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Structure of the keratan sulphate chains attached to fibromodulin isolated from bovine tracheal cartilage

Oligosaccharides generated by keratanase digestion

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The structure of the repeat region and chain caps of the N-linked keratan sulphate chains attached to bovine tracheal cartilage fibromodulin has been examined. The chains were fragmented by keratanase digestion, the resultant oligosaccharides isolated by strong anion-exchange chromatography, and their structures determined using high-field ^{1}H-n.m.r. spectroscopy. The chains were found to possess the following general structure:

All of the capping oligosaccharides isolated terminate with α(2-3)-linked *N*-acetylneuraminic acid. No α(2-6)-linked *N*-acetylneuraminic acid chain terminators, nor any fucose, α(1-3)-linked to *N*-acetylglucosamine along the repeat region, were detected. This work demonstrates that the structure of the repeat region and chain caps of N-linked keratan sulphate attached to fibromodulin isolated from bovine tracheal cartilage is identical with that of O-linked keratan sulphate chains attached to aggrecan derived from non-articular cartilage.

INTRODUCTION

Fibromodulin is a member of a group of small, leucine-rich, interstitial proteoglycans and is found in articular cartilage along with all other connective tissues which have been examined (Heinegård et al., 1986). The group includes decorin, biglycan and lumican. Fibromodulin was first isolated (Heinegård et al., 1986) from bovine articular cartilage, and subsequently cloned and sequenced (Oldberg et al., 1989) from bovine tracheal cartilage, a tissue known to be rich in fibromodulin (Heinegård et al., 1986). It has a tyrosine-rich domain at its N-terminus, in which some of the tyrosine residues are sulphated (Antonsson et al., 1991) and recent work (Plaas et al., 1990) has confirmed that the keratan sulphate (KS) chains attached to fibromodulin are N-linked to the protein core. In fibromodulin from 3-month-old bovine articular cartilage only four out of the five potential sites were N-glycosylated with either KS chains or N-linked oligosaccharides.

There is evidence that fibromodulin is involved in regulating the formation of the network of collagen fibrils which makes up the extracellular matrix of cartilage. Fibromodulin inhibits the formation of collagen fibrils *in vitro* (Hedbom and Heinegård, 1989), and binds to collagen types I and II (Hedbom and Heinegård, 1989; Novori et al., 1992).

Keratan sulphates have been classified according to their mode of linkage to protein as KS-I for N-linked chains, and KS-II for the O-linked chains from skeletal tissues such as cartilage (Bray et al., 1967). A third type, O-linked from mannose to serine or threonine, has been isolated from brain tissue (Krusius et al., 1986).

KS chains are known to be based upon a repeating *N*-acetyllactosamine sequence of -4GlcNAcβ1-3Galβ1- which is usually sulphated on C-6 of *N*-acetylglucosamine, and further sulphate groups may be present on C-6 of galactose (Bhavanandan and Meyer, 1968). Knowledge of the detailed structure of O-linked KS is increasing, following studies by Dickenson et al. (1990, 1991, 1992), Tai et al. (1993) and Brown et al. (1994). Using a combination of enzymic or chemical fragmentation, and high-field n.m.r. spectroscopy the structures of many oligosaccharides derived from O-linked KSs have been elucidated.

Previous studies (Nieduszynski et al., 1990) have suggested that, for O-linked KS chains from aggrecan, there is a distinction between chains from articular cartilage (KS-II-A), which contain α(1-3)-linked fucose and α(2-6)-linked *N*-acetylneuraminic acid, and those from non-articular cartilage (KS-II-B) which contain neither of these features.

KS chains, both N- and O-linked, can conveniently be considered to have three domains: the chain cap, a linkage region which connects the chain to the protein core, and an intervening repeat region.

Keratanase cleaves KS chains at an unsulphated galactose residue adjacent to a sulphated *N*-acetylglucosamine residue (Nakazawa and Suzuki, 1975). Several oligosaccharides generated by digestion of O-linked KS-II with keratanase have been characterized (Dickenson et al., 1990, 1991, 1992; Tai et al., 1993), and the substrate specificity of keratanase is now more completely understood (Tai et al., 1993).

In preliminary studies fibromodulin was isolated from bovine articular cartilage, but the full characterization of the KS chains requires amounts greater than are readily available via this approach. In this study fibromodulin was isolated using ethanol precipitation from bovine tracheal cartilage, and the KS chains were degraded by keratanase. The oligosaccharides generated were isolated using strong anion-exchange chromatography and their structures then determined by high-field ^{1}H-n.m.r. spectroscopy.

