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A sub-population of keratan sulphates derived from bovine articular cartilage is capped with α(2–6)-linked *N*-acetylneuraminic acid residues

Affinity chromatography using immobilized *Sambucus nigra* lectin and characterization using ^{1}H n.m.r. spectroscopy

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Alkaline borohydride-reduced keratan sulphate (KS) chains derived from bovine femoral head cartilage were fractionated by lectin affinity chromatography with *Sambucus nigra* agglutinin (SNA) into binding and non-binding populations. Analysis of the SNA-binding and non-binding KS chains using 600 MHz ^{1}H n.m.r. spectroscopy showed that the former population contained α(2–6)-*N*-acetylneuraminic acid residues and the latter contained primarily α(2–3)-*N*-acetylneuraminic acid residues as chain terminators. Both populations contained a similar proportion of α(2–3)-*N*-acetylneuraminic acid residues within their protein-linkage regions, and similar sulphation and fucosylation levels. Analysis of these two fractions by gel-permeation chromatography (g.p.c.) on a TSK-30 XL column showed them to have the same size distributions. It was concluded from the n.m.r. spectra and g.p.c. data that the populations differed primarily in the mode of linkage of the chain-terminating sialic acids.

INTRODUCTION

Keratan sulphate (KS) species have been classified according to their linkage to protein as KS-I for the *N*-linked chains derived from cornea [or cartilage fibromodulin (Plaas *et al.*, 1990)] and KS-II for the *O*-linked chains from skeletal tissues such as cartilage (Bray *et al.*, 1967). A further type which is also *O*-linked, but from mannose to serine or threonine, has been isolated from brain tissue (Krusius *et al.*, 1986).

Skeletal KSs occur mostly within the so-called KS-rich region of the large cartilage proteoglycan, or aggrecan, whose entire protein-core sequence is now known (Doege *et al.*, 1987, 1991; Antonsson *et al.*, 1989). These molecules have assumed particular significance with the advent of various anti-KS monoclonal antibodies (Caterson *et al.*, 1983; Zanetti *et al.*, 1985; Funderburgh *et al.*, 1987; Keiser & Diamond, 1987; Hoadley *et al.*, 1990) and clinical assays for KS in body fluids, which are being used as monitors of, and markers for, various arthritic diseases (Thonar *et al.*, 1985; Pavelka & Seibel, 1989; Poole *et al.*, 1990; Mehraban *et al.*, 1991).

However, there is still uncertainty about the entire primary structure of KS-II chains (for reviews see Stuhlsatz *et al.*, 1989; Nieduszynski *et al.*, 1990*a*). In particular, it is not clear whether branching occurs within the main KS chain, or whether discrete sub-populations of chain types exist. Recent studies (Nieduszynski *et al.*, 1990*b*) have suggested that a structural distinction should be made between chains from articular cartilage (KS-II-A) that contain both α(1–3)-linked fucose and α(2–6)-linked *N*-acetylneuraminic acid, and those from non-articular cartilages (KS-II-B) that contain neither of these features. It is now known that the α(1–3)-fucose residues occur on the main poly-*N*-acetyl-lactosamine repeat sequence (Tai *et al.*, 1991), and that the α(2–6)-*N*-acetylneuraminic acid residues may occur (Dickenson *et al.*, 1992) in non-reducing termini of structure:

NeuAcα2–6Galβ1–4GlcNAc(6SO$_3$)β1–3Gal–

In this study, the immobilized lectin, *Sambucus nigra* agglutinin (SNA), which is known to bind selectively to α2–6NeuAcGal/GalNAc (Broekart *et al.*, 1984; Shibuya *et al.*, 1987*a*,*b*), has been used to examine the distribution of this structural feature within the KS chain populations.

MATERIALS AND METHODS

Materials

The immobilized SNA gel (SNA–agarose) was purchased from EY Laboratories (via Bradsure Biologicals, Market Harborough, Leics., U.K.). The Mono-Q HR 10/10 column and the Sephadex G-50 column were from Pharmacia (Uppsala, Sweden). The Bio-Gel TSK 30 XL column was from Bio-Rad Laboratories Ltd. (Watford, Herts., U.K.).

