Carbohydrate Research, 197 (1990) 159–169
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

ISOLATION AND SOME STRUCTURE ANALYSES OF A COPOLYMERIC CHONDROITIN SULFATE–DERMATAN SULFATE PROTEOGLYCAN FROM POST-BURN, HUMAN HYPERTROPHIC SCAR*

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(Received July 7th, 1988; accepted for publication in revised form, April 6th, 1989)

ABSTRACT

A D-glucuronic acid rich, copolymeric chondroitin sulfate (CS)–dermatan sulfate (DS) proteoglycan (PG) from post-burn hypertrophic scar tissue (HSc) was obtained by DEAE-cellulose chromatography and differential ethanol fractionation, and further purified on a Sepharose CL-6B column. CS–DS-PG protein content was 14% (w/w). The amino-terminal amino acid sequence of the first ten residues was as follows: NH_2-Asp-Glu-Ala-B-Gly-Ile-Gly-Pro-Glu-Val. This sequence is identical to that of human embryonic fibroblast cell (IMR-90) CS–DS-PG, as well as to human HSc–DS-PG. After chondroitinase ABC treatment, two peptides (M_r 22 000 and 16 000 daltons) were detected by sodium dodecyl sulfate–(polyacryl)amide gel electrophoresis (SDS-PAGE). ELISA analysis using rabbit antiserum raised against a synthetic peptide that contained 15 amino acids in the same sequence as the amino terminus of human fetal membrane PG showed significant reactivity with HSc CS–DS-PG. HSc CS-DS-PG had an apparent M_r of ~78 000 daltons, as determined by Sepharose CL-6B chromatography and SDS-PAGE. Alkaline borohydride treatment of CS–DS-PG liberated CS–DS glycosaminoglycan (GAG) chains having an M_r of 29 000 daltons. The conversion of xylose to xylitol indicated that the GAG chains are attached to the PG protein core at O-3 through a xylosyl–seryl linkage. CS–DS-PG also contained both *N* and *O*-linked oligosaccharides and did not aggregate with hyaluronic acid. These results, together with those reported previously, showed that HSc CS–DS-PG and DS-PG have the same A_1–A_{15} amino acid sequence at the amino terminus but different protein cores. HSc CS–DS-PG was completely digested with chondroitinase AC and is, therefore, distinctly different from HSc DS-PG.

*This work was supported by research funds from the Shriners Hospitals for Crippled Children and presented, in part, at the IXth International Symposium on Glycoconjugates, Lille, France, July 6–11, 1987.

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0008-6215/90/$ 03.50

INTRODUCTION

Proteoglycans (PGs) are important components of the extracellular matrix, and alterations in either their amounts or types may precede changes in collagen fibrillogenesis. Elevated PG levels[1-3] have been found in different types of human scar tissue when compared to normal human skin. To understand the role of these macromolecules in different types of scar formation, our studies have focused on the isolation and characterization of PGs from human post-burn scar tissue, specifically: (a) the core protein of PGs and their amino-terminal amino acid sequence, (b) the position and the amount of sulfation of 2-amino-2-deoxy-D-galactose residues in glycosaminoglycan (GAG) chains, and (c) PG size and the nature of GAG chains. In a previous paper[4], the isolation and characterization of dermatan sulfate (DS) PGs from normal and hypertrophic scar (NSc, HSc) tissue were reported. This communication reports the isolation and partial characterization of the D-glucuronic acid rich, copolymeric chondroitin sulfate–dermatan sulfate proteoglycan (CS–DS-PG) from HSc tissue.

EXPERIMENTAL

Preparation of HSc CS–DS-PG. — PG was isolated from HSc tissue of patients of various ages at the Shriners Burns Institute, Boston Unit, as previously described[3,4]. CS–DS-PG was obtained from PG precipitated with 75% ethanol in the first ethanol-precipitated fraction (PG75) by sequential precipitation with 50, 60, and 75% (v/v) ethanol[5,6], as summarized in Table I. Briefly, PG75 (Table II, ref. 4) was dissolved in 4M guanidinium chloride buffer (20 mL) containing proteinase inhibitors, and 95% ethanol was added to achieve a final concentration of 50% (v/v) of ethanol. The mixture was kept overnight at 4° and the precipitate (PG 75/50) collected by centrifugation (38 000 r.p.m., Beckman 60 Ti rotor) for 30 min. The ethanol concentration of the supernatant was increased to 60% (v/v), and again the precipitate (PG 75/60) was collected by centrifugation. Finally, this procedure was repeated with an ethanol concentration of 75% (v/v) to give PG 75/75.

