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Determination and structural characterisation of dermatan sulfate in the presence of other galactosaminoglycans

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Abstract

Chondroitin sulfate and dermatan sulfate are galactosaminoglycans that have similar size and charge density thus making difficult their separation and accurate determination from tissue preparations. A procedure was developed, which was based on the specific action of chondroitinase B, that allowed the determination of dermatan sulfate content in a mixture of chondroitin sulfate/dermatan sulfate, its molecular mass (M_r), and iduronic acid content and distribution throughout the chain. According to this procedure, the galactosaminoglycan sample was treated with chondroitinase B and its profile, upon gel chromatography on Sepharose CL-6B, was compared to that of the initial sample. The differences in uronic acid content of the fractions of the gel chromatographies were plotted and a secondary profile was constructed, which corresponded to the elution profile of intact dermatan sulfate in the sample. From this profile, the size distribution of dermatan sulfate was obtained and its M_r was calculated. In addition, the accurate content of dermatan sulfate in the sample was determined. The digest contained oligosaccharides of variable size that were separated on BioGel P-10. From the separated oligosaccharides the distribution of iduronic acid throughout the dermatan sulfate chains was determined. The procedure was applied to the determination and partial characterisation of dermatan sulfate from sheep nasal cartilage, in which it is reported for the first time that it contains a significant proportion of dermatan sulfate chains of low iduronic acid content.

Keywords: Dermatan sulfate; Glycosaminoglycans; Nasal cartilage

1. Introduction

Various types of connective tissue contain diverse amounts of dermatan sulfate, as a part of different proteoglycans [1]. In cartilage, for example, the most abundant proteoglycan is aggrecan, which consists of a core protein onto which chondroitin sulfate, keratan sulfate and oligosaccharides are covalently attached [2–4]. However, it also contains small proteoglycans, such as biglycan and decorin, carrying two and one side chains, respectively, either chondroitin sulfate or dermatan sulfate, depending on the tissue source [5,6]. Chondroitin sulfate and dermatan sulfate are both galactosaminoglycans, and have similar backbone structures. All of the uronic acid of chondroitin sulfate is in the form of

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glucuronic acid (GlcA), while that in dermatan sulfate is a mixture of glucuronic acid and its C_5 epimer, iduronic acid (IdoA), in varying proportions. Thus, dermatan sulfate is a hybrid galactosaminoglycan [1,7,8] made up by two types of disaccharide repeating units, namely [GlcA(β1→3)GalNAc] and [IdoA(α1→3)GalNAc]. The IdoA residues are derived from the epimerization of GlcA residues on the growing chondroitin sulfate chain and may comprise from 0 to 80% of the chain total uronic acid. Small proteoglycans from skin have been found to contain the highest proportion of IdoA residues [9], whereas small proteoglycans from bovine tendon contain about 35% [10]. About equimolar amounts of both hexuronic acid residues have been observed in bovine sclera [11] and arterial tissue [12] proteoglycans. Small proteoglycans from articular cartilage have been found to contain significant proportions of iduronosyl residues [9], while those from bovine nasal or pig laryngeal cartilage relatively few [13] or not at all [14], respectively, on the basis of enzymatic susceptibility.

On the other hand, there is a considerable confusion regarding the distinction between chondroitin sulfate and dermatan sulfate. Generally, this distinction has been based on the extent of degradation of a galactosaminoglycan sample by bacterial lyases, i.e., chondroitinases AC, ABC and B. Chondroitinase AC cleaves the *N*-acetyl-hexosaminyl (β1→4) glucuronyl bonds of glycosaminoglycans and chondroitinase B the 4-sulfated *N*-acetyl-galactosaminyl (β1→4) iduronosyl bonds of dermatan sulfate. Chondroitinase ABC has the activities of both previous chondroitinases. All three enzymes produce disaccharides or oligosaccharides, depending on the enzyme and the substrate used, containing a Δ-4,5 unsaturated uronic acid residue at the non-reducing end, which absorbs UV with a maximum at 232 nm and a molar coefficient of 5500 M^{-1} cm^{-1}. By measuring the absorbance of a digested preparation of galactosaminoglycans and using this molar coefficient the amount of double bonds produced is obtained. Another methodology involves the analytical determination of the amounts of IdoA and GlcA by high-performance liquid chromatography (HPLC), after specific hydrolysis and derivatization of the sample [15]. In both cases, the measured amounts of hexuronic acids are compared with the generally accepted determination of total uronic acid by the borate–carbazole assay [16]. However, with the current methodology, the amounts of GlcA and IdoA are determined and not the amount of the glycosaminoglycan itself, which may contain variable percentage of these constituents, depending on the tissue source [9–14]. It becomes apparent that there is a significant difficulty for, at least, the quantitative determination and size estimation of the intact dermatan sulfate chains present in a mixture of galactosaminoglycans of low dermatan sulfate content.

In this study, we describe the development of a procedure that overcomes these difficulties. This procedure is based on the specified action of chondroitin lyases on galactosaminoglycan mixtures, followed by the characterisation of the degradation products with the use of analytical gel chromatography. With this methodology, we were able to establish the presence of dermatan sulfate in tissues so far believed to be free of dermatan sulfate, such as sheep nasal cartilage, and to derive information regarding its size and structure.

2. Experimental

2.1. Materials

Sepharose CL-6B and DEAE-Sephacel were obtained from Pharmacia (Uppsala, Sweden) and BioGel P-10 from Bio-Rad Labs. Chondroitin sulfate (from whale cartilage), dermatan sulfate (from bovine mucosa), papain (twice crystallised), chondroitinases AC I and B, and standard preparations of chondroitin disaccharides, ΔDi-4S, ΔDi-6S and ΔDi-OS were purchased from Sigma (St. Louis, MO, USA). All other chemicals used throughout this study were of the best available grade.