Chondroitin ABC lyase was purchased from Seikagaku Kogyo Co. (via ICN Biomedicals Ltd., High Wycombe, Bucks., U.K.), and papain was from Sigma (Poole, Dorset, U.K.).

1,9-Dimethylmethylene Blue, $LiClO_4$ and anhydrous piperazine were from Koch–Light (Haverhill, Suffolk, U.K.), Aldrich Chemical Co. (Poole, Dorset, U.K.) and Fluka (Glossop, Derbs., U.K.) respectively. Phosphate-buffered saline (PBS; 137 mM-NaCl/2.7 mM-KCl/10 mM-sodium/potassium phosphate, pH 7.4), ethylenediamine (EDA) and $NaBH_4$ were from Sigma (Poole, Dorset, U.K.).

All other chemicals and reagents used were analytical grade, except for the industrial-grade ethanol used for fractionation.

Abbreviations used: KS, keratan sulphate; SNA, *Sambucus nigra* agglutinin; g.p.c., gel-permeation chromatography; PBS, phosphate-buffered saline; EDA, ethylenediamine; TSP, 3-trimethylsilyl[$^2H_4$]propionic acid.

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Analytical methods

KS concentrations were monitored on microtitre plates using a 1,9-Dimethylmethylene Blue assay (Klompmakers & Hendriks, 1986).

H.p.l.c. system

H.p.l.c. was performed on a Bio-Rad series 700 HRLC titanium gradient and isocratic system using u.v., refractive index and conductivity detectors.

Preparation of KS chains

Diced bovine (6–8-year-old animals) femoral head cartilage (47.8 g) was added to 200 ml of 0.2 M-NaCl/50 mM-EDTA/10 mM-cysteine hydrochloride/50 mM-NaH_2PO_4, pH 7.0, and the temperature was raised to 65 °C. Papain (480 units) was added at intervals over 24 h. After centrifugation at 17000 *g* for 4 h in an MSE HI-SPIN 21 centrifuge, the supernatant was recovered. The glycans in the supernatant were precipitated using 4 vol. of ethanol and were left at 4 °C for 48 h, after which the precipitate was dissolved in 200 ml of 0.2 M-sodium acetate and filtered through Whatman glass microfibre filters. The resultant solution was then subjected to a 1.25% (v/v) ethanol precipitation, and left overnight at 4 °C. The chondroitin sulphate-rich precipitate was discarded and the resulting supernatant was subjected to a 4% (v/v) ethanol precipitation. This KS-enriched material was dissolved in 0.2 M-NaCl, dialysed against distilled water, and lyophilized.

The crude KS (1.6 g) was dissolved in 100 ml of 0.1 M-Tris acetate, pH 7.3, warmed to 37 °C, and digested with 2 units of chondroitin ABC lyase for 24 h. The product was recovered by lyophilization after extensive dialysis against water.

This peptido-KS (1.25 g) was then subjected to alkaline borohydride reduction (Carlson, 1968) in 1.0 M-$NaBH_4$/0.05 M-NaOH for 24 h at 45 °C, neutralized by dropwise addition of 4 M-acetic acid in an ice-bath, and then dialysed against water and lyophilized. Separate experiments showed that there was no significant loss of sialic acid under these conditions provided that the acid neutralization step was carried out carefully.

The reduced KS chains (600 mg) were chromatographed on a Sephadex G-50 column (82 cm × 1.5 cm) eluted in water. The KS-rich fraction as assessed by the anthrone and 1,9-Dimethylmethylene Blue (Klompmakers & Hendriks, 1986) assays was pooled and lyophilized.

The reduced KS chains (390 mg) were further purified on a Mono-Q HR 10/10 column eluting with a linear gradient of 0–0.5 M-lithium perchlorate/10 mM-piperazine, pH 5.0. The KS-containing fractions were pooled, lyophilized and then desalted on a Bio-Gel P-2 column, and recovered by lyophilization.

SNA lectin affinity chromatography

KS (1 mg in 100 μl of PBS) was applied to a pre-equilibrated SNA–agarose column (8.5 cm × 0.66 cm) at 4 °C. The column was eluted at 0.2 ml/min with PBS to elute non-binding material and then with 20 mM unbuffered EDA (Shibuya *et al.*, 1987*b*). Fractions of size 0.4 ml were assayed for KS using 1,9-Dimethylmethylene Blue. The EDA-eluted material was then immediately neutralized with phosphate buffer, pH 6.5. Binding and non-binding-KS was recovered after desalting on Bio-Gel P-2, and was then lyophilized.

