Fucose content of keratan sulphates from bovine articular cartilage

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Alkaline-borohydride-reduced keratan sulphate chains were isolated from bovine articular cartilage (6–8-year-old animals). Nine keratan sulphate fractions of increasing molecular weight were prepared by gel-permeation chromatography on a calibrated column of TSK 30 XL. The samples were analysed for fucose and galactose contents (% by wt. of keratan sulphate) and fucose/galactose ratio. The fucose content increased with molecular size, but the galactose content remained constant. It was concluded that the $\alpha(1\rightarrow3)$ -linked fucose [Thornton, Morris, Cockin, Huckerby, Nieduszynski, Carlstedt, Hardingham & Ratcliffe (1989) *Biochem. J.* **260**, 277–282] was located within the poly-*N*-acetyl-lactosamine repeat sequence of articular-cartilage keratan sulphate.

INTRODUCTION

Keratan sulphate was first isolated from bovine cornea by Meyer *et al.* (1953) and has subsequently been discovered in many tissues, including human cartilage (Meyer *et al.*, 1958) and nucleus pulposus (Gardell, 1955). Following the notation of Meyer (1970), keratan sulphates have been subclassified into two groups, which differ in their mode of linkage to protein. KS-I is found in the cornea and is *N*-linked via *N*-acetylglucosamine to asparagine (Baker *et al.*, 1969). KS-II is found in skeletal tissues such as cartilage and is *O*-linked via *N*-acetyl-galactosamine to either serine or threonine in the protein core (Bray *et al.*, 1967).

The structure of keratan sulphate is poorly understood, but is known to be based upon a repeating disaccharide sequence comprising the sequence (1→3)- β -D-galactose-(1→4)- β -D-*N*-acetylglucosamine. Sulphation of the repeat unit usually occurs on C-6 of *N*-acetylglucosamine, but further sulphate groups may also be present on C-6 of galactose (Bhavanandan & Meyer, 1968).

Both fucose and sialic acid have been reported as minor but significant components of keratan sulphates, apparently occupying terminal positions (Seno *et al.*, 1965; Gardell, 1957). Sialic acid is known to occur in the linkage region (Hopwood & Robinson, 1974; Dickenson *et al.*, 1990a) and as a capping residue at the non-reducing end of the keratan sulphate chain (Nieduszynski *et al.*, 1990b). The position of the fucose residue (s) in the keratan sulphate structure is not known, although previous n.m.r. studies (Thornton *et al.*, 1989a,b) have shown this residue to be $\alpha(1\rightarrow3)$ -linked. Recent studies by Nieduszynski *et al.* (1990a) have suggested that skeletal keratan sulphates should be subclassified into KS-II-A and KS-II-B. Keratan sulphates from articular cartilage (KS-II-A) contain fucose and $\alpha(2\rightarrow6)$ -linked sialic acid, whereas those from non-articular cartilage (KS-II-B) lack these residues.

In the present study, a population of keratan sulphate chains was isolated from bovine femoral-head cartilage and fractionated by gel-permeation chromatography in order to investigate the fucose distribution in keratan sulphates as a function of molecular size.

EXPERIMENTAL

Materials

Chemicals and enzymes used in this study are as described previously (Thornton *et al.*, 1989a). Chondroitin ABC lyase (*Proteus vulgaris*, EC 4.2.2.4) was purchased from ICN Bio-medicals, High Wycombe, Bucks., U.K. 2-Cyanoacetamide (> 98%) was obtained from Fluka Chemicals, Glossop, Derbyshire, U.K. The Mono-Q HR 10/10 column was from Pharmacia (Uppsala, Sweden), and the Bio-Gel TSK 30 XL and Aminex HPX-87H columns were from Bio-Rad Laboratories, Watford, Herts., U.K.

Preparation of peptido-keratan sulphates

Peptido-keratan sulphate fragments from the keratan sulphate-rich region (Heinegård & Axelsson, 1977) of the proteoglycans were prepared from bovine femoral-head cartilage (6–8-year-old) essentially by the method described by Dickenson *et al.* (1990a).

H.p.l.c.

H.p.l.c. was performed by using a Bio-Rad series 700 h.p.l.c. titanium gradient system with u.v. and refractive-index detectors.

Isolation and purification of keratan sulphate chains

Keratan sulphate chains were subsequently prepared by alkaline borohydride reduction (Carlson, 1968) followed by chondroitin ABC lyase digestion and gel-permeation chromatography on a column (82 cm $\times$ 1.5 cm) of Sephadex G-50 eluted with 0.2 M-NH₄HCO₃. After extensive dialysis against 1.0 M-NaCl and then water, the keratan sulphate chains were recovered by freeze-drying and then purified further from any remaining *O*-linked oligosaccharides on a Pharmacia Mono-Q HR 10/10 column eluted with a linear gradient of 0–0.5 M-LiClO₄/10 mM-piperazine, pH 5.0.

