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Structure of the complement-activating proteoglycan from the pilose antler of *Cervus nippon* Temminck

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ABSTRACT

An anti-complementary polysaccharide, DWA-2, isolated from an unossified pilose antler of *C. nippon* Temminck by digestion with pronase, gel filtration, and affinity chromatography, consisted mainly of GalNAc, GlcA, IdoA, and sulfate in the molar ratios 1.0:0.6:0.3:0.8, and small proportions of Man, Gal, GlcNAc, and protein (4.5%). Methylation analysis, NMR spectroscopy, and degradation with enzymes indicated that DWA-2 contained chondroitin sulfate A-, B-, and C-like moieties. DWA-2 showed potent anti-complementary activity, and crossed immunoelectrophoresis indicated that it cleaved complement C3 in the absence of Ca^{2+} ion. Digestion of DWA-2 with chondroitinase ABC or ACI reduced the anti-complementary activity to a low level, but digestion with chondroitinase B reduced the activity by ~ 40% and the enzyme-resistant fraction still showed a significant activity.

INTRODUCTION

The unossified pilose antler of *C. nippon* Temminck (Japanese name Rokujo) is an animal drug used in traditional oriental herbal medicine for the treatment of anemia in women, impotence, seminal emission, and premature ejaculation. However, little is known of the pharmacologically active substances. A crude polysaccharide isolated from the pilose antler was reported[1] to have anti-ulcer activity. We have found that a high molecular weight fraction in a hot-water extract of the antler has anti-complementary activity and we now report on the purification and structure of the complement-activating proteoglycan.

EXPERIMENTAL

Materials.—The unossified pilose antler of *C. nippon* Temminck was cultivated in Jilin Province, China. DEAE-TOYOPEARL was obtained from Toyo Soda Co.

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Ltd.; Sephadex G-50 and G-100, and Sepharose CL-6B from Pharmacia; and Sep-pak C_{18} cartridges from Waters Associates. Chondroitinase ACI, chondroitinase B, chondroitinase ABC, chondro-4-sulfatase, chondro-6-sulfatase, the disaccharides 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose (Di-0S), 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 4-sulfate (Di-4S), and 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 6-sulfate (Di-6S), RCA_{120}-coupled agarose, heparin, hyaluronic acid (hog skin), and chondroitin sulfates A (whale cartilage), B (hog skin), and C (shark cartilage) were obtained from Seikagaku Kogyo Co. Ltd., Japan.

General methods.—Uronic acid, protein, hexosamine, and sulfate were assayed by the *m*-hydroxybiphenyl[2], Lowry[3], indole–HCl[4], and Dodgson methods[5], respectively, using GlcA, bovine serum albumin, GalN, and K_2SO_4 as the respective standards. NAc was assayed[6] after hydrolysis. Carbohydrates in column eluates were assayed by the phenol–H_2SO_4 method[7]. Ratios of GlcA and IdoA were assayed using orcinol–HCl[8] and carbazole–H_2SO_4[9]. Polysaccharide was hydrolysed with trifluoroacetic acid, 2 M at 121° for 1.5 h or M at 100° for 2 h. The hydrolysates were analysed by TLC on cellulose, using EtOAc–pyridine–acetic acid–H_2O (5:5:1:3). Reducing sugars were detected with alkaline silver nitrate[10] and uronic acids with *p*-anisidine hydrochloride[11]. Uronic acids, neutral sugars, and amino sugars were converted into the corresponding alditol acetates, and analysed by GLC at 120 → 195° (3°/min) using a Shimadzu GL-6A gas chromatograph equipped with a flame-ionisation detector and an SPB-5 capillary column (0.25 mm i.d. × 30 m, 0.25-μm film thickness, Supelco). HPLC of polysaccharides was performed with a Waters Model ALC/GPC 244 instrument equipped with columns (0.76 × 50 cm) of Asahi-pak GS-510 + GS-320 (Asahi Chemical Industry Co. Ltd.) and elution with 0.2 M NaCl. The molecular weight of the polysaccharide was estimated from the calibration curve of the elution volumes of standard pullulans (P-400, 200, 100, 50, 20, and 5; Showa Denko Co. Ltd.) in HPLC. Electrophoresis was performed on cellulose acetate membrane (Fuji Film Co. Ltd.) in 0.08 M pyridine–0.04 M acetate buffer (pH 5.4) at 70 V/cm for 30 min with detection using Toluidine Blue. Optical rotations were determined at 23° with a JASCO DIP digital polarimeter.

