced keratanase II oligosaccharides derived from the N-linked keratan sulphate attached to bovine tracheal cartilage fibromodulin (preparation I) were separated by strong anion exchange chromatography on a

Table 1. Results of analysis of HPAE chromatograms of oligosaccharides derived by keratanase II digestion of bovine tracheal cartilage fibromodulin preparations I, II, III and IV.

(a) Relative molar abundance of each oligosaccharide. The values are referenced to C1 as unity. It was from preparation I that oligosaccharides were isolated by strong anion exchange chromatography and examined by high field ^{1}H-NMR spectroscopy as well as HPAE chromatography.

	Preparation				
	I	*II*	*III*	*IV*	
R1	1.79	2.27	1.05	2.39	Gal-GlcNAc-ol(6S)
R3	3.12	4.35	1.61	5.43	Gal(6S)-GlcNAc-ol(6S)
R2	0.57	0.59	0.45	1.34	Gal-GlcNAc(6S)-Gal-GlcNAc-ol(6S)
R4	0.55	0.54	0.38	1.00	Gal(6S)-GlcNAc(6S)-Gal-GlcNAc-ol(6S)
R5	0.29	0.35	0.26	0.58	Gal-GlcNAc(6S)-Gal(6S)-GlcNAc-ol(6S)
R6	0.59	0.78	0.67	1.08	Gal(6S)-GlcNAc(6S)-Gal(6S)-GlcNAc-ol(6S)
C1	1.00	1.00	1.00	1.00	NeuAc3-Gal-GlcNAc(6S)-Gal-GlcNAc-ol(6S)
C2	1.01	1.10	1.02	1.99	NeuAc3-Gal-GlcNAc(6S)-Gal(6S)-GlcNAc-ol(6S)
C3	0.02	0.04	0.04	0.02	NeuAc3-Gal(6S)-GlcNAc(6S)-Gal(6S)-GlcNAc-ol(6S)

(b) Relative molar abundance of each oligosaccharide type i.e. disaccharides, tetrasaccharides and capping fragments. The average number of disaccharides isolated per *N*-acetyl-neuraminic acid residue was determined by examination of the ratio of repeat region oligosaccharides to capping oligosaccharides. Each pentasaccharide cap and repeat region tetrasaccharide comprises two disaccharides. Therefore the average number of disaccharides $(\text{-Gal-GlcNAc})_n$ isolated per *N*-acetyl-neuraminic acid residue is calculated by:

$$n = (\text{disaccharide}) + (\text{tetrasaccharide} \times 2) + (\text{pentasaccharide cap} \times 2).$$

	Preparation			
Oligosaccharide	*I*	*II*	*III*	*IV*
Repeat region disaccharide	2.41	3.08	1.29	2.60
Repeat region tetrasaccharide	0.98	1.06	0.85	1.33
Pentasaccharide cap	1.00	1.00	1.00	1.00
Average number of disaccharides per *n*-acetyl-neuraminic acid	6.37	7.20	4.99	7.26

Nucleosil 5SB column. The chromatogram of these oligosaccharides (Fig. 1) is simple, showing few dominant oligosaccharides each of which has been isolated and examined by both HPAE chromatography (Fig. 2) and ^{1}H-NMR spectroscopy (Fig. 3). The structures have been categorised as deriving from the cap region [C], or from the repeat region [R] of the parent N-linked keratan sulphate (Fig. 4). Linkage region fragments have not been isolated in this study. The oligosaccharides have been numbered incrementally according to their elution order on Nucleosil 5SB. The nomenclature, i.e. C1–C3 and R1–R6, is used solely to aid reference to these oligosaccharides within this study.

A feature common to all of the oligosaccharides is the reducing terminal sulphated *N*-acetylglucosaminitol derived by reduction from the 6-*O*-sulphated, β(1-3)-linked *N*-acetylglucosamine residue within the parent keratan sulphate at which keratanase II cleaves.

