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There are two major types of skeletal keratan sulphates

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High-field ^{1}H-n.m.r.-spectroscopic studies supported by chemical carbohydrate analyses show that skeletal keratan sulphates (KS-II) of bovine origin may be sub-classified into two groups. Keratan sulphate chains from articular and intervertebral-disc cartilage (KS-II-A) contain two structural features, namely $\alpha(1\rightarrow3)$-fucose and $\alpha(2\rightarrow6)$-linked *N*-acetylneuraminic acid residues, that are absent from keratan sulphates from tracheal or nasal-septum cartilage (KS-II-B).

INTRODUCTION

Keratan sulphates (KS) are glycosaminoglycans that were originally classified into two major types according to their mode of linkage to protein (Bray *et al.*, 1967). Thus KS-I represented carbohydrate chains, isolated from cornea, that were *N*-linked from *N*-acetylglucosamine to asparagine, and KS-II were chains, from skeletal tissues such as cartilage, that were *O*-linked from *N*-acetylgalactosamine to serine/threonine. For a review of structures see Stuhlsatz *et al.* (1989). A further type of KS has been isolated from brain tissue that is apparently *O*-linked from mannose to serine/threonine (Krusius *et al.*, 1986).

Skeletal keratan sulphates occur within large cartilage proteoglycans (see, e.g., Hardingham, 1986), and the chains are attached at serine or threonine residues that occur within a repeating hexapeptide motif (Antonsson *et al.*, 1989).

EXPERIMENTAL

Materials and general methods

The chemicals, enzymes and chromatographic media used in this investigation were as described in Dickenson *et al.* (1990).

Keratan sulphate chains were isolated from four bovine tissues, namely femoral-head cartilage (6–8-year-old animals), nucleus pulposus of intervertebral disc (6–8-year-old animals), tracheal rings (15–18-month-old animals) and nasal septum (6–8-year-old animals), by using methods previously described (Thornton *et al.*, 1989*a*,*b*). In brief, the tissues were comminuted and extracted in 4 M-guanidinium chloride in the presence of proteinase inhibitors, then subjected to an associative CsCl-density-gradient centrifugation, and the bottom fractions containing proteoglycan aggregates were recovered. These were digested with chondroitin ABC lyase followed by diphenylcarbamoyl chloride-treated trypsin, and the large peptido-KS fragments, the so-called KS-rich regions (Heinegård & Axelsson, 1977), were isolated on a Sepharose CL-6B column. The peptido-KS fragments were reduced with alkaline borohydride, and the KS chains were separated from *O*-linked oligosaccharides and chondroitin sulphate-derived oligosaccharides on Sephadex G-50. These KS chain preparations were then applied to a Pharmacia Mono-Q column, which provided further purification, and typically yielded six to eight KS fractions (Thornton *et al.*, 1989*b*) that were significantly more homogeneous in molecular size and charge density than the pre-fractionated material. The molecular sizes of these KS fractions were determined by gel-permeation chromatography on a Bio-Gel TSK-30XL column that had been pre-calibrated with KS oligosaccharides of known size.

The individual KS fractions from each preparation were examined by chemical carbohydrate analysis and by high-field ^{1}H-n.m.r. spectroscopy.

Carbohydrate analyses were performed under conditions similar to those previously described (Lohmander, 1986). Keratan sulphate samples (up to 0.1 mg) were hydrolysed for 4 h at 94 °C in 0.2 ml of 4 M-trifluoroacetic acid, which was subsequently removed on a rotary evaporator. To each hydrolysate was added 0.2 ml of 5 mM-H_2SO_4 (0.5 ml in the case of the tracheal sample), and the solution was filtered before injection of 20 μl portions on to the column. Galactose and fucose contents and ratios were determined on a Bio-Rad Laboratories HRLC 700 chromatography system with an Aminex HPX-87H column, maintained at 40 °C and eluted with 5 mM-H_2SO_4. High sensitivity was achieved by post-column derivative formation (Honda *et al.*, 1981) with a mixture of 1% (v/v) 2-cyanoacetamide and 0.2 M-borate buffer, pH 10.0, and monitoring at 276 nm.