Isolation of the water-soluble polysaccharide fraction.—The pilose antlers (10 kg) were extracted with EtOH at room temperature for 24 h and the residue was extracted with water (3 × 4 vol) at 100° for 2 h. The combined hot-water extracts were concentrated to 1/3 volume, EtOH (4 vol) was added, and the precipitate was collected and dried to give fraction DW-1 (6.5%). A solution of DW-1 in water was dialysed against running water for 4 days and distilled water for 4 days, then centrifuged, and EtOH (4 vol) was added to give fraction DW-4 (26.3% from DW-1).

Digestion of DW-4 with pronase.—To a solution of DW-4 (50 g) in 50 mM Tris–HCl buffer (500 mL, pH 7.9) containing 10 mM $CaCl_2$ was added pronase (1

g). The mixture was incubated at 37° for 24 h in the presence of toluene, the pH was readjusted to 7.9 with 0.2 M NaOH, more pronase (0.5 g) was added, and the digestion was repeated. The mixture was heated at 100° for 5 min, EtOH (4 vol) was added, and a solution of the precipitate in water was lyophilised to give fraction DW-5 (13.0 g, 26% from DW-4).

Purification of the anti-complementary polysaccharides.—(a) Ion-exchange chromatography. DW-5 was applied to a column (3 × 32 cm) of DEAE-TOYOPEARL (Cl^- form) equilibrated with 5 mM NaCl. The column was washed with 5 mM NaCl until carbohydrate was no longer detected in the eluate, and the absorbed polysaccharides were eluted with 0.5 M and then M NaCl. The unabsorbed fraction (DW5-I, 32.0%) and the major (DW5-IIa, 31.1%) and minor (DW5-IIb, 0.5%) absorbed fractions were obtained after dialysis by lyophilisation.

(b) Gel-filtration on Sephadex G-50 and G-100. Fraction DW5-IIa from (*a*) was eluted from a column (6 × 110 cm) of Sephadex G-50 with 0.2 M NaCl to give fraction DW5-IIa1 (19.1%) in the void volume, and lower molecular weight fractions (DW5-IIa2 and -IIa3: 35.0 and 48.4%, respectively). DW5-IIa1 was eluted from a column (2.7 × 96 cm) of Sephadex G-100 with 0.2 M NaCl to give fraction DW5-IIa1A (35.6%) in the void volume, and lower molecular weight fractions (DW5-IIa1B and -IIa1C: 16.7 and 19.9%, respectively).

(c) Affinity chromatography. Fraction DW5-IIa1A from (*b*) was dissolved in 0.01 M sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl, and eluted from a column (2.5 × 6 cm) of RCA_{120}-coupled agarose to give the unbound fraction (92%). Elution with the same buffer containing M lactose gave the bound fraction (6.9%). Each fraction was dialysed and lyophilised.

(d) Gel-filtration on Sepharose CL-6B. The unbound fraction from (*c*) was eluted from a column (2.7 × 96 cm) of Sepharose CL-6B with 0.2 M NaCl to give fraction DWA-1 (6.6%) in the void volume, an intermediate fraction (DWA-2, 68.7%), and the lowest molecular weight fraction (DWA-3, 8.7%).

Methylation analysis of the polysaccharide.—The polysaccharide was methylated once by the modified method of Hakomori[12] in order to prevent[13] β-elimination. The product was recovered using a Sep-pak C_{18} cartridge (Waters Associates) by the procedure of Waeghe and Albersheim[14], except that the samples were eluted with EtOH and the uronic acid moieties were reduced[14,15] with $NaBD_4$ in tetrahydrofuran–EtOH (7:3) for 18 h at room temperature followed by incubation for 1 h at 70°. The product was desulfated[16] in H_2O–Me_2SO (1:9) at 80° for 3 h, then remethylated with CD_3I. The methylated polysaccharide was hydrolysed (2 M trifluoroacetic acid, 1.5 h, 121°), and the products were converted into the alditol acetates which were then analysed by GLC and GLC–MS. GLC was performed on a Hewlett–Packard model 5840A gas chromatograph equipped with an SPB-1 capillary column (25 m × 0.25 mm i.d., 0.25-μm film thickness, Supelco) with splitless injection. The carrier gas was He at 0.9 mL/min, and the temperature programme was 60° for 1 min, 60 → 150° at 18°/min, then 150 → 230° at 2°/min.