2.2. Preparation of galactosaminoglycans from sheep nasal cartilage

Galactosaminoglycans from sheep (3 years old) nasal cartilage were prepared essentially as described before [17]. In brief, the tissue was solubilized by proteolysis with papain. The digest was treated with 0.05 *M* sodium hydroxide–1 *M* sodium borohydride at 45°C for 48 h under vacuum [18] and the glycosaminoglycans were precipitated with four vol-

umes of ethanol. The glycosaminoglycan preparation was then dissolved in 0.005 *M* sodium sulfate and excess cetylpyridinium chloride (CPC) was added [17]. Keratan sulfate remained in the supernatant and the mixture of glycosaminoglycans in the precipitate was chromatographed on a DEAE-Sephacel column eluted with 0.1 to 1 *M* sodium chloride linear gradient. The galactosaminoglycans were recovered from the 0.7 *M* sodium chloride fractions [19].

2.3. Gel chromatography

Samples of galactosaminoglycans from sheep nasal cartilage, standard solutions of chondroitin sulfate, dermatan sulfate, alone and as a mixture, were chromatographed on an analytical column of Sepharose CL-6B (110×0.6 cm) in 0.5 *M* sodium acetate, pH 7.0 before and after treatment with specific degradative enzymes. The molecular mass (M_r) of the chains was calculated from Sepharose CL-6B chromatography data [20] using standards of chondroitin sulfate of known M_r (60 000, 25 000 and 16 000) [21]. The size of dermatan sulfate-derived Δ-oligosaccharides by chondroitinase B was determined by chromatography on BioGel P-10 (160×0.6 cm) in 1.0 *M* ammonium acetate, pH 7.0 using standard preparations of Δ-oligosaccharides [22]. The void (V_0) and total volumes (V_t) of each column were determined with sheep nasal cartilage proteoglycan aggregates and tritiated water, respectively.

2.4. Enzymatic digestions

Digestion of cartilage with papain was performed at 60°C for 18 h using 0.2 mg of enzyme per g wet mass of tissue in 0.1 *M* Tris–HCl, pH 7.2 containing 0.01 *M* Na_2EDTA and 0.005 *M* cysteine·HCl. Digestion of the galactosaminoglycans with chondroitinase AC was performed in 0.1 *M* Tris–acetate, pH 7.3 at 37°C for 24 h using 0.5 units of enzyme per mg uronic acid and with chondroitinase B in 0.05 *M* Tris–HCl, pH 8.0, containing 0.05% (w/v) bovine serum albumin, at 37°C for 24 h, using 0.3 units enzyme per mg uronic acid. Chondroitinases digestion was terminated by heating the solutions at 100°C for 3 min. The digestions were monitored by measuring the absorbance of the digest at 232 nm. It was found that the digestions were completed after 24 h.

2.5. Analytical methods

Hexuronic acid was determined by an automated version [23] of the borate–carbazole reaction [16]. GlcA and IdoA were determined by the method of Karamanos et al. [15]. In brief, the galactosaminoglycans were reacted with EDAC and then subjected to reduction of their hexuronic acid to the respective alditols. Finally, the samples were hydrolyzed and the monosaccharides were liberated, the alditols were purified using ion-exchange chromatography, and transformed to the respective perbenzoyl derivatives, which were separated and quantitated by HPLC.

The quantification of each Δ-oligosaccharide pool produced by the action of chondroitinases and isolated by gel chromatography, besides hexuronic acid determination, was based on their absorbance at 232 nm ($E_{232}=5500\ M^{-1}\ cm^{-1}$) owed to the C_4 double bond of the uronic acid at the non-reducing end. The accuracy of the assay was tested by using internal standards of Δ-disaccharides.

3. Results

3.1. Distribution of dermatan sulfate upon gel chromatography in a standard mixture of galactosaminoglycans

The standard dermatan sulfate and chondroitin sulfate preparations were analysed by HPLC [15] for their GlcA and IdoA content. The ratio of GlcA/IdoA in dermatan sulfate was found to be 2:3, thus the ratio of IdoA/total uronic acid was 0.6. Chondroitin sulfate contained solely GlcA. Known amounts, based on uronic acid analysis (borate–carbazole reaction), of standard dermatan sulfate and chondroitin sulfate were chromatographed separately on Sepharose CL-6B in 0.5 *M* sodium acetate, pH 7.0 (Fig. 1A). The galactosaminoglycans had different size distribution on the gel, however an overlapping was observed. In another experiment, a synthetic mixture of these galactosaminoglycans was chromatographed under the same conditions and eluted as a single asymmetric peak, without allowing the separation of its components. In each fraction the uronic acid content was determined by the borate–carbazole assay and expressed as nmol and % of

