High-field n.m.r. studies of keratan sulphates

^1H and ^{13}C assignments of keratan sulphate from shark cartilage

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Keratan sulphate was extracted from a shark/whale cartilage preparation and examined by 400 MHz ^1H - and 100 MHz ^{13}C -n.m.r. spectroscopy. Assignment of the majority of the resonances was facilitated by two-dimensional ^{13}C - ^1H correlation by using a modified COLOC procedure and a COSY-45 experiment. The spectra are consistent with an *N*-acetyl-lactosamine repeating unit that is predominantly sulphated at C-6 of both galactose and *N*-acetylglucosamine. Gel chromatography of a keratanase digest of the shark keratan sulphate confirmed the high degree of galactose sulphation.

INTRODUCTION

Keratan sulphate, a glycosaminoglycan, was first isolated from bovine cornea by Meyer *et al.* (1953) and was subsequently reported to be present in human cartilage (Meyer *et al.*, 1958) and nucleus pulposus (Gardell, 1955). Keratan sulphates occur covalently linked to protein as components of proteoglycans and have been classified into corneal (type I), which is *N*-linked via glucosamine to asparagine (Baker *et al.*, 1969), and skeletal (type II), with an *O*-glycosidic linkage between galactosamine and serine or threonine (Bray *et al.*, 1967).

Keratan sulphate, which has a repeating disaccharide unit of (1→3)- β -D-galactose-(1→4)- β -D-*N*-acetylglucosamine, is generally sulphated at C-6 of the hexosamine and may also be sulphated at C-6 of the galactose (see, e.g., Bhavanandan & Meyer, 1968). Other unsulphated polymers with poly-*N*-acetyl-lactosamine backbones occur upon the surfaces of erythrocytes (Viitala & Järnefelt, 1985). The degree of sulphation of keratan sulphate is known to vary with age (Kaplan & Meyer, 1959; Roughley & White, 1980) and cartilage type. Shark and whale cartilages are predominantly sulphated (Furuhashi, 1961) on both galactose and glucosamine residues, and this structure is shown in Fig. 1.

The enzyme keratanase, an endo- β -D-galactosidase, has proved to be an important tool (Oike *et al.*, 1980) in structural studies. Keratanase only cleaves at the reducing end of an unsulphated galactose, and hence the gel-filtration profile of the oligosaccharides produced is indicative of the degree of galactose sulphation in the parent polymer.

In the present study the high-field ^{13}C - and ^1H -n.m.r. spectra of the regularly sulphated keratan sulphate from shark cartilage have been recorded and assigned.

MATERIALS AND METHODS

Chemicals and enzymes

Crude chondroitin sulphate (grade II) from whale or shark cartilage, papain (EC 3.4.22.2) (type 3, 2× crystallized) from *Papaya latex* and chondroitinase

Fig. 1. Structure of shark cartilage keratan sulphate repeating unit

ABC (EC 4.2.2.4) from *Proteus vulgaris* were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Keratanase (EC 3.2.1.23) from *Pseudomonas* sp. was obtained from Miles Scientific (Slough, Berks., U.K.), Sephadex G-75 from Pharmacia Fine Chemicals (Uppsala, Sweden), Bio-Gel P-10 (200–400 mesh) from Bio-Rad Laboratories (Richmond, CA, U.S.A.) and Spectropor membrane tubing (M_r cut-off 6000–8000) from Orme Scientific Ltd. (Manchester, U.K.). $^2\text{H}_2\text{O}$ (> 99.8 atom% ^2H) for routine n.m.r. use was obtained from Nuclear Magnetic Resonance Ltd. (High Wycombe, Bucks., U.K.) and $^2\text{H}_2\text{O}$ (100.0 atom% ^2H) for high-field n.m.r. studies from Aldrich Fine Chemicals (Gillingham, Dorset, U.K.).

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

Papain digestion

Chondroitin sulphate powder (100 g) was added gradually with stirring to 0.2 M-NaCl/50 mM-EDTA/10 mM-cysteine hydrochloride/50 mM- NaH_2PO_4 buffer at pH 7 (Cöster & Fransson, 1981) and the temperature raised to 65 °C by using a water bath. Papain (1 ml containing 27 mg of protein/ml; 24 units/mg) at 65 °C was added to the chondroitin sulphate mixture and the digestion was continued, with stirring, for 5 days. Further additions of papain were made after 2 days (1.5 ml) and 4 days (1 ml). After centrifugation at 32000 g for 2 h in

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an MSE PrepSpin 50 centrifuge, the supernatant was collected, freeze-dried and redissolved in 0.2 M-sodium acetate.

Ethanol fractionation

The glycans precipitated at between 75% and 80% (v/v) ethanol in water were recovered, air-dried to remove the ethanol, dissolved in water and freeze-dried.

Chondroitinase ABC digestion

The glycans were dissolved in 80 ml of 67 mM- Na_2HPO_4 , pH 7.4, and 2 units of chondroitinase ABC were added. Digestion was carried out at 37 °C for 48 h in Spectropor dialysis tubing against 67 mM- Na_2HPO_4 buffer, pH 7.4. Dialysis was continued, against frequent changes of water, for 48 h and the non-diffusible material was freeze-dried.