The spectra of oligosaccharides C1–C3 (a representative spectrum for C1 is shown in Fig. 3) were found to have distinctive signals at ca. 1.80 and 2.76 ppm corresponding to H(3ax) and H(3eq) protons, respectively, of *N*-acetyl-neuraminic acid linked to galactose in an α(2-3)-linkage [16]. No signals characteristic of galactose or *N*-acetylglucosamine residues at the non-reducing terminus were detected. This clearly indicates that oligosaccharides C1–C3 have their non-reducing termini capped by α(2-3)-linked *N*-acetyl-neuraminic acid.

The spectra of oligosaccharides R1–R6 (a representative spectrum for R2 is shown in Fig. 3) lack the α(2-3)-linked *N*-acetyl-neuraminic acid signals observed in oligosaccharides C1–C3. These oligosaccharides have signals indicative of non-reducing terminal galactose residues, as would be expected for oligosaccharides derived from the repeat region.

These oligosaccharides elute from the Nucleosil 5SB

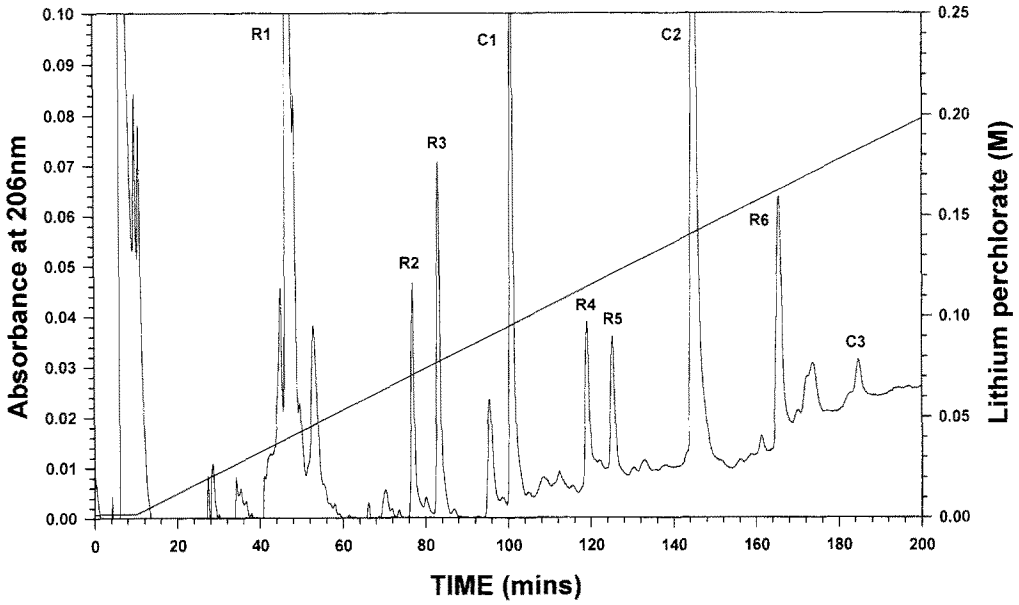

Figure 1. Nucleosil 5SB strong anion exchange chromatography of reduced oligosaccharides generated from bovine tracheal cartilage fibromodulin preparation I by keratanase II digestion. The keratanase II derived oligosaccharides were applied to a Nucleosil 5SB column (250 mm × 10 mm) and eluted at a flow rate of 2 ml min^{-1}. The eluate was monitored on line using UV detection at 206 nm. The gradient program was as follows: 10 min of buffer A (2 mM $LiClO_4$, pH 5.0) and then 240 min of 0–100% buffer B (250 mM $LiClO_4$, pH 5.0).