GLC–MS (70 eV) was performed on a JEOL DX-300 instrument equipped with an SPB-1 capillary column.

Enzymic digestions.—(a) Chondroitinase ACI, B, and ABC. DWA-2 (10 mg) was digested with chondroitinase ACI (1 U) or B (0.1 U) in 50 mM Tris–HCl buffer (5 mL, pH 7.3 or 8.0) at 37° for 24 h. Each mixture was neutralised, heated at 100° for 3 min to inactivate the enzyme, and eluted from a column of Sepharose CL-6B with 0.2 M NaCl to give fractions ACI-1–3 or B-1–2, respectively. B-1 was digested with chondroitinase ACI (0.3 U) in 50 mM Tris–HCl buffer (3 mL, pH 7.3) at 37° for 24 h. ACI-2 was digested with chondroitinase B (0.02 U) in 50 mM Tris–HCl buffer (3 mL, pH 8.0) at 37° for 24 h.

For assay of anti-complementary activity, DWA-2 (5 mg) was digested severally with chondroitinase ABC (10 U), ACI (0.5 U), or B (0.05 U) in 50 mM Tris–HCl buffer (5 mL, pH 8.0, 7.3, or 8.0) at 37° for 3 h. Each mixture was neutralised, heated at 100° for 5 min, then desalted with Microacylizer model G1 (Asahi Chemical Industry).

(b) Chondro-4- and -6-sulfatases. Samples (75 μg each) were digested with chondro-4- (0.2 U) or -6-sulfatase (0.2 U) in 0.1 M Tris–HCl buffer (pH 7.9, 75 μL) containing 0.1 M NaOAc at 37° for 3 h. The products were analysed by HPLC.

Analysis of unsaturated oligosaccharides by HPLC.—HPLC was performed on a Waters Model ALC/GPC 244 instrument equipped with a column (250 × 4.6 mm i.d., JASCO) of Nucleosil $5NH_2$ and developed with 0.5 M ammonium formate buffer(pH 4.8)–MeOH (85:15) at 1.5 mL/min. The unsaturated oligosaccharides were detected at 254 nm and identified by comparison with authentic Di-0S, Di-4S, and Di-6S.

NMR studies.—The ^{13}C-NMR (100 MHz) spectra of DWA-2 and chondroitin sulfates A–C were obtained for 0.5% solutions in D_2O at 25°, using a Varian XL-400 FT spectrometer. Chemical shifts are expressed relative to that of sodium 3-(trimethylsilyl)propane-l-sulphonate-d_4 (TSP).

Anti-complementary activity.—Anti-complementary activity was measured as described[17].

Crossed immunoelectrophoresis.—Normal human serum (NHS, 100 μL) was incubated with a solution (50 μL) of DWA-2 (1 mg/mL) in gelatin–veronal-buffered saline (pH 7.4) containing 500 μM Mg^{2+} and 150 μM Ca^{2+} (GVB^{2+}), or 10 mM EGTA containing 2 mM $MgCl_2$ in GVB^{2-} (Mg^{2+}–EGTA–GVB^{2-}), at 37° for 30 min. Crossed immunoelectrophoresis for the treated NHS was performed[18] using rabbit anti-human C3 serum.