Alkaline borohydride treatment

Alkaline borohydride reduction of the keratan sulphate was carried out with 5 mg of keratan sulphate/ml of reaction mixture, 50 mM- NaOH /1 M- NaBH_4 , at 45 °C for 48 h (Carlson, 1968). The reaction was terminated by the dropwise addition of acetic acid, and the mixture was dialysed against frequent changes of water for 2 days.

Keratanase digestion

Borohydride-reduced keratan sulphate (7.2 mg) was dissolved in 200 μl of 0.1 M-Tris/HCl containing 10 mM-EDTA, 10 mM-*N*-ethylmaleimide, 0.36 mM-pepstatin, 100 mM-6-aminohexanoate, 5 mM-benzamidine hydrochloride, 30 mM-sodium acetate and 1% bovine serum albumin (Oike *et al.*, 1980). The pH was adjusted to 7.4, 2 units of keratanase were added, and the digestion was carried out for 24 h at 37 °C and then stopped by bringing the solution to the boil for 2 min.

Chromatographic methods

The borohydride-reduced keratan sulphate (140 mg) was dissolved in 0.2 M- NH_4HCO_3 , pH 8.05, and fractionated by gel filtration on a column (66.5 cm $\times$ 1 cm) of Sephadex G-75. The eluent was 0.2 M- NH_4HCO_3 , pH 8.05, and 0.5 ml fractions were collected at a flow rate of 6 ml/h. Bovine serum albumin and Cl^- were used as V_0 (void-volume) and V_t (total-volume) markers respectively. The effluent was analysed for hexose by the automated anthrone method (Heinegård, 1973). Fractions eluted between K_{av} 0.12 and K_{av} 0.71 were pooled, dialysed against frequent changes of water and freeze-dried.

The oligosaccharides produced by the keratanase treatment of the keratan sulphate were fractionated on a column (83 cm $\times$ 1 cm) of Bio-Gel P-10. The eluent was 0.2 M- NH_4HCO_3 , pH 8.05, and 650 μl fractions were collected at a flow rate of 1.31 ml/h. A keratan sulphate sample was chromatographed under similar conditions for control purposes. The effluent was assayed for hexose as indicated above.

^{13}C - and ^1H -n.m.r. spectroscopy

High-field spectra were determined on a Bruker WH400 instrument with a 5 mm variable-temperature probe at 60 °C operating at 400.14 MHz for ^1H and at 100.61 MHz for ^{13}C . The borohydride-reduced keratan sulphate samples (80–100 mg) were buffered to pH 7 with phosphate, dissolved in $^2\text{H}_2\text{O}$ (0.5 ml for 5 mm probe,

1.1 ml for 10 mm probe), and 3-trimethylsilyl[$^2\text{H}_4$]propionic acid sodium salt was added as an internal reference (Huckerby, 1983). The samples for ^1H n.m.r. were exchanged several times with $^2\text{H}_2\text{O}$ and finally dissolved in 100.0 atom% $^2\text{H}_2\text{O}$. Two-dimensional homonuclear correlations were performed by using the COSY-45 method (Bax & Freeman, 1981). The ^{13}C - ^1H two-dimensional correlation was achieved by using the approach of Reynolds *et al.* (1985), based on the COLOC procedure (Kessler *et al.*, 1984) and with the standard COLOC phase cycling provided in the Bruker 2-D software. An average $^1J(\text{CH})$ of 160 Hz was assumed, and a T of 0.01055 s was employed to induce a vector rotation of 270° for polarization transfer. The delay was set to 0.3/ $J(\text{CH})$ (Bax, 1984).

RESULTS

Keratanase digestion

Shark keratan sulphate was subjected to extensive keratanase digestion. The parent polymer is completely excluded from a Bio-Gel P-10 column and the digest only contains oligosaccharides larger than decasaccharide. It may therefore be concluded that the galactose residues are predominantly sulphated.

N.m.r. spectroscopy

The 400 MHz ^1H , 100 MHz ^{13}C , ^{13}C - ^1H two-dimensional correlation and COSY-45 spectra for the shark keratan sulphate are presented in Fig. 2. These may be interpreted in terms of a simple disulphated disaccharide repeat unit, for which assignments are discussed below.

Glucosamine 6-sulphate

The resonance at δ 57.90 in the carbon spectrum is assigned to the glucosamine C-2, which is substituted with nitrogen. This resonance connects with a proton signal at δ 3.83, and the COSY-45 plot indicates that this proton is spin-coupled with the lowest field resonance at δ 4.76, which may therefore be assigned to H-1. This latter resonance correlates with a C-1 resonance at δ 105.27.