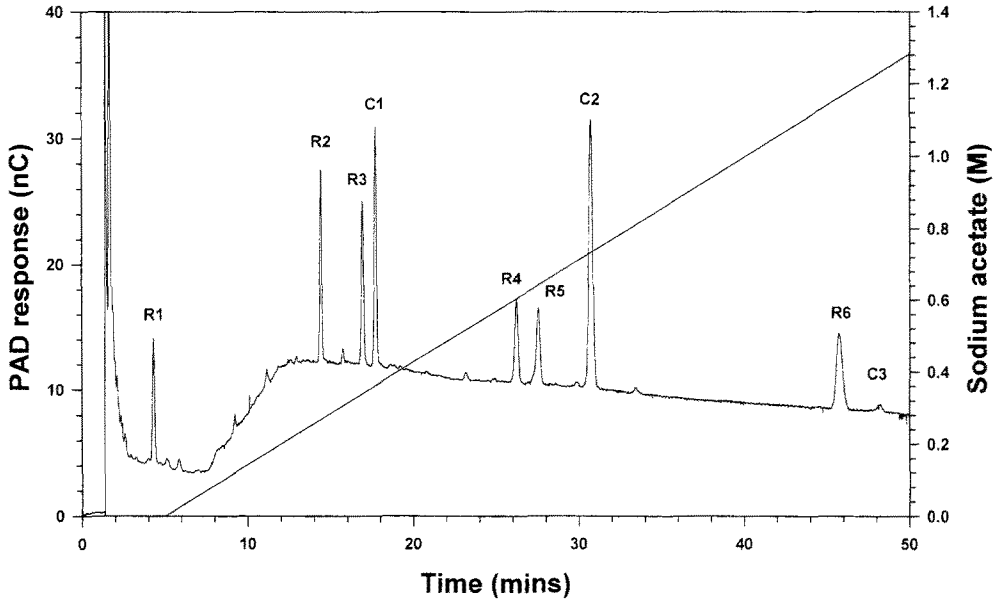

Figure 2. HPAE chromatography of keratanase II derived oligosaccharides from fibromodulin preparation I. A 20 μl aliquot of the reduced oligosaccharides from fibromodulin preparation I was resuspended in water and applied to an AS4A-SC column (4 mm × 250 mm) and the oligosaccharides separated by high pH anion exchange (HPAE) chromatography on a Dionex chromatography system, and monitored on-line by a pulsed electrochemical detector using integrated amperometry. An isocratic period of 5% 1 M NaOH/95% water, was followed by a linear gradient of 0–95% 1.5 M sodium acetate and constant 5% NaOH over 50 min.

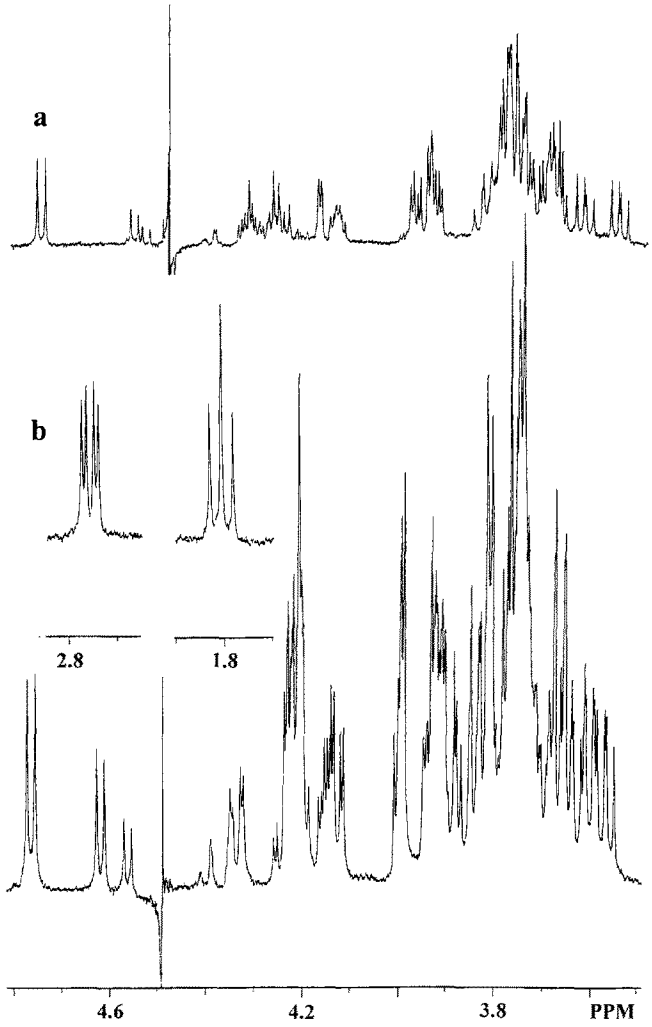

Figure 3. Partial 500 MHz proton NMR spectra of reduced oligosaccharides derived by keratanase II digestion from keratan sulphate chains isolated from bovine tracheal cartilage. a) Repeat region fragment R2 – disulphated tetrasaccharide. b) Capping region fragment C1 – disulphated pentasaccharide. The x scale 3.5–4.8 ppm relates to both a and b. All of the oligosaccharides isolated in this work have been examined, and their structures confirmed, by high field ^{1}H-NMR spectroscopy. However, for illustrative purposes only two spectra are shown.

