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Structural Studies of Two Populations of Keratan Sulphate Chains from Mature Bovine Articular Cartilage

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Two discrete peptido-keratan sulphate fragments were isolated *via* chondroitinase ABC and trypsin digestion of a proteoglycan aggregate fraction prepared from bovine femoral head cartilage (six year old animals). The larger fragments (K_{av} = 0.07, CL-6B) contained peptides substituted with several keratan sulphate (KS) chains from the KS-rich region of the proteoglycan and the smaller fragments (K_{av} = 0.5, CL-6B) contained peptides with, perhaps, only one KS chain and the stubs of post-chondroitinase-treated chondroitin sulphate chains.

The two peptido-KS samples and the KS chains derived from these by alkaline borohydride reduction were characterised by ^{13}C-NMR spectroscopy. The two populations of KS chains were also examined by chromatography (Sephadex G-75), and keratanase digestion followed by chromatography on Bio-Gel P-10. From the results it was concluded that the KS chains from the two major trypsin-derived peptido-KS fragments had similar sulphation levels, distributions of hydrodynamic sizes and susceptibilities to keratanase.

The large cartilage proteoglycans are known to contain considerable numbers of both chondroitin sulphate and keratan sulphate chains [1]. Recently, the complete amino-acid sequence of the cartilage proteoglycan core protein from Swarm rat chondrosarcoma has been reported [2] and this permits the identification of the probable sites of chondroitin sulphate chain attachment. However, the Swarm rat chondrosarcoma proteoglycan does

Abbreviations: KS, keratan sulphate; A1, proteoglycan aggregate; T, diphenyl carbamyl chloride (DPCC)-trypsin-treated; CB, chondroitinase ABC-treated; C, chymotrypsin-treated; P, papain-treated; R, alkaline borohydride-reduced; TSP, sodium 3-trimethylsilylpropionate.

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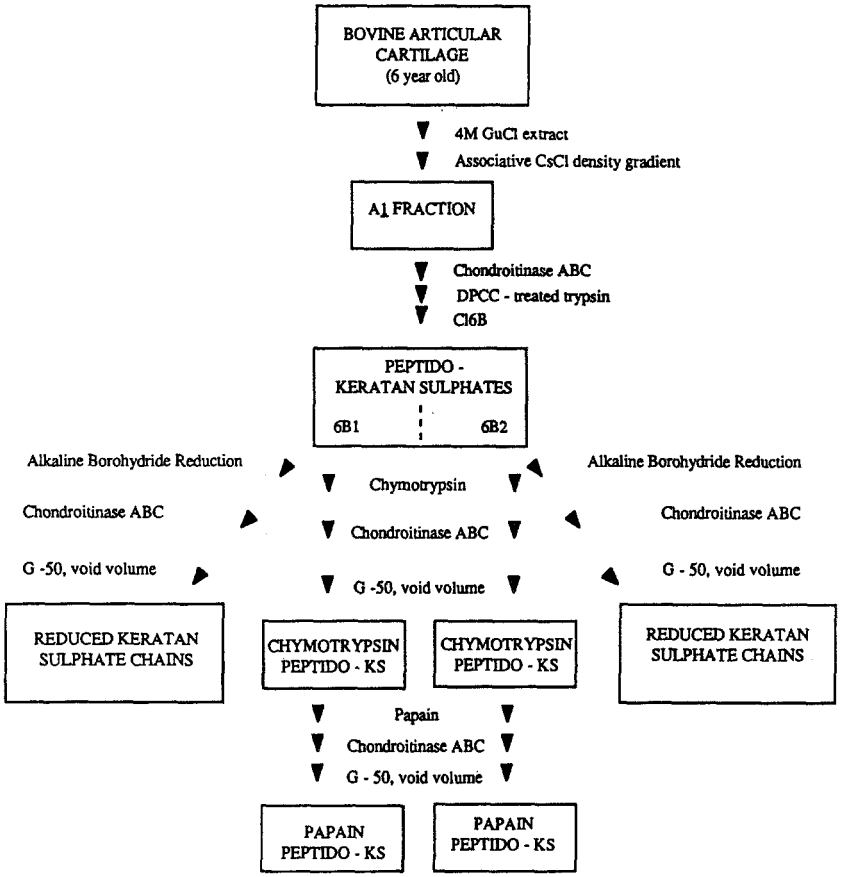