Bio-Gel TSK-30 XL chromatography

The parent KS as well as both the SNA-binding and non-binding fractions were chromatographed (Dickenson *et al.*, 1990*a*) on a Bio-Gel TSK-30 XL column (300 mm × 7.8 mm) eluted with 0.2 M-NaCl, at a flow rate of 0.5 ml/min. The eluate was monitored with a Bio-Rad 1755 refractive index detector.

N.m.r. spectroscopy

KS samples were initially dissolved in 0.5 ml of ^2H_2O (99.8%), buffered to pH 7 with phosphate, and then filtered by centrifugation in a microfilterfuge tube (0.45 μm pore size). The internal reference 3-trimethylsilyl[$^2H_4$]propionic acid (TSP) was then added, and the samples were exchanged three times with 99.8% and then once with 100% ^2H_2O to minimize the residual HOD signal before dissolution in 100% ^2H_2O (0.5 ml). ^1H n.m.r. spectra were determined at 60 °C and 600.14 MHz on a Bruker AMX600 spectrometer.

RESULTS

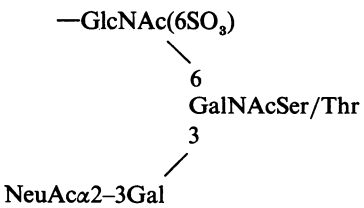
KS (1 mg) was applied to the SNA–agarose column in PBS at 4 °C. Preliminary experiments had determined that this loading would not exceed the capacity of the column. The column was first washed with sufficient PBS to elute all non-binding material, and subsequently with 20 mM-EDA. In other experiments in which SNA-binding KS was eluted with 0.1 M-lactose rather than with EDA the peak was very dispersed and therefore more difficult to recover. Therefore EDA was used to elute the binding fraction. After the elution was complete the column was washed with PBS. The elution profile, as monitored with 1,9-Dimethylmethylene Blue (Fig. 1), shows two peaks, a larger breakthrough fraction corresponding to non-binding chains and a smaller binding fraction. Both fractions were recovered (see above) for subsequent chromatography and spectroscopic analysis.

The SNA-binding and non-binding KS species and the parent KS sample were subjected to g.p.c. on Bio-Gel TSK-30 XL (Dickenson *et al.*, 1990*a*) eluted in 0.2 M-NaCl at 30 °C (see Fig. 2). It is clear that there was no difference in the molecular-size distributions between SNA-binding and non-binding KS species. Both populations have mass-average relative M_r values of about 7900.

The structural characteristics of these two fractions were then examined by 600 MHz ^1H n.m.r. spectroscopy. The partial spectra (Fig. 3) show that the main differences lie in the sialic acid resonances. Thus the SNA-binding KS sample shows resonances characteristic of α(2–6)-linked *N*-acetylneuraminic acid, such as those at 1.700 p.p.m. and at 2.695 p.p.m. (results not shown), whereas the non-binding material does not. Both samples show resonances typical of α(2–3)-linked *N*-acetylneuraminic acids, such as those at 1.787 and 1.807 p.p.m., but the non-binding material clearly contains a higher proportion of such sialic acids. It is already known from keratanase-derived fragments that there are basically two α(2–3)-NeuAc environments in skeletal KS chains: one from a chain cap (Dickenson *et al.*, 1991):

NeuAcα2–3Galβ1–4GlcNAc(6SO_3)β1–3Gal–

and one from a linkage region (Dickenson *et al.*, 1990*b*):



Closer examination of the signals corresponding to α(2–3)-NeuAc shows that both binding and non-binding fractions contain the linkage region sialic acid [H-3_{ax} at 1.790 p.p.m., 60 °C (J. M. Dickenson, T. N. Huckerby & I. A. Nieduszynski,