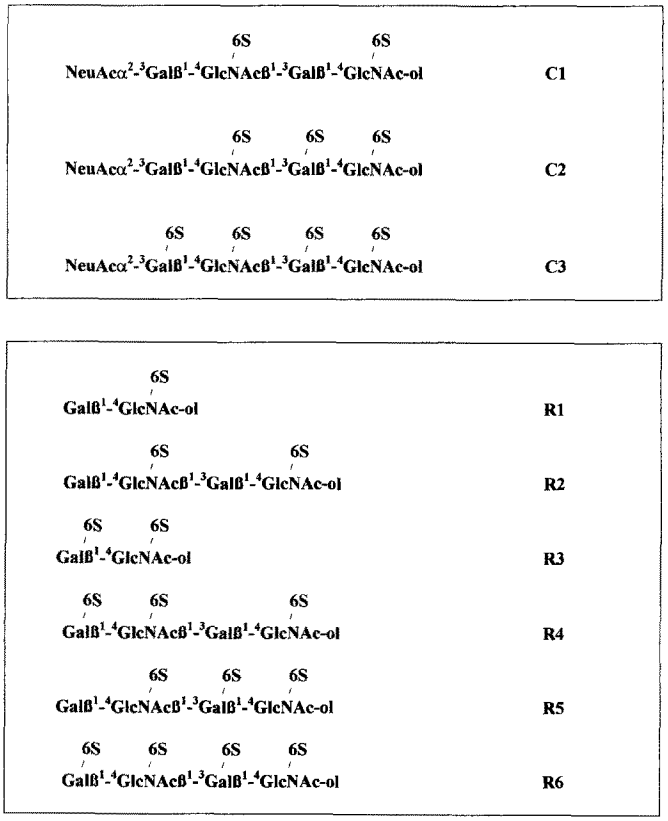

Figure 4. Oligosaccharides derived from the N-linked keratan sulphate attached to fibromodulin from bovine tracheal cartilage. The *N*-acetyl-neuraminic acid capping the non-reducing termini is always α(2-3)-linked to the adjacent galactose.

ion exchange column at progressively higher salt concentrations, indicating a steady increase in the number of charges carried.

Integration of peaks following Dionex chromatography

The keratan sulphate from four separate preparations of fibromodulin, each isolated from bovine tracheal cartilage, have been subjected to keratanase II digestion, and the oligosaccharides generated examined by HPAE chro-

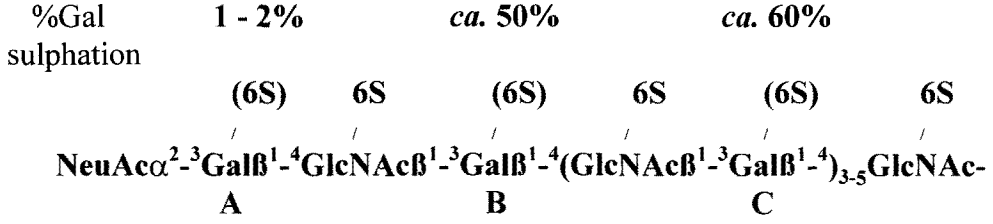

Figure 5. Proposed structure, and galactose sulphation levels, of the cap and repeat region of an antenna from the N-linked keratan sulphate attached to bovine tracheal cartilage fibromodulin.

matography (Table 1b and Fig. 2). The abundance of each oligosaccharide has been estimated by scaling the peak area by the individual oligosaccharides response factor (as described in Methods). The results of these analyses are displayed in Table 1a, b and Fig. 5.