Abbreviations used: KS, keratan sulphate; 6S/(6S), *O*-ester sulphate group on C-6 present/sometimes present; TSP-d_4, sodium 3-trimethylsilyl[$^2H_4$]propionate.

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EXPERIMENTAL

Materials

Sepharose CL-6B, Q-Sepharose and a Superose 6 HR 10/30 column were purchased from Pharmacia (Uppsala, Sweden). A Nucleosil 5SB column was purchased from Technicol Ltd. (Stockport, Cheshire, U.K.) and a Brownlee C4 column was supplied by Alltech Associates (Carnforth, Lancs., U.K.). Lithium perchlorate (A.C.S. grade) was from Aldrich Chemical Co. (Poole, Dorset, U.K.) Keratanase (EC 3.2.1.103; from *Pseudomonas* sp.) was from ICN Biomedicals (High Wycombe, Bucks., U.K.). Boehringer–Mannheim GmbH (Germany) supplied 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid. Bovine (tracheal) cartilage powder, guanidine hydrochloride (practical grade), Tween 20, PBS tablets, 3,3′,5,5′-tetramethylbenzidine dihydrochloride and peroxidase-conjugated goat anti-(rabbit IgG) antibodies were from Sigma Chemical Co. (Poole, Dorset, U.K.). The antibody to the protein core of fibromodulin used in this study was a kind gift from Dr. A. H. K. Plaas (Shriners Hospital, Tampa, FL, U.S.A.). All other chemicals and reagents were of analytical grade.

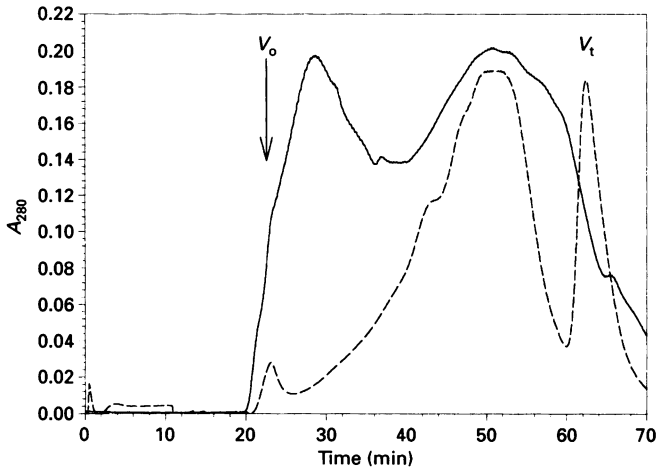


Figure 1 Superose 6 size-exclusion chromatography

Samples of the 2-vol. (——) and 4-vol. (– – –) ethanol precipitates were resuspended in a minimum volume of 4 M guanidine hydrochloride/50 mM Tris/HCl (pH 7.3) and subjected to size-exclusion chromatography on a Superose 6 column (30 cm × 1 cm) running in 4 M guanidine hydrochloride/50 mM Tris/HCl (pH 7.3) at 0.3 ml/min. The absorbance of the eluate was monitored at 280 nm.

Preliminary ethanol precipitation experiments

Preliminary investigations of the utility of ethanol in differentially precipitating the large aggregating cartilage-specific proteoglycan (aggrecan) and fibromodulin from a 4 M guanidine hydrochloride solution were performed. It was found that aggrecan (mainly chondroitin sulphate-substituted) was precipitated by the addition of 2 vol. of ethanol, whereas fibromodulin (exclusively KS-substituted) was not precipitated until 3–4 vol. were added (results not shown). Ethanol precipitation was therefore identified as a useful first stage in the purification of large (> 100 mg) amounts of fibromodulin.

Isolation of fibromodulin from cartilage powder

Bovine tracheal cartilage powder (100 g) was suspended in 3 litres of 4 M guanidine hydrochloride containing 50 mM sodium acetate, 0.1 M 6-aminohexanoic acid, 10 mM EDTA and 5 mM