N.m.r. spectroscopy

^{1}H-n.m.r. spectra were determined at 60 °C with a Bruker AM500 spectrometer. Samples (1–2 mg) were buffered to pH 7 with phosphate, referenced with internal sodium trimethylsilyl[$^2H_4$]propionate and dissolved in 99.96% ^2H_2O (0.5 ml) after micro-filtration and several exchanges with 99.8% ^2H_2O. Preliminary n.m.r. determinations were made with a Bruker WH400 instrument under similar conditions. The residual ^{2}HOH resonance was suppressed by using gated decoupling, and responses were sampled into 4096 complex points. Data were transferred to a VAX 11-785 computer and re-processed for display with the computer program suite NMR1 (Lab One NMR1 Spectroscopic Data Analysis System, Release 3.92; New Methods Research, Syracuse, NY, U.S.A.) and zero-filling to 32000 complex points before Fourier transformation.

RESULTS AND DISCUSSION

We have examined KS fractions from many cartilage preparations using ^{1}H-n.m.r. spectroscopy, which is a technique that is particularly suited to the recognition of minor carbohydrate components in such molecules. Partial n.m.r. spectra from

Abbreviations used: KS, keratan sulphate.

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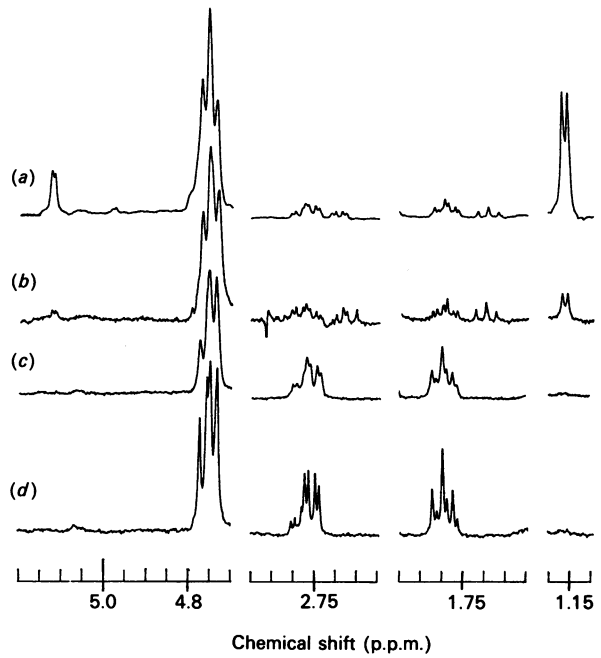

Fig. 1. Partial 500 MHz proton n.m.r. spectra for KS chain fractions isolated from bovine cartilage tissues

(*a*) Femoral-head cartilage, M_r 5600; (*b*) intervertebral disc, nucleus pulposus, M_r 5700; (*c*) tracheal-ring cartilage, M_r 3300; (*d*) nasal septum, M_r 3400. Chemical shifts are shown in p.p.m. relative to sodium trimethylsilyl[$^2H_4$]propionate, with scale divisions at 0.05 p.p.m. intervals. Signal components from within each fraction are shown on a common vertical scale.

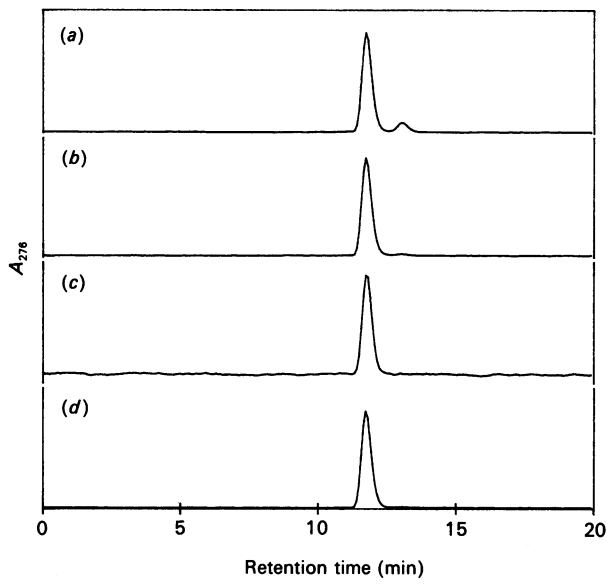

Fig. 2. Chromatograms on an Aminex HPX-87H column, monitored at 276 nm, showing the galactose (retention time 11.74 min) and fucose (retention time 13.02 min) contents of KS fractions derived from bovine cartilage tissues

