N.m.r. spectroscopic studies of fucose-containing oligosaccharides derived from keratanase digestion of articular cartilage keratan sulphates

Influence of fucose residues on keratanase cleavage

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Keratan sulphate chains from bovine articular cartilage were fully digested with keratanase from *Pseudomonas* sp. and the products were reduced with alkaline borohydride. The resultant fragments were fractionated on a Nucleosil 5SB column and the earliest eluting fucose-containing oligosaccharides were isolated. Structural analysis using ¹H n.m.r. spectroscopy (600 MHz) showed the two least-charged species to have the following structure: GlcNAc(6S)β1-3Galβ1-4(Fucα1-3)GlcNAc(6S)β1-3Galβ1-4GlcNAc(6S)β1-3Gal-ol and GlcNAc(6S)β1-3Galβ1-4(Fucα1-3)GlcNAc(6S)β1-3Galβ1-4GlcNAc(6S)β1-6(Galβ1-3)GalNAc-ol. Both galactoses adjacent to the fucosylated *N*-acetylglucosamine residue are unsulphated. Therefore, it can be

deduced from these structures that the presence of fucose on *N*-acetylglucosamine residues in keratan sulphates protects both of the adjacent unsulphated galactose residues from keratanase cleavage. This result has implications for the interpretation of keratanase fingerprints, because in articular cartilage keratan sulphates the keratanase-resistant blocks are not solely those with fully sulphated galactose residues, but also include the fucosylated sequences, which have unsulphated galactoses. It is, therefore, not possible to estimate their galactose sulphation or the size of the fully sulphated disaccharide-repeat sequences from keratan sulphates that contain fucose.

INTRODUCTION

Skeletal keratan sulphates are glycosaminoglycan components of the large proteoglycans (aggrecans) in cartilage (Hardingham and Fosang, 1992), and they may contribute up to 2–5% of the dry wt. of that tissue. During normal turnover of cartilage and in disease, proteoglycan fragments are released into the synovial fluid and ultimately into the blood (for a review see Lohmander, 1991). The monoclonal antibody, 5-D-4 (Caterson et al., 1983), which recognizes a fully sulphated hexasaccharide sequence (Mehmet et al., 1986) in keratan sulphate has been used in immunoassays of body fluids as a potential early marker for the disease of osteoarthritis (Thonar et al., 1985).

The structure of keratan sulphate is based upon a repeating disaccharide sequence of (–3Galβ1-4GlcNAcβ1–). Sulphation may occur on C-6 of both galactose and *N*-acetylglucosamine residues. According to the type of linkage to protein, keratan sulphates can be classified (Meyer, 1970) into keratan sulphate-I (N-linked keratan sulphate derived from cornea or fibromodulin) and keratan sulphate-II (O-linked keratan sulphate from skeletal tissues). Further studies (Nieduszyński et al., 1990) showed that skeletal keratan sulphates can be subclassified into articular, keratan sulphate-II-A, and non-articular, keratan sulphate-II-B. In articular keratan sulphate fucose is a minor component typically comprising 1–2% of the total weight (Gardell, 1957; Seno et al., 1965; Tai et al., 1991). A fragmentation study using the hydrazinolysis/nitrous acid procedure (Brown et al., 1992) has demonstrated that fucose is α(1-3)-linked to a sulphated *N*-acetylglucosamine residue in the main repeating sequence, and that the galactose on the non-reducing side is always unsulphated, but the one on the reducing side may or may not be sulphated.

There are three major keratan sulphate fragmentation methods available at present: hydrazinolysis/nitrous acid (Hopwood and Elliott, 1983; Brown et al., 1992), keratanase (Nakazawa and Suzuki, 1975) and keratanase II (Nakazawa et al., 1989). Keratanase (and/or endo-β-galactosidase) has been the most widely used. Keratanase from *Pseudomonas* sp. is known to cleave at an unsulphated galactose residue adjacent to a sulphated *N*-acetylglucosamine residue (Nakazawa and Suzuki, 1975). Several oligosaccharides produced from such digestion have been characterized (Hounsell et al., 1986; Huckerby et al., 1990; Dickenson et al., 1990b, 1991, 1992).