Column chromatography. — A Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, NJ) analytical column (1.0 × 142 cm) was equilibrated and eluted with 3M guanidinium chloride. The flow rate was 4 mL/h, and 2-mL fractions were collected. V_o and V_t represent the elution positions of Blue Dextran and D-glucurono-1,4-lactone, respectively.

The copolymeric CS–DS-GAG and oligosaccharide constituents obtained from HSc CS–DS-PG by alkaline borohydride treatment were fractionated on a Bio-Gel P-10 column (1.5 × 150 cm) (Bio-Rad) eluted with 0.1M ammonium formate. The flow rate was 10 mL/h, and 5-mL fractions were collected.

Analytical procedures. — The uronic acid content of the CS–DS-PG and GAG column fractions were determined by the carbazole reaction[7]. Amino acid analyses were performed by ion-exchange chromatography using an automated

amino acid analyzer[8] after hydrolysis of the CS–DS-PG samples with 6M HCl for 24 h at 106°. Neutral sugars (except galactose, *N*-acetylneuraminic acid, and hexosamines) were determined by gas–liquid chromatography[9] using an OV-17 column. (These analyses were performed, in part, by K. Linsley in the Carbohydrate Research Laboratory, Massachusetts General Hospital, Boston, Dr. R. W. Jeanloz, Director.) The galactose, and the glucuronic and iduronic acid contents were determined by g.l.c. using an SE-30 column. Total sulfate content was determined by the method of Antonopoulos[10]. Amino-terminal amino acid sequence data were obtained by use of a model 470 gas-phase protein sequencer (ref. 11, Applied Biosystems, Foster City, CA). The phenylthiohydantoin amino acids were identified by h.p.l.c. (Hewlett–Packard 1090) with a 1040 diode-array detector equipped with Zorbax-C_{18} column (Dupont) by Mr. William S. Lane at the Harvard University Microchemical Facility, Cambridge, MA.

Isolation of copolymeric CS–DS-GAG chains. — CS–DS-PG samples (2 mg) were treated with 50mM NaOH–M $NaBH_4$ (500 μL) under the conditions of Carlson[12].

SDS–poly(acrylamide)-gel electrophoresis. — Electrophoresis was performed with 7% poly(acrylamide) gels in the presence of 0.1% sodium dodecyl sulfate[13].

M, *Determination of the core protein.* — CS–DS-PG was digested with chondroitinase ABC (CS ABCase) in the presence of proteinase inhibitors[14]. The reaction mixture was analyzed by SDS-PAGE. Gels were stained with Coomassie Blue to detect peptides and protein.

Chondroitinase AC digestion. — CS–DS-PG was treated with chondroitinase AC under the same conditions as used for CS ABCase, as described above, except that proteinase inhibitors were not added.

Electrophoresis on cellulose acetate. — GAG chains released by alkaline borohydride treatment of CS–DS-PG were analyzed on cellulose acetate membrane[15]. Staining was performed with Alcian Blue.

Analysis of CS–DS-PG by gel electrophoresis. — Before and after treatment with testicular hyaluronidase (Worthington, 12.6 units of enzyme/100 μg of sample, 90 min at 37°) in 0.05M sodium acetate buffer, pH 5.0, containing 0.15M NaCl, samples of CS–DS-PG were analyzed by the gel electrophoresis method of Cowman *et al.*[16].

Quantitative determination of nonsulfated, and of 4- and 6-sulfated disaccharides. — Quantification of the 4′,5′-unsaturated disaccharides generated from CS–PG sample (25 μg) after treatment with CS ABCase (0.5 unit; Sigma) in 0.1M sodium fluoride (0.25 mL) for 18 h at 37° was performed by the method described in a previous paper[4].

