Structural and immunological studies of keratan sulphates from mature bovine articular cartilage

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Two populations of alkaline-borohydride-reduced keratan sulphate (KS) chains were prepared from the two peptido-keratan sulphate trypsin fragments of proteoglycan aggregates isolated from bovine femoral head cartilage (6-year-old animals). Each population was separated by high-performance ion-exchange chromatography on a Pharmacia Mono-Q column into eight pools, Q1–Q8. These were analysed by gel permeation chromatography, radioimmunoassay with the monoclonal antibody MZ15, and 500 MHz ¹H n.m.r. spectroscopy. Upon chromatography on Sephadex G-75 the Mono-Q fractions were shown to increase in hydrodynamic size progressively from Q1 to Q8 for both KS populations. For each population the strongest antigenic response with the anti-KS monoclonal antibody MZ15 was expressed by the two fractions of greatest size and charge density, Q7 and Q8. Proton n.m.r. spectroscopic studies on the two series of fractions demonstrated: (i) a progressive increase in the level of galactose sulphation from Q1 to Q8, (ii) the presence of approximately one $\alpha(1\text{--}3)$ -linked fucose residue per chain in every sample, and (iii) the presence of *N*-acetylneuraminic acids in three discrete environments, two $\alpha(2\text{--}3)$ - and one $\alpha(2\text{--}6)$ -linked in every sample. The results are discussed in terms of a possible heterogeneity in the carbohydrate–protein linkage region of keratan sulphates from bovine articular cartilage.

INTRODUCTION

Cartilage proteoglycans contain a protein core, whose complete primary structure has recently been deduced from cDNA clones (for Swarm rat chondrosarcoma; Doege *et al.*, 1987), upon which numerous chondroitin sulphate and keratan sulphate chains as well as *O*-linked and *N*-linked oligosaccharides occur (Hardingham, 1986).

The keratan sulphate content of cartilage increases with age (Mathews & Glagov, 1966), as does the chain size (Theocharis *et al.*, 1985) and level of sulphation (Greiling & Baumann, 1973).

Most keratan sulphate chains are believed to be located within a cluster upon a single domain, the so-called keratan sulphate-rich region, of the protein core (Heinegård & Axelsson, 1977), although some isolated chains may occur within the chondroitin sulphate-rich regions (see, e.g., Sheehan *et al.*, 1987). Keratan sulphate chains recovered from both the KS-rich and the chondroitin sulphate-rich regions are similar both in size (Sweet *et al.*, 1979) and in structure.

The complete primary structure of cartilage keratan sulphate (skeletal KSII, after the notation of Meyer, 1970) is not fully understood, but may be considered in terms of a linkage region, a repeating disaccharide unit

and a capping sialic acid residue. The chains are *O*-linked from *N*-acetylgalactosamine to either threonine or serine (Bray *et al.*, 1967) in the protein core, and the disaccharide *N*-acetylneuraminyl-galactose is attached to C-3 of the *N*-acetylgalactosamine residue (Hopwood & Robinson, 1974). The major part of the keratan sulphate chain is believed to be linked to C-6 of the *N*-acetylgalactosamine residue (Lohmander *et al.*, 1980), and the repeating component is the disaccharide *N*-acetyl-lactosamine which is usually sulphated at the C-6 positions of most of the *N*-acetylglucosamine residues and some of the galactose residues (Roden, 1980). Sialic acid (Seno *et al.*, 1965), fucose (Gardell, 1957; Seno *et al.*, 1965) and mannose (Bhavanandan & Meyer, 1968; Hascall & Riolo, 1972) have all been reported as minor components of skeletal keratan sulphates.

Monoclonal antibodies to keratan sulphates have been produced in several laboratories (Caterson *et al.*, 1983; Zanetti *et al.*, 1985; Keiser & Diamond, 1987) and the antigenic determinants recognized by some of these have been examined (Mehmet *et al.*, 1986; Tang *et al.*, 1986). These antibodies have also been used in clinical investigations (Thonar *et al.*, 1985, 1988; Williams *et al.*, 1988; Sweet *et al.*, 1988) to monitor keratan sulphate concentrations in body fluids.

Abbreviations used: KS, keratan sulphate; Q, Mono-Q fractionated; TSP, sodium 3-trimethylsilylpropionate.

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There have been previous high-field n.m.r. spectroscopic studies of keratan sulphates; a ^1H investigation of corneal KS (Hounsell *et al.*, 1986) and a ^1H and ^{13}C examination of shark KS (Cockin *et al.*, 1986). In this study the heterogeneity of skeletal KS structure is addressed and the ^1H n.m.r. spectroscopic assignments for peripheral sialic acid and fucose residues are reported.