Figure 1. Summary of preparative procedures.

not contain keratan sulphate chains and thus the understanding of the distribution of keratan sulphate chains along the protein core comes largely from studies of proteolytic fragments derived from proteoglycans. Studies using the enzymes trypsin and chymotrypsin [3], have indicate that keratan sulphate chains occur in blocks in the so-called KS-rich region of the protein core and also as isolated chains amongst the chondroitin sulphate-rich regions [4].

Recent immuno-electron microscopic studies [5] using the anti-KS monoclonal antibodies Mz15 [6] and 5-D-4 [7], showed different apparent distributions of the keratan sulphate chains. These data suggested that it might be important to examine the structures of keratan sulphate chains from different locations within the proteoglycan or from different populations of proteoglycans.

Materials and Methods

Materials

Guanidinium chloride (practical grade) was purchased from the Sigma Chemical Co. (Poole, U.K.) and further purified as previously described [8].

Benzamidine hydrochloride, *N*-ethylmaleimide and sodium borohydride were also purchased from Sigma. Caesium chloride, disodium EDTA and 6-amino-hexanoic acid were obtained from BDH Chemicals (Poole, U.K.). 1,9-Dimethylmethylene blue was purchased from Koch-Light Laboratories (Haverhill, U.K.) and sodium 3-trimethylsilyl-($^2H_4$)propionate [($^2H_4$)TSP] from Lancaster Synthesis (Lancaster, U.K.).

Sepharose CL-6B, Sephadex G-50 and G-75 were from Pharmacia (Uppsala, Sweden). Bio-Gels P-2 and P-10 and Aminex A-9 were from Bio-Rad Laboratories Ltd. (Watford, U.K.). The enzymes, DPCC-treated trypsin (bovine pancreas, EC 3.4.21.4), chondroitinase ABC (*Proteus vulgaris*, EC 4.2.2.4), chymotrypsin (Type I-S, bovine pancreas, EC 3.4.21.1), papain (Papaya Latex, Type III, EC 3.4.22.2) and keratanase (pseudomonas species, EC 3.2.1.103) were purchased from Sigma.

Analytical Methods

Hexose was determined by an automated anthrone assay [9] with galactose as the standard. Sialic acid was assayed as in [10]. Uronate was assayed by an automated procedure [9] based on the modified carbazole assay [11] with glucuronolactone as the standard. Keratan sulphate was assayed using 1,9-dimethylmethylene blue [12]. Hexosamines were determined after hydrolysis in 4 M HCl for 7 h at 105°C under N_2, followed by isocratic elution in 0.35 M sodium citrate, pH 5.28, on a Bio-Rad Aminex A9 column (25 cm x 4.6 mm) at 72°C, and detected and quantified using a Bio-Rad Model 1755 refractive index monitor.

Preparation of Peptido-Keratan Sulphates

Proteoglycan aggregates (A1 fraction) were prepared from bovine femoral head cartilage (six year old animals) as previously described [8]. The preparative procedures are shown in Fig. 1. The A1 fraction (approx. 7.5 mg of proteoglycan/ml) was dialysed into 0.1 M Tris acetate, pH 7.3 and then digested with chondroitinase-ABC (2.5 units/g proteoglycan) followed by DPCC-treated trypsin (2 mg/g proteoglycan) as described in [3]. The digest was partially freeze-dried and then chromatographed on a column of Sepharose CL-6B (152 cm x 3.2 cm) eluted with 0.5 M sodium acetate/10 mM EDTA, pH 6.8 [13]. The two peptido-keratan sulphate-containing fractions were pooled as indicated (Fig. 2), dialysed against distilled water and lyophilised.