Gel-permeation chromatography

The total population of purified keratan sulphate chains was then chromatographed in three separate and identical runs (to prevent overloading of the column) on a Bio-Gel TSK 30 XL

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Fig. 1. Preparative and analytical TSK 30 XL high-performance gel-permeation chromatography profiles of reduced keratan sulphate chains

(a) The column (300 mm $\times$ 7.8 mm) was eluted at a flow rate of 0.5 ml/min with 0.2 M-NaCl and 0.25 ml fractions were collected. The eluate was monitored by refractive index. Fractions of increasing M_w (M1-M9) were pooled as indicated by the vertical bars. (b) The individual TSK 30 XL profiles of fractions M1-M9. The column (300 mm $\times$ 7.8 mm) was eluted at a flow rate of 0.5 ml/min with 0.2 M-NaCl and the eluate was monitored by refractive index.

column eluted with 0.2 M-NaCl to fractionate the chains according to their molecular size. This column had previously been calibrated for M_r by using oligosaccharides of known size derived from keratan sulphate (Dickenson *et al.*, 1990b). Fractions were combined from each of the three runs, pooled as indicated (Fig. 1a) and then rechromatographed on the column to calculate their weight-average M_r values and to estimate their degree of polydispersity (Fig. 1b).

Carbohydrate analysis

Carbohydrate analyses were performed with the use of con-

ditions similar to those described by Lohmander (1986). Keratan sulphate samples were desiccated for 48 h. Samples (approx. 0.1 mg) were weighed to within 0.1 μ g on a Cahn 29 automatic electrobalance and then hydrolysed for 4 h at 94 $^{\circ}$ C in 0.2 ml of 4 M-trifluoroacetic acid, which was subsequently removed by rotary evaporation at less than 40 $^{\circ}$ C. These hydrolysis conditions had been determined by prior experimentation to give optimum release and recovery of fucose and galactose. To each hydrolysate 0.2 ml of 5 mM- H_2SO_4 was added and the solution filtered. Galactose and fucose contents and ratios were determined by using an Aminex HPX-87H column maintained at 40 $^{\circ}$ C and eluted with 5 mM- H_2SO_4 . High sensitivity was obtained by post-column formation of derivatives (Honda *et al.*, 1981) with a mixture of 1% 2-cyanoacetamide and 0.2 M-borate buffer, pH 10.0, with monitoring at 276 nm. The responses given by fucose and galactose had previously been calibrated so that the weight of each sugar and hence the molar ratio could be accurately determined.

N.m.r. spectroscopy

Proton-n.m.r. spectra were determined at 60 $^{\circ}$ C with a Bruker AM500 spectrometer. Samples (0.5–2 mg) were buffered to pH 7 with phosphate, referenced with internal sodium trimethylsilyl[2H_4]propionate and dissolved in 0.5 ml of 99.96% 2H_2O after micro-filtration and several previous exchanges with 99.8% 2H_2O and one with 99.96% 2H_2O . The residual $^1H^2HO$ resonance was suppressed by using gated decoupling, and spectra were reprocessed for display with the program suite NMR1 (Lab One NMR1 Spectroscopic Data Analysis System, Release 3.95; New Methods Research, Syracuse, NY, U.S.A.).

RESULTS

The reduced keratan sulphate chains before gel-permeation chromatography were free from O-linked oligosaccharides and chondroitin sulphates as judged by 500 MHz n.m.r. spectroscopy (spectrum not shown).

The distribution of molecular sizes of reduced keratan sulphate chains as seen on TSK 30 XL yielded nine fractions of increasing molecular size, M1–M9 (see Fig. 1b). The elution profiles on TSK 30 XL for each fraction were monitored by refractive index and gave normal Gaussian distributions. The K_{av} value for each fraction was calculated from the maximum of each peak, and weight-average M_r values (M_w) (see Table 1) were calculated by using the equation shown below (Dickenson *et al.*, 1990b):

$$\log M_r = 4.588 - (2.128 \times K_{av})$$

This method of detection readily yields weight-average M_r values, M_w . Number-average M_r values, M_n , would allow easier comparison with the carbohydrate analyses; however, it seems

Table 1. Carbohydrate analyses for keratan sulphate chains as a function of M_w

Fraction	M_w	Fucose/ galactose molar ratio	Galactose (% by wt of keratan sulphate)	Fucose (% by wt. of keratan sulphate)
M1	3750	0.087	20.4	1.61
M2	4100	0.085	20.4	1.58
M3	4850	0.102	20.8	1.93
M4	6100	0.108	20.4	2.01
M5	7200	0.113	20.7	2.13
M6	7600	0.117	20.7	2.23
M7	8800	0.125	20.7	2.35
M8	10900	0.124	20.4	2.31
M9	13600	0.137	—	—

preferable to use the directly determined M_w data, rather than any numerically derived M_n values, as the degree of polydispersity of the individual fractions is low.