RESULTS

Purification of the anti-complementary polysaccharide.—A crude, high molecular weight fraction (DW-4) was obtained from the pilose antler by extraction with boiling water, followed by precipitation with EtOH and dialysis. DW-4 was digested with pronase in order to remove protein and gave a crude polysaccharide

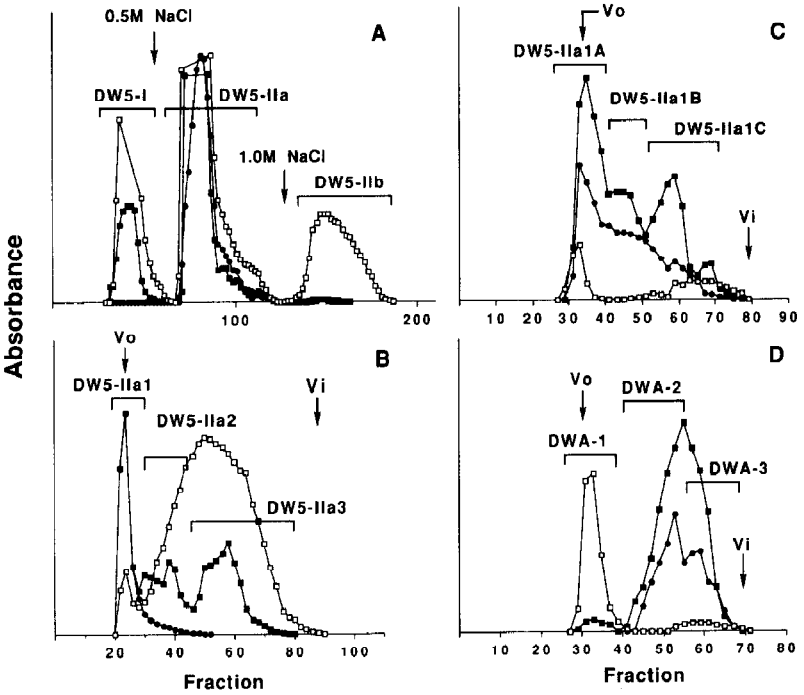

Fig. 1. A, Anion-exchange chromatography of DW5 on DEAE-TOYOPEARL (Cl^- form); B, gel filtration (0.2 M NaCl) of DW5IIa from A on Sephadex G-50; C, gel filtration of DW5IIa1 from *B* on Sephadex G-100; D, gel filtration of DW5IIa1A from C on Sepharose CL-6B: hexosamine (492 nm), ■; uronic acid (520 nm), •; protein (280 nm), □; Vo, void volume; Vi, inner volume.

fraction (DW-5) that had anti-complementary activity higher than that of DW-4 (data not shown). DW-5 was fractionated on DEAE-TOYOPEARL (Cl^- form) to give an unabsorbed (DW-5I) and two absorbed fractions (DW-5IIa and -5IIb) (Fig. 1A), of which DW-5IIa had the highest anti-complementary activity (Table I). Gel filtration of DW-5IIa on Sephadex G-50 in 0.2M NaCl gave three fractions (DW-5IIa1, -5IIa2, and -5IIa3) (Fig. 1B) of which DW-5IIa1 had the highest anti-complementary activity (Table I). On fractionation of DW-5IIa1 on Sephadex G-100, the most-active fraction (DW-5IIa1A) was eluted in the void volume (Fig. 1C and Table I). DW5-IIa1A gave GalN, Glc, Man, Gal, and GlcN in the molar ratios 5.6:1.0:0.02:1.1:0.8 together with a large proportion of uronic acid on hydrolysis. Since DW5-IIa1A contained Gal, DW5-IIa1A was subjected to affinity chromatography on RCA_{120}-coupled agarose, and unbound and bound fractions were obtained in the ratio 92.0:6.9 and with similar anti-complementary activities (data not shown). The major fraction was fractionated on Sepharose CL-6B to give fractions DWA-1, DWA-2, and DWA-3 in the ratios 4.4:68.7:8.7 (Fig. 1D). The order of anti-complementary activity was DWA-1 > DWA-2 > DWA-3 (Table I). DWA-1–DWA-3 each gave mainly GalN and uronic acids on hydrolysis.