A proportion of each of the peptido-keratan sulphate fragments was further digested with chymotrypsin (0.12 units/mg) in 0.1 M sodium phosphate, pH 7.0, for 5 h at 25°C, followed by further chondroitinase ABC digestion and recovery in the void volume of a Sephadex G-50 column. Subsequent papain treatment was performed as described in [14].

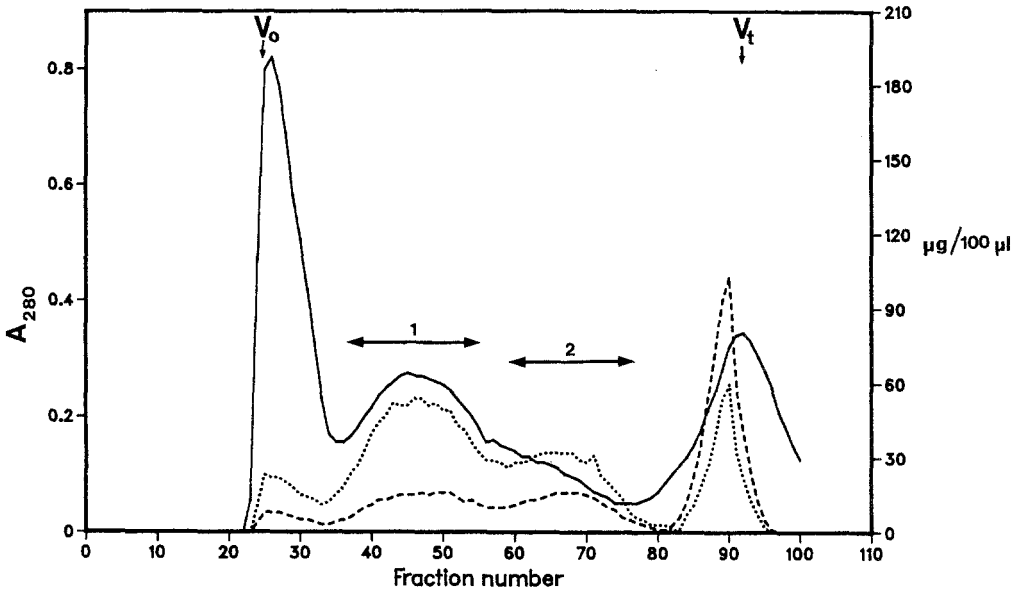

Figure 2. Preparative Sepharose CL-6B chromatography of chondroitinase ABC- and trypsin-treated proteoglycan aggregates.
The chondroitin ABC- and trypsin-treated A1 fraction was chromatographed on Sepharose CL-6B (152 cm x 3.2 cm), eluted with 0.5 M sodium acetate/10 mM EDTA, pH 6.8; flow rate 24 ml/h. Fractions (12 ml) were analysed for uronate (——), hexose (.....) and absorbance at 280 nm (____). The V_o and V_t were determined using Dextran Blue and glucuronolactone, respectively. Fractions were pooled as indicated.

Preparation of Keratan Sulphate Chains

Keratan sulphate chains were prepared by alkaline borohydride reduction [15] of the peptido-keratan sulphates (5 mg peptido-KS/ml 1 M sodium borohydride in 0.05 M sodium hydroxide) followed by desalting on a column of Bio-Gel P-2 (82 cm x 1.5 cm). The preparation was then digested with chondroitinase ABC (2 units/g) and the keratan sulphate was finally recovered by lyophilisation after desalting on a column of Sephadex G-50 (82 cm x 1.5 cm) eluted with 0.2 M NH_4HCO_3.

NMR Spectroscopy

Carbon NMR spectra of samples (50-100 mg), buffered to pH 7 with sodium phosphate and referenced to internal ($^2H_4$)TSP as previously described [16], were obtained at 25 MHz and 60°C on a JEOL FX-100 system equipped with a dual $^1H/^{13}C$ 5 mm V.T. probe.

Table 1. Analytical data for keratan sulphate fractions .