The carbohydrate analyses on these fractions yielded two types of data, namely fucose/galactose molar ratios (which are more accurate because they are not subject to any error associated with differential hydration of the samples) and also the percentages of galactose and fucose by weight, which are shown in Fig. 2. This indicates that the fucose content increases with molecular size, but the proportion of galactose is constant, which makes this a good measure of keratan sulphate content in this preparation. A clearer indication of the distribution of fucose residues in keratan sulphate chains of articular cartilage can be obtained by examining the behaviour of the fucose/galactose ratio versus molecular size. Because the galactose content does not vary with molecular size, the plot of (fucose/galactose) $\times M_w$ versus M_w (Fig. 3) essentially gives a measure of changing fucose content, and this graph requires no assumptions concerning structure. As was seen before, the fucose content clearly rises with M_w ; moreover, extrapolation of the plot to zero fucose content reveals the presence of a non-fucosylated structure that has an approximate M_w of 2000. The fact that the plot is a straight line shows that the degree of fucosylation in the fucosylatable part of the molecule is constant, with one fucose residue per 6000 in M_w of the sulphated poly-*N*-acetylglucosamine repeat sequence in this sample. Studies of keratan sulphates from human articular cartilage in this laboratory indicate that the extent of fucosylation may vary between preparations.

Fig. 2. Galactose and fucose weight percentages as a function of molecular size

Fig. 3. Fucose content as a function of molecular size for bovine articular cartilage keratan sulphate

The right-hand axis represents the number of fucose residues per chain, as calculated by the following formula:

$$\text{Fucose residues/chain} = \frac{\text{Fucose (\% by wt.)} \times M_w}{100 \times M_r \text{ of fucose}}$$

Fig. 4. Partial 500 MHz proton-n.m.r. spectra for four keratan sulphate fractions

(a) M1, $M_w = 3750$; (b) M3, $M_w = 4850$; (c) M5, $M_w = 7200$; (d) M7, $M_w = 8800$.

Partial proton-n.m.r. spectra of several fractions are shown in Fig. 4. The region of the spectra shown contains signals from the anomeric protons in both fucose (about 5.12 p.p.m.) and *N*-acetylglucosamine residues (about 4.74–4.76 p.p.m.). The H-5 quartet from fucose is also known to contribute to the signals in the 4.74–4.76 p.p.m. region. The data in Fig. 4 show the relative fucose/*N*-acetylglucosamine ratios for four fractions of increas-

ing molecular size. Although these ratios are not as accurate as those obtained from the carbohydrate analysis, they are, nonetheless, broadly consistent with those shown in Table 1.

DISCUSSION

It is noteworthy that the galactose content of these fractions of keratan sulphate chains remains constant with increasing M_w . This would indicate that the degree of sulphation within the keratan sulphate chains must also remain constant with increasing molecular size, as an increase in sulphation would cause a concomitant decrease in galactose weight percentage. This finding is confirmed by n.m.r. spectroscopy, where it is clearly seen that the pattern of signals at 4.74–4.76 p.p.m. (from *N*-acetylglucosamine H-1) is similar in each spectrum. These signals are known to be sensitive to the degree of galactose sulphation in their immediate environment (Thornton *et al.*, 1989a) and would clearly show any changes in the sulphation pattern. In the study of pig corneal keratan sulphate by Oeben *et al.* (1987), a different result was found, namely that the extent of sulphation increased with molecular size.

The rise in fucose content with increasing molecular size shows that the fucose residues must be located within the poly-*N*-acetyl-lactosamine major repeat sequence of the molecule (or that of a proportion of a molecules). Our previous work (Thornton *et al.*, 1989a) has shown that the fucose residues were $\alpha(1\rightarrow3)$ -linked and the only available 3-position at which it may be located is on *N*-acetylglucosamine. Hence the sequence is identical with that of the SSEA-1 antigen (Hounsell, 1987), but is probably sulphated:

The suggestion that the fucose is located within the major repeat sequence is consistent with the M_w value of about 2000 obtained by the extrapolation to zero fucose content shown in Fig. 3. This number approximately represents the sum of the M_w values for a linkage-region fragment (I) (Dickenson *et al.*, 1990a) and a capping oligosaccharide sequence (II) (Nieduszynski *et al.*, 1990b):

When keratan sulphate chains are fractionated by means of Mono-Q ion-exchange chromatography as in the study by Thornton *et al.* (1989a) rather than by molecular size, the fucose content is seen to be slightly enriched in those molecules that are eluted in the middle of the salt gradient. It is, nonetheless, unclear whether the fucose residues are randomly distributed among all of the skeletal keratan sulphate chains in the cartilage

or are present within a subpopulation of the chains or, indeed, a subset of the proteoglycan molecules.

