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Note

Hydrophobic-interaction chromatography of glycosaminoglycuronans: fractionation of *N*-acetylchondrosine homopolymers having different degrees of polymerization on Phenyl-Sepharose CL-4B gels

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Recently, we reported[1] the fractionation of heparin into two distinct groups, one having high affinity and another low affinity for agarose-gel carrying hydrophobic ligands, and the striking differences in chemical and biological properties between these two groups. It was also reported that both the *N*-acetyl content and the molecular size of the heparin fractions obtained increased with increasing degree of affinity for the hydrophobic gel. The present report describes a relationship between chain-length of *N*-acetylchondrosine homopolymers and their affinity for a hydrophobic gel, as a basis for fractionation of natural glycosaminoglycuronans according to molecular size.

EXPERIMENTAL

Preparation of N-acetylchondrosine homopolymer fractions having different degrees of polymerization. — The pyridinium salt of chondroitin 6-sulfate was prepared by passing the sodium salt of the polysaccharide (Seikagaku Kogyo Co., Tokyo) through a column of Dowex 50W(H^+), followed by neutralization with pyridine, and by lyophilization. A solution of pyridinium chondroitin 6-sulfate (400 mg) in dimethyl sulfoxide containing 10% of water (100 mL) was heated for 3 h at 90°, cooled, and treated as described previously[2]. After dialysis against distilled water (6 × 20 L) for 48 h, the solution obtained was lyophilized to give the sodium salt of depolymerized chondroitin (348 mg); S content 0.18%.

A solution of depolymerized chondroitin (210 mg) in 0.1M ammonium hydrogencarbonate (5 mL) was applied to a column (2.6 × 94 cm) of Sephadex G-100 prepared in 0.1M ammonium hydrogencarbonate, and eluted with the same solvent at a flow rate of 32 mL/h at 20–25°. The eluate was collected in 5-mL fractions, and each fraction was analyzed for uronic acid content. The material eluted was pooled to give

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nine main fractions of equal volume, which were lyophilized. Each of the residues was dissolved in water (2 mL) and subjected to gel-filtration on the Sephadex G-100 column, and the elution was monitored by the uronic acid assay. Rechromatography of the pooled samples under identical conditions yielded sharp peaks for each fraction. Each residue from the rechromatography was dissolved in a small volume of water and passed through a column (0.6 × 6 cm) of Dowex 50W(Na^+), which was eluted with water. Each eluate was collected and lyophilized to give the sodium salt of the product as a white powder. The nine preparations thus obtained were analyzed for uronic acid and reducing 2-acetamido-2-deoxy-D-galactose content, and were chromatographed on Sephadex G-100 gel (Table I).

Standards of N-*acetylchondrosine and its oligomers.* — *N*-Acetylchondrosine was prepared by acetylation of chondrosine (Seikagaku Kogyo Co., Tokyo) according to the method of Danishefsky *et al.*[3]. Standards of hexameric, heptameric, and octameric *N*-acetylchondrosine have been described by Inoue and Nagasawa[2].

Analytical methods. — Uronic acid content was determined by the modified method of Bitter and Muir[4], reducing 2-acetamido-2-deoxy-D-galactose content by the method of Reissig *et al.*[5], and sulfate content by a turbidimetric method[6].

Analytical gel-chromatography of N-*acetylchondrosine homopolymers on Sephadex G-100.* — Each sample (~1 mg) dissolved in 0.1M sodium chloride (1 mL) was