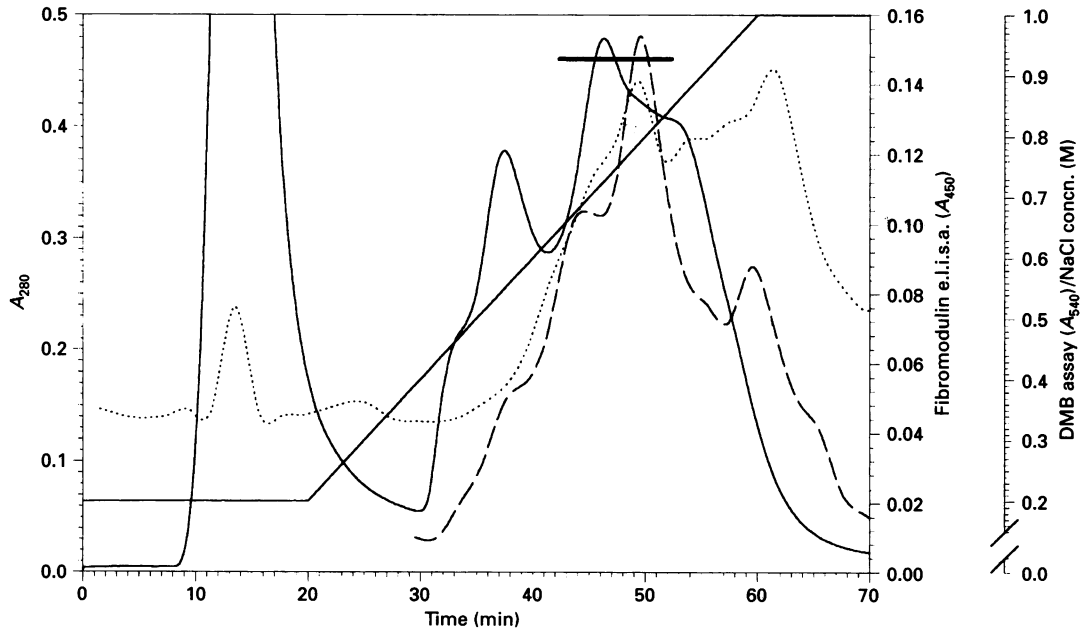


Figure 2 Q-Sepharose chromatography of 4-vol. ethanol precipitate

The 4-vol. ethanol precipitate containing fibromodulin was applied to a Q-Sepharose column (12 cm × 2 cm) previously equilibrated with buffer A (0.15 M NaCl/6 M urea/50 mM Tris/HCl, pH 7.0). Bound material was eluted with a linear gradient of 0–100% buffer B (1 M NaCl/6 M urea/50 mM Tris/HCl, pH 7.0) over 60 min at a flow rate of 2 ml/min. The eluate was monitored at 280 nm (——), by an e.l.i.s.a for fibromodulin (– – –), and total glycosaminoglycans were measured by the 1,9-Dimethylmethylene Blue (DMB) assay (· · ·). Pooled fractions are indicated by the solid horizontal line.

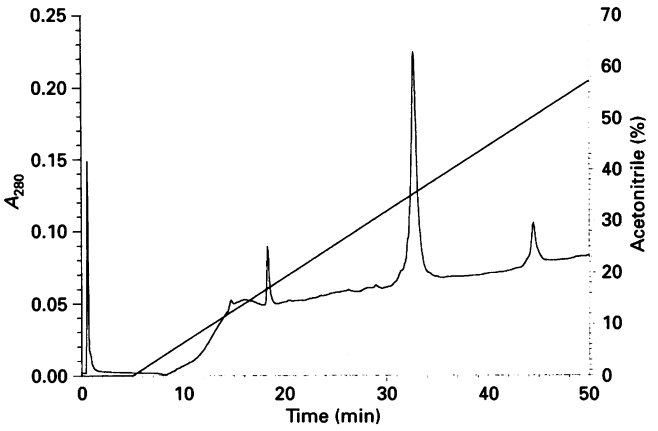


Figure 3 C4 reverse-phase chromatography of fibromodulin

An aliquot of purified fibromodulin, resuspended in 0.1% trifluoroacetic acid (TFA), was applied to a C-4 reverse-phase column and eluted at a flow rate of 1 ml/min. The eluate was monitored at 280 nm. The gradient program was as follows: buffer A (0.1% TFA) for 5 min and then 60 min of a gradient of 0–100% buffer B [70% (v/v) acetonitrile in 0.1% TFA]. Peaks at approx. 18 min and 44.5 min are the result of impurities in the buffers and are seen in a control experiment.

benzamidine hydrochloride, pH 6.8, at 4 °C for 48 h. Insoluble material was removed by filtration and soluble material precipitated at 2 and 4 vol. of ethanol.

Aliquots of material precipitated by 2 and 4 vol. of ethanol were subjected to size-exclusion chromatography as described in Figure 1. The 2 vol. precipitate was shown to have a bi-modal size distribution, with small material ($M_r < 1 \times 10^4$; M_r is expressed in terms of globular protein standards) present together with very large molecules ($M_r > 7.5 \times 10^5$). In contrast, the 4 vol. precipitate gave a single broad unresolved peak (all $M_r < 4 \times 10^5$) with a broad leading shoulder on which a peak at the position of fibromodulin (M_r approx. 1.5×10^5) can be clearly seen.