The presence of fucose in articular-cartilage-derived keratan sulphates has potential relevance to the degradation of the molecules. First, there are both human and bovine hepatic receptors that specifically bind fucosyl- $\alpha(1\rightarrow3)$ -*N*-acetylglucosamine-containing oligosaccharides (Prieels *et al.*, 1978) and thus there may be an alternative mode of clearance to that involving the galactose receptor. Secondly, in the genetic disorder fucosidosis, in which there is a deficiency of the lysosomal enzyme α -L-fucosidase, there is increased excretion of keratan sulphate in the urine (Greiling *et al.*, 1978).

The presence of occasional fucose residues, in addition to sialic acid residues in as many as five discrete environments (Nieduszynski *et al.*, 1990b), in articular-cartilage keratan sulphate samples shows great diversity. Such structural diversity is reminiscent of the carbohydrate differentiation antigens (Feizi, 1985) and may give rise to a large number of potentially biologically active oligosaccharide sequences.

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REFERENCES

- Baker, J. R., Cifonelli, J. A. & Rodén, L. (1969) *Biochem. J.* **115**, 11P.
- Bhavanandan, V. P. & Meyer, K. (1968) *J. Biol. Chem.* **243**, 1052–1059.
- Bray, B. A., Lieberman, R. & Meyer, K. (1967) *J. Biol. Chem.* **242**, 3373–3380.
- Carlson, D. M. (1968) *J. Biol. Chem.* **243**, 616–626.
- Dickenson, J. M., Huckerby, T. N. & Nieduszynski, I. A. (1990a) *Biochem. J.* **269**, 55–59.
- Dickenson, J. M., Morris, H. G., Nieduszynski, I. A. & Huckerby, T. N. (1990b) *Anal. Biochem.* **190**, 271–275.
- Feizi, T. (1985) *Nature (London)* **314**, 53–57.
- Gardell, S. (1955) *Acta Chem. Scand.* **9**, 1035.
- Gardell, S. (1957) *Acta Chem. Scand.* **11**, 668–675.
- Greiling, H., Stuhlsatz, H. W., Cantz, M. & Gehler, J. (1978) *J. Clin. Chem. Clin. Biochem.* **16**, 329–334.
- Heinegård, D. & Axelsson, I. (1977) *J. Biol. Chem.* **252**, 1971–1979.
- Honda, S., Takahashi, M., Kakehi, K. & Ganno, S. (1981) *Anal. Biochem.* **113**, 130–138.
- Hopwood, J. J. & Robinson, H. C. (1974) *Biochem. J.* **141**, 57–69.
- Hounsell, E. F. (1987) *Chem. Soc. Rev.* **16**, 161–185.
- Lohmander, L. S. (1986) *Anal. Biochem.* **154**, 75–84.
- Meyer, K. (1970) in *Chemistry and Molecular Biology of the Intercellular Matrix* (Balazs, E. A., ed.), vol. 1, p. 15, Academic Press, New York.
- Meyer, K., Linker, A., Davidson, E. A. & Weissman, B. (1953) *J. Biol. Chem.* **205**, 611.
- Meyer, K., Hoffman, P. & Linker, A. (1958) *Science* **128**, 896.
- Nieduszynski, I. A., Huckerby, T. N., Dickenson, J. M., Brown, G. M., Tai, G.-H., Morris, H. G. & Eady, S. (1990a) *Biochem. J.* **271**, 243–245.
- Nieduszynski, I. A., Huckerby, T. N., Dickenson, J. M., Brown, G. M., Tai, G.-H. & Bayliss, M. T. (1990b) *Biochem. Soc. Trans.* **18**, 792–793.
- Oeben, M., Keller, R., Stuhlsatz, H. W. & Greiling, H. (1987) *Biochem. J.* **248**, 85–93.
- Prieels, J.-P., Pizzo, S. V., Glasgow, L. R., Paulson, J. C. & Hill, R. L. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2215–2219.
- Seno, N., Meyer, K., Anderson, B. & Hoffman, P. (1965) *J. Biol. Chem.* **240**, 1005–1010.
- Thornton, D. J., Morris, H. G., Cockin, G. H., Huckerby, T. N., Nieduszynski, I. A., Carlstedt, I., Hardingham, T. E. & Ratcliffe, A. (1989a) *Biochem. J.* **260**, 277–282.
- Thornton, D. J., Morris, H. G., Cockin, G. H., Huckerby, I. A. & Nieduszynski, I. A. (1989b) *Glycoconjugate J.* **6**, 209–218.