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Note

Fractionation of whale cartilage chondroitin sulfate on sepharose CL-4B in the presence of high concentration of ammonium sulfate. Relationship between molecular size and unit-disaccharide composition of the polysaccharide

AKIRA OGAMO, ATSUKO METORI, TOSHIHARU YAMADA*, AND KINZO NAGASAWA†

School of Pharmaceutical Sciences, Kitasato University, 9-1, Shirokane 5 chome, Minato-ku, Tokyo 108 (Japan)

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We reported[1,2] that Sepharose CL-4B gel can retain sulfated glycosaminoglycuronans in concentrated ammonium sulfate solution at low temperature, resulting in an unique separation of the molecular species by stepwise elution of decreasing concentration of ammonium sulfate. The fractionation of shark cartilage chondroitin sulfate by this procedure[2] indicated that the chondroitin sulfate molecules having the larger molecular size contain generally more chondroitin 6-sulfate than chondroitin 4-sulfate units. Reciprocally, chondroitin sulfate having a smaller molecular size contains more chondroitin 4-sulfate than chondroitin 6-sulfate units[3]. We describe herein the fractionation of whale cartilage chondroitin sulfate on a column of Sepharose CL-4B with a reverse linear gradient of 3.25–1.7M ammonium sulfate in 10mM HCl at 4°. The results confirme the correlation between molecular size and unit-disaccharide composition of the polysaccharide.

EXPERIMENTAL

Materials. — Whale cartilage chondroitin sulfate (special grade, lot No. W9082), chondroitinase ABC from *Proteus vulgaris* (chondroitin sulfate lyase, EC 4.2.99.6), and 4′,5′-unsaturated disaccharide standards [ΔDi, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose; ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 4-sulfate; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 6-sulfate; and ΔDi-diS_D, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid 2- or 3-sulfate)-D-

*Chief research member of Kanagawa Prefectural Public Health Laboratories.
†To whom correspondence should be addressed.

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galactose 6-sulfate] were obtained from Seikagaku Kogyo Co. Ltd. (Tokyo). Standard polysaccharides with M_r 18 000 and 10 000 for electrophoresis were prepared from shark cartilage chondroitin sulfate according to the procedure described[4]. Sepharose CL-4B and Sephadex G-200 were products of Pharmacia LKB Biotechnology Inc. (Uppsala). The gel electrophoresis apparatus (SJ-1060) and silver staining reagents were obtained from Atto Corp. (Tokyo) and Bio-Rad Laboratories (Richmond, CA), respectively.

Analytical methods. — Uronic acid content was determined by the method of Bitter and Muir[5], and sulfur content by the method of Dodgson and Price[6]. Analytical gel filtration on Sephadex G-200 was carried out by the following procedure. Each sample (2 mg), dissolved in 0.2M NaCl (1 mL), was loaded onto a column (1.5 × 95 cm) of the gel, and eluted with the same solvent at 20°. The flow rate was 10 mL/h, and 2 mL-fractions were collected, each of which was analyzed for uronic acid (A_{530}).

Determination of constitutional disaccharide composition in whale cartilage chondroitin sulfate fractions was carried out by the same procedure described previously[7], which is based on the procedure of Seldin *et al.*[8].

Poly(acrylamide) gel electrophoresis of the fractions was performed as follows. The poly(acrylamide) gel composition was 10% acrylamide, 0.33% bis-(acrylamide), 0.1M Tris·borate, and 1mM EDTA·Na_2, pH 8.3, prepared as described[9]. Slabs (10 × 7 × 0.1 cm) were cast with 12-sample wells (1.2 × 0.3 cm). For electrophoresis, samples containing 25 μg of material in 10 μL of water were mixed with one-fifth volume of 2M sucrose in Tris·borate–EDTA buffer, and a portion (2 μL) of this solution was applied to the gel. The reservoir buffer was Tris·borate–EDTA. A current of 5 mA was applied for ~60 min, until Bromphenol Blue migrated to the other side of the gel. Immediately after electrophoresis, the gel was stained by Alcian Blue–silver according to Min and Cowman[10].