EXPERIMENTAL

Materials

Guanidinium chloride (practical grade) was purchased from Sigma and further purified as described in Thornton *et al.* (1987).

Benzamide hydrochloride, *N*-ethylmaleimide and sodium borohydride were also purchased from Sigma. Caesium chloride, disodium EDTA and 6-amino-hexanoic acid were obtained from BDH. Lithium perchlorate (A.C.S. grade) and piperazine (99%) were from Aldrich. 1,9-DimethylMethylene Blue was purchased from Koch-Light and sodium 3-trimethylsilyl($^2\text{H}_4$)-propionate [$(^2\text{H}_4)$ TSP] from Lancaster Synthesis.

Sepharose CL-6B, Sephadex G-50 and G-75 and the Mono-Q HR 10/10 column were purchased from Pharmacia. Bio-Gel P-10 was from Bio-Rad. The enzymes diphenylcarbamoyl chloride (DPCC)-treated trypsin (bovine pancreas, EC 3.4.21.4), chondroitinase ABC (*Proteus vulgaris*, EC 4.2.2.4) and keratanase (*Pseudomonas* species, EC 3.2.1.103) were purchased from Sigma.

Analytical methods

Hexose was determined by an automated anthrone assay (Heinegård, 1973) with galactose as the standard. Sialic acid was assayed by the procedure of Lohmander *et al.* (1980). Uronate was determined by an automated procedure (Heinegård, 1973) based on the modified carbazole assay (Bitter & Muir, 1962) with glucuronolactone as the standard. KS was assayed using 1,9-dimethylMethylene Blue (Farndale *et al.*, 1982).

The monoclonal antibody MZ15 was used in radioimmunoassay as described previously (Zanetti *et al.*, 1985). It has been shown (Mehmet *et al.*, 1986) that MZ15 binds to a pentasulphated hexasaccharide and larger related oligosaccharides. The keratan sulphate epitope was determined by competition with a purified proteoglycan monomer from pig laryngeal cartilage and results are therefore expressed in mol of proteoglycan (Zanetti *et al.*, 1985).

Proteoglycan aggregates (A1 fraction) were prepared from bovine femoral head cartilage (6-year-old animals) essentially as described by Thornton *et al.* (1987). The A1 fraction (approx. 7.5 mg of proteoglycan/ml) was dialysed into 0.1 M-Tris/acetate, pH 7.3, and then digested with chondroitinase ABC (2.5 units/g of proteoglycan) followed by DPCC-treated trypsin (2 mg/g of proteoglycan). The digest was partially freeze-dried and then chromatographed on a column of Sepharose CL-6B eluted with 0.5 M-sodium acetate/10 mM-EDTA, pH 6.8. Peptido-keratan sulphates of two sizes (labelled 6B1 and 6B2) were isolated, dialysed against water and lyophilized.

KS chains were prepared by alkaline borohydride reduction (Carlson, 1968) of each of the 6B1- and 6B2-series peptido-keratan sulphates (5 mg of peptido-KS/ml of 1 M-sodium borohydride in 0.05 M-NaOH) followed

by desalting on Bio-Gel P2. The preparation was then digested with chondroitinase ABC (2 units/g) and the keratan sulphates were finally recovered by lyophilization after desalting on a column of Sephadex G-50 eluted with 0.2 M- NH_4HCO_3 .

The KS chains were subjected to high-performance ion-exchange chromatography on a Mono-Q HR 10/10 column eluted with a linear gradient of 0–0.5 M-lithium perchlorate/10 mM-piperazine, buffered to pH 5.0, and fractions (Mono-Q 1–8) were pooled as indicated (see Fig. 1). These samples were desalted by gel filtration on Sephadex G-75 (67 cm $\times$ 1.0 cm), eluted with 0.2 M- NH_4HCO_3 and then freeze-dried.

N.m.r. spectroscopy

Individual fractions (0.5–5 mg) were buffered to pH 7 and referenced using ($^2\text{H}_4$)TSP as internal standard for ^1H n.m.r. spectroscopy as previously described (Sander-son *et al.*, 1987). Proton n.m.r. spectra were determined at 400 or 500 MHz using Bruker WH400 and AM500 spectrometers respectively using 5 mm V.T. probes at 60 °C. Spectra were re-processed for presentation using the computer program NMR1 (Lab One NMR1 Spectroscopic Data Analysis System, Release 3.8; New Methods Research Inc., Syracuse, NY, U.S.A.).

Keratanase digestion

KS was dissolved in 0.2 M-sodium acetate, pH 7.4, and digested for 24 h with keratanase (1 unit/2.8 mg of sample) at 37 °C. Digests were chromatographed in 0.2 M- NH_4HCO_3 on a Bio-Gel P-10 column (81 cm $\times$ 1.0 cm) eluted at 2 ml/h.