Fraction	Uronate[a]	Hexose[a]	Hexose/ Uronate[b]	GlcNAc/ GalNAc
A1-CB-T-6B1	7.8	25.0	3.2	-
A1-CB-T-6B2	14.8	30.7	2.1	-
A1-CB-T-6B1-C-CB-P-CB	-	-	-	7.4
A1-CB-T-6B2-C-CB-P-CB	-	-	-	9.4

[a] Percentage of dry weight.
[b] Weight ratio.

Keratanase Digestion

Keratan sulphate chains were borohydride-reduced, dissolved in 0.2 M sodium acetate, pH 7.4, and digested for 24 h with keratanase (1 unit/2.8 mg sample) at 37°C. Digests were chromatographed in 0.2 M NH_4HCO_3 on a Bio-Gel P-10 column (81 cm x 1.0 cm) eluted at 2 ml/h.

Results and Discussion

The preparative Sepharose CL-6B chromatography profile of the trypsin digest of chondroitinase-treated proteoglycan A1 fraction is shown in Fig. 2. The two major anthrone-positive peaks were pooled separately as shown and the analytical data are summarised in Table 1. Aliquots of the combined fractions (A1-CB-T-6B1 and -6B2) were re-chromatographed on an analytical Sepharose CL-6B column [see Fig. 3(i)]. The elution position of the A1-CB-T-6B1 fraction after further chondroitinase ABC and trypsin digestion [see Fig. 3(ii)] was unchanged. Thus, it is clear that no fragments of the size of A1-CB-T-6B2 were produced from A1-CB-T-6B1. Alkaline borohydride reduction of the A1-CB-T-6B2 fraction yielded KS chains whose elution position showed them to be smaller than the parent material [Fig. 3(iii)]. Hexosamine analyses (Table 1) were performed on papain-derived peptido-keratan sulphates of both the 6B1- and 6B2-series (see Fig. 1) which were shown to be free of chondroitin sulphate contamination by ^{13}C-NMR spectroscopic analysis. On the assumption that there is one *N*-acetylgalactosamine residue per chain, it becomes clear from the hexosamine data that the average M_r of the keratan sulphate chains is 5000 - 6000.

Representative ^{13}C-NMR spectra for the peptido-keratan sulphates and derived keratan sulphate chains are shown in Fig. 4. The obvious difference between A1-CB-T-6B1 and -6B2 [spectra (i) and (ii)] is that the latter contains a much greater proportion of chondroitin-4- and -6-sulphate chains (as indicated by the anomeric carbon signals at 104-104.5 and 106.4-