Viscosity measurements. — Viscosity measurements were performed as previously described[17,18].

ELISA. — Antiserum against the peptide having an *N*-terminal 15 amino acid sequence of human fetal membrane (HFM) DS-PG[19] was raised in rabbits and utilized as described earlier[20]. The analyses were performed by Dr. T. Krusius in

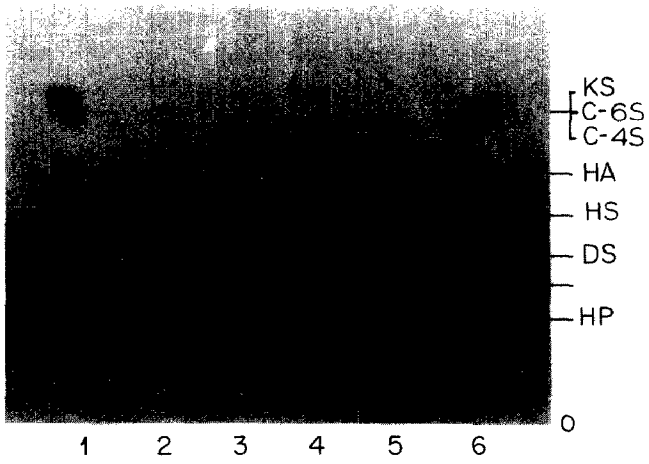

Fig. 1. Cellulose acetate electrophoresis of: Lanes 1 and 6, mixtures of reference GAGs; lane 2, purified HSc CS–DS-PG plus CS ACase; lane 3, purified HSc CS–DS-PG; lane 4, B fraction (Fig. 1, Ref. 3); and lane 5, one-time 75% EtOH-precipitated fraction. The origin (0) and the mobility of the reference GAGs, *i.e.*, heparin (HP), heparin sulfate (HS), hyaluronic acid (HA), dermatan sulfate (DS), keratan sulfate (KS), and chondroitin 4- and 6-sulfate (C-4S, C-6S) are indicated. The reference heparin also contained an additional GAG component, indicated by an unmarked line.

Dr. Ruoslahti's laboratory in La Jolla, CA, and in his laboratory at the Department of Medical Chemistry, University of Helsinki, Finland.

RESULTS AND DISCUSSION

Two major PGs, copolymeric CS–DS and DS, were present in the B fraction (Fig. 1, Lane 4) of the 4M guanidinium chloride extract of post-burn scar tissue[3] (Fig. 1). The possibility of the degradation of these PGs during isolation procedures

TABLE I

SECOND FRACTIONATION OF THE PG75 FRACTION BY A DIFFERENTIAL ETHANOL-PRECIPITATION PROCEDURE SHOWING THE DISTRIBUTION AND RECOVERY OF PG CONSTITUENTS

Fraction	*PG profile*[a]				*Percent (w/w) of PG75*	*Percent of total*
	DS	*HS*	*HA/DS'*	*CS or copolymeric CS/DS*		
PG75/50[b]	+	Trace	Trace	Trace	9.95	10.58
PG75/60		Trace		+	38.49	40.96
PG75/75				+	41.47	44.13
PG75/75 (supernatant)	Trace	(+)		(+)	4.05	4.33

[a]Abbreviations: DS, dermatan sulfate; HS, heparan sulfate; HA, hyaluronic acid; and CS, chondroitin sulfate. [b]Per cent of ethanol in second fractionation.