Examination of the keratanase II-susceptible portion of fibromodulin from bovine tracheal cartilage can provide an average ratio of capping oligosaccharides to repeat region oligosaccharides. Using data from Table 1a, b it can be seen that there are five to seven disaccharides $(\text{-Gal-GlcNAc})_n$ isolated per *N*-acetyl-neuraminic acid residue.

The level of galactose sulphation within the repeat region and the cap is shown in Fig. 5. In the capping fragments only 1–2% of the galactose residues adjacent to the non-reducing *N*-acetyl-neuraminic acid (Gal A) are sulphated. However, ca. 50% of the galactose residues adjacent to the reduced *N*-acetylglucosaminitol (Gal B) are sulphated. In the repeat region, on average, 59–63% of the galactose residues are sulphated.

Discussion

The keratan sulphate attached to bovine tracheal cartilage fibromodulin has been fragmented by keratanase II, the resulting oligosaccharides isolated by strong anion exchange chromatography, and their structures determined by high field ^{1}H-NMR spectroscopy, or, by reference to their elution position following HPAE chromatography [13]. Three sialylated capping oligosaccharides, all pentasaccharides, and six repeat region oligosaccharides, two disaccharides and four tetrasaccharides, have been isolated and characterised in this study. The structures of these oligosaccharide are shown in Fig. 4.

Each capping oligosaccharide isolated terminates with *N*-acetyl-neuraminic acid α(2-3)-linked to a galactose, which may be sulphated. *N*-acetyl-neuraminic acid with an α(2-6)-linkage was not detected in this study. It has been demonstrated [17] that, in the case of O-linked keratan sulphate chains from aggrecan, chains isolated from articular cartilage (KS-II-A) contain α(2-3)- and α(2-6)-linked *N*-acetyl-neuraminic acid chain terminators, while those from non-articular cartilages, such as tracheal, (KS-II-B) terminate solely with α(2-3)-linked *N*-acetyl-neuraminic acid. Further, *N*-acetyl-neuraminic acid with an α(2-3)-linkage to a galactose was the sole chain termination sequence found in a previous study of the N-linked keratan sulphate attached to fibromodulin from bovine tracheal cartilage [9].

It is notable that the three caps isolated are all pentasaccharides. It has previously been determined that the linkage of the internal sulphated *N*-acetylglucosamine in such species is resistant to keratanase II cleavage, presumably because of the proximity of the α(2-3)-linked *N*-acetyl-neuraminic acid residue [13].

Oligosaccharide C3 reveals the presence of a structural feature hitherto unknown for both fibromodulin in particular and non-skeletal keratan sulphates in general, namely the presence of *N*-acetyl-neuraminic acid α(2-3)-linked to a sulphated galactose. However, this structure has been previously identified in O-linked keratan sulphates from articular cartilage [18], and in oligosaccharides isolated from recombinant human tissue plasminogen activator expressed in mouse epithelial cells [19]. Following keratanase digestion of fibromodulin [9], a large number of capping oligosaccharides were produced as a result of the mechanism of action of that enzyme. In consequence, this rare structure (present in only 1–2% of the caps) would have been distributed amongst many oligosaccharides, making confident identification impossible. However, as discussed above digestion of bovine tracheal cartilage fibromodulin with keratanase II generates only three pentasaccharide caps, allowing the isolation and identification of the component containing this structural feature.

Six repeat region oligosaccharides, comprising two disaccharides together with four tetrasaccharides, have been isolated and characterised in this study. The activity of keratanase II is dependant upon the size of the substrate oligosaccharide, and within a tetrasaccharide further cleavage of the otherwise susceptible linkage of the internal sulphated *N*-acetylglucosamine, to generate two disaccharides, is not possible [21].

It is important to note that following the use of keratanase II repeat region tetrasaccharides are indistinguishable from the fragments which would be generated from a keratan sulphate capped by galactose *in vivo*. Keratanase digestion is the only degradative technique which can detect these species. Both hydrazinolysis [22] and keratanase II [13] generate repeat region fragments indistinguishable from asialo capping fragments. Previous data [9] suggests that, if present *in vivo*, such caps would represent a very minor percentage of those present on fibromodulin.