Beyond H-2 the glucosamine protons form a complex second-order group of signals at δ 3.7–3.85 that are not readily assignable at 400 MHz. C-4, however, is displaced characteristically downfield, being the site of the glycosidic linkage, and appears at δ 81.41, in accord with the data of Torchia *et al.* (1981). The ^{13}C - ^1H correlation suggests that the H-4 shift is δ approx. 3.8. Two closely spaced carbon resonances at δ approx. 75 arise from C-3 and C-5, but are not definitively assigned. The corresponding protons fall at δ approx. 3.8. C-6, assignable as a methylene group from a DEPT-135 measurement, is at δ 69.33, and correlates with an AB multiplet at δ 4.32–4.39 attributable to the H-6 and H-6' protons. These assignments, together with those for the carbonyl and methyl resonances, are summarized in Table 1.

Galactose 6-sulphate

The other anomeric carbon at δ 105.39 must be assigned to C-1 in galactose. This correlates with a proton resonance at δ 4.54, which may therefore be assigned to the corresponding H-1. This is in accord with literature data for a β -linked galactose (Hounsell *et al.*, 1986). This

Fig. 2. ^{13}C - ^1H correlation (upper) and ^1H -COSY spectrum (lower) for shark keratan sulphate

Insets show one-dimensional ^1H and ^{13}C spectra; carbonyl and methyl resonances have been excluded.

proton signal shows a COSY connection to an H-2 resonance at δ 3.605, which itself connects to a carbon resonance at δ 72.59. There is a homonuclear correlation to H-3 at δ 3.79. This signal falls beneath a complex envelope of glucosamine resonances, but the ^{13}C - ^1H

correlation shows a connection to a resonance at δ 84.87, which is known to correspond to C-3 (Torchia *et al.*, 1981). This downfield perturbation is characteristic for galactose linked at C-3 (Gagnaire *et al.*, 1978). From H-3 a COSY connection is observed at δ 4.22, which must be

Table 1. ^{13}C and ^1H δ values for shark keratan sulphate

^1H and ^{13}C chemical shifts are given in p.p.m. from internal 3-trimethylsilyl[$^2\text{H}_4$]propionic acid.

Ring position	^{13}C chemical shift (p.p.m.)	^1H chemical shift (p.p.m.)
<i>N</i> -Acetylglucosamine		
1	105.27	4.76
2	57.90	3.83
3	74.87/75.15*	~ 3.8
4	81.41	~ 3.8
5	74.87/75.15*	~ 3.8
6†	69.33	4.32/4.39 (6,6')
CH_3	25.09	2.053
CO	177.54	—
Galactose		
1	105.39	4.54
2	72.59	3.605
3	84.87	3.79
4	70.87	4.22
5	75.42	3.97
6‡	70.34	4.22 (6,6')

* Assignments uncertain.

† C-6 of minor unsulphated *N*-acetylglucosamine component at δ 62.88.

‡ C-6 of minor unsulphated galactose component at δ 63.69.

H-4, and this, in turn, connects to H-5 at δ 3.97. This resonance shows an off-diagonal connection to H-6 protons at δ 4.22. The two ^{13}C - ^1H correlations from the protons at δ 4.22 may be unambiguously assigned to C-4 at δ 70.87 and C-6 at δ 70.34 because the latter shows an inverted signal in a DEPT-135 experiment. The isolated H-5 resonance shows a clear connection to C-5 at δ 75.42. These assignments are summarized in Table 1.

DISCUSSION

Attempts to perform decoupling difference measurements on keratan sulphate samples proved unsuccessful. Similarly, no responses were obtained from conventional ^{13}C - ^1H correlation measurements by using the method of Bax (1983). These failures may be attributed to the presence of an efficient spin-diffusion process resulting, for the correlation experiment, in a rapid decay of information during the period over which the pulse sequence is operating. Proton-proton connections are possible in the COSY experiment because this is not a spin-spin-decoupling procedure, although it leads to information concerning spin-coupled nuclei. In the COLOC ^{13}C - ^1H correlation experiment the first evolution delay time is incorporated into the incremental t_1 period, which is itself considerably attenuated compared with that of the normal Bax (1983) method. Loss of information content through T_2 processes is thus minimized, and successful correlations are possible for this type of polymeric system. The available resolution along the f_1 axis is very poor (Reynolds *et al.*, 1985), but the high signal dispersion available with 400 MHz (^1H) measurements provides partial compensation.

It is clear from the lack of complexity of the ^{13}C spectrum that there is a high degree of uniformity in the extent of sulphation of the shark keratan sulphate. The two minor $-\text{CH}_2\text{OH}$ signals at δ 63.69 (galactose) and δ 62.88 (*N*-acetylglucosamine) indicate the low content of unsulphated units. Similarly, the small extent of keratanase cleavage testifies to the high degree of galactose sulphation. It is evident that ^{13}C -n.m.r. spectroscopy holds considerable promise for the identification of the positions and degrees of sulphation in the keratan sulphate family. Unfortunately, the more sensitive ^1H -n.m.r. spectroscopy gives rise to broad and overlapped resonances, from which a quantitative analysis of sulphation would be extremely difficult.

The unambiguous assignment of the n.m.r. spectra from fully sulphated blocks of keratan sulphate should facilitate the analysis of co-polymeric structure in more complex keratan sulphates.

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