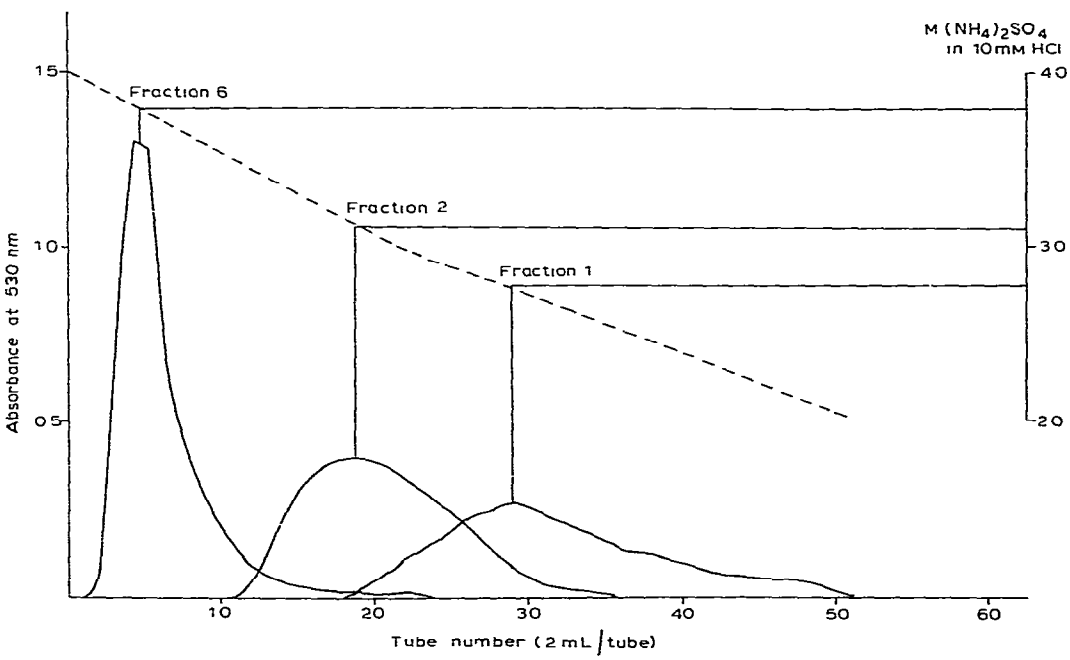

Fig. 1. Elution, on Phenyl-Sepharose CL-4B, of some *N*-acetylchondrosine homopolymer fractions having different d.p. values; fractions 1 (d p. 50), 2 (d p. 34), and 6 (d p. 17) had been obtained by repeated gel-filtration of depolymerized chondroitin on Sephadex G-100: carbazole reaction (———), and concentration of ammonium sulfate in 10mM hydrochloric acid (– – – – –).

applied to a column (1.5 × 95 cm) of Sephadex G-100, and eluted with the same solvent at 20°. The flow rate was 20 mL/h, and 2-mL fractions were collected, each of which was analyzed for uronic acid. V_o and V_t were determined by elution of Blue dextran and by conductivity measurement of sodium chloride, respectively.

Hydrophobic-interaction chromatography of N-*acetylchondrosine homopolymer preparations on Phenyl-Sepharose CL-4B.* — Each sample (~1.7 mg) was applied to a column (0.6 × 6 cm) of Phenyl-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala) prepared in 4.0M ammonium sulfate in 10mM hydrochloric acid, pH 3.4, at 20°. The column was eluted with a reversed linear-gradient (100 mL, 4.0–2.0M ammonium sulfate in 10mM hydrochloric acid). The flow rate was 8.0 mL/h, and 2-mL fractions were collected. An aliquot (0.5 mL) was taken for the carbazole reaction and ionic-strength measurement. Each fraction was characterized by the concentration of ammonium sulfate at the peak of elution (Fig. 1).

Separation of depolymerized chondroitin (a mixture of N-*acetylchondrosine homopolymers) into fractions having and lacking affinity for Phenyl-Sepharose CL-4B.* — A solution of depolymerized chondroitin (sodium salt, 20 mg) in 4.0M ammonium