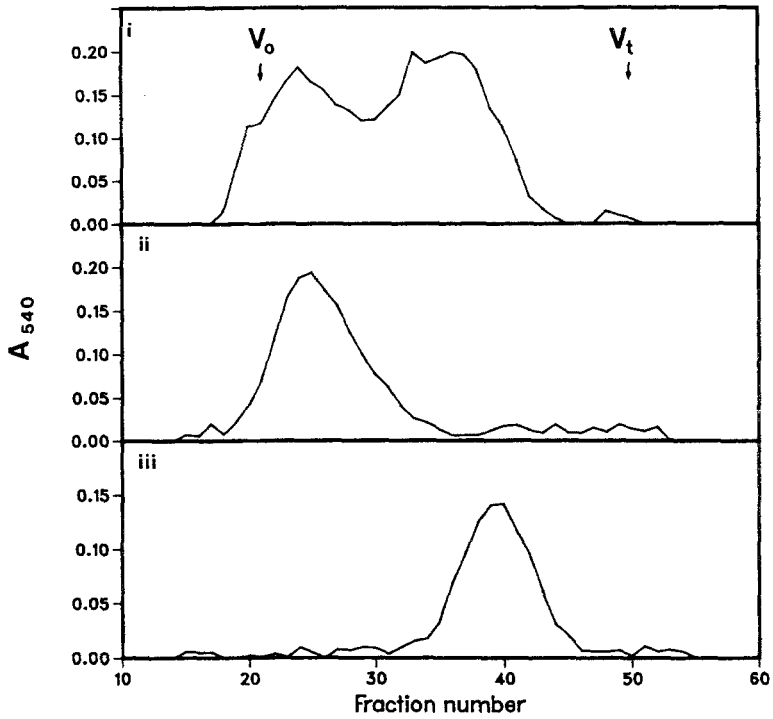

Figure 3. Analytical Sepharose CL-6B chromatography profiles of peptido-keratan sulphates.
(i). Peptido-keratan sulphates (A1-CB-T-6B1 and A1-CB-T-6B2); (ii) Peptido-keratan sulphate A1-CB-T-6B1 after further chondroitinase ABC and trypsin digestion; (iii) Keratan sulphate chains derived from A1-CB-T-6B2 after alkaline borohydride reduction.
The column (570 mm x 10 mm) was eluted at a flow rate of 12 ml/h with 0.5 M sodium acetate/10 mM EDTA, pH 6.8 and fractions (0.85 ml) were assayed with 1,9-dimethylmethylene blue (A_{540}). The positions of V_o and V_t were determined using bovine nasal septum proteoglycan and sodium chloride, respectively.

106.8 ppm) together with significantly increased peptide content (many resonances in the range 20-50 ppm). In the spectrum of A1-CB-T-6B2 [Fig. 4(ii)], a significant signal at *ca.* 110 ppm arising from C(4) in a terminal unsaturated uronic acid residue [17] confirms that, following chondroitinase ABC digestion, the chondroitin sulphate chains are now present as short stubs. The major unresolved resonances between 105 and 106 ppm arise from the two main anomeric carbons in the keratan sulphate moiety. Spectra (iii-iv) show that following reduction, the removal of both peptide and residual chondroitin sulphate components is essentially complete. The methyl carbon of the acetyl group is seen at *ca.* 25 ppm, C(2) from *N*-acetylglucosamine is found at *ca.* 58 ppm and two unsubstituted and two