The 4 vol. precipitate (0.98 g) was subjected to ion-exchange chromatography as described in Figure 2. Fibromodulin was assayed by an e.l.i.s.a. using an antibody specific to its protein core and was shown to be eluted between 0.45 and 0.75 M NaCl.

Fibromodulin-containing fractions were pooled, exchanged into 4 M guanidine/50 mM Tris/HCl (pH 7.3) and subjected to size-exclusion chromatography on a Sepharose CL-6B column (152 cm × 2.7 cm) running in 4 M guanidine/50 mM Tris/HCl (pH 7.3) at a flow rate of 16 ml/min over 48 h; fractions were collected every 30 min. The absorbance of each fraction was determined at 280 nm and the presence of fibromodulin confirmed by an e.l.i.s.a. A single peak of fibromodulin with a K_{av} of 0.5 (the position in which fibromodulin is known to elute) and a minor peak of smaller material was detected. The fibromodulin was pooled, dialysed against 0.2 M NaCl overnight, then dialysed extensively against water before being recovered by lyophilization.

The purity of the fibromodulin prepared in this way was confirmed by 400 MHz ^{1}H-n.m.r. spectroscopy (results not shown), and by reverse-phase chromatography on a Brownlee C4 column. Fibromodulin eluted from the C4 column (Figure 3) in a single peak (peaks at approx. 18 min and 44.5 min are buffer contaminants) with a retention time of approx. 32 min. This is identical with the elution position of fibromodulin isolated from articular cartilage by the method of Plaas et al. (1989).

Analytical methods

Glycosaminoglycan concentrations were monitored on microtitre plates using a 1,9-Dimethylmethylene Blue assay (Farndale et al., 1982). Fibromodulin levels were determined with a microtitre plate e.l.i.s.a. using an antibody specific to the protein core of fibromodulin. A 5 μl aliquot of each fraction of column eluate was added to 195 μl of 20 mM sodium carbonate, pH 9.6, in the well of a microtitre plate and coated overnight at room temperature. Immunoreactivity was determined as previously described (Plaas et al., 1990).

Keratanase digestion

Purified fibromodulin (200 mg) was digested using 7 units of keratanase (EC 3.2.1.103) in 0.2 M sodium acetate, pH 7.2, in

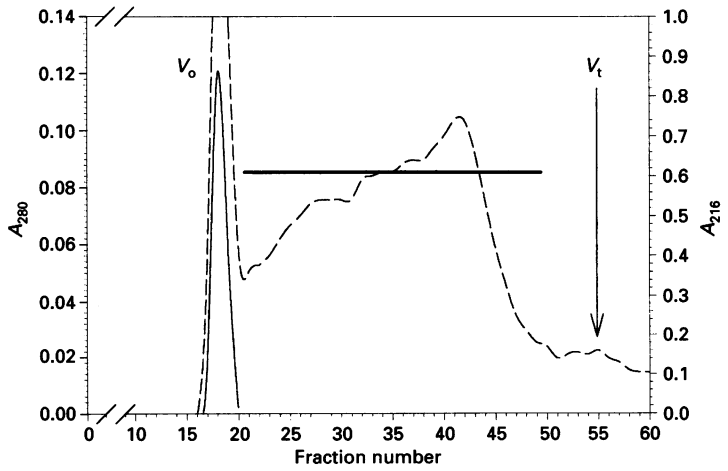


Figure 4 Size-exclusion chromatography of reduced oligosaccharides generated by enzymic digestion

The reduced keratanase digest was applied to a Sephadex G-50 (medium) column (84 cm × 15 mm) and eluted in 0.15 M NaCl at a flow rate of 9 ml/min, fractions being collected over 20 min intervals. The absorbance of the fractions was monitored at 216 nm (– – –) and 280 nm (——). Pooled fractions are indicated by the solid horizontal line.