(*a*) Femoral-head cartilage, galactose 14.87 nmol, fucose 1.88 nmol; (*b*) intervertebral disc, nucleus pulposus, galactose 11.45 nmol, fucose 0.21 nmol; (*c*) tracheal-ring cartilage, galactose 1.36 nmol, fucose not detected; (*d*) nasal septum, galactose 13.96 nmol, fucose 0.16 nmol. The limit of fucose detection is 0.05 nmol with this technique; however, fucose was not detected in any of seven tracheal samples, most of which contained 5 nmol of galactose. Thus the fucose content (if any) of the tracheal samples is less than 1 % of the galactose content.

representative fractions of purified keratan sulphates derived from four tissues are shown in Fig. 1. The major resonances to be observed (Thornton *et al.*, 1989*b*) are the anomeric signals for fucose and *N*-acetylglucosamine at 5.12 p.p.m. and 4.74–4.76 p.p.m. respectively, the H-3 equatorial and axial signals for *N*-acetylneuraminic acids at approx. 2.75 p.p.m. and approx. 1.75 p.p.m. respectively, and the fucose methyl signal at 1.17 p.p.m. Examination of Fig. 1 shows that the spectra have many common signals, but, in addition, the spectra from samples from femoral-head cartilage and intervertebral disc show signals that are absent from the spectra of KS from tracheal rings and nasal septum. Thus all of the spectra demonstrate the presence of *N*-acetylglucosamine residues adjacent to both sulphated and non-sulphated galactose residues, and at least two environments of *N*-acetylneuraminic acid residues that are $\alpha(2\rightarrow3)$-linked (see signals at 2.79 p.p.m., 2.76 p.p.m. and at 1.80 p.p.m.). However, the femoral-head and intervertebral-disc KS samples in addition show the presence of $\alpha(1\rightarrow3)$-linked fucose (see signals at 5.12 p.p.m. and 1.17 p.p.m.) and $\alpha(2\rightarrow6)$-linked *N*-acetylneuraminic acid (see signals at 2.695 p.p.m. and 1.70 p.p.m.).

Confirmation of the relative galactose and fucose contents of these KS samples was derived from the sensitive carbohydrate analyses shown in Fig. 2. A similar pattern of behaviour is noted to that shown in the n.m.r.-spectroscopic data, with fucose clearly observable in the femoral-head- and intervertebral-disc-derived KS samples. However, no fucose was detected in the tracheal-ring KS, and only a minute quantity in the nasal-septum KS. The molar ratios of galactose to fucose were 7.9:1, 53.7:1 and 85.5:1 for KS from femoral head, intervertebral disc and nasal septum respectively. It is believed that the fucose detected in this particular nasal-septum sample (some other fractions contained no detectable fucose), which is highly sub-stoichiometric (perhaps one fucose residue per 20 KS chains), represents a contaminant, possibly another KS type, but either from a small KS proteoglycan (Plaas *et al.*, 1989) or from the hyaluronan-binding region (Baker, 1989). A small shoulder is sometimes observed on the low-field side of the fucose anomeric signal at 5.12 p.p.m. in our articular-cartilage-derived KS samples, which may represent the same contaminant.

It should be emphasized that the fucose residues and each of the three (or more) discrete *N*-acetylneuraminic acid residues are non-reducing termini (Dickenson *et al.*, 1990). The relative sialic acid contents with respect to galactose or *N*-acetylglucosamine are probably related to the molecular sizes of the KS chains. The estimated M_r values of the fractions studied are quoted in the legend to Fig. 1.

It is concluded that there are two structurally distinct types of skeletal keratan sulphates, and it is suggested that they be sub-classified as KS-II-A (Articular) for the femoral-head and intervertebral-disc materials, and as KS-II-B (Basic) for the tracheal and nasal-septum keratan sulphates. It is, as yet, unknown whether this structural distinction is related to function, but it may be noted that the KS-II-A molecules are isolated from load-bearing tissues, unlike the KS-II-B ones.

The two types of skeletal KS are believed to be closely related. Preliminary studies directed at elucidating the stoichiometry and positions of the fucose and sialic acid residues suggest that the detailed KS molecular structure is dependent upon the tissue of origin. It seems likely that fucose and the $\alpha(2\rightarrow6)$-linked *N*-acetylneuraminic acid in KS-II-A may be parts of recognition or binding sites for interactions with other articular-cartilage macromolecules.

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