Structure of DWA-2.—DWA-2 gave a single spot in electrophoresis on cellulose acetate membrane (staining with Toludine Blue), was eluted from Sepharose

TABLE I
Anti-complementary activity (%) of subfractions and purified polysaccharides [a]

Sample	Concentration (μg/mL)		
	333	167	33
DW5	86.0	64.0	31.0
DW5-I	58.2	30.5	8.0
DW5-IIa	88.0	79.0	46.0
DW5-IIb	63.0	52.0	7.4
DW5-IIa1	88.0	89.0	84.0
DW5-IIa2	49.5	38.0	32.0
DW5-IIa3	17.0	12.0	11.0
DW5-IIa1A	84.0	80.0	62.5
DW5-IIa1B	63.0	52.0	25.5
DW5-IIa1C	30.0	24.0	10.0
DWA-1	100.0	100.0	100.0
DWA-2	98.5	92.0	88.0
DWA-3	68.0	62.5	40.0

[a] See Experimental for identification of the fractions.

CL-6B as a single peak, gave a single peak (mol wt 64000) in HPLC using Asahi-pak GS-510 + GS-320 in 0.2 M NaCl, had $[\alpha]_D$ −20° (*c* 1, H_2O), and contained 25.7% of GlcA, 13.1% of IdoA, 42.4% of GalN, 18.0% of sulfate, 11.5% of NAc (molar proportions of 0.6:0.3:1.0:0.8:0.9), and 4.5% of protein.

DWA-2 was methylated once with MeI, the uronic acids were then reduced with $NaBD_4$, and the reduced product was desulfated by heating in H_2O–Me_2SO (1:9) for 3 h at 80°, then re-methylated with CD_3I. This methylated polysaccharide was hydrolysed, and the products were converted into the alditol acetate derivatives, and analysed by GLC and GLC–MS. The major derivatives were 1,3,5-tri-*O*-acetyl-2-deoxy-6-*O*-methyl-2-(*N*-methylacetamido)-4-*O*-trideuteriomethylgalactitol, 1,3,5-tri-*O*-acetyl-2-deoxy-4-*O*-methyl-2-(*N*-methylacetamido)-6-*O*-trideuteriomethylgalactitol, 1,3,5-tri-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)galactitol, and 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-6-*O*-trideuteriomethylglucitol-*6,6-d*$_2$, indicating that DWA-2 consisted mainly of 4-sulfated 3-linked GalN, 6-sulfated 3-linked GalN, 3-linked GalN, and 4-linked GlcA.

^{13}C-NMR studies.—The spectrum of DWA-2 in D_2O contained a signal at 25.190 ppm for $NCOCH_3$; seven signals at 103–107 ppm due to C-1β, the chemical shifts of which agreed with those for the commercial chondroitin sulfates (Table II); a signal at 72.587 ppm assigned to C-2 or C-5 of IdoA by comparison with signals[19] (72.289 and 72.532 ppm) for chondroitin sulfate B; and signals at 63.934 and 70.389 ppm due[19] to C-6 of GalN and C-6 of 6-sulfated GalN or C-4 of GalN.

Enzymic digestions of DWA-2.—DWA-2 was digested with chondroitinase B and the products were fractionated on Sepharose CL-6B to give B-1 and B-2 in the

TABLE II

^{13}C-NMR spectra of DWA-2 and glycosaminoglycans

Assignment	Chemical shift (ppm)			
	DWA-2	Chondroitin sulfate [a]		
		A	B	C
GalA-1	106.733	106.622	106.117	106.647
(IdoA-1)	107.252	107.141	106.558	107.150
GalN-1	103.937	103.778	103.400	
	104.084			
	104.395	104.407		104.238
	104.532			104.430
	105.167		104.948	
GalN-6 (free)	63.934	64.002	63.841	
GalN-6 (6-sulfated) / GalN-4 (free)	70.389	70.416		70.434
IdoA-2 / IdoA-5	72.587		72.289	
			72.532	

[a] Reported by Hamer and Perlin[19].

ratio 2.7:1.0 (Fig. 2A). HPLC of B-2 before and after digestion with chondro-4-sulfatase (Fig. 3, A and B), indicated the presence of Di-0S and Di-4S in the ratio 1.0:6.0. When B-1 was digested with chondroitinase ACI, HPLC of the products, before and after digestion with chondro-4- or -6-sulfatase (Fig. 3, C), indicated that B-1 consisted of Di-0S, Di-4S, and Di-6S in the ratios 1.0:1.1:0.6.