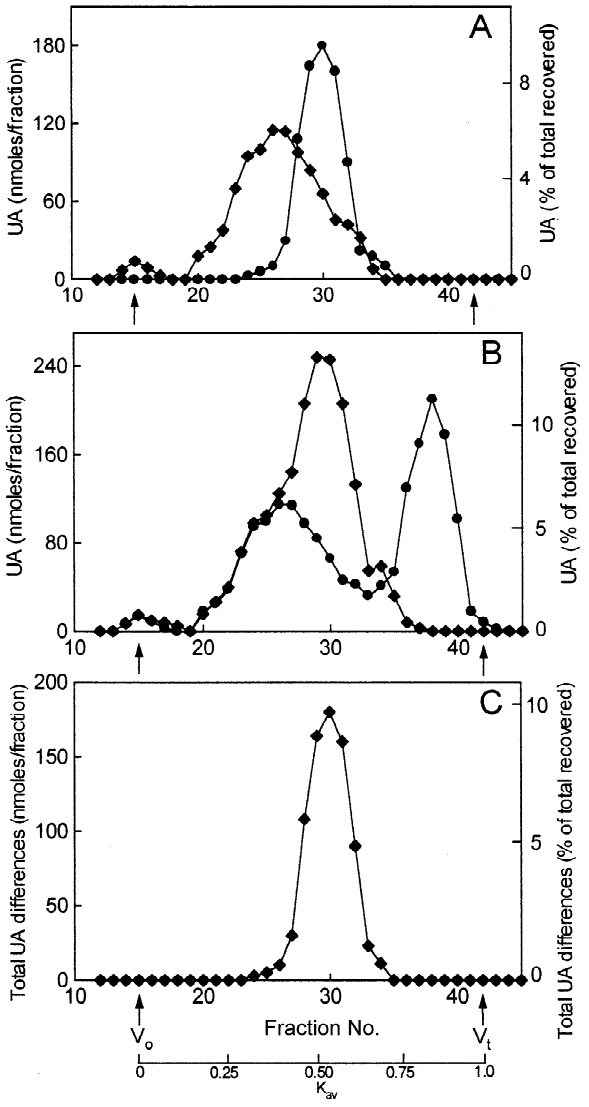

Fig. 1. Gel chromatography of standard galactosaminoglycans on an analytical column of Sepharose CL-6B. The column was eluted with 0.5 *M* sodium acetate, pH 7.0 and the uronic acid content of each fraction was measured by the borate–carbazole reaction. (A) Chondroitin sulfate (♦) and dermatan sulfate (●). (B) Mixture of chondroitin sulfate and dermatan sulfate before (♦) and after (●) digestion with chondroitinase B. (C) Secondary profile of dermatan sulfate, obtained from the positive differences of uronic acid in each fraction of the sample before and after digestion with chondroitinase B. For details see text.

total recovered (Table 1). These data were used to draw the elution profile as shown in Fig. 1B. An equal amount of the same mixture of galactosaminoglycans was subjected to exhaustive digestion with chondroitinase B, which cleaves the β(1→4) galactosaminyl–iduronic acid bonds. The extent of the digestion was estimated by measuring the absorbance at 232 nm (A_{232}), from which the nmol of liberated unsaturated oligosaccharides were calculated and found to correspond to 98% of the IdoA content of the mixture analysed by the method of Karamanos et al. [15], before the digestion; the finding suggesting that the digestion was complete. The digest was then applied on the same column and eluted with 0.5 *M* CH_3COONa, pH 7.0. Analysis of the fractions by the borate–carbazole assay revealed the presence of two peaks well separated. The uronic acid was expressed as nmol and % of total recovered (Table 1). It should be noticed that the sample recovery was greater than 98%. The uronic acid profile is shown in Fig. 1B, and it was constructed as for the undigested sample. As was expected, a part of the standard mixture, corresponding to chondroitin sulfate, was resistant to chondroitinase B and its size distribution remained unaltered after the treatment with the enzyme. The remainder of the mixture, corresponding to dermatan sulfate, was degraded by chondroitinase B and the produced oligosaccharides were eluted as a peak close to the total volume of the column. More than 95% of the A_{232} applied on the column was found in fractions corresponding to that peak. The absolute amounts (nmol) or percentage of uronic acid measured in each fraction of the chromatographies of the mixture before and after treatment with chondroitinase B were subtracted, fraction by fraction, and the differences were obtained (Table 1). The positive differences were used to construct a new profile (Fig. 1C), which represented the uronic acid content of fractions removed after the enzymatic digestion, i.e., the elution profile of intact dermatan sulfate chains. This new profile was called secondary profile and it was an exact image of the profile obtained when dermatan sulfate was chromatographed alone (Fig. 1A), showing the distribution of the chains upon gel chromatography. Thus the secondary profile allowed the determination of the range of the size of dermatan sulfate in the mixture. The calibration of the column with reference glycosaminoglycans of known M_r allowed also the calculation of the M_r of dermatan sulfate in the mixture, and of chondroitin sulfate, as well. In addition, by

Table 1
Distribution of uronic acid in each chromatographic fraction (Sepharose CL-6B) of the standard mixture of chondroitin sulfate (CS) and dermatan sulfate (DS) before and after digestion by chondroitinase B, expressed as nmol or percentage of total recovered[a]

Fraction No.	Initial mixture of CS and DS		Digested mixture of CS and DS		Differences before–after digestion	
	Uronic acid		Uronic acid		Uronic acid	
	nmol	% of total	nmol	% of total	nmol	% of total
12	0	0	0	0	0	0
13	0	0	0	0	0	0
14	7	0.37	7	0.37	0	0
15	15	0.80	15	0.80	0	0
16	10	0.53	10	0.53	0	0
17	8	0.43	8	0.43	0	0
18	5	0.29	5	0.29	0	0
18	0	0	0	0	0	0
20	16	0.86	16	0.86	0	0
21	27	1.44	25	1.37	2	0.10
22	40	2.14	38	2.03	2	0.10
23	72	3.85	70	3.74	2	0.10
24	98	5.24	95	5.08	3	0.16
25	105	5.61	100	5.30	5	0.29
26	125	6.68	115	6.10	10	0.58
27	144	7.70	114	6.10	30	1.60
28	206	11.10	98	5.20	108	5.90
29	248	13.30	84	4.50	164	8.80
30	246	13.15	66	3.53	180	9.62
31	206	11.01	46	2.46	160	8.50
32	133	7.11	42	2.25	90	4.85
33	55	2.94	32	1.70	22	1.24
34	59	3.15	41	2.45	18	0.70
35	32	1.71	54	2.94	−22	−1.10
36	8	0.43	130	7.00	−122	−5.57
37	3	0.16	170	9.09	−167	−9.97
38	0	0	210	11.30	−210	−11.30
39	0	0	178	9.50	−178	−9.50
40	0	0	102	5.40	−102	−5.40
41	0	0	18	0.96	−18	−0.96
42	0	0	10	0.53	−10	−0.53

[a] Values are the mean of three separate experiments.

calculating the total uronic acid provided by the secondary profile, the amount of dermatan sulfate (as degradable material) was obtained.