Purification of whale cartilage chondroitin sulfate. — A solution of the chondroitin sulfate (1.31 g, sodium salt) in water (25 mL) was applied to a column (2.6 × 90.5 cm) of AG 1-X2 (Cl^-) anion-exchange resin, equilibrated in 1.25M NaCl. The column was eluted at 20° successively with the same solvent (1 L), 1.5M NaCl (2 L), 1.75M NaCl (2 L), and 2.5M NaCl (1 L). The fractions eluted with 1.5M NaCl (84% of total material fractionated) were collected and concentrated *in vacuo* to a small volume. The solution was dialyzed against running tap water overnight, then against distilled water (20 L × 5) overnight at room temperature, concentrated *in vacuo* to a small volume, and freeze-dried (1.10 g).

Fractionation of purified whale cartilage chondroitin sulfate on Sepharose CL-4B in ammonium sulfate solution at 4°. — A solution of purified chondroitin sulfate (129 mg) in 3.25M $(NH_4)_2SO_4$ in 10mM HCl (25 mL) was applied to a column (1.5 × 24.2 cm) of Sepharose CL-4B prepared in the same solvent at 4°. The column was eluted with 250 mL of the same solvent, and then with a reverse linear-gradient (1 L) of 3.25–1.7M $(NH_4)_2SO_4$ in 10mM HCl at 4°. The eluate was collected in

12.5-mL fractions, each of which was analyzed for uronic acid content. The fractions [Fractions 1–7 in Table I(i)] indicated by the length of the braces in Fig. 1 were separately dialyzed against distilled water (3 × 20 L) for one day at room temperature, concentrated *in vacuo* to a small volume, dialyzed again, and freeze-dried after the pH had been adjusted to 6.5 by the addition of 1M NaOH. The yield (mg) of each fraction isolated was as follows: Fraction 1, 9.7; Fraction 2, 8.1; Fraction 3, 17.0; Fraction 4, 18.9; Fraction 5, 15.0; Fraction 6, 17.6; and Fraction 7, 17.7. The total recovery was 81%.

Fractionation of purified chondroitin sulfate on Sephadex G-200 in 0.2M NaCl at 20°. — A solution of purified chondroitin sulfate (183.8 mg) in 0.2M NaCl (5 mL) was applied to a Sephadex G-200 column (2.5 × 94 cm) prepared in 0.2M NaCl. The column was eluted at 20° with the same solvent at a flow rate of 45 mL/h. The eluate was collected in 5.3-mL fractions, and each fraction was analyzed for uronic acid. The elution diagram based on A_{530} was divided into seven fractions of equal peak area [Fractions 1–7 in Table I(ii)]. Each of the fractions was dialyzed against distilled water (4 × 20 L) for 24 h at room temperature, evaporated *in vacuo* to a small volume, and freeze-dried.

RESULTS AND DISCUSSION

A fraction of whale cartilage chondroitin sulfate was eluted with 1.5M NaCl from an AG 1-X2 (Cl^-) column (84% of total material fractionated) and further fractionated on a column of Sepharose CL-4B gel in ammonium sulfate–10mM hydrochloric acid at 4° to give mainly two fractions, one retained and one not retained by the gel at a concentration of 3.25M ammonium sulfate (Fig. 1). The first