When DWA-2 was digested with chondroitinase ACI and the products were fractionated on Sepharose CL-6B, low (ACI-3) and high molecular weight fractions (ACI-1 and ACI-2) were obtained in the ratios 7.0:1.0:1.2 (Fig. 2B). HPLC of ACI-3, before and after digestion with chondro-4- or -6-sulfatase, indicated that it contained Di-0S, Di-6S, and Di-4S in the ratios 1.0:0.7:1.1 (Fig. 4, A–C). Hydrolysis of ACI-2 gave GalN and uronic acid; therefore, ACI-2 was digested with condroitinase B. HPLC of the products, before and after digestion with chondro-4-sulfatase indicated that the products from ACI-2 consisted of Di-0S and Di-4S in the ratio 1.0:6.0 (Fig. 4D). ACI-1 contained a large proportion of protein together with Xyl, Man, Glc, Gal, GlcN, GalN, and uronic acid, but could not be analysed further because of the small amount available.

Anti-complementary activity of DWA-2.—DWA-2 showed potent anti-complementary activity at 333–33 μg/mL (Table III), whereas hyaluronic acid, heparin, and chondroitin sulfates A–C had weak activities (Table III). When crossed immunoelectrophoresis was carried out after the incubation of normal human serum (NHS) in GVB^{2+}, in order to determine whether C3 activation had

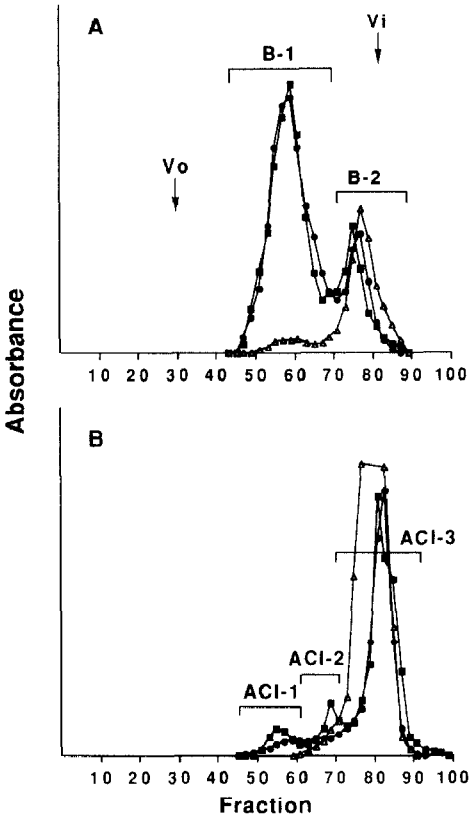

Fig. 2. Gel filtration on Sepharose CL-6B (0.2 M NaCl) of the products of digestion of DWA-2 with A, chondroitinase B; and B, chondroitinase ACI: hexosamine (492 nm), ■; uronic acid (520 nm), ●; 4,5-unsaturated uronic acid (232 nm), △; Vo, void volume; Vi, inner volume.

occurred, cleavage of the C3 precipitin line was obtained in the serum treated with DWA-2 (Fig. 5A). However, this cleavage was not observed in the serum treated with heparin (Fig. 5B). The complement system can be activated through alternative and/or classical pathways. When NHS was incubated with DWA-2 in Ca^{2+}-depleted buffer (Mg^{2+}–EGTA–GVB^{2-}) and subjected to crossed immunoelectrophoresis, cleavage of the C3 precipitin line was observed, as effected with the AR-arabinogalactan mixture[17] as an activator of the alternative pathway (Fig. 5, C and D).

Effects of treatments with enzymes on the anti-complementary activity of DWA-2. —When DWA-2 was digested severally with condroitinase ABC, ACI, and B, the anti-complementary activities were reduced by 70, 66, and 38%, respectively, at a concentration of 333 μg/mL (Table IV). The product (B-1 in Fig. 2A) from chondroitinase B-digested DWA-2 showed a potent anti-complementary activity similar to that of DWA-2 at 333 and 167 μg/mL, whereas the product (B-2), which contained unsaturated chondrodisaccharides (Di-0S and Di-4S), had a weak activity (Table IV).