3.2. Distribution of iduronic and glucuronic acid residues within the dermatan sulfate chains

Chromatography of known amounts of standard dermatan sulfate on BioGel P-10 after digestion with chondroitinase B (Fig. 2A) or chondroitinase AC I (Fig. 2B) resolved the degradation products to populations of different size. Chondroitinase B produced six (a–f) and AC I ten (a–j) populations, based on total uronic acid analysis of the column fractions, which were pooled as it is shown by the horizontal bars. For each pool of Δ-oligosaccharides, the uronic acid content and absorbance at 232 nm were determined, and expressed as nmol. The nmol of double bonds reflected the IdoA or GlcA residues at the non-reducing end of the oligosaccharides, depending on the enzyme used. From the values of the molar ratios (uronic acid/double bonds), the size of the Δ-oligosaccharides was determined (Table 2), and it was found to be in good agreement with that obtained from a calibration curve with standard oligosaccharides (not shown). The digestion of der-

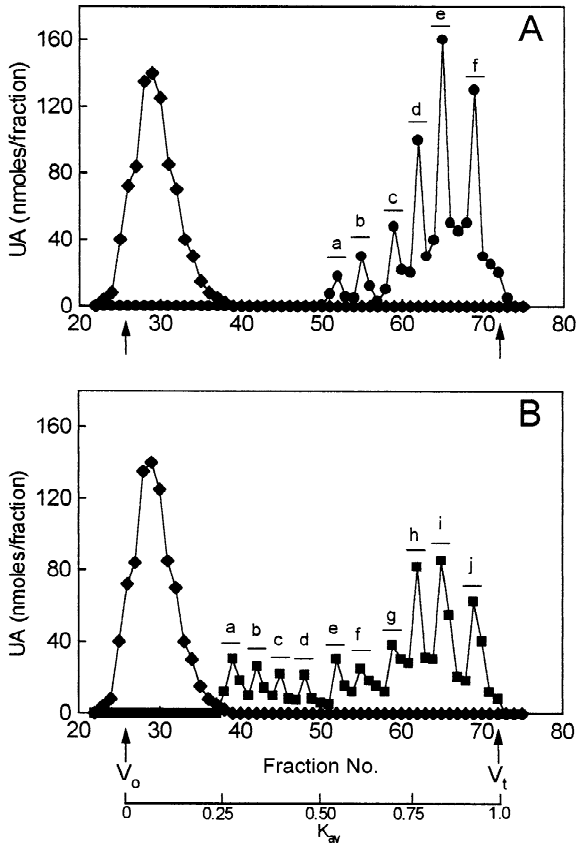

Fig. 2. Gel chromatography of galactosaminoglycans on an analytical column of BioGel P-10. The column was eluted with 1.0 *M* ammonium acetate, pH 7.0, and fractions of 1 ml were collected and their uronic acid was measured. (A) Standard dermatan sulfate before (♦) and after (●) digestion with chondroitinase B. (B) Standard dermatan sulfate before (♦) and after (■) digestion with chondroitinase AC. The oligosaccharide pools were isolated and their A_{232} was measured.

matan sulfate with chondroitinase B produced Δ-dodecasaccharides (Δ-12s) down to Δ-disaccharides (Δ-2s) and with chondroitinase AC I produced Δ-eicosasaccharides (Δ-20s) down to Δ-disaccharides. The presence of chondroitin sulfate was not found to affect the action of chondroitinase B on dermatan sulfate chains, regarding the extent and the size of oligosaccharides produced (not shown). The recovery of uronic acid as assessed by the borate–carbazole assay was greater than 95% in both cases.

In order to quantitate each pool of Δ-oligosaccharides derived from the digestion of dermatan sulfate with either chondroitinase B or chondroitinase AC, the total uronic acid, IdoA and GlcA were determined. Total uronic acid was measured by the borate–carbazole assay. IdoA and GlcA in intact dermatan sulfate were quantitated by HPLC. IdoA or GlcA in dermatan sulfate oligosaccharides derived after digestion with chondroitinase B or AC I, respectively, was estimated from the absorbance at 232 nm, using $E_{232}=5500\ M^{-1}\ cm^{-1}$, with internal standard the reference Δ-disaccharides. It should be noted that the sum of nmol of IdoA or GlcA, measured in the oligosaccharide pools from the double bonds content, was equal to the nmol of the respective sugars measured in intact dermatan sulfate chains by HPLC. The content of total uronic acid, IdoA or GlcA was expressed as percentage of the respective total amounts in the degradable material (Fig. 3A and B). It was observed that, after the digestion with chondroitinase B, the majority of IdoA, about 60%, was found in the Δ-disaccharide pool (Fig. 3A). This finding indicated that the greater proportion of IdoA in this sample of dermatan sulfate was present in the form of one or more clusters, i.e., continuous repeats of IdoA-containing disaccharides. This pool of Δ-disaccharides comprised 34% of the total uronic acid, suggesting that the IdoA disaccharide repeats in this sample occupy about one third of the length of the chain. About 40% of the IdoA, was distributed in Δ-oligosaccharides larger than disaccharides, containing one or more GlcA–GalNAc(SO_4) repeats. On the other hand, after digestion of dermatan sulfate with chondroitinase AC I (Fig. 3B) the Δ-disaccharide pool contained 40% of GlcA, comprising 17% of the total uronic acid in the starting material. This finding indicated that the larger proportion of GlcA (60%) was copolymerised with one or more of IdoA-containing repeats. The distribution of GlcA and uronic acid in Δ-oligosaccharide pools derived by chondroitinase AC I was in agreement with that obtained by chondroitinase B.