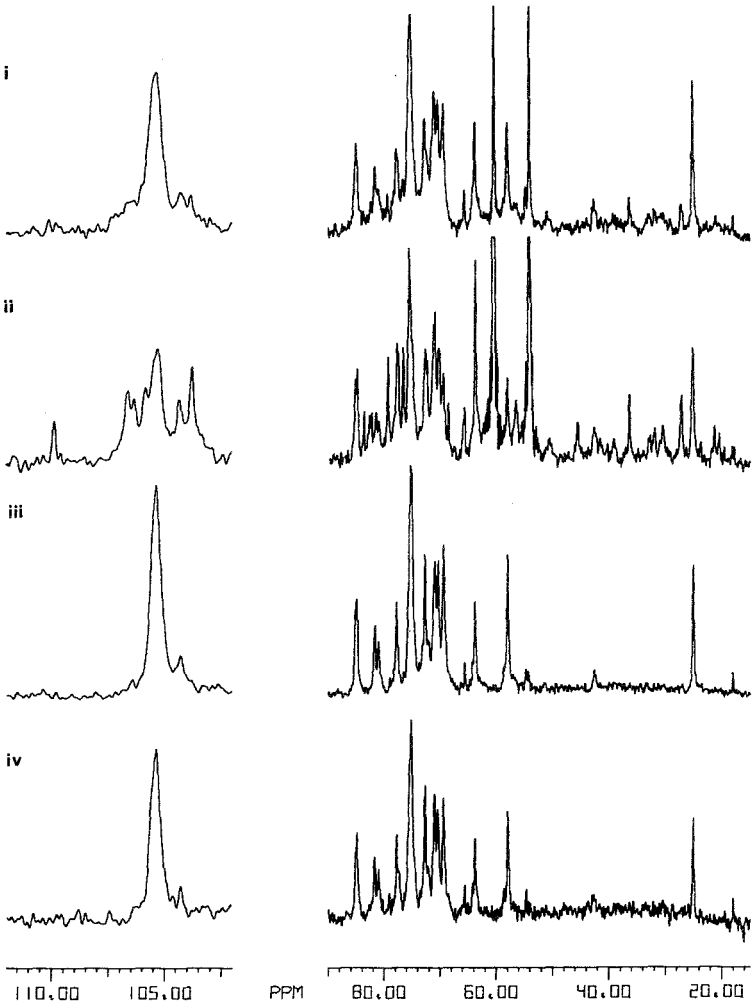

Figure 4. Partial ^{13}C-NMR spectra obtained at 25 MHz of: (i) Peptido-keratan sulphate, A1-CB-T-6B1; (ii) Peptido-keratan sulphate, A1-CB-T-6B2; (iii) Keratan sulphate chains, A1-CB-T-6B1-R-CB; (iv) Keratan sulphate chains, A1-CB-T-6B2-R-CB.

substituted methylene carbons are found at *ca.* 63 and 70 ppm, respectively [14]. Groups of signals at 81-82 and 85 ppm, respectively, represent C(4) in *N*-acetylglucosamine and C(3) in galactose residues. Minor but significant signals indicate the presence of fucose (CH_3- at *ca.* 18 ppm) and sialic acid (43, 54, and 66 ppm) in the keratan sulphate chains.

The NMR spectra (not shown) of peptido-keratan sulphates treated with chymotrypsin followed by chondroitinase ABC (A1-CB-T-6B1-C-CB and -6B2-C-CB, see Fig. 1) were closely similar to those shown in Fig. 4(i) and (ii) indicating that the chymotrypsin had had

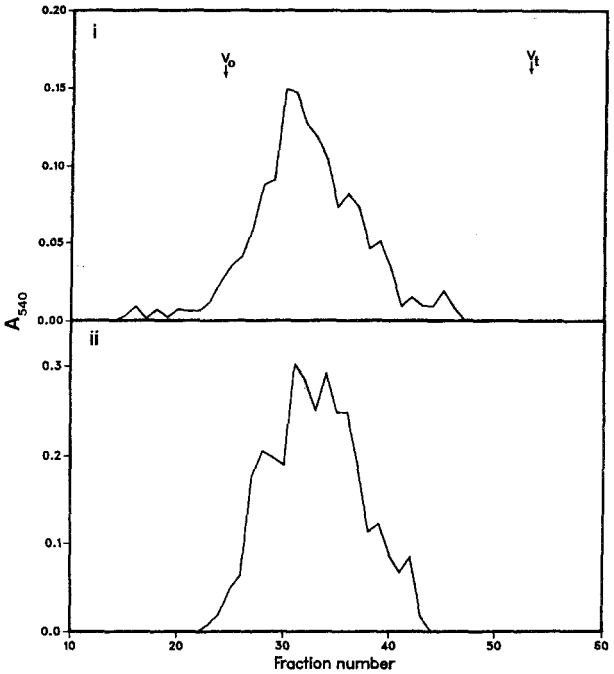

Figure 5. Analytical Sephadex G-75 chromatography profiles of keratan sulphate chains.
(i) A1-CB-T-6B1-R-CB; (ii) A1-CB-T-6B2-R-CB.
The column (67 cm x 1.0 cm) was eluted at a flow rate of 4 ml/h with 0.2 M ammonium bicarbonate and fractions (1.0 ml) were assayed with 1,9-dimethylmethylene blue (A_{540}). The V_o and V_t were determined using bovine articular cartilage proteoglycan and sodium chloride, respectively.

little or no effect. Subsequent treatment of these materials with papain followed by chondroitinase ABC yielded samples whose NMR spectra showed the presence of keratan sulphates with a similar structure to those shown in Fig. 4(iii) and (iv). It should be noted that the sulphation levels, as determined from the relative proportions of methylene signals, are not different from those observed in the alkaline borohydride-treated preparations; this indicates that the latter treatment does not result in significant removal of ester sulphate residues. There was, however, some residual peptide present.

The Sephadex G-75 chromatograms of the KS chains A1-CB-T-6B1-R-CB and -6B2-R-CB are shown in Fig. 5(i) and (ii). Both profiles appear to contain some partially resolved peaks.