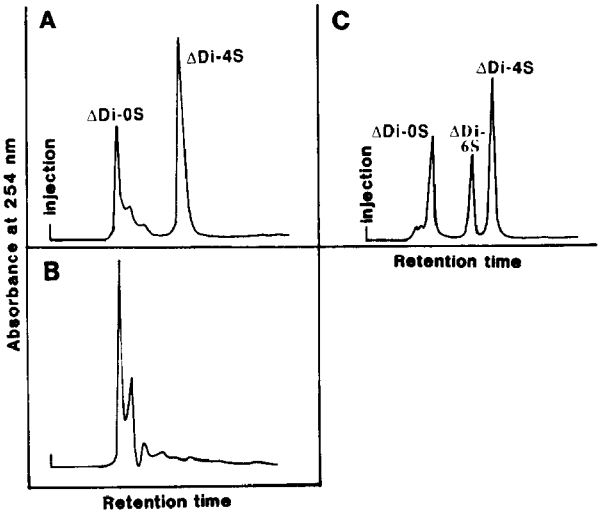

Fig. 3. HPLC of B-2 from Fig. 2A before (A) and after (B) digestion with chondro-4-sulfatase; C, HPLC of B-1 after digestion with chondroitinase ACI.

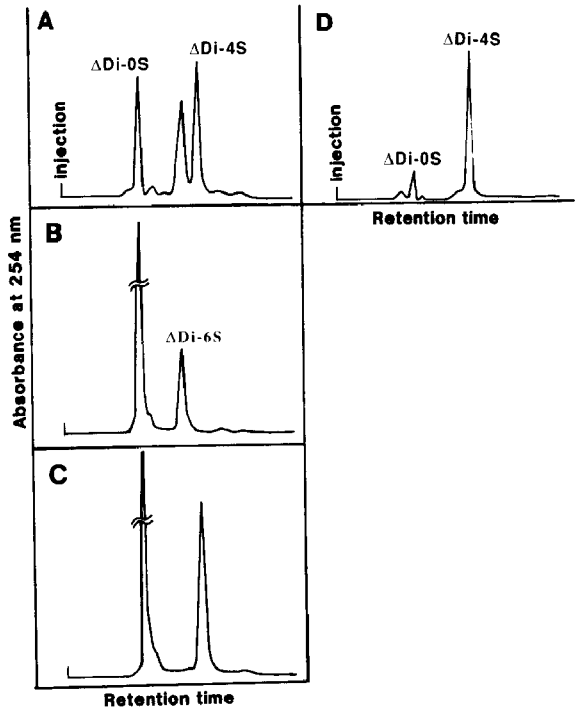

Fig. 4. HPLC of ACI-3 from Fig. 2B before (A) and after digestion with chondro-4-sulfatase (B) and chondro-6-sulfatase (C); D, HPLC of ACI-2 after digestion with chondroitinase B.

TABLE III

Anti-complementary activity (%) of DWA-2 and glycosaminoglycans

Sample	Concentration (μg/mL)		
	333	167	33
DWA-2	95.0	93.2	74.2
Hyaluronic acid	25.8	27.8	21.0
Heparin	19.2	16.8	9.0
Chondroitin sulfate A	34.8	26.5	23.4
Chondroitin sulfate B	26.0	24.2	15.2
Chondroitin sulfate C	12.8	14.8	5.8

DISCUSSION

The purified, high molecular weight, anti-complementary component (DWA-2) of pilose antler of *C. nippon* consisted mainly of GlcA, IdoA, and GalNAc.

Methylation analysis, NMR spectroscopy, and digestion with enzymes indicated that DWA-2 consisted mainly of glycosaminoglycan chains with 72.9% of chondroitin sulfate A- and C-like moieties. These moieties comprised 29.7% of D-GlcA-