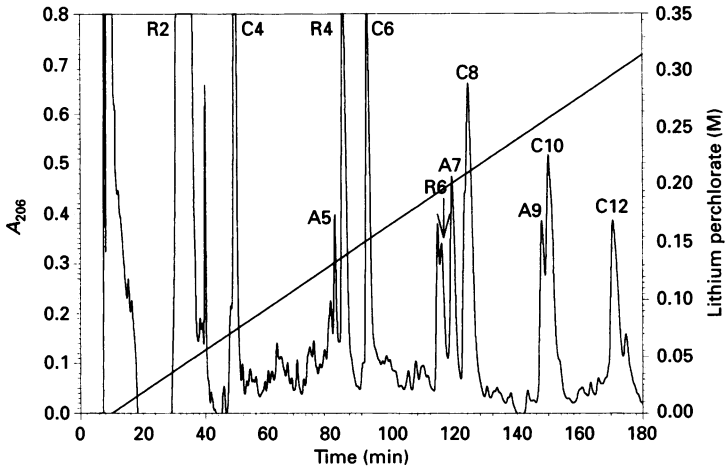


Figure 5 Nucleosil 5SB strong anion-exchange chromatography of reduced oligosaccharides generated from bovine tracheal cartilage fibromodulin by keratanase digestion

The keratanase-derived oligosaccharides were applied to a Nucleosil 5SB column (250 mm × 10 mm) and eluted at a flow rate of 2 ml/min. The eluate was monitored 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 (500 mM $LiClO_4$, pH 5.0). The peak containing oligosaccharide R2 also contains acetate which absorbs strongly at 206 nm.

the presence of 5 mM 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (a sialidase inhibitor) for 24 h at 37 °C.

Alkaline borohydride reduction

The oligosaccharides generated by enzymic digestion were reduced by the addition of $NaBH_4$ (1 M final concentration) at room temperature for 3 h and stopped by stepwise addition of acetic acid.

Sephadex G-50 size-exclusion chromatography

Following reduction the oligosaccharides were separated from the protein core by size-exclusion chromatography on a Sephadex G-50 (medium) column as described in Figure 4. Oligosaccharides were pooled as shown, desalted on a Bio-Gel P-2 column (11 cm × 1 cm) by elution with water at a flow rate of 12 ml/h, then lyophilized.

Strong anion-exchange chromatography

The reduced oligosaccharides were separated by ion-exchange chromatography (Figure 5) on a semi-preparative Nucleosil 5SB column (25 cm × 1 cm) using a Bio-Rad 700 h.p.l.c. titanium gradient system with u.v.- and conductivity-detectors. Oligosaccharides were eluted with a linear gradient of 0–0.5 M lithium perchlorate at a flow-rate of 2 ml/min over 200 min, pooled, desalted on a Bio-Gel P-2 column (11 cm × 1 cm) by elution with water at 12 ml/h, and then lyophilized.

N.m.r. spectroscopy

Samples were buffered to pH 7 with sodium phosphate, referenced with sodium 3-trimethylsilyl[$^2H_4$]propionate (TSP-d_4) as internal standard and dissolved in 0.5 ml of 99.96% ^2H_2O after microfiltration, several previous exchanges with 99.8% ^2H_2O and one using 99.96% ^2H_2O. ^{1}H-n.m.r. spectra were determined at 60 °C using a Bruker AM500 spectrometer with 5-mm probe. All chemical shifts are quoted relative to internal TSP-d_4 at 0.00 p.p.m.

Spectra were reprocessed for presentation using the NMR1 (V 1-4-1) software package supplied by New Methods Research Inc. (Syracuse N.Y., U.S.A.).

RESULTS

The reduced keratanase oligosaccharides derived from KS chains attached to bovine tracheal cartilage fibromodulin were separated by strong anion-exchange chromatography on a Nucleosil 5SB column. The chromatographic profile of these keratanase-derived oligosaccharides (Figure 5) is relatively simple, showing the presence of only a few dominant oligosaccharides. These have been isolated and their structures determined (Figure 6) using ^{1}H-n.m.r. spectroscopy, after examination of the spectra (Figures 7a, 7b and 7c) and comparison with standards. They have been categorized as deriving from the cap region (C, cap; A, asialo cap), and from the repeat region (R, repeat) of the parent KS chain. The number assigned to each oligosaccharide indicates the number of residues present.

A feature common to all of the oligosaccharides is a reducing terminal unsulphated Gal-ol, derived after borohydride reduction of the galactose at which keratanase has cleaved.

Cap region

The partial 500 MHz spectrum of a representative cap-region (C) oligosaccharide is shown in Figure 7(a). The spectra of oligosaccharides C4–C12 were found to have the distinctive signals at approx. 1.80 and 2.76 p.p.m. corresponding to $H(3_{ax.})$ and $H(3_{eq.})$ protons respectively, of *N*-acetylneuraminic acid attached to unsulphated galactose by an α(2-3)-linkage (Dickenson et al., 1991). No signals characteristic of non-reducing terminal galactose or *N*-acetylglucosamine were detected, clearly indicating that each oligosaccharide has its non-reducing terminus capped by α(2-3)-linked *N*-acetylneuraminic acid.