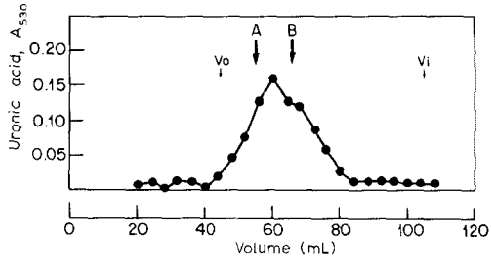

Fig. 2. Sepharose CL-6B column (1.0 × 142 cm) chromatography of HSc CS–DS-PG with 3M guanidinium chloride and 0.05M sodium acetate buffer, pH 5.8. The column fractions were analyzed for uronic acid content. The arrows marked V_o and V_i indicate the elution position of Dextran Blue 2000 and uronic acid, respectively. The elution positions of β-D-galactosidase (M_r 130 000 daltons) and bovine serum albumin (M_r 66 000 daltons) are indicated by arrows A and B, respectively.

was examined earlier[4] by implementing the various isolation procedures described by Pearson and Gibson[21], as well as by Damle *et al.*[22]. PGs isolated by these diverse methods were of similar sizes. DS-PG was separated by differential ethanol precipitation[4], leaving mainly copolymeric CS–DS-PG in the PG75 fraction. Pure copolymeric CS–DS-PG was isolated by a second sequential precipitation with 50, 60, and 75% (v/v) ethanol from PG75. The yields of PG present in various fractions are given in Table I. PG75/75 contained 44% of the total PG present in the PG75 fraction. Cellulose acetate plate electrophoresis (Fig. 1, Lane 3), which showed a single Alcian Blue staining band with a mobility similar to the reference C-4S–C-6S band, revealed this to be pure copolymeric CS–DS-PG. The same results (data not given) were obtained by CS–DS-GAG chains released after alkaline borohydride treatment. This clearly illustrates the copolymeric nature of the CS–DS-PG.

In comparison to HSc, relatively small amounts of CS–DS-PG were present in human normal skin or NSc tissue. Further characterization of CS–DS-PG involved treatment with CS ACase; no Alcian Blue positive band was observed (Fig. 1, Lane 2). On a Sepharose CL-6B column (Fig. 2), HSc CS–DS-PG had a K_{av} of 0.25, whereas D-galactose oxidase (M_r 130 000 daltons) and bovine serum albumin

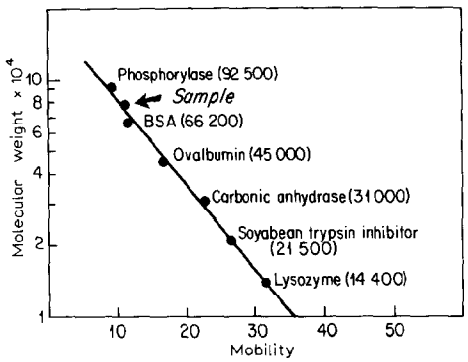

Fig. 3. Molecular-weight determination of HSc CS–DS-PG by SDS-PAGE.

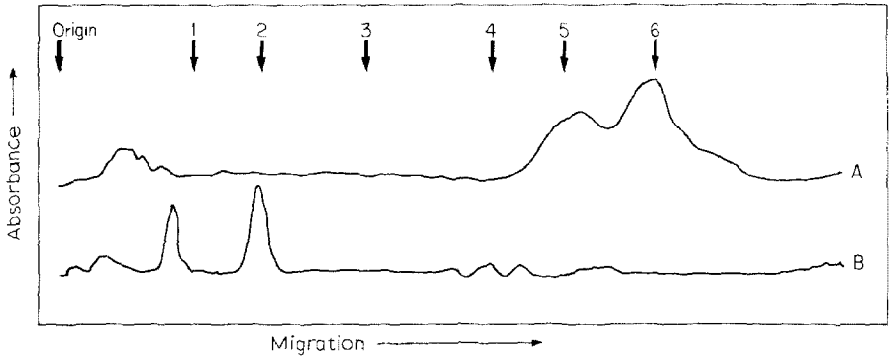

Fig. 4. Densitometric tracing of: (A) SDS-PAGE analysis of protein core of HSc CS–DS-PG sample obtained by treatment with CS ABCase; (B) CS ABCase alone. The arrows marked 1, 2, 3, 4, and 5 indicate the peak mobility of reference peptides having M_r values of 66 200, 45 000, 31 000, 21 500, and 16 000 daltons, respectively.