3.3. Galactosaminoglycans from sheep nasal cartilage

In order to establish the applicability of this methodology to mixtures of galactosaminoglycans from tissues, the glycosaminoglycans from sheep nasal cartilage were isolated and the galac-

Table 2
Composition of dermatan sulfate-derived Δ-oligosaccharides isolated after BioGel P-10 gel chromatography[a]

Dermatan sulfate Δ-oligosaccharides derived from chondroitinase B			Dermatan sulfate Δ-oligosaccharides derived from chondroitinase AC I		
Peak No.	Uronic acid[b]/double bonds[c] (molar ratio)	Size	Peak No.	Uronic acid[b]/double bonds[c] (molar ratio)	Size
a	6.20	Δ-12s	a	10.01	Δ-20s
b	4.95	Δ-10s	b	8.95	Δ-18s
c	4.15	Δ-8s	c	7.97	Δ-16s
d	3.13	Δ-6s	d	6.95	Δ-14s
e	1.97	Δ-4s	e	6.20	Δ-12s
f	1.02	Δ-2s	f	5.03	Δ-10s
			g	4.20	Δ-8s
			h	2.97	Δ-6s
			I	1.98	Δ-4s
			j	1.01	Δ-2s

[a] Values are the mean of three separate experiments.
[b] Uronic acid was determined by the borate–carbazole assay.
[c] Double bonds were estimated by the absorbance at 232 nm of the oligosaccharide pools isolated from BioGel P-10 using E_{232}=5500 M^{-1} cm^{-1}.

tosaminoglycans were purified by DEAE-Sephacel ion-exchange chromatography (Fig. 4A, inset). In the purified galactosaminoglycans, the ratio of GlcA to IdoA was determined by HPLC and found to be 96:4. An aliquot of the sample was digested with chondroitinase B. The digestion was complete and this was estimated as in the case of standard mixture. The galactosaminoglycans from sheep nasal cartilage were chromatographed on an analytical column of Sepharose CL-6B before and after digestion with chondroitinase B (Fig. 4A). The enzyme degraded appreciable amounts of the galactosaminoglycans, suggesting the presence of dermatan sulfate in the mixture; the majority of the sample, being chondroitin sulfate, remained unaltered. By determining the K_{av} of the resistant material, the M_r of chondroitin sulfate was calculated to be 18 000. The secondary profile of dermatan sulfate from sheep nasal cartilage was constructed (Fig. 4B) as previously described in details for the case of standard dermatan sulfate. From this secondary profile the relative amount of dermatan sulfate was calculated to be 22% of total galactosaminoglycans. The M_r of dermatan sulfate was determined to be 22 000. Thus the relative content of chondroitin sulfate and dermatan sulfate from a tissue digest and the respective M_r may be determined by this procedure.

Aliquots from the same samples, i.e., before and after digestion with chondroitinase B, were also chromatographed on an analytical column of BioGel P-10 and the elution profiles are shown in Fig. 5A. All of the degradation products were retarded by the gel, since no absorbance at 232 nm was measured in the V_0 fractions. From the amounts of uronic acid in the various Δ-oligosaccharide pools (belonging to dermatan sulfate) and in the V_0 fractions (belonging to chondroitin sulfate), the relative content of chondroitin sulfate and dermatan sulfate in the sample was calculated and found to be 78% and 22%, respectively, as it was also estimated by the secondary profile. The IdoA content of the degraded material was determined via the double bonds and was found to be 14% of the total uronic acid. Thus, the dermatan sulfate of sheep nasal cartilage was characterised as dermatan sulfate of low IdoA content. The action of chondroitinase B on sheep dermatan sulfate chains produced eight types of Δ-oligosaccharides, i.e., Δ-eicosasaccharides down to Δ-tetrasaccharides, with Δ-eicosasaccharides predominant (Fig. 5A and B). It is noteworthy that neither Δ-disaccharides nor Δ-hexasaccharides were produced with this sample. The absence of Δ-disaccharides might suggest the absence of IdoA-containing clusters in nasal cartilage.