In this study we report the structures of fucose-containing oligosaccharides derived from skeletal keratan sulphate by keratanase (*Pseudomonas* sp.) digestion and discuss the influence of fucose residues upon keratanase action.

MATERIALS AND METHODS

Chemicals and enzymes

The chemicals and enzymes used in this study were as described by Dickenson et al. (1991), except that the sialidase inhibitor, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid, was from Boehringer-Mannheim U.K. (Bell Lane, Lewes, East Sussex, U.K.) and chondroitin lyase AC (*Arthrobacter aurescens*) was from Sigma (Poole, Dorset, U.K.).

Keratan sulphate preparation

Keratan sulphate chains were prepared from bovine femoral head cartilage (6–8-year-old animals) essentially by the method described by Dickenson et al. (1990a). This involved 4M guanidine hydrochloride extraction of proteoglycans from comminuted

Abbreviations used: Gal(6S), β-D-galactose 6-O-sulphate; GlcNAc(6S), β-D-*N*-acetylglucosamine 6-O-sulphate; Gal-ol, β-D-galactitol; GalNAc-ol, *N*-acetylglucosaminol; COSY, two-dimensional homonuclear chemical-shift correlation spectroscopy.

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Partial one-dimensional ^1H -n.m.r. spectra for the fucose-containing oligosaccharides **7**, **8** and **12** are shown in Figure 2. In each case signals at approx. 1.17 p.p.m. (H-6) and 5.12 p.p.m. (H-1) confirm the presence of fucose. Inspection of the spectra in the region between 2.0 and 2.1 p.p.m. shows 3, 4 and 3 *N*-acetylmethyl resonances in oligosaccharides **7**, **8** and **12** respectively. The spectra from all three oligosaccharides show three doublet resonances attributable to H-1 in *N*-acetylglucosamine in the range 4.55–4.8 p.p.m. The spectrum for oligosaccharide **8** shows two signals at 3.51 p.p.m. (H-4) and

Figure 2 Partial 600 MHz ^1H n.m.r. spectra at 55 °C for (a) oligosaccharide 7 and (b) oligosaccharide 8; and (c) partial 500 MHz n.m.r. spectrum at 55 °C for oligosaccharide 12

4.06 p.p.m. (H-3) which are characteristic (Dickenson et al., 1990b) for a 3,6-linked *N*-acetylgalactosaminitol residue. By contrast, a triplet of doublets at approx. 4.10 p.p.m. (H-5) characterizes the presence of galactitols in oligosaccharides 7 and 12. The three *N*-acetylglucosamine methyl resonances are representative of a hexasaccharide, excised by the action of keratanase (Nakazawa and Suzuki, 1975), to which one fucose residue is attached. Clearly, oligosaccharides 7 and 12 are both heptasaccharides; their galactitols are characteristic of (reduced)

Figure 3 Partial 600 MHz COSY-45 spectrum at 55 °C of oligosaccharide 7

The spectral width was 2645.5 Hz, and 32 acquisitions for each of 960 increments were sampled into 1024 complex points. Data were transferred to a VAX 4000 computer and then processed for presentation and detailed analysis using the software package NMR2 (New Methods Research Inc., Syracuse, NY, U.S.A.). The array was zero-filled to 2048 $\times$ 2048 complex points and transformed in each dimension after application of a 2% offset sinebell window function.

Figure 4 Partial 600 MHz COSY-45 spectrum at 55 °C of oligosaccharide 8

The spectral width was 2551 Hz, and 80 acquisitions for each of 480 increments were sampled into 1024 complex points. Data were transferred and processed as described for Figure 3. The array was zero-filled to 2048 $\times$ 2048 complex points and transformed in each dimension after application of a 2% offset sinebell window function.

Table 1 Chemical-shift data for oligosaccharides 7, 8 and 12

Chemical-shift values in p.p.m. are measured at 55 °C relative to internal 3-trimethylsilyl[²H₄]propionate.

