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A STUDY ON HETEROGENEITY IN MOLECULAR SPECIES OF SHARK CARTILAGE CHONDROITIN SULFATE C. FRACTIONATION OF THE POLYSACCHARIDE ON SEPHAROSE CL-4B IN THE PRESENCE OF HIGH CONCENTRATIONS OF AMMONIUM SULFATE

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ABSTRACT

Shark cartilage chondroitin sulfate C was fractionated by chromatography on Sepharose CL-4B–2.5 to 1.5M ammonium sulfate in 10mM hydrochloric acid at 4°. Both unit-disaccharide composition and molecular-size distribution clearly affected the fractionation. Comparison of this fractionation with the fractionation on Sepharose 6B gel in 0.2M sodium chloride revealed that the former is distinctly superior to the latter. The fractionation on Sepharose CL-4B in the presence of ammonium sulfate also showed that the chondroitin sulfate C molecules having a larger molecular size contain generally more chondroitin 6-sulfate units (as major constituent) and less chondroitin disulfate units (D type, as minor constituent) than those having a smaller molecular size.

INTRODUCTION

We reported[1–3] that the chromatographic behavior of glycosaminoglycuronans on a hydrophobic gel, such as Phenyl-Sepharose CL-4B, in the presence of a high concentration of ammonium sulfate in 10mM hydrochloric acid depends on the proportion of *N*-acetyl groups and the molecular size of the polysaccharide molecule. Recently, we reported[4] that the bound sulfate groups and uronic acid residues in glycosaminoglycuronans strongly affect their solubility properties in ammonium sulfate solution, which result in a different chromatographic behavior on hydrophobic gels. Previously, we observed[5] that Sepharose CL-4B, which does not possess specific ligands with hydrophobic property, can retain heparin in highly concentrated ammonium sulfate solutions, especially at low temperatures. This phenomenon was considered to depend on factors which are different from those of gel filtration, and may depend mainly on the solubility of the polysaccharide, as well as

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on the hydrophobic interaction between the gel and polysaccharide molecule in highly concentrated ammonium sulfate solutions[6].

The present report describes the separation of shark cartilage chondroitin sulfate C on Sepharose CL-4B gel in the presence of a high concentration of ammonium sulfate in 10mM hydrochloric acid at 4°, which indicates an interesting relationship between the molecular-size distribution and the composition among chondroitin 6- and 4-sulfate units (as major constituent), and chondroitin disulfate unit (D type, as minor constituent) within the copolymeric polysaccharide molecules[7,8].

EXPERIMENTAL

Materials. — Chondroitin sulfate C (shark cartilage, special reagent grade, Lot No. N9103), chondroitinase ABC from *Proteus vulgaris* (chondroitin sulfate lyase, EC 4.2.99.6), and 4,5-unsaturated disaccharide standards (ΔDi, ΔDi-4*S*, and ΔDi-6*S*)* were obtained from Seikagaku Kogyo Co. Ltd. (Tokyo). ΔDi–diS_D was prepared[9] by the chondroitinase ABC digestion of sulfated chondroitin sulfate C.

Analytical methods. — Uronic acid content was determined by the method of Bitter and Muir[10], and sulfur content by the method of Dodgson and Price[11]. Analytical gel filtration on Sepharose 6B was carried out by the same procedure described previously[2], modified by increasing the NaCl concentration to 0.2M. Cellulose acetate membrane-electrophoresis was performed on Separax strips (Fuji Photo-Film Co., Tokyo) in 0.3M calcium acetate (pH 7.25), with a current of 1 mA/cm for 2 h. The strips were stained with 0.5% Alcian Blue in 3% acetic acid.

Determination of the components of disaccharides in the chondroitin sulfate C fractions, separated on Sepharose CL-4B gel in $(NH_4)_2SO_4$ solutions at 4° or on Sepharose 6B gel in 0.2M NaCl at 20°, was carried out by the same procedure described previously[9], which is based on the procedure of Seldin *et al.*[12].

Purification of chondroitin sulfate C. — A solution of the chondroitin sulfate C (1.02 g, sodium salt) in water (25 mL) was applied to a column (2.6 × 92 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 1.75M NaCl (81.7% 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, evaporated *in vacuo* to a small volume, and freeze-dried (833 mg). The preparation was shown, by electrophoresis on Separax strips, to be homogeneous as chondroitin sulfate C species (data not shown).