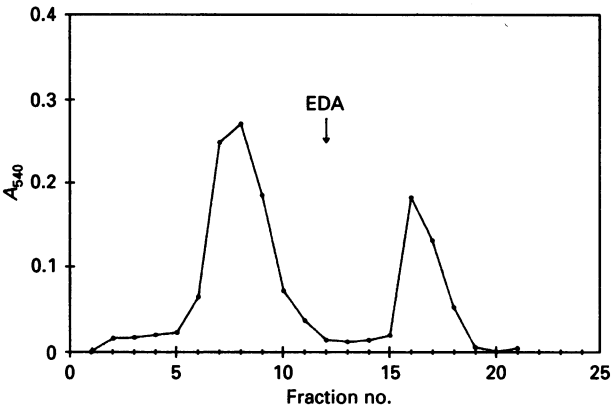


Fig. 1. Elution of KS species from SNA–agarose

KS (1 mg) was applied to an SNA–agarose column (3 ml) in PBS at 4 °C. The column was initially eluted in PBS, pH 7.4, and then the bound material was eluted in 20 mM-EDA as indicated. The column flow rate was 0.2 ml/min, the fraction size was 0.4 ml, and the eluent was monitored with 1,9-Dimethylmethylene Blue.

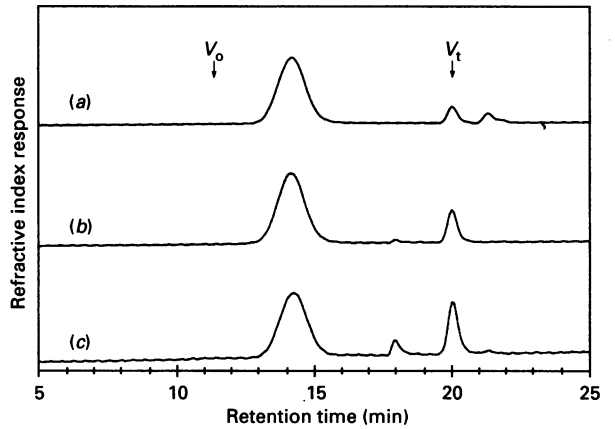


Fig. 2. G.p.c. of SNA-binding and non-binding KS species

The samples parent KS (*a*), SNA-non-binding fraction (*b*), and SNA-binding fraction (*c*) were chromatographed on Bio-Gel TSK 30 XL column. The column (300 mm × 7.8 mm) was eluted at a flow rate of 0.5 ml/min with 0.2 M-NaCl at 30 °C and the elution profile was monitored by measuring the refractive index. The small peak at 18 min corresponds to TSP remaining after n.m.r. spectroscopy.

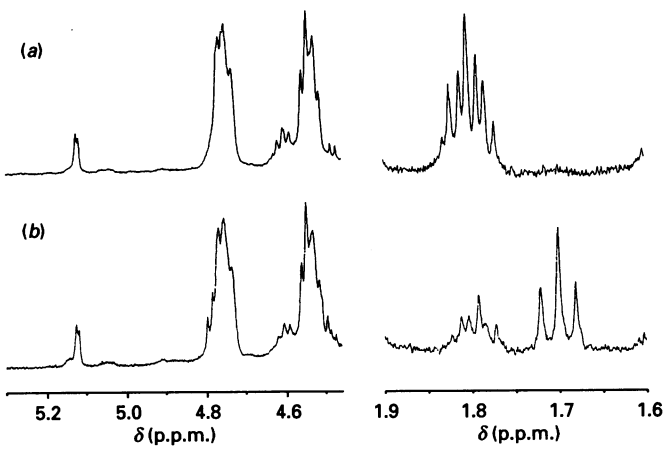


Fig. 3. Partial 600 MHz ^{1}H n.m.r. spectra acquired at 60 °C of (*a*) SNA-non-binding fraction and (*b*) SNA-binding fraction

NeuAc H-3_{ax} signals (1.6–1.9 p.p.m.) and anomeric signals (4.5–5.2 p.p.m.) are shown. Chemical shifts are shown in p.p.m. relative to internal TSP. Signal components within separate ranges shown are not on a common vertical scale.

unpublished work)], but only the non-binding KS fraction contains a major proportion of capping α(2–3)-NeuAc [H-3_{ax} at 1.8 p.p.m. (Dickenson *et al.*, 1991)]. Therefore the two fractions differ primarily in the mode of linkage of the capping sialic acid.