(M_r 66 000 daltons) had K_{av} of 0.20 and 0.39, respectively. The M_r of HSc CS–DS-PG was further determined by use of a logarithmic plot of the M_r of standard proteins and found to be ~78 000 daltons (Fig. 3). The M_r values of small DS-PGs were determined earlier by SDS-PAGE and found to be similar to those determined by sedimentation equilibrium[21].

The amino acid compositions of HSc CS–DS-PG, DS-PG, and pig skin PG[22] were found to differ. The protein content of HSc CS–DS-PG is higher at 13.6% than that of pig skin CS-PG (4–5%). In HSc CS–DS-PG, the proportions of glycine, leucine, and valine residues are lower, whereas those of aspartic acid and threonine residues are higher than those found in pig skin PG.

After CS ABCase digestion in the presence of proteinase inhibitors, HSc CS–DS-PG gave two peptides with M_r values of 21 500 and 16 000 daltons (Fig. 4). The first 10 amino acids of the amino-terminus amino acid sequence (NH_2-Asp-Glu-Ala-O-Gly-Ile-Gly-Pro-Glu-Val-) of HSc CS–DS-PG, HSc DS-PG[4], and cloned cDNA of CS–DS-PG[20] from human embryonic fibroblast are identical. However, the amino acid composition of the PGs varies; this could be due to their

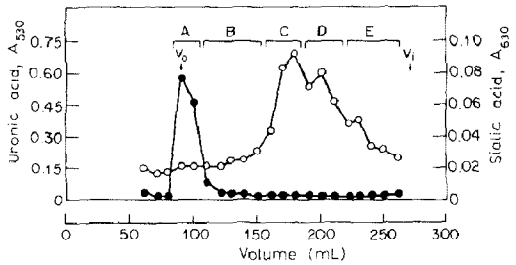

Fig. 5. Bio-Gel P-10 chromatography of HSc CS–DS-PG after treatment with alkaline borohydride: (—●—●—●) uronic acid (A_{530}), (—○—○—○) sialic acid (A_{630}). The column fractions indicated were pooled.

TABLE I I

CARBOHYDRATE COMPOSITION OF HSc CS–DS-PG BEFORE AND AFTER ALKALINE BOROHYDRIDE TREATMENT, AND COMPOSITION OF 4′,5′-UNSATURATED DISACCHARIDES OBTAINED

Carbohydrate component (%, w/w)	*Alkaline treatment*	
	Before	*After*[a]
2-Acetamido-2-deoxyglucose	1.69	0.45
2-Acetamido-2-deoxygalactose	12.71	10.80
Glucuronic acid	9.54	10.94
Iduronic acid	1.42	[b]
Xylitol	[b]	0.21
Xylose	0.33	0.09
Mannose	0.16	0.26
Galactose	4.24	1.17
N-Acetylneuraminic acid	4.09	0.54
Sulfate ester	10.6	
4′,5′-Unsaturated disaccharides	*Percent of total*	*Percent of HSc DS-PG*[c]
4-Sulfate	53	92
6-Sulfate	38	2
Nonsulfated	8.5	4
4,6-Disulfate	[b]	2

[a]GAG chains; see Fig. 5, peak A. [b]Not detected. [c]Ref. 4.

various protein cores. The possibility of the degradation of the protein core of human scar PGs was examined earlier[4], and the presence of two peptides in the protein core of HSc PGs was clearly shown. Moreover, previous studies[21,23] clearly demonstrated that small DS-PGs from different tissues within the same species, differing in amino acid compositions, could have identical amino-terminus amino acid sequences. Two possible explanations for the variations in the structure of protein cores are: (a) *in vivo* proteolytic cleavage occurs, or (b) synthesis of a slightly different protein core takes place. In the latter case, it is possible that the protein core may have a role in regulating different aspects of the biosynthesis of GAG chains (*i.e.*, polymerization, epimerization, or elongation). The fourth amino acid of the amino-terminus appears to be the attachment site of copolymeric CS–DS-GAG chain to the protein core of HSc CS–DS-PG. GAG chains in bovine skin DS-PG[24], sclera DS-PG-II[23], and cartilage DS-PG-II[25] were also reported to be attached to the fourth amino acid of the protein core.