No oligosaccharides have been isolated in this study which contain α(1-3)-linked fucose, confirming the results of a previous examination [9]. This structural feature has been found to be absent from O-linked keratan sulphate from non-articular cartilage, although it is found as a branching unit on some *N*-acetylglucosamine residues in O-linked keratan sulphate from articular cartilage [17].

Preliminary examination of the HPAE chromatography profile of the N-linked keratan sulphate from bovine tracheal cartilage fibromodulin (Fig. 2), demonstrates that the keratan sulphate chains are short, the relative abundance of the capping fragments C1–C3, clearly exceeding that of the repeat region oligosac-

charides R1–R6. This indicates that a significant portion of the parent keratan sulphate is found in the pentasaccharide caps. This is not so for O-linked keratan sulphate [13] from articular cartilage, in which the abundance of repeat region oligosaccharides greatly exceeds that of pentasaccharide capping fragments.

A detailed examination of the results of HPAE chromatographic analysis of the oligosaccharides derived from four separate preparations of bovine tracheal cartilage fibromodulin (Table 1b) shows that, on average, there are five to seven disaccharides per *N*-acetyl-neuraminic acid residue. In our previous study of bovine tracheal cartilage fibromodulin [9] the enzyme keratanase was used and α(2-3)-linked *N*-acetyl-neuraminic acid capped oligosaccharides up to 6 disaccharides in length were isolated. These data are consistent with the interpretation that the keratanase II oligosaccharides isolated in this study derive largely from a single antenna, capped with α(2-3)-linked *N*-acetyl-neuraminic acid and with a keratanase II susceptible length of five to seven disaccharides. The existence *in vivo* of chains capped with galactose would result in an overestimation of chain length; following keratanase II digestion asialo caps are indistinguishable from repeat region tetrasaccharides. The close agreement of the chain length estimated in this work and previous studies [9] reinforces our view that chains capped by galactose, if present *in vivo*, represent a very minor percentage of the total.

The structure of O-linked keratan sulphate chains from bovine nasal septum has been examined by Stuhlstatz *et al.* [23] who reported the presence of a repeat region seven to nine disaccharides in length. However, Oeben *et al.* [10], in a study of N-linked keratan sulphate from pig cornea, found chains with repeat regions ranging from 10–52 disaccharides in length. These workers were unable to observe any *N*-acetyl-neuraminic acid caps, and so the chains may be even longer *in vivo*.

The repeat region and cap of the N-linked keratan sulphate attached to fibromodulin from bovine tracheal cartilage has been found to have the structure shown in Fig. 5.

The average level of galactose sulphation (Fig. 5) has been found to be higher in the oligosaccharides derived from the repeat region ca. 60%, than in those from the pentasaccharide caps, in which Gal A and B are sulphated in 1–2% and ca. 50% of the chains respectively. These data contrast with that obtained for O-linked keratan sulphate from articular cartilage [20] in which a higher level of galactose sulphation was observed; Gal A was sulphated in 13%, and Gal B in 66% of chains capped with α(2-3)-linked *N*-acetyl-neuraminic acid.

Because of the nature of the oligosaccharides generated by keratanase II digestion it is not possible, from these data, to gain any further information regarding the distribution of galactose sulphation within the repeat region. However, previous work [9] demonstrated a progressive increase in the level of galactose sulphation towards the non-reducing terminus, i.e. towards the cap. Taken together these data suggest that the level of galactose sulphation may increase towards the non-reducing ends of the antennae (the caps), but, falls sharply at the galactose adjacent to the terminal *N*-acetyl-neuraminic acid residue. These data are generally in agreement with those of Oeben *et al.* [10], who reported increased galactose sulphation along the repeat region, towards the chain cap, of pig corneal N-linked keratan sulphate. The finding of a non-identical level of galactose sulphation along a keratan sulphate chain contrasts with the results obtained by Tai *et al.* [24] who found constant levels in O-linked keratan sulphate derived from aggrecan.

Although these data point to general structural similarities between O-linked keratan sulphate derived from aggrecan, and the N-linked keratan sulphate which derives from fibromodulin, there are important differences between these two keratan sulphate types. The N-linked keratan sulphate chains are, on average, shorter and have a lower level of galactose sulphation at, or near to, the cap.

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