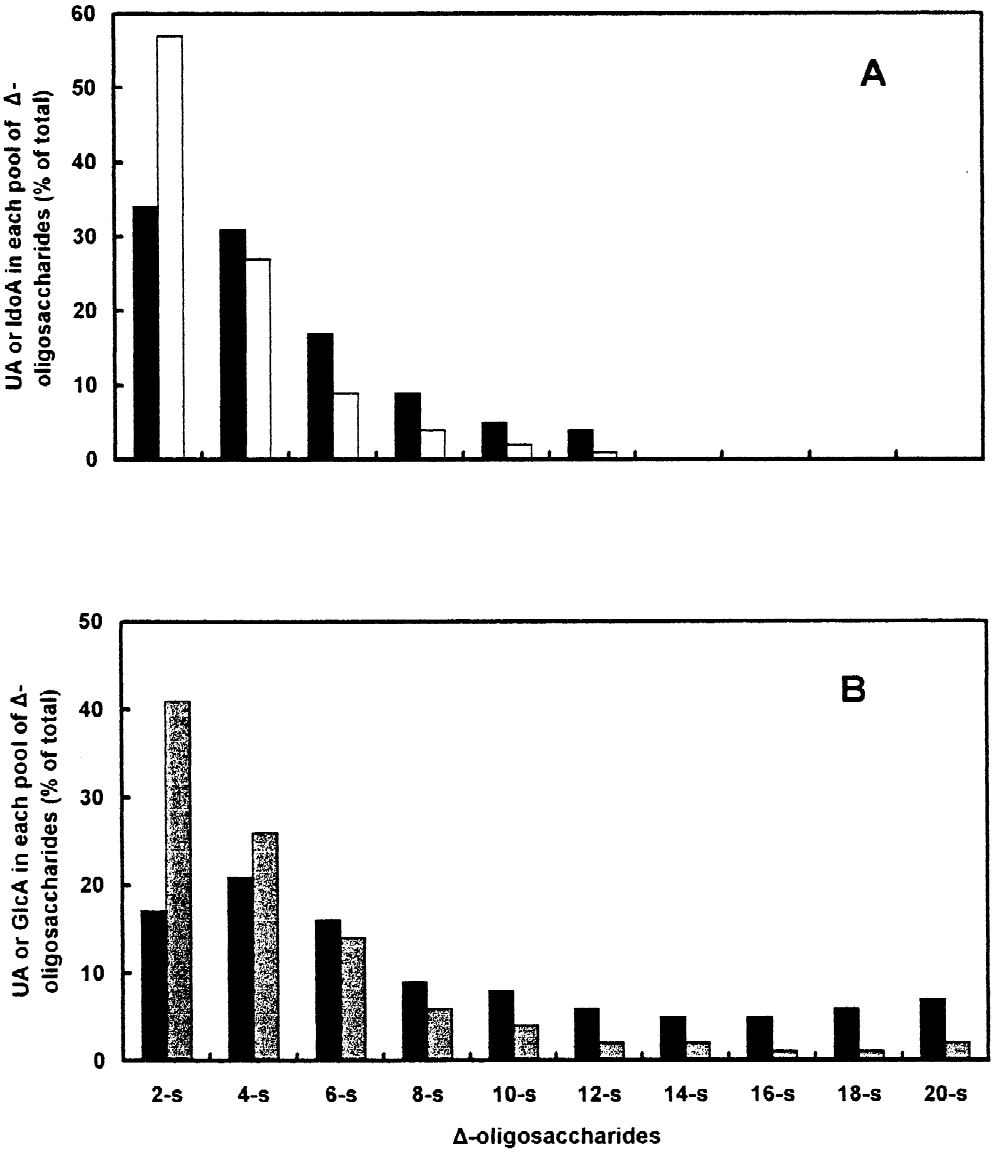

Fig. 3. Distribution of hexuronic acids in each Δ-oligosaccharide pool produced by the action of chondroitinase B (A) and of chondroitinase AC (B) on dermatan sulfate chains. Iduronic acid and glucuronic acid contents were calculated from A_{232} of the chondroitinase B or the chondroitinase AC digest, respectively. Filled boxes: uronic acid. Shadowed boxes: glucuronic acid. Open boxes: iduronic acid.

4. Discussion

The present study was undertaken with the aim to develop a procedure through which the presence, the amounts and the structural features of dermatan sulfate in tissues poor in this galactosaminoglycan might be determined. The outlined procedure, summarised in the Appendix, utilised the action of chondroitinase B, which cleaves β(1→4) galactosaminyl–iduronic acid bonds, and analytical gel chromatography of the degradation products. The produced oligosaccharides bore 4,5-unsaturated IdoA at their non-reducing end and absorbed UV light, therefore they could easily be quantitated by measur-