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* Abbreviations used: ΔDi, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic-acid)-D-galactose; ΔDi-4*S*, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 4-sulfate; ΔDi-6*S*, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 6-sulfate; ΔDi-diS_D, 2-ACETAMIDO-2-DEOXY-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid 2- or 3-sulfate)-D-galactose 6-sulfate.

Fractionation of purified chondroitin sulfate C on Sepharose CL-4B in ammonium sulfate solutions at 4°. — A solution of purified chondroitin sulfate C (300 mg) in 2.5M ammonium sulfate in 10mM HCl (10 mL) was applied to a column (2.5 × 20 cm) of Sepharose CL-4B equilibrated with the same solvent. The elution of the column was performed stepwise at a flow-rate of 60 mL/h with 2.5M (1 L), 2.25M (1 L), 2.0M (1 L), 1.75M (1 L), and 1.5M (1 L) $(NH_4)_2SO_4$ 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 (2.5M, 875 mL; 2.25M, 438 mL; 2.0M, 338 mL; 1.75M, 238 mL; and 1.5M, 113 mL) indicated by the length of the braces in Fig. 1 were separately dialyzed against running tap water for 2 days, then against distilled water (8 × 20 L) for 72 h at room temperature, evaporated *in vacuo* to a small volume, and freeze-dried. The yields (mg) of each fraction isolated were as follows: 2.5M, 21.7; 2.25M, 70.0; 2.0M, 93.2; 1.75M, 73.0; and 1.5M, 13.0. The total recovery was 90.3%.

Fractionation of purified chondroitin sulfate C on Sepharose 6B gel in 0.2M NaCl at 20°. — A solution of purified chondroitin sulfate C (151.5 mg) in 0.2M NaCl (5 mL) was applied to a Sepharose 6B column (2.6 × 88 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 6-mL fractions, and each fraction was analyzed for uronic acid. The elution diagram based on A_{530} was divided into five fractions of equal peak area (Fractions 1–5 in Table I). Each of the fractions was dialyzed against running tap water overnight, then against distilled water (20 L × 4) for 24 h at room temperature, evaporated *in vacuo* to a small volume, and freeze-dried.

RESULTS AND DISCUSSION

A preparation of shark cartilage chondroitin sulfate C, fractionated previous-

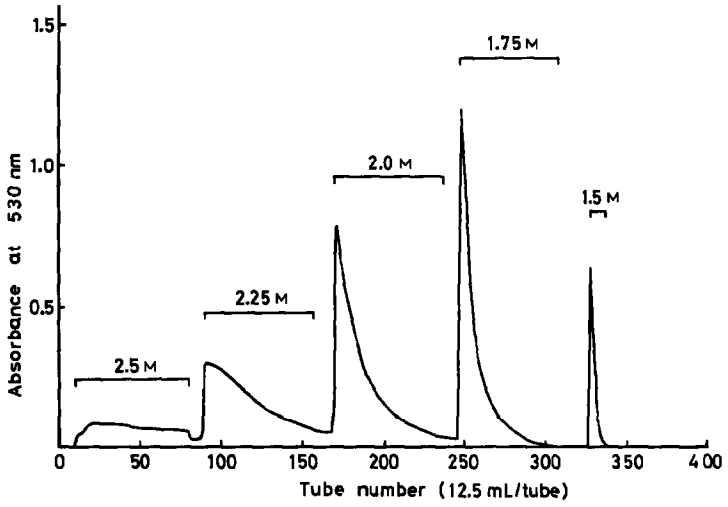

Fig. 1. Separation of chondroitin sulfate C on Sepharose CL-4B in the presence of ammonium sulfate. The purified chondroitin sulfate C (300 mg) was chromatographed on a Sepharose CL-4B column (2.5 × 20 cm) by using stepwise elution with ammonium sulfate (2.5 to 1.5M) in 10mM hydrochloric acid at 4°. Fractions (12.5 mL) were analyzed for uronic acid content (200-μL sample). Each of the pooled fractions circled by braces was subjected to isolation.