The protein cores of both HSc CS–DS-PG and DS-PG showed cross-reactivity with a rabbit antiserum raised against the synthetic peptide (15 amino acids) having the sequence of PG-40 isolated from HFM (data not shown)[19]. This suggests an identical A_1–A_{14} amino acid sequence of the amino terminus sequence of HSc CS–DS and DS-PG. The carbohydrate composition of CS–DS-PG before and after alkaline borohydride treatment (Fig. 5, peak A), as well as the content of the 4,5-unsaturated disaccharides liberated after CS ABCase treatment of CS–DS-PG, is

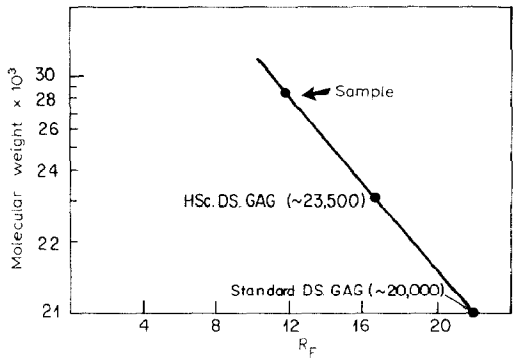

Fig. 6. Molecular-weight determination of CS–DS-GAG chains by Cowman gel electrophoresis.

given in Table II. The presence of *N*-acetylneuraminic acid and 2-amino-2-deoxyglucose (~12% of the total hexosamine) suggests that CS–DS-PG contains *N*-linked oligosaccharides[25]. Both 4′,5′-unsaturated disaccharide 4- and 6-sulfate (ratio 1.4:1) were present in HSc CS–DS-PG, whereas 92% of 4′,5′-unsaturated disaccharide 4-sulfate was present in HSc DS-PG[4]. The alkaline borohydride treatment of CS–DS-PG destroyed 73% of xylose, suggesting a xylosyl–serine, carbohydrate–protein linkage[26]. The loss of galactose in the Bio-Gel P-10 column A peak after alkaline treatment also suggested the presence of *N*-oligosaccharides.

The β-elimination products were fractionated on a Bio-Gel P-10 column (Fig. 5), and the void volume, uronic acid-positive peak (A) of GAG chains was analyzed on a Sepharose CL-6B column. The logarithm of the M_r value of NSc and HSc GAG chains[4] was plotted against the K_{av} value (Fig. 6). The M_r value of CS–DS-GAG was approximately 28 800. The carbohydrate composition of the three oligosaccharides fractions obtained by Bio-Gel P-10 column chromatography (B, C, and

TABLE III

COMPOSITION[a] OF OLIGOSACCHARIDE FRACTIONS (B, C, AND D) OBTAINED BY BIO-GEL P-10 CHROMATOGRAPHY[b] OF ALKALINE BOROHYDRIDE-TREATED HSc CS–DS-PG

Carbohydrate component	*Fractions*		
	B	*C*	*D*
Xylitol and fucose	0.59	0.07	0.15
2-Amino-2-deoxygalactitol	[c]	1.06	1.20
Xylose	0.30	0.28	0.11
Galactose	7.70	1.86	2.55
Mannose	4.53	1.34	1.77
2-Acetamido-2-deoxyglucose	1.00	1.00	1.00
2-Acetamido-2-deoxygalactose	6.82	0.19	0.14
N-Acetylneuraminic acid	1.98	1.05	0.59
Glucuronic acid	9.30	0.32	[c]

[a]Residue per residue of 2-acetamido-2-deoxyglucose. [b]See Fig. 5. [c]Not detected.