	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
	GlcNAc(6S)β1-3Galβ1-4GlcNAc(6S)β1-3Gal-ol			GlcNAc(6S)β1-3Galβ1-4GlcNAc(6S)β1-3Gal-ol			GlcNAc(6S)β1-3Galβ1-4GlcNAc(6S)β1-3Gal-ol			GlcNAc(6S)β1-3Galβ1-4GlcNAc(6S)β1-3Gal-ol			GlcNAc(6S)β1-3Galβ1-4GlcNAc(6S)β1-3Gal-ol		
	Fucal			Fucal			Fucal			Fucal			Fucal		
	Galβ1			Galβ1			Galβ1			Galβ1			Galβ1		
	3			3			3			3			3		
	GalNAc-ol			GalNAc-ol			GalNAc-ol			GalNAc-ol			GalNAc-ol		
	3			3			3			3			3		
Proton	Residue			Residue			Residue			Residue			Residue		
H-1	GlcNAc(6S)	1	4.715	GlcNAc(6S)	1	4.712	GlcNAc(6S)	1	4.712	GlcNAc(6S)	1	4.712	GlcNAc(6S)	1	4.712
H-2	3.788	3.981	4.770	3.788	3.981	4.770	3.788	3.981	4.770	3.788	3.981	4.770	3.788	3.981	4.770
H-3	3.591	3.918	3.793	3.588	3.788	3.983	3.588	3.788	3.983	3.588	3.788	3.983	3.588	3.788	3.983
H-4	3.538	3.981	3.725	3.538	3.725	3.913	3.538	3.725	3.913	3.538	3.725	3.913	3.538	3.725	3.913
H-5	3.661	3.810	3.841	3.661	3.810	3.806	3.661	3.810	3.806	3.661	3.810	3.806	3.661	3.810	3.806
H-6	4.243	4.333	4.272	4.243	4.333	4.243	4.243	4.333	4.243	4.243	4.333	4.243	4.243	4.333	4.243
H-6'	4.324	4.384	4.478	4.323	4.384	4.478	4.323	4.384	4.478	4.323	4.384	4.478	4.323	4.384	4.478
	Gal	Gal	Gal	Gal	Gal	Gal	Gal	Gal	Gal	Gal	Gal	Gal	Gal	Gal	Gal
H-1	1	2	4.515	1	2	4.515	1	2	4.515	1	2	4.515	1	2	4.515
H-2	3.528	3.599	4.502	3.527	3.599	4.502	3.527	3.599	4.502	3.527	3.599	4.502	3.527	3.599	4.502
H-3	3.709	3.719	3.719	3.709	3.719	3.721	3.709	3.719	3.721	3.709	3.719	3.721	3.709	3.719	3.721
H-4	4.137	4.182	4.182	4.136	4.182	4.181	4.136	4.182	4.181	4.136	4.182	4.181	4.136	4.182	4.181
H-5	3.617	3.613	3.613	3.613	3.613	3.613	3.613	3.613	3.613	3.613	3.613	3.613	3.613	3.613	3.613
H-6	3.737	3.735	3.735	3.735	3.735	3.735	3.735	3.735	3.735	3.735	3.735	3.735	3.735	3.735	3.735
H-6'	3.737	3.735	3.735	3.735	3.735	3.735	3.735	3.735	3.735	3.735	3.735	3.735	3.735	3.735	3.735
	Fuc	Fuc	Fuc	Fuc	Fuc	Fuc	Fuc	Fuc	Fuc	Fuc	Fuc	Fuc	Fuc	Fuc	Fuc
H-1	5.122	5.122	5.122	5.122	5.122	5.122	5.122	5.122	5.122	5.122	5.122	5.122	5.122	5.122	5.122
H-2	3.698	3.698	3.698	3.695	3.698	3.695	3.695	3.698	3.695	3.695	3.698	3.695	3.695	3.698	3.695
H-3	3.910	3.910	3.910	3.911	3.910	3.911	3.911	3.910	3.911	3.911	3.910	3.911	3.911	3.910	3.911
H-4	3.793	3.793	3.793	3.792	3.793	3.792	3.792	3.793	3.792	3.792	3.793	3.792	3.792	3.793	3.792
H-5	4.784	4.784	4.784	4.791	4.784	4.791	4.791	4.784	4.791	4.791	4.784	4.791	4.791	4.784	4.791
H-6 (CH ₃)	1.166	1.167	1.167	1.167	1.167	1.167	1.167	1.167	1.167	1.167	1.167	1.167	1.167	1.167	1.167
	Gal-ol	Gal-ol	Gal-ol	Gal-ol	Gal-ol	Gal-ol	Gal-ol	Gal-ol	Gal-ol	Gal-ol	Gal-ol	Gal-ol	Gal-ol	Gal-ol	Gal-ol
H-1	3.659	3.659	3.659	3.659	3.659	3.659	3.659	3.659	3.659	3.659	3.659	3.659	3.659	3.659	3.659
H-1'	3.659	3.659	3.659	3.659	3.659	3.659	3.659	3.659	3.659	3.659	3.659	3.659	3.659	3.659	3.659
H-2	4.001	4.001	4.001	4.001	4.001	4.001	4.001	4.001	4.001	4.001	4.001	4.001	4.001	4.001	4.001
H-3	3.889	3.889	3.889	3.889	3.889	3.889	3.889	3.889	3.889	3.889	3.889	3.889	3.889	3.889	3.889
H-4	3.838	3.838	3.838	3.838	3.838	3.838	3.838	3.838	3.838	3.838	3.838	3.838	3.838	3.838	3.838
H-5	4.097	4.097	4.097	4.097	4.097	4.097	4.097	4.097	4.097	4.097	4.097	4.097	4.097	4.097	4.097
H-6	3.677	3.677	3.677	3.677	3.677	3.677	3.677	3.677	3.677	3.677	3.677	3.677	3.677	3.677	3.677
H-6'	3.677	3.677	3.677	3.677	3.677	3.677	3.677	3.677	3.677	3.677	3.677	3.677	3.677	3.677	3.677
N-acetyl/methyl signals (p.p.m.)	2.035	2.035	2.035	2.036	2.035	2.036	2.036	2.035	2.036	2.036	2.035	2.036	2.036	2.035	2.036
	2.043	2.043	2.043	2.044	2.043	2.044	2.044	2.043	2.044	2.044	2.043	2.044	2.044	2.043	2.044
	2.070	2.070	2.070	2.064	2.070	2.064	2.064	2.070	2.064	2.064	2.070	2.064	2.064	2.070	2.064