TABLE I

ANALYTICAL DATA OF CHONDROITIN SULFATE C FRACTIONS SEPARATED ON SEPHAROSE CL-4B GEL IN 2.5 TO 1.5M $(NH_4)_2SO_4$–10mM HCl AT 4° OR SEPHAROSE 6B IN 0.2M NaCl AT 20°

Chondroitin sulfate C fraction	*S (%)*	K_{av} *on Sepharose 6B*	*Unsaturated disacchride-unit composition (%)*				*Distribution of fractions (%)*
			ΔDi	*ΔDi-6S*	*ΔDi-4S*	*ΔDi-diS$_D$*	
Separation on Sepharose CL-4B in 2.5 to 1.5M $(NH_4)_2SO_4$							
Purified chondroitin sulfate C	6.78	0.37	1.5	69.5	21.0	8.0	
2.5 M fraction	6.88	0.63	2.1	53.1	32.6	11.9	11.4
2.25M fraction	6.67	0.50	1.3	63.6	25.6	9.5	25.9
2.0 M fraction	6.77	0.40	0.9	72.8	19.0	7.3	34.4
1.75M fraction	6.72	0.29	0.8	78.8	15.3	5.1	24.2
1.5 M fraction	6.59	0.21	0.5	81.5	12.4	5.6	4.1
Separation on Sepharose 6B in 0.2M NaCl							
Fraction 1	6.82	0.28	1.0	78.1	14.5	6.4	20.5
Fraction 2	6.76	0.35	1.1	74.8	17.0	7.2	22.3
Fraction 3	6.85	0.40	1.5	69.2	20.4	8.9	17.6
Fraction 4	6.91	0.42	1.4	67.5	21.1	10.0	20.5
Fraction 5	6.96	0.48	1.7	66.3	21.1	10.9	19.1

ly by conventional chromatographic procedures, was fractionated on a column of Sepharose CL-4B prepared in 2.5M ammonium sulfate in 10mM hydrochloric acid by a stepwise elution with decreasing concentrations of ammonium sulfate (2.5 to 1.5M) in 10mM hydrochloric acid at 4° (Fig. 1). The five fractions having different retention property for the gel were analyzed to identify the variety of molecular species of the polysaccharide. The purified chondroitin sulfate C that was subjected to this fractionation is a copolymer which consists of chondroitin 6-sulfate units (70%), 4-sulfate units (21%), and D type disulfate units (8%) on the average (Table I).

From the chromatogram shown in Fig. 1, the distribution of these fractions in percentage of total applied chondroitin sulfate C, based on uronic acid assay, is summarized in Table I. Partition of glycosaminoglycans by the Sepharose CL-4B (with or without hydrophobic ligand)–ammonium sulfate procedure is influenced by various factors, such as column dimensions, amount of sample applied, flow-rate, acidity of elution medium, and especially temperature, as previously reported[5,6]. Reproducibility of the partition of the chondroitin sulfate C sample by the Sepharose CL-4B–ammonium sulfate procedure was reasonably good provided the same set of conditions were employed, as indicated by a partition of the 2.0M material (Table I) into 2.5–2.25M fraction (~10%) and 2.0M fraction (~90%) by rechromatography. The pooled fractions, as indicated in Fig. 1, were dialyzed against water and the chondroitin sulfate C fractions were isolated as sodium salts (yields described in the Experimental section). Although there existed only minor difference in the sulfate content among these fractions, the difference in molecular size was significant, together with the difference in unit-disaccharide composition as discussed later. The K_{av} values determined on a Sepharose 6B gel indicated that the increase in retention property of the fractions for Sepharose CL-4B gel is related to the increase in molecular size of the polysaccharide, as discussed in previous reports[1–4,6]. To understand the difference in unit-disaccharide composition among these fractions, they were digested separately with chondroitinase ABC and the digestion products obtained were analyzed for unit-disaccharide composition by the liquid chromatography technique[12]. As shown in Table I, the data clearly indicate that the increase in retention property of these fractions for the gel is related to the increase in the proportion of chondroitin 6-sulfate units, and necessarily to the decrease in the proportion of chondroitin 4-sulfate units within the polysaccharide molecule. The data also indicate that the proportion of chondroitin D type disulfate units, decreased with increasing retention of the polysaccharide molecule by the gel.