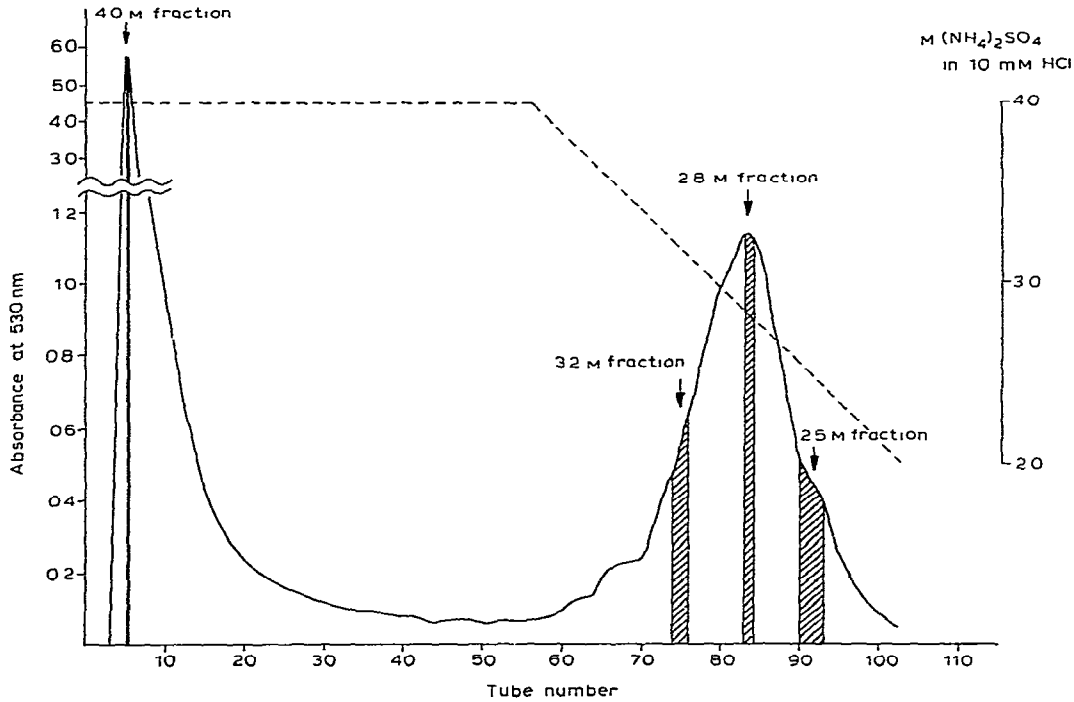

Fig. 2. Fractionation of depolymerized chondroitin (a mixture of *N*-acetylchondrosine homopolymers) into fractions having and lacking affinity for Phenyl-Sepharose CL-4B: carbazole reaction (———); and concentration of ammonium sulfate in 10mM hydrochloric acid (– – – – –). Volume of eluent; tube numbers 1–52, 5 mL/tube; and 53–102, 2 mL/tube.