Limit keratanase digests of both populations of KS chains were chromatographed on Bio-Gel P-10 and the elution profiles are shown in Fig. 6(i) and (ii). The arrows indicate the elution positions of the pentasulphated hexasaccharide (6), the trisulphated tetrasaccharide (4) and

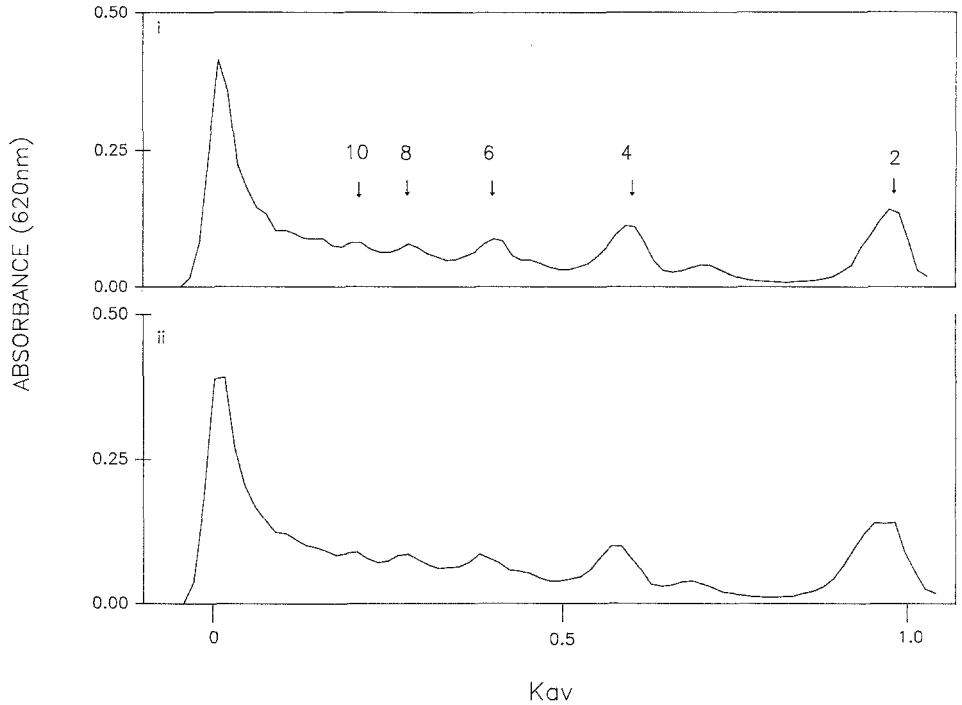

Figure 6. Analytical Bio-Gel P-10 chromatography profiles of keratanase digests of: (i) Keratan sulphate chains, A1-CB-T-6B1-R-CB; (ii) Keratan sulphate chains, A1-CB-T-6B2-R-CB.
The column (81 cm x 1.0 cm) was eluted at a flow rate of 2 ml/h with 0.2 M ammonium acetate and fractions (0.5 ml) were assayed for hexose (A_{620}). The V_o and V_t were determined using bovine serum albumin and sodium chloride, respectively. There is a change in column length between these two experiments. The arrows indicate the elution positions of the pentasulphated hexasaccharide (6), the trisulphated tetrasaccharide (4) and the monosulphated disaccharide (2) derived from corneal keratan sulphate [18].

the monosulphated disaccharide (2) derived from corneal keratan sulphate [18]. Both profiles are similar and show that substantial proportions of keratanase-resistant fragments are of dodecasaccharide size or greater.

Conclusion

Keratan sulphate chains isolated from both of the larger and smaller trypsin-derived fragments of proteoglycans were shown to be of similar molecular size and size distribution as judged, respectively, by their hexosamine ratios (estimated average M_r 5000-6000) and their elution behaviours upon gel permeation chromatography on Sephadex G-75. They also appeared to be of similar structure and sulphation level as shown by their ^{13}C-NMR spectra and the elution profiles of their keratanase digests.

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