To evaluate the fractionation by the Sepharose CL-4B–ammonium sulfate system, the correlation between the molecular size and unit-disaccharide composition of the chondroitin sulfate C fractions separated by the usual gel-filtration procedure was investigated. The purified chondroitin sulfate C was chromatographed on Sepharose 6B gel in 0.2M sodium chloride. The elution diagram based on A_{530} was divided into five fractions of equal area (elution diagram not shown). Each of the materials isolated was analyzed for sulfur content, K_{av} value on Sepharose 6B

gel, and composition of disaccharide unit. The data showed a correlation between the molecular size and unit-disaccharide composition that is similar to that previously obtained, as shown in Table I. Accordingly, these results strongly indicate that the chondroitin sulfate C molecules of larger molecular size were generally more abundant in chondroitin 6-sulfate units (as major constituent) and less in chondroitin disulfate units (D type, as minor constituent) than those of smaller molecular size.

As shown by comparison of the respective analytical data of chondroitin sulfate C fractions separated on Sepharose CL-4B–ammonium sulfate in 10mM hydrochloric acid at 4° and Sepharose 6B in 0.2M NaCl at 20° (Table I), the separation on Sepharose CL-4B was distinctly superior to that on Sepharose 6B in terms of discrimination of molecular species of chondroitin sulfate C. The data collected in Table I are not entirely consistent with those reported by Seno *et al.*[13] indicating that all the ethanol fractions (35, 40, and 50%) of chondroitin sulfate C from shark cartilage consist of 4- and 6-sulfated disaccharides residues in the ratio of 1.0:1.7.

In conclusion, the present study has shown that (a) the shark cartilage chondroitin sulfate C fraction eluted with 1.75M sodium chloride on Dowex 1 – X2 (Cl^-) anion-exchange resin consists of a variety of molecular species distinctly different both in molecular size and in unit-disaccharide composition, (b) there exists an interesting correlation between the molecular size and unit-disaccharide composition of the polysaccharide molecule, and (c) as reported previously[1,3,6], the chromatography 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 a variety of molecular species existing in a single glycosaminoglycuronan.

REFERENCES

1 A. OGAMO, H. UCHIYAMA, AND K. NAGASAWA, *Biochim. Biophys. Acta*, 626 (1980) 477–485.
2 H. UCHIYAMA, A. OGAMO, AND K. NAGASAWA, *Carbohydr. Res.*, 99 (1982) 87–92.
3 K. NAGASAWA, A. OGAMO, H. UCHIYAMA, AND K. MATSUZAKI, *Carbohydr. Res.*, 111 (1983) 273–281.
4 H. UCHIYAMA, K. OKOUCHI, AND K. NAGASAWA, *Carbohydr. Res.*, 140 (1985) 239–249.
5 A. OGAMO, K. MATSUZAKI, H. UCHIYAMA, AND K. NAGASAWA, *J. Chromatogr.*, 213 (1981) 439–451.
6 H. UCHIYAMA, N. FUJIMOTO, K. SAKURAI, AND K. NAGASAWA, *J. Chromatogr.*, 287 (1984) 55–66.
7 N. SENO, F. AKIYAMA, AND K. ANNO, *Biochim. Biophys. Acta*, 362 (1974) 290–298.
8 C. R. FALTYNEK AND J. E. SILBERT, *J. Biol. Chem.*, 253 (1978) 7646–7649.
9 K. NAGASAWA, H. UCHIYAMA, AND N. WAJIMA, *Carbohydr. Res.*, 158 (1986) 183–190.
10 T. BITTER AND H. M. MUIR, *Anal. Biochem.*, 4 (1962) 330–334.
11 K. S. DODGSON AND R. G. PRICE, *Biochem. J.*, 84 (1962) 106–110.
12 D. C. SELDIN, N. SENO, K. F. AUSTEN, AND R. L. STEVENS, *Anal. Biochem.*, 141 (1984) 291–300.
13 N. SENO, K. ANNO, Y. YAEGASHI, AND T. OKUYAMA, *Connect. Tissue Res.*, 3 (1975) 87–96.