The oligosaccharides C4–C12 elute from the Nucleosil 5SB ion-exchange column at progressively higher salt concentrations. The increasing length of the oligosaccharide is shown by the changing ratio of signals for *N*-acetylglucosamine in different environments within the oligosaccharide (results not shown).

The partial 500 MHz spectrum of a representative asialo-cap

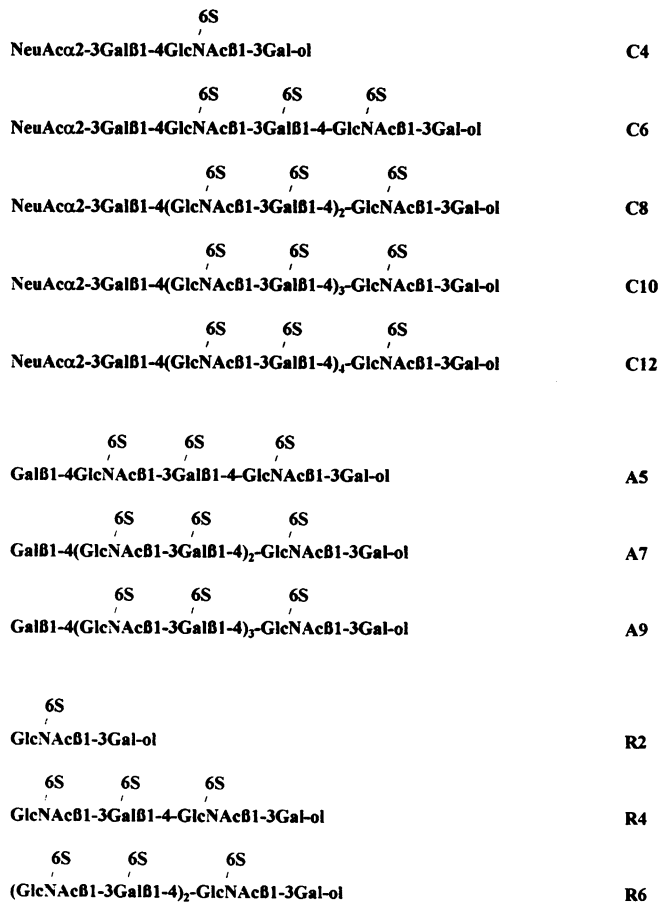


Figure 6 Oligosaccharides derived from the N-linked KS chains attached to fibromodulin from bovine tracheal cartilage which have been isolated and characterized in this study

The *N*-acetylneuraminic acid capping the non-reducing termini is always α(2-3)-linked to the adjacent galactose.

region (A) oligosaccharide is shown in Figure 7(b). The spectra of oligosaccharides A5–A9 lack the α(2-3)-linked *N*-acetylneuraminic acid signals present in oligosaccharides C4–C12 (Figure 7a). However, they have signals at approx. 3.95 p.p.m. from H(4) of a non-reducing terminal unsulphated galactose residue. Oligosaccharides derived from the repeat region by keratanase digestion will have a non-reducing terminal *N*-acetylglucosamine. The oligosaccharides A5–A9 therefore derive from the cap region, but lack the α(2-3)-linked *N*-acetylneuraminic acid of C4–C12.

Each of these asialo oligosaccharides A5–A9 are eluted from the Nucleosil 5SB column at a lower salt concentration than their respective capping fragments, C6–C10. This suggests that they each have a lower charge than the related capping fragments, which possess a charged *N*-acetylneuraminic acid residue. However, it is important to emphasize that the precise order of elution of these oligosaccharide fragments is dependent upon the strong anion-exchange column used.