Other spectral differences appear to relate to the different modes of linkage of sialic acid in the two fractions. Hence comparison of the signals at 4.7–4.8 p.p.m., corresponding to sulphated *N*-acetylglucosamine, shows the presence of a small doublet at 4.79 p.p.m. in the SNA-binding material. This may be assigned to H-1 of the specific GlcNAc(6SO_3) in the sequence:

NeuAcα2–6Galβ1–4GlcNAc(6SO_3)–

by comparison with analytical data derived from the oligosaccharide NeuAcα2–6Galβ1–4GlcNAc(6SO_3)β1–3Gal-ol (Dickenson *et al.*, 1992). The remaining GlcNAc(6SO_3) H-1 signals that occur in both populations lie within the envelope of signals at 4.74–4.76 p.p.m. There are also other minor differences in the spectra attributable to resonances from the galactose residues adjacent to the terminal sialic aids.

Further comparison of the spectra of the two KS fractions shows considerable similarities. Thus both the SNA-binding fraction and the non-binding fraction appear to contain similar fucose contents (GlcNAc/Fuc = 11.6 and 11.2 respectively), as determined from the relative areas of the fucose (5.117 p.p.m.) and *N*-acetylglucosamine (4.74–4.76 p.p.m.) anomeric signals (see Fig. 3). Sulphation levels for each fraction can be estimated by the pattern of GlcNAc anomeric signals at approx. 4.74–4.76 (Thornton *et al.*, 1989), and these give similar results of around 1.7 sulphates per disaccharide. Also, the signals between 3.85 and 4.0 p.p.m. which are sensitive to sulphation levels (Thornton *et al.*, 1989) show no significant differences between the SNA-binding and non-binding KSs.

DISCUSSION

Previous studies (Shibuya *et al.*, 1987*a*) of SNA binding specificity have shown that this lectin binds NeuAcα(2–6)-Gal/GalNac strongly, but has low affinity for NeuAcα(2–3)-Gal/GalNAc. Similar specificity was observed in this study, in which all KS chains containing NeuAcα(2–6)-Gal– were firmly bound to the SNA–agarose column. This sub-population represented about one-third of the KS chains. By contrast, the non-binding material is dominated by α(2–3)-NeuAc capped chains and may possibly also contain asialo chains or chains with other caps. There is a need for additional probes (whether lectin or antibody) for the recognition and further fractionation of these chains.

Articular cartilage KS species contain both α(2–6)-NeuAc and α(1–3)-fucose residues in addition to those found in non-articular cartilage KS species. However, it is clear from Fig. 3 that both SNA-binding and non-binding KS chains contain equivalent amounts of fucose. Thus the two features unique to articular cartilage KS chains are not co-distributed.

The n.m.r. spectroscopic study of the SNA-binding population also showed (very small) sub-stoichiometric amounts of non-reducing terminal α(2–3)-NeuAc, which suggests the presence of two chain caps in one molecule. It is unclear whether this results from incomplete papain digestion and borohydride reduction or the presence of branching in KS. The 600 MHz n.m.r. spectra do

provide evidence for the presence of small quantities of residual peptide units, e.g. at 0.8–1.0 p.p.m. (not shown).

It is important to emphasize that the molecular size of the parent KS preparation was biased towards larger chains as a result of the pooling strategy at the Sephadex G-50 and Mono-Q chromatography stages. Thus the results reported here on the two fractions (their proportions, molecular-size distributions and sulphation and fucosylation) relate specifically to the larger chains studied here. By contrast Merkle & Cummings (1987), who studied an unsulphated polylactosaminoglycan system, found that smaller chains had a higher proportion of α(2–6)-linked sialic acid caps.

The functions of discrete KS sub-populations with differently glycosylated caps are not understood, and further studies on their topographical locations and distributions within proteoglycans are required. It is possible that the α(2–3)- and α(2–6)-linked sialic acid caps may have different labilities, thus affecting the rates of clearance of KS-rich proteoglycan fragments via the hepatic asialoglycoprotein receptor system. Perhaps specific recognition sequences involving, say, the α(2–6)-*N*-acetylneuraminic acid residues and other rare structural features such as the α(1–3)-linked fucose residues are involved in interactions with other matrix macromolecules. The fact that the two above mentioned structural features are only found in articular cartilage suggests that their expression is related to loading of the tissue.

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