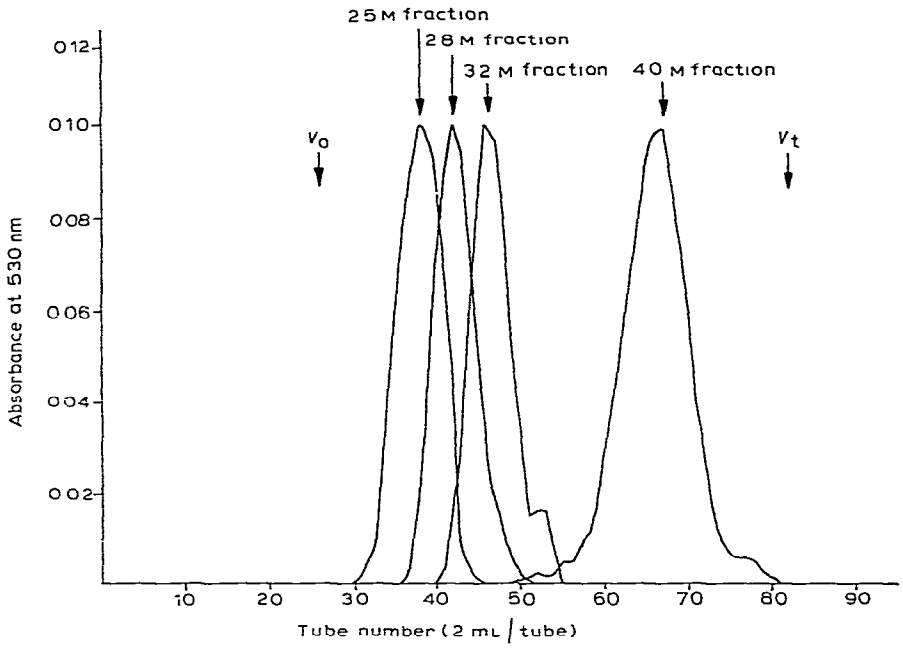

Fig. 3. Elution, on Sephadex G-100, of *N*-acetylchondrosine homopolymer fractions (see Fig. 2) having different affinities for Phenyl-Sepharose CL-4B

sulfate in 10mM hydrochloric acid (5 mL) was applied to a column (1.0 × 23 cm) of Phenyl-Sepharose CL-4B prepared in 4.0M ammonium sulfate in 10mM hydrochloric acid at 20°. The column was washed with 260 mL of the same solvent, and then eluted with a reversed linear-gradient (100 mL, 4.0–2.0M ammonium sulfate in 10mM hydrochloric acid) at a flow rate of 35 mL/h. The eluate was collected in 5-mL and 2-mL fractions for the 4.0M ammonium sulfate and the reversed linear-gradient elution, respectively. Each fraction was analyzed for uronic acid and ionic strength (Fig. 2).

Gel-filtration, on Sephadex G-100, of N*-acetylchondrosine homopolymer fractions having different affinity for Phenyl-Sepharose CL-4B.* — Fractions corresponding to the four peaks shown in Fig. 2 (tube 5 for 4.0M, tubes 74, 75, and 76 for 3.2M, tubes 83 and 84 for 2.8M, and tubes 90, 91, 92, and 93 for 2.5M ammonium sulfate), were pooled separately. Each pooled fraction was concentrated under diminished pressure and freed from the bulk of ammonium sulfate by passage through a column (1.0 × 23 cm) of Sephadex G-15, prepared in 0.1M ammonium hydrogencarbonate, and eluted with the same solvent. The eluate was lyophilized and the residue redissolved in 0.1M sodium chloride (1 mL). The solution was applied to a column (1.5 × 90 cm) of Sephadex G-100 prepared in 0.1M sodium chloride. The flow rate was 20 mL/h and 2-mL fractions were collected. An aliquot (0.5 mL) was taken for uronic acid assay (Fig. 3).

TABLE I

ANALYTICAL DATA OF *N*-ACETYLCHONDROSINE HOMOPOLYMER FRACTIONS

Fraction No.	*Uronic acid content (%)*	*Molar ratio of reducing 2-acetamido-2-deoxy-D-galactose to uronic acid*[a]	*D.p* $_{av}$[b]	K_{av} *on Sephadex G-100*	*Position of elution on Phenyl-Sepharose CL-4B (M ammonium sulfate)*[c]
1	46 9	0.019 (0 020)[d]	50	0 09 (1 6.8)[e]	2.80
2	42.7	0.028 (0 029)	34	0.15 (1 .7.3)	3.10
3	46.7	0.034 (0 034)	29	0 22 (1:7 5)	3 58
4	42.7	0 040 (0 038)	26	0 26 (1:8 3)	3.60
5	43 3	0 047 (0 048)	21	0 35 (1:6.4)	3.78
6	44 6	0.060 (0 058)	17	0.43 (1:6 4)	3 80
7	43.1	0.080 (0.077)	13	0 50 (1:6 4)	3.90
8	44.3	0.10 (0.09)	11	0.57 (1:6 5)	4.00
9	42.5	0.13 (0.14)	7	0.64 (1:6 4)	4 00
Octameric *N*-acetylchondrosine[f]		0 12 (0.13)	8		
Heptameric *N*-acetylchondrosine[f]		0.15 (0.14)	7		
Hexameric *N*-acetylchondrosine[f]		0.17 (0.17)	6		
N-Acetylchondrosine[f]		1.00 (1.00)	1		