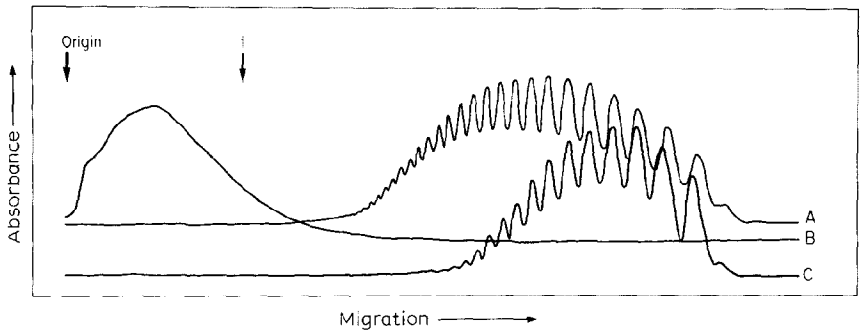

Fig. 7. Gel electrophoresis[16] of HSc–CS-PG with and without testicular hyaluronidase: (A) HSc CS–DS-PG treated with testicular hyaluronidase, (B) HSc CS–DS-PG and (C) standard CS-GAG (Sigma) treated with testicular hyaluronidase. Arrow 1 indicates the migration position of standard chondroitin 4-sulfate (Sigma).

D) was determined by g.l.c. (Table III). The absence of 2-amino-2-deoxygalactitol and the presence of 2-acetamido-2-deoxyglucose, mannose, and *N*-acetylneuraminic acid in the B fraction indicated that this fraction contains mainly *N*-oligosaccharides. The presence of glucuronic acid and xylitol is due to incomplete separations from the CS–DS-GAG chains. Fractions C and D contained 2-amino-2-deoxygalactitol, which indicated that *O*-linked oligosaccharides are present in these fractions. These data suggested that CS–DS-PG contains CS–DS-GAG chains along with *N*- and *O*-glycosyl-linked chains.

The distribution of iduronic acid present in CS–DS-PG (Table II) was examined by treating HSc CS–DS-PG with testicular hyaluronidase. The patterns of the oligosaccharides liberated from standard (Sigma) chondroitin 4- and 6-sulfate, and HSc CS–DS-PG are shown in Fig. 7. The highest-mol. wt. oligosaccharide from HSc CS–DS-PG contains ~25 disaccharide units, which showed that the GAG chains are of hybrid structure and contain 8–10% of the total uronic acid as iduronic acid. Attempts to obtain the oligosaccharide by treatment with CS ABCase remained unsuccessful.

Viscosity experiments performed as described previously[27] (data not given) indicated that HSc–CS-PG did not aggregate with hyaluronic acid.

DISCUSSION

CS–DS-PG only recently has been isolated from bovine aorta[28,29] and HFM[19], whereas the presence of CS–DS-PG in mammalian skin was reported by Toole and Lowther[30], and by Öbrink[31]. The results of recent studies concerned with the characterization of small, nonaggregating CS–DS-PG and CS-PG are summarized in Table IV. The PG described herein has a relatively low M_r value and only a few GAG chains containing either glucuronic or iduronic acid; this indicated that its function is different from that of the high-mol. wt. cartilage CS-PG. DS-PG is closely associated with Type 1 collagen[33,39], and the observations that the amount

TABLE IV

LOW-MOL. WT. COPOLYMERIC CONDROITIN SULFATE-DERMATAN SULFATE AND CHONDROITIN SULFATE PROTEOGLYCANS

Tissue	*Mol. wt.*	*GAG chain*		*Core protein mol. wt. 10^{-3}*	*Ref.*
		Number	*Mol. wt. 10^{-3}*		
Bovine					
Aorta	180	12			29
Cartilage					
Articular		1		44	13
Nasal	76	2–3		42	32
Bone					
Mature diaphyses	74	1–2	33.7	46	33
Compact	80–120	1–2	25–35	45	34
Fetal calf	80–120	1–2	40	38	35
Pig					
Skin	1000	50	20	38	35
Laryngeal cartilage	110–130	1–2		40–42	37
Rat					
Bone	80	1–2	40	38	35
Rabbit					
Muscle	95	2	21	50	36
Human fetal					
Bone	80–120	1–2	40	38	35

of copolymeric CS–DS-PG is elevated in HSc scar tissue, as compared to normal skin or NSc, may be related to the disorganized arrangement of the collagen fibers in scar tissue. However, the precise role of CS–DS-PG in hypertrophic scarring has yet to be established.

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