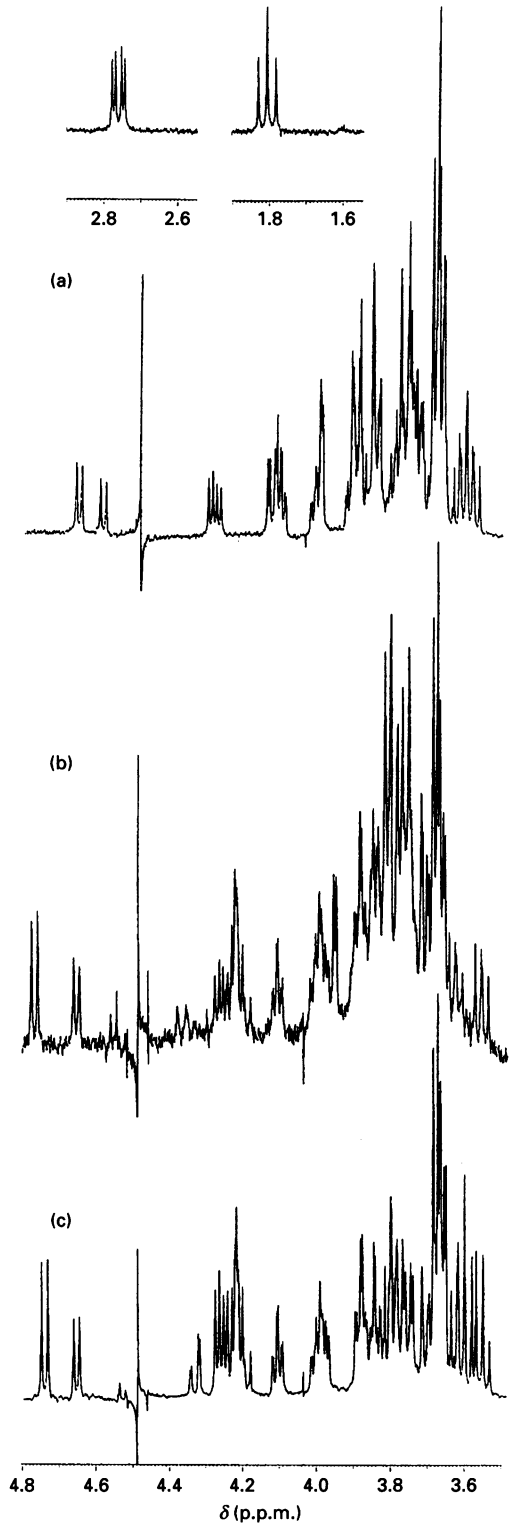


Figure 7 Partial 500 MHz n.m.r. spectra of reduced KS chain fragments isolated from bovine tracheal cartilage and subjected to keratanase digestion

This shows typical examples of the three regions (**a**) capping region fragment C4, a monosulphated tetrasaccharide; (**b**) asialo capping region fragment A5, a trisulphated pentasaccharide and (**c**) repeat region fragment R4, a trisulphated tetrasaccharide.

Repeat region

The partial 500 MHz spectrum of a representative repeat-region (R) oligosaccharide is shown in Figure 7(c). The spectra of oligosaccharides R2–R6 all have signals at approx. 4.75 p.p.m. characteristic of non-reducing-terminal-sulphated *N*-acetyl-

glucosamine residues, as expected for oligosaccharides derived from the repeat region by keratanase digestion. The absence of signals from non-reducing terminal unsulphated galactose residues and *N*-acetylneuraminic acid (Figure 7c) clearly demonstrates that these oligosaccharides derive from the repeat region of the parent KS chain. These oligosaccharides elute from the Nucleosil 5SB ion-exchange column at progressively higher salt concentrations.

DISCUSSION

The procedure used in this study to isolate fibromodulin differs from that of other workers (Plaas et al., 1989) only in its first step. Conventional methodology uses CsCl density-gradient centrifugation to separate fibromodulin from aggrecan, which has a higher buoyant density. In this study the differing solubilities of the two molecules in ethanol solutions have been used to effect separation. The success of this method is shown in Figure 1, which demonstrates the absence of high-buoyant-density material in the fibromodulin-rich material precipitated by 4 vol. of ethanol. The same subsequent procedures, i.e. ion-exchange and size-exclusion chromatography, are used in both methods to isolate pure fibromodulin.

The identity of the fibromodulin thus isolated is confirmed by immunoreactivity with an antibody raised to the core protein of fibromodulin and behaviour during ion-exchange, size-exclusion and reverse-phase chromatography which mirrors that known for fibromodulin. Reverse-phase chromatography (Figure 3) also demonstrates the purity of the fibromodulin, which elutes in a single peak.

The chromatographic profile of these keratanase-derived oligosaccharides (Figure 5) is relatively simple, partly because of the absence of linkage-region fragments, which were not studied in this work. All of the prominent oligosaccharides have been isolated and their structures determined using ^{1}H-n.m.r. spectroscopy by comparison with standards.

Three types of oligosaccharides were isolated and characterized in this study, namely sialylated chain caps, asialo chain caps and repeat-region fragments. Five sialylated chain caps were isolated, each having *N*-acetylneuraminic acid as the chain terminator, α(2-3)-linked to the adjacent unsulphated galactose. *N*-Acetylneuraminic acid with an α(2-6)-linkage was not detected in this study. *N*-Acetylneuraminic acid with an α(2-3)-linkage has been found to be the sole chain-terminating structure on O-linked KS-II-B chains from non-articular cartilage aggrecan (Nieduszynski et al., 1990).