Figure 6 Keratanase (*Pseudomonas* sp.) specificity

Keratanase cleavage sites on an idealized keratan sulphate chain from articular cartilage are indicated by arrows. Parentheses indicate may or may not occur.

protects the cleavage site. Larger keratanase-resistant fragments may contain either fucosylated (and consequently less sulphated) or galactose-sulphated regions, or both. Therefore, the pattern of keratanase fragmentation cannot be used to calculate the level of galactose sulphation in the presence of fucosylation.

A partial keratanase digest profile is shown in Figure 1. Twelve oligosaccharides are identified (Figure 1a). These are three fragments, 3, 6 and 8, from the linkage region; five fragments, 1, 4, 5, 9 and 11, from the capping region (the sialidase inhibitor remaining in the preparation eluted at the same position as oligosaccharide 1); and four fragments 2, 7, 10 and 12, from the repeating sequence. Oligosaccharide 2, the mono-sulphated disaccharide, is under-represented in the profile. Most of it was removed in the desalting procedure; it would otherwise have been one of the major peaks in the chromatogram.

The introduction of fucose residues into keratan sulphate significantly increases the structural diversity. In the oligosaccharides studied here the fucose is observed in a 'central repeat' environment and associated with the linkage region. It is possible that there are additional environments for fucose involving the cap structures of keratan sulphate. Thus, sialylated and sulphated, fucosylated Lewis X-containing oligosaccharides are important ligands for the selectins, but do they occur in the keratan sulphates?

There is a keen general interest in keratan sulphates as markers for osteoarthritis, but so far no studies have been addressed to the structures of the keratan sulphates in synovial fluids during the development of disease. A more detailed understanding of the specificity of the enzymes, keratanase and keratanase II, and

facile identification of their characterized oligosaccharide fragments are essential prerequisites to such studies.

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