RESULTS

The reduced KS chains (of the 6B1 and 6B2 series) were again treated with chondroitinase ABC and fractionated on Sephadex G-50 (results not shown). The V_0 fractions, which were substantially free of the smaller *O*-linked oligosaccharides and chondroitin sulphate-derived oligosaccharides, were pooled and fractionated by high-performance ion-exchange chromatography on a Mono-Q column. The elution profile (for KS chains from the 6B1 series) is shown in Fig. 1. Similar results were obtained for KS chains of the 6B2 series (results not shown). Indeed, there was no distinction between 6B1- and 6B2-derived KS fractions in any of the experimentation. Eight individual peaks were most clearly observed in the sialic acid assay and fractions 6B1-Q(1–8) were pooled as indicated (Fig. 1). In a parallel experiment on the 6B2 material eight peaks were again observed and the fractions 6B2-Q(1–8) were collected. Each of these individual fractions Q(1–8) of both series were desalted and sized by gel filtration on Sephadex G-75 and the elution profiles for the 6B1 series are shown in Fig. 2. In the cases of 6B1-Q1 and 6B2-Q1 this fractionation also permitted the removal of residual *O*-linked oligosaccharides.

The fractions from the Mono-Q column were also monitored by radioimmunoassay using the monoclonal antibody MZ15 and it is clear from Fig. 1 that the strongest antigenic response arises in the fractions Q7 and Q8. Identical results were obtained in the Mono-Q run on the 6B2-derived KS.

Keratanase digestion followed by gel-permeation chromatography on Bio-Gel P-10 was only performed

Fig. 1. High-performance ion-exchange chromatography profile of KS chains

KS chains were chromatographed on a Mono-Q HR 10/10 column at a flow rate of 4.0 ml/min. The gradient programme was as follows: 4.0 min of buffer A (10 mM-piperazine, pH 5.0) and then 20 min of 0–50 % buffer B (1.0 M-lithium perchlorate, pH 5.0). Fractions (1.0 ml) were assayed for hexose (A_{620} , —, upper trace), sialic acid (A_{620} , ·····, lower trace) and MZ15 anti-KS monoclonal antibody activity (expressed as pmol/100 μ l; ·····). The broken line indicates the percentage of buffer B (as programmed). Fractions were pooled as shown.

Fig. 2. Sephadex G-75 gel-chromatography profiles of KS fractions of 6B1 series from the Mono-Q column

The fractions from the Mono-Q column (Q1–Q8) were chromatographed on Sephadex G-75 [(a) Q8–(h) Q1]. The column (67 cm $\times$ 1.0 cm) was eluted at a flow rate of 4.0 ml/h with 0.2 M- NH_4HCO_3 and fractions (1.0 ml) were assayed with 1,9-dimethylMethylene Blue (A_{540}). The V_0 and V_t were determined using bovine articular cartilage proteoglycan and NaCl, respectively.

on fraction 6B1-Q4 and the results showed that a significant proportion of the keratanase-resistant oligosaccharides were of dodecasaccharide size or larger. Further digestions were not performed because the chromatographic technique was not sensitive enough for adequate sizing of the largest keratanase-resistant fragments formed.

Each of the discrete fractions Q(1–8) was subjected to 400 or 500 MHz ^1H n.m.r. spectroscopy and a typical spectrum (for 6B2-Q5) is shown in Fig. 3. There are several regions of interest in these spectra that relate to the presence of fucose and sialic acid residues and to the degree of sulphation.

First, every fraction shows a clear doublet resonance at 1.17 p.p.m. (which has a coupling of about 6 Hz) and another at 5.12 p.p.m. These peaks are characteristic of $-\text{CH}_3$ and H-1 resonances, respectively, in fucose residues. The peak at 1.17 p.p.m. is seen in a two-dimensional correlated-spectroscopy (2D-COSY) spectrum to be coupled to another resonance at about 4.75 p.p.m. (fucose H-5), from which it seems most probable that the fucose residues are linked $\alpha(1-3)$ (Vliegthart *et al.*, 1983). The relative peak areas of the fucose resonances decrease from fraction Q1 to Q8, and are consistent with the presence of one fucose residue per KS chain.

Secondly, the spectrum of each fraction (see, for example, Fig. 4) shows resonances, in two groups, at 1.69, 1.785 and 1.795 p.p.m. and at 2.69, 2.76 and 2.78 p.p.m. These peaks are clearly recognizable as families of H-3_{ax} and H-3_{eq} signals from NeuNAc residues in three different environments (Vliegthart *et al.*, 1983); their assignments are again confirmed by 2D-COSY experiments. They correspond to $\alpha(2-6)$ -linked NeuNAc in one environment and $\alpha(2-3)$ -linked NeuNAc residues in two separate environments. The relative proportion of the $\alpha(2-6)$ -linked NeuNAc decreases with respect to the $\alpha(2-3)$ -linked NeuNAc between fractions Q1 and Q8.