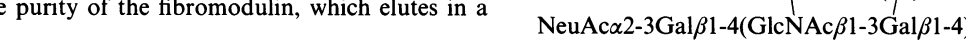
Three cap-region oligosaccharides which lack an *N*-acetylneuraminic acid chain terminator have been characterized. These asialo caps are probably attributable to neuraminidase activity, previously observed in the keratanase used in this study (Dickenson et al., 1992). The *N*-acetylneuraminic acid analogue, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid, included in the keratanase incubation mixture, is a competitive inhibitor and cannot completely abolish neuraminidase activity. It is, however, possible that the *N*-acetylneuraminic acid was either never added to the chain during synthesis, or was removed, *in vivo*, before isolation. It is important to note that keratanase digestion is the only degradative technique which can be used to detect these asialo species. Both hydrazinolysis (Brown et al., 1992) and keratanase II (Brown et al., 1994), because they cleave at the reducing side of an *N*-acetylglucosamine residue, generate repeat-region fragments indistinguishable from asialo capping fragments. Examination of the parent fibromodulin by ^{1}H-n.m.r. spectroscopy does not allow unequivocable determination of the presence or not of asialo caps before keratanase digestion, as the portion of the spectrum containing the characteristic signals (approx. 3.95 p.p.m.) is obscured by signals from the protein core.

Significantly, no repeat-region fragments have been isolated in this study which contain α(1-3)-linked fucose. This structure has also been found to be absent from O-linked KSs from non-articular cartilage aggrecan, although it is found on chains from articular cartilage aggrecan (Nieduszynski et al., 1990).

As for KSs derived from aggrecan, N-linked chains attached to bovine tracheal cartilage fibromodulin possess a repeat region which is always sulphated on C-6 of *N*-acetylglucosamine and may be sulphated on the C-6 of galactose. These data agree with those of Hounsell et al. (1986) who examined the repeat region of N-linked KS chains isolated from bovine cornea. None of the studies performed in this laboratory has yielded any evidence for unsulphated *N*-acetylglucosamine along the main chain repeat region of either N- or O-linked KSs, so it is reasonable to assume that this residue is rarely unsulphated *in vivo*.

Oligosaccharides C4–C12, A5–A9 and R2–R6 have been previously isolated from the O-linked KSs of aggrecan. Full n.m.r. assignments of the following oligosaccharides have been previously published: C4 (Dickenson et al., 1991), C6 (Huckerby et al., 1992), A5 (Huckerby et al., 1992) and R4 (Huckerby et al., 1993).

The repeat region and chain caps of KS chains attached to fibromodulin from bovine tracheal cartilage have been found to have the following general structure:



Each of the three groups of oligosaccharides isolated in this study represent a homologous series, arising from the inability of keratanase to cleave at a sulphated galactose residue adjacent to a sulphated *N*-acetylglucosamine (Nakazawa and Suzuki, 1975). In this study the largest keratanase-resistant oligosaccharides isolated and characterized in each series are a nonasulphated dodecasaccharide sialylated cap; a heptasulphated nonasaccharide asialo cap and a pentasulphated hexasaccharide repeat-region fragment. Gel-permeation chromatography on a Sephadex G-50 column during preparation will have set an upper size limit on the structures studied, as material voided on this column was excluded from further study. Therefore, *in vivo*, the KS chains attached to fibromodulin may contain oligosaccharides longer than a dodecasaccharide.

In this study repeat-region oligosaccharides with eight, ten, or 12 residues were not found, despite the isolation of capping-region fragments with this number of residues. The longest keratanase-resistant blocks therefore occur in the capping region rather than the repeat region. The keratanase profile thus gives a measure of the distribution of sulphated galactose, demonstrating a higher level of galactose sulphation towards the non-reducing terminus than towards the reducing terminus. Oeben et al. (1987) also found an increasing level of galactose sulphation towards the non-reducing terminus of the N-linked KS chains from pig cornea.

KS chains N-linked to bovine tracheal cartilage fibromodulin and O-linked chains from non-articular aggrecan appear to have similar structures in respect of their capping and repeat regions, despite having different modes of linkage to different core proteins. These results highlight the importance of tissue-specific expression of sialyl-transferases and fucosyl-transferases in the elaboration of KSs.

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n.m.r. facility at Leicester University and for funding towards the purchase of a 400 MH⁻ n.m.r. spectrometer at Lancaster.

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