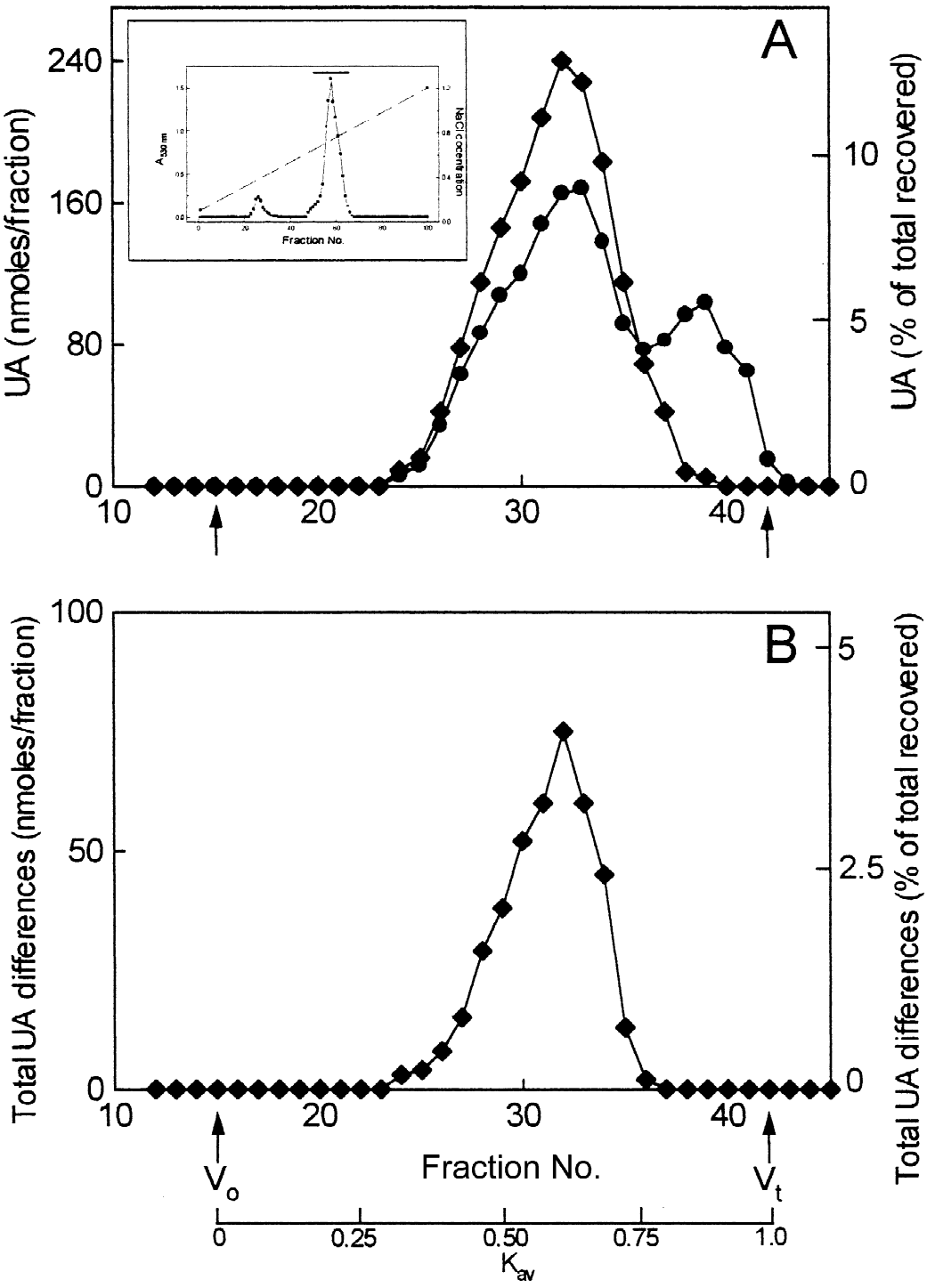

Fig. 4. (A) Gel chromatography of galactosaminoglycans isolated from sheep nasal cartilage before (♦) and after (●) digestion with chondroitinase B. Inset, purification of galactosaminoglycans of sheep nasal cartilage by DEAE-Sephacel, recovered from the fractions shown by the solid bar. (B) Secondary profile for dermatan sulfate of sheep nasal cartilage, constructed as in Fig. 1C.

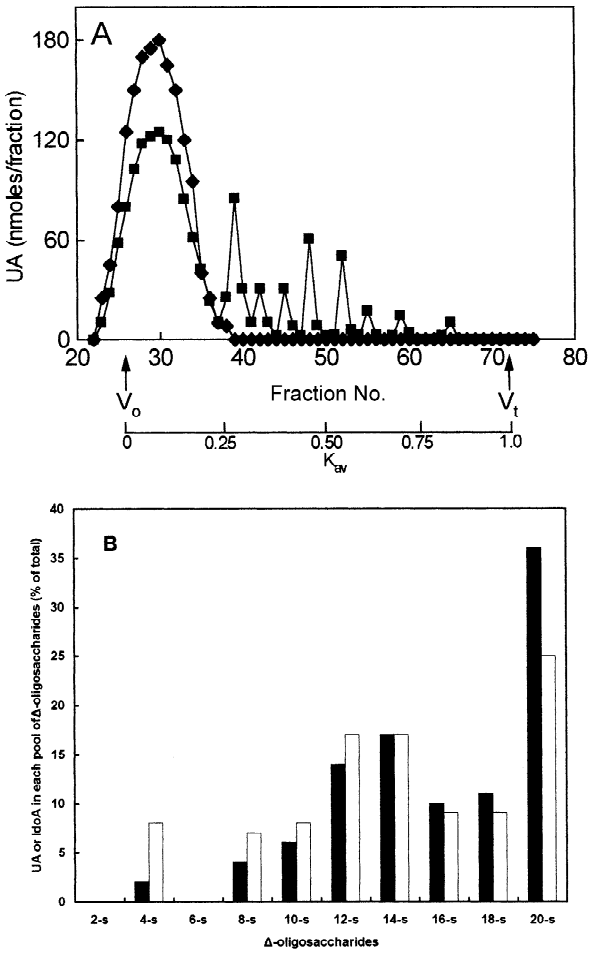

Fig. 5. (A) Gel chromatography of galactosaminoglycans, isolated from sheep nasal cartilage, on BioGel P-10 before (♦) and after (■) digestion with chondroitinase B. For details see Fig. 2. (B) Distribution of hexuronic acids in each Δ-oligosaccharide pool produced by the action of chondroitinase B on galactosaminoglycans isolated from sheep nasal cartilage. Iduronic acid content was calculated from A_{232} of the chondroitinase B digest. Filled boxes: uronic acid. Open boxes: iduronic acid.

ing their A_{232}. By using the data of the chromatography of galactosaminoglycans on Sepharose CL-6B, before and after digestion with chondroitinase B, a secondary profile could be constructed, which corresponds to the profile of intact dermatan sulfate chains. From the secondary profile, the total amounts and size of dermatan sulfate could be estimated. The construction of the secondary profile, is the only way to determine the M_r of dermatan sulfate when in a mixture with chondroitin sulfate or other glycosaminoglycans. However, it requires good quality of the chromatographic runs, i.e., no overloading of the column, quantitative recovery of the applied sample, and also the application to the column of exactly the same amounts of sample before and after each treatment. The secondary profile may also be constructed by using the difference of the percentages of uronic acid in each fraction before and after treatment with the enzyme. The idea of secondary profile may have a large variety of applications, provided that the compound of interest in a mixture is either sensitive or resistant to a specific treatment and an appropriate chromatographic system is available. In addition, by using the data of chromatography on BioGel P-10, before and after digestion with chondroitinase B, the amounts of each type of the derived Δ-oligosaccharides could be estimated.

The procedure shown in the Appendix could be applied when the digestion with chondroitinase B produced Δ-oligosaccharides, which were well separated from the undigested sample. However, the degradation of dermatan sulfate by chondroitinase B is dependent on the number and distribution along the chain of IdoA residues. When IdoA is the major constituent of dermatan sulfate or when it is well distributed within the chain, the degradation products are expected to be of very small size as compared to that of the intact dermatan sulfate chains. When dermatan sulfate is of low IdoA content and the latter is localised at one end of the chain, it may be difficult to interpret the results due to the overlap of resistant and partially degraded chains. This may be overcome by measuring, in each column fraction, the total uronic acid content (borate–carbazole reaction) and the A_{232}, before and after digestion with chondroitinase B. Uronic acid analysis will demonstrate the uronic acid shift to fractions of smaller hydrodynamic volume and the A_{232} the presence of products due to the enzyme action. So, by chromatographing, on Sepharose CL-6B, known amounts of chondroitin sulfate and dermatan sulfate as a mixture, before and after digestion with chondroitinase B, followed by determination in each fraction of both total uronic acid and A_{232}, a secondary profile could be constructed, which would give the distribution of intact chains in the sample. Assuming x nmol of dermatan sulfate and y nmol of chondroitin sulfate in a chromatographic fraction, the sum, $x+y=a$, is