Fig. 3. 500 MHz ^1H n.m.r. spectrum of KS fraction 6B2-Q5

Fig. 4. Detail of ^1H n.m.r. spectrum of KS fraction 6B1-Q3 showing NeuAc resonances

Thirdly, there are several signals in the ^1H n.m.r. spectra which are sensitive to the level of sulphation in the individual fractions. The majority of the *N*-acetylglucosamine residues in these samples may be assumed to be sulphated as judged by the ^{13}C n.m.r. spectrum (not shown) of the parent KS material prior to fractionation on Mono-Q. However, it is clear that the gross level of sulphation of the galactose residues increases progressively throughout the fractions Q1–8. This is seen in Fig. 5, where it can be observed that the resonance at 3.97 p.p.m., which is characteristic of sulphated galactose H-5, increases in relative proportion from Q1 to Q8 until at Q8 it is almost equivalent to that observed in the keratanase-resistant fully sulphated shark KS. A further interesting feature is seen in the resonances at 4.74–4.76 p.p.m. These correspond to H-1 in sulphated *N*-acetylglucosamine. These resonances are sensitive to their immediate environment, for in 6B2-Q2 a doublet occurs at 4.74 p.p.m. but in 6B2-Q8 this doublet is seen at 4.76 p.p.m. In an intermediate sample such as 6B2-Q4 the two overlapped doublets occur. It seems likely that this reflects the presence or absence of sulphate on the adjacent galactose on the reducing side.

A further feature of the spectra is the presence of a doublet at 4.98 p.p.m. of unknown origin that decreases in relative intensity between Q1 and Q4 and then increases again between Q5 and Q8 for both the 6B1 and 6B2 KS chain series.

DISCUSSION

The Mono-Q fractionation separated both the 6B1- and 6B2-derived materials into eight partly resolved components. (Structured profiles have since also been

Fig. 5. Details from ^1H n.m.r. spectra from KS fractions

(a) 6B2-Q2, (b) 6B2-Q4, (c) 6B2-Q8, and (d) KS from shark cartilage (fully sulphated).

observed for KS samples isolated from other sources.) It is clear from the gel-filtration results that the fractions Q1 to Q8 increase progressively in size, and equally apparent from the ^1H n.m.r. spectroscopic results that the gross galactose sulphation level increases from Q1 to Q8. It is perhaps for this reason that the Mono-Q column may have effected a separation, as the resolution would be the consequence of the combined effects of size and of charge density. There is evidence from ^{13}C n.m.r. spectroscopy that most of the *N*-acetylglucosamine residues in the unfractionated 6B1- and 6B2-derived materials are 6-sulphated. Thus, the increasing chain size does seem to correspond to the addition of further disulphated disaccharide units, as is found for corneal KS (Oeben *et al.*, 1987).

The observation of three sialic acid environments is similar to the situation found in the three major *O*-linked oligosaccharides (Lohmander *et al.*, 1980). This suggests that KS chains from bovine articular cartilage may have several different carbohydrate structures adjacent to the linkage to protein.

Each KS chain would appear to have one fucose residue in it, unlike the *O*-linked oligosaccharides that, from n.m.r. spectroscopic evidence, do not appear to contain fucose (results not shown). The chemical shift data indicate that the fucose is clearly $\alpha(1\text{--}3)$ -linked (Vliegthart *et al.*, 1983), and this may be of significance to KS catabolism as hepatocyte receptors exist for fucose

linked in this manner to *N*-acetylglucosamine (Prieels *et al.*, 1978).

The strong response of the largest and most highly charged KS chains in the radioimmunoassay with the monoclonal antibody MZ15 perhaps indicates that this antibody requires two epitopes in one chain for strong interaction, as it is known (Mehmet *et al.*, 1986) that the smallest fragment to elicit any response with this antibody is the linear pentasulphated hexasaccharide. The results of keratanase (from *Pseudomonas* species) digestion of 6B1-Q4 support this idea. On examination of the digestion pattern on Bio-Gel P-10 it is clear that approximately half of the anthrone-positive material is of dodecasaccharide size or larger. If the enzyme is specific for cleavage in blocks of -GlcNAc-Gal-GlcNAc and not -GlcNAc(SO₃)-Gal-GlcNAc then clearly dodecasaccharide-sized blocks of fully or partially sulphated KS occur in this fraction. It may, therefore, be assumed that fractions Q7 and Q8 will have significantly larger keratanase-resistant blocks with a consequent increase in the probability that two antigenic sites will be present.

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