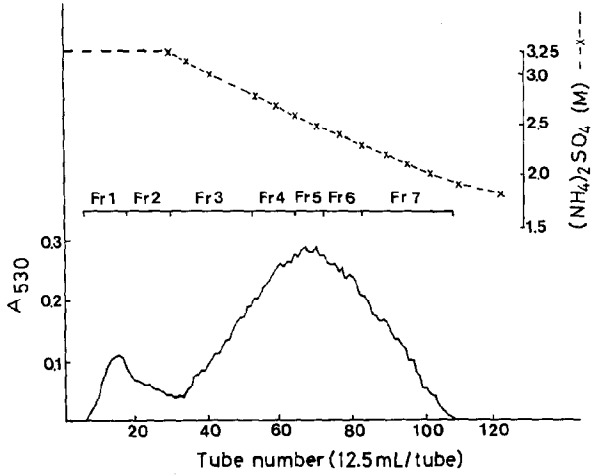

Fig. 1. Separation of whale cartilage chondroitin sulfate on Sepharose CL-4B in the presence of $(NH_4)_2SO_4$. Fractions (12.5 mL) were analyzed for uronic acid content (100-μL sample). Each of the pooled fractions indicated by the length of the braces was subjected to isolation.

TABLE I

ANALYTICAL DATA OF WHALE CARTILAGE CHONDROITIN SULFATE FRACTIONS SEPARATED ON SEPHAROSE CL-4B GEL IN A REVERSE LINEAR GRADIENT OF 3.25→1.7M AMMONIUM SULFATE–10mM HYDROCHLORIC ACID AT 4° OR ON SEPHADEX G-200 IN 0.2M SODIUM CHLORIDE AT 20°

Chondroitin sulfate fraction No.	*S*[a] *(mol)*	K_{av} *Sephadex G-200*	*Unsaturated disaccharide-unit composition (%)*				*Distribution of fraction (%)*
			ΔDi	*ΔDi-4S*	*ΔDi-6S*	*ΔDi-diS$_D$*	
(i) Separation on Sepharose CL-4B in reverse linear gradient of 3.25–1.7M $(NH_4)_2SO_4$							
Starting chondroitin sulfate	0.974	0.26	3.1	80.4	16.0	0.5	
1	0.978	0.37	3.2	73.1	22.9	0.9	9.3
2	0.983	0.31	2.8	77.3	19.6	0.7	7.8
3	0.980	0.28	2.7	79.4	17.6	0.5	16.3
4	0.980	0.26	2.2	81.6	16.0	0.2	18.2
5	0.977	0.22	2.3	82.3	15.4	0	14.4
6	0.978	0.19	2.2	84.9	12.9	0	16.9
7	0.977	0.13	2.3	88.4	9.3	0	17.0
(ii) Separation on Sephadex G-200 in 0.2M NaCl							
1	0.977	0.15	2.5	84.1	13.2	0.2	13.2
2	0.983	0.21	2.3	82.8	14.3	0.9	13.5
3	0.984	0.24	2.1	82.1	15.3	0.5	14.1
4	0.983	0.26	2.2	81.3	16.0	0.5	15.7
5	0.986	0.28	2.1	80.5	16.9	0.6	15.7
6	0.984	0.30	2.2	79.5	17.7	0.6	13.3
7	0.989	0.34	2.0	77.4	19.9	0.8	14.5

[a]Sulfur content (mol/repeating unit).

small peak and the second large peak were further fractionated into two and five fractions of equal peak area, respectively. These fractions were subjected to chemical and physical analyses. As shown in Table I(i), only minor differences in the sulfate content were found between the fractions. The differences in molecular size, however, were significant. The K_{av} values indicated that the increase in retention property on the Sepharose CL-4B column is related to the increase in molecular size, as had been observed for the fractionation of heparin[1] and shark cartilage chondroitin sulfate[2]. The composition of the unsaturated disaccharides obtained by digestion of each fraction with chondroitinase ABC was analyzed by liquid chromatography[7,8]. This indicated that the fractionation on the Sepharose CL-4B column correlates with both the change in molecular size and the change in chondroitin 4- and 6-sulfation composition. Probably, the sulfation pattern of the polysaccharide chains is related to the size of the chains, and the partition on the column is related to the size. Although the proportions of chondroitin and chondroitin disulfate units were extremely small, they suggested that the amount of both units decreases with increasing molecular size of the polysaccharide.