calculated from the total uronic acid content of the fraction divided by the M_r of the chains, derived from calibration curves. After digestion with chondroitinase B, in the same fraction, there will be x' nmol of degraded dermatan sulfate and y nmol of chondroitin sulfate, and the sum, $x'+y=b$, is calculated as before. The value of x' is calculated from the A_{232} using $E_{232}=5500\ M^{-1}\ cm^{-1}$, and therefore, the nmol of chondroitin sulfate, y, are given from the difference $b-x'$. The nmol of intact dermatan sulfate, x, are finally calculated from the difference $a-y$, thus providing the nmol of dermatan sulfate eluted in each fraction before the digestion. From these data, the initial amount of each galactosaminoglycan in the sample and its M_r and weight-average molecular mass (M_w) are calculated. The application of this procedure is limited in only one case, that is, when the GlcA-containing degradation products obtained after the digestion of dermatan sulfate with chondroitinase B and derived from the non-reducing portion of the digested chain (which cannot be detected by A_{232}) are of similar hydrodynamic size with the resistant chains.

The procedure described was quite useful in determining the relative amounts and the size of dermatan sulfate in galactosaminoglycan preparations of a given tissue, together with the distribution of IdoA within the dermatan sulfate chains. Thus, it was found that sheep nasal hyaline cartilage contained appreciable amounts of structures degradable by chondroitinase B, indicative of dermatan sulfate. Although the IdoA content of the sample was very low, about 4% of the total uronic acid, the degradable material was quite high, about 22% of the total uronic acid, which made this procedure more reliable. The analysis of IdoA is a multistep procedure which increases the experimental error, whereas the application of the enzymatic–gel chromatography procedure is simpler and more sensitive. It is likely, however, to have restricted degradation of the dermatan sulfate structures if the IdoA residues are linked to non-sulfated galactosamine [24] and therefore misleading results may be obtained, regarding mainly the size and composition of the degradation products. However, for a detailed study of the primary structure of a given dermatan sulfate preparation, a combination of the present with other procedures [13,22] becomes necessary.

As outlined in the Results, by the present procedure, the size and relative content of chondroitin sulfate and dermatan sulfate may be determined in a mixture of galactosaminoglycans. However, if one is interested only in dermatan sulfate, the method may be directly applied to unfractionated tissue digests, without prior separation of the galactosaminoglycans. In such samples, information derived only from direct analysis of IdoA would be misleading, since heparan sulfate and/or heparin might be present.

The documentation of the presence of dermatan sulfate in hyaline cartilage from various sources has interested several investigators [9,13,14,25–27]. So far, only articular cartilage has been shown to contain dermatan sulfate in the form of biglycan and decorin, of about 40% IdoA content [9]. The data reported in this study established the presence of dermatan sulfate in another type of cartilage, nasal cartilage, and agree with a previous report [13] that a small amount of the side chains of biglycan from bovine nasal cartilage was sensitive to chondroitinase B digestion. The large differences in IdoA content between the nasal and articular cartilage dermatan sulfate may be related to the different functions of the respective tissues.

The extent and the distribution of uronosyl epimerization in dermatan sulfate chains seems to be different for different tissues. Dermatan sulfate derived from bovine mucosa appears to contain 60% of its IdoA in long IdoA–GalNAc repeats; the remainder being rather randomly distributed. Similarly, it has been shown that the majority of IdoA in dermatan sulfate from pig skin appears in IdoA–GalNAc repeats [22,28]. However, the dermatan sulfate of nasal hyaline cartilage seemed to have almost all of its IdoA randomly distributed, since all of the IdoA in the sample was recovered in oligosaccharides ranging from tetra-, octa- to eicosasaccharides long. It seems that, in tissues relatively poor in IdoA, the C_5-epimerase activity is expressed at long intervals. The difference in IdoA distribution may be elucidated by studying the turnover and kinetic properties of the C_5-epimerase in different tissues.

5. Nomenclature

GlcA	Glucuronic acid
IdoA	Iduronic acid

GalNAc	2-Acetamido-2-deoxy-β-D-galactose
ΔDi-OS	2-Acetamido-2-deoxy-3-*O*-(β-D-gluco-4-ene-pyranosyluronic acid)-D-galactose
ΔDi-4S and ΔDi-6S	Derivatives of ΔDi-OS bearing one sulfate at C_4 or at C_6 of the galactosamine, respectively
EDAC	1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide

Appendix A

We recommend for dermatan sulfate determination in an unknown preparation the following steps, which should be performed on a synthetic standard mixture in parallel. The synthetic standard mixture plays the role of internal reference.

1. Determination of total uronic acid (borate–carbazole reaction) and GlcA and IdoA by HPLC, in the intact sample.
2. Exhaustive digestion of a part of the sample with chondroitinase B and determination of total uronic acid and A_{232} in the digest, followed by the calculation of the molar ratio of uronic acid to double bonds. From the A_{232} the success of the digestion procedure may be obtained.
3. Gel chromatography on Sepharose CL-6B of aliquots from both the intact and the digested samples, followed by the determination of nmol of total uronic acid per fraction or the percentage distribution of total uronic acid per fraction. The uronic acid that may be measured, after the chromatography of the digested sample in fractions of higher K_{av} values compared to the intact sample, corresponds to dermatan sulfate oligosaccharides.
4. Calculation of the differences in total uronic acid content of the fractions obtained from the chromatography of intact and digested sample.
5. Construction of the secondary profile using the positive differences of 4 (Table 1), which corresponds to the chromatographic behaviour of the intact dermatan sulfate chains.
6. Gel chromatography on BioGel P-10 of aliquots from both the intact and the digested samples, followed by the determination of nmol of total uronic acid per fraction. From the chromatogram, the approximate number and size of oligosaccharides produced may be obtained.
7. Isolation of the oligosaccharide pools and determination of their A_{232} and uronic acid. Then, calculation of the molar ratios of uronic acid to double bonds, which give the size of oligosaccharides (see Table 2). Using this step, the distribution of IdoA and GlcA within the dermatan sulfate chains may be obtained.

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