[a]Expressed as a ratio relative to the molar ratio of 2-acetamido-2-deoxy-hexose to uronic acid in a standard of *N*-acetylchondrosine; the content of 2-acetamido-2-deoxy-D-galactose of the standard determined by the Morgan–Elson reaction was 90.7%, *i.e.*, 1.93 times the calculated value (55 7%). [b]The average degree of polymerization was calculated from the molar ratio of reducing 2-acetamido-2-deoxy-D-galactose to uronic acid based on the standard of *N*-acetylchondrosine, 1.00 (d p 1) [c]Expressed as the concentration of ammonium sulfate eluent at the peak of elution [d]In parentheses, calculated values [e]In parentheses, ratio of width to height for each peak as an indication of the elution profile. [f]Standards, see Experimental section

RESULTS AND DISCUSSION

Our previous observation on the heparin fractions having both different molecular size and hydrophobicity[1] suggested a possible relationship between chain-length of the glycosaminoglycuronans and behavior on hydrophobic gels, such as Phenyl-Sepharose CL-4B. A mixture of depolymerized chondroitin homopolymers was prepared by desulfating depolymerization of chondroitin 6-sulfate with dimethyl sulfoxide containing water[2]. By repeated gel-filtration on Sephadex G-100, the product was fractionated into nine fractions having different d.p. The preparations obtained were *N*-acetylchondrosine homopolymers having a 2-acetamido-2-deoxy-D-galactose residue at the reducing end[2], and their analytical data are shown in Table I.

Each fraction of *N*-acetylchondrosine homopolymers (fractions 1–9) was chromatographed on Phenyl-Sepharose CL-4B with a reversed, linear-gradient elution (Fig. 1). Since temperature seriously influences the separation of heparin by hydrophobic-interaction chromatography[7], the temperature was maintained at

20° during the chromatography. The position of each peak was determined by the molar concentration of ammonium sulfate in 10mM hydrochloric acid (Fig. 1). The affinity of *N*-acetylchondrosine homopolymers for Phenyl-Sepharose CL-4B decreased with decreasing degree of polymerization (Table I). The homopolymers having a d.p. <11 did not show any affinity for the gel in 4.0M ammonium sulfate in 10mM hydrochloric acid. Those having d.p. >17 showed an increasing affinity for the gel with increasing d.p., and their elution diagrams were broadened also with increasing d.p. (see Fig. 1) Comparison of the elution diagrams of the homopolymer fractions with higher d.p. (>17) on Phenyl-Sepharose CL-4B (Fig. 1) with those on Sephadex G-100 (Table I) shows that the former diagram reflects more faithfully the distribution in molecular size among the fractions than did the latter. These data and those obtained for heparin[1] indicate that a glycosaminoglycuronan containing homopolymeric 2-acetamido-2-deoxyglycopyranosylglycosyluronic units, such as chondroitin, hyaluronic acid, and chondroitin 4- and 6-sulfate, may be isolated according to its molecular size on the basis of its affinity for a hydrophobic gel.

The depolymerized chondroitin (a mixture of homopolymers having d.p. 7–50) was examined by Phenyl-Sepharose CL-4B column chromatography (Fig. 2). The homopolymers having no affinity for the gel were eluted with 4.0M ammonium sulfate, and those having some affinity for the gel, with ammonium sulfate at a concentration <4.0M. The fractions obtained from four different peaks of elution (see Fig. 2; 4.0, 3.2, 2.8, and 2.5M ammonium sulfate) were separately chromatographed on Sephadex G-100 (Fig. 3). The elution diagrams indicated that the fractions having a higher affinity for the hydrophobic gel were composed of compounds having a larger molecular-size, and the discrimination in molecular size between the *N*-acetylchondrosine homopolymers having and lacking affinity for the gel was remarkable.

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