The purified whale cartilage chondroitin sulfate was chromatographed by the usual gel-filtration procedure, and the elution diagram (based on A_{530}) was divided into seven fractions of equal area (elution diagram not shown). The analytical data [Table I(ii)] obtained for these fractions were compared with those obtained for the fractionation on Sepharose CL-4B in ammonium sulfate–10mM hydrochloric acid.

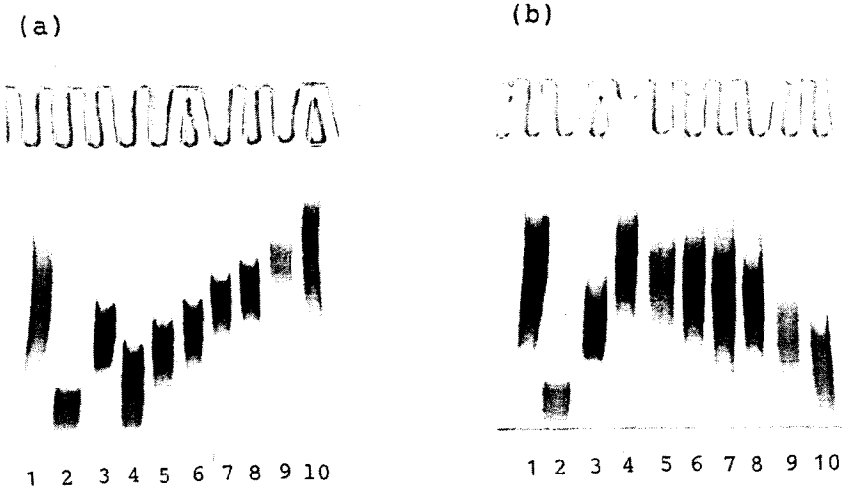

Fig. 2. Poly(acrylamide) gel electrophoresis of whale cartilage chondroitin sulfate fractions separated by Sepharose CL-4B–$(NH_4)_2SO_4$ (a) or by Sephadex G-200–0.2M NaCl (b). The gels were fixed with 0.5% Alcian Blue in 2% acetic acid, then subjected to silver staining: (a) Lane 1, starting chondroitin sulfate; lane 2, standard polysaccharide (M_r 10 000); lane 3, standard polysaccharide (M_r 18 000); lanes 4–10, Fractions 1–7 in Table I(i), respectively. (b) Lanes 1–3, see (a); lanes 4–10, Fractions 1–7 in Table I(ii), respectively.

They showed a correlation between molecular size and unit-disaccharide composition that is similar to that shown in Table I(i). Comparison of the data of both procedures [Table I, (i) and (ii)] showed that the separation on Sepharose CL-4B is distinctly superior to that on Sephadex G-200. To evaluate further the fractionation by Sepharose CL-4B–ammonium sulfate, the whale cartilage chondroitin sulfate fractions obtained by both procedures were analyzed by electrophoresis on a poly(acrylamide) gel and detected by a sensitive combined Alcian Blue and silver staining method[10]. As shown in Fig. 2, the separation on Sepharose CL-4B clearly discriminated better the molecular species, especially in term of molecular size.

In conclusion, the present study has shown that (a) the whale cartilage chondroitin sulfate fraction eluted with 1.5M sodium chloride from a column of AG 1-X2(Cl^-) anion-exchange resin consists of various molecular species distinctly different both in molecular size and in unit-disaccharide composition, (b) there exists a correlation between molecular size and unit-disaccharide composition of the polysaccharide molecule, and (c), as reported previously[1,2,11–13], the chromatography of glycosaminoglycuronans on a hydrophobic gel such as phenyl-Sepharose CL-4B at room temperature, or on cross-linked hydrogel such as Sepharose CL-4B at lower temperature, in the presence of high concentrations of ammonium sulfate, is useful for the discrimination of the molecular species existing in a single glycosaminoglycuronan.

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