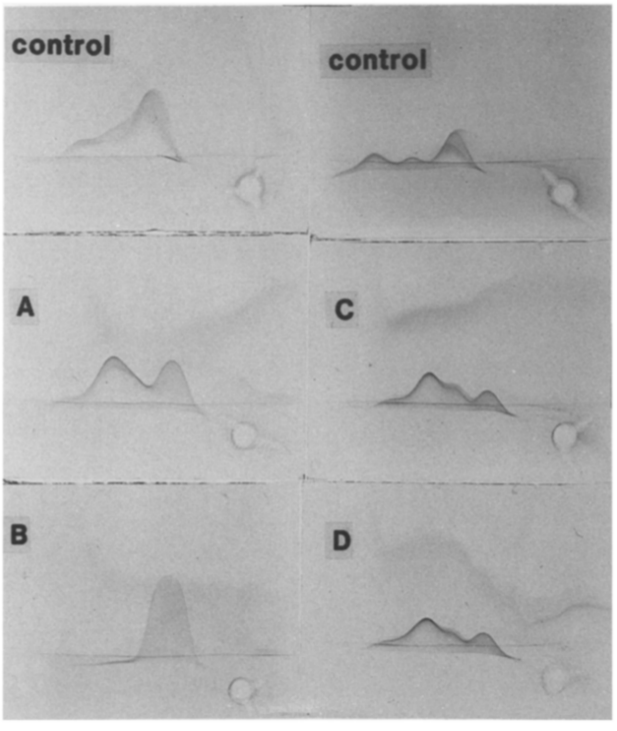

Fig. 5. Crossed immunoelectrophoresis of NHS incubated with DWA-2 (A, C), heparin (B), and AR-arabinogalactan (D) in GVB^{2+} (A, B) or Mg^{2+}–EGTA–GVB^{2-} (C, D).

TABLE IV

Anti-complementary activity (%) of enzymic digestion products from DWA-2

Sample		Concentration (μg/mL)		
		333	167	33
DWA-2	Original	90.5	79.8	66.4
	Chondroitinase ACI [a]	30.8	25.0	7.2
	Chondroitinase B [b]	56.5	46.0	21.5
	Chondroitinase ABC [c]	26.5	16.6	6.2
DWA-2	Original	63.0	50.0	41.5
	B-1 [d]	66.5	52.5	25.0
	B-2 [d]	25.0	11.0	5.0

[a] Chondroitinase ACI-digested DWA-2. [b] Chondroitinase B-digested DWA-2. [c] Chondroitinase ABC-digested DWA-2. [d] Products of chondroitinase B-digested DWA-2 obtained from Fig. 2A.

(1 → 3)-D-GalNAc 4-sulfate, 16.2% of D-GlcA-(1 → 3)-D-GalNAc 6-sulfate, and 27.0% of *N*-acetylchondrosine [D-GlcA-(1 → 3)-D-GalNAc] units.

Hamer and Perlin have reported[19] that chondroitin sulfates A and C consist of D-GlcA-(1 → 3)-D-GalNAc 6- and 4-sulfate units in various proportions (NMR data), and the results of enzymic studies now reported suggested that DWA-2 might be a copolymer of chondroitin sulfate A- and C-like moieties.

Enzymic digestion indicated that DWA-2 also consisted of 16.2% of a chondroitin sulfate B-like moiety which comprised L-IdoA-(1 → 3)-D-GalNAc 4-sulfate and *N*-acetyldermosine [L-IdoA-(1 → 3)-D-GalNAc] units in the ratio 6.0 : 1.0. One of the chondroitinase ACI-resistant fractions (ACI-2, which contained a chondroitin sulfate B-like moiety) had a molecular weight lower than that of DWA-2 and contained no protein. Digestion of DWA-2 with chondroitinase ACI also gave an enzyme-resistant fragment (ACI-1), which contained mainly protein in addition to Xyl and Gal. These results indicated that chondroitin sulfate A- and C-like moieties were attached to a protein moiety, and that a chondroitin sulfate B-like moiety was attached to the non-reducing terminals of chondroitin sulfate A- and/or C-like moieties in DWA-2.

Radhakrishnamurthy et al.[20] have proposed that, in the chondroitin sulfate–dermatan sulfate proteoglycan from bovine aorta, dermatan sulfate chains are attached to non-reducing terminals of chondroitin sulfate A chains. The present studies do not indicate whether the chondroitin sulfate B-like moiety in DWA-2 is attached to chondroitin sulfate A- or C-like moieties.

Enzymic digestion indicated that the proteoglycan moiety of DWA-2 had anti-complementary activity, and suggests that the chondroitin sulfate A- and C-like moieties mainly contributed to this activity. The chondroitin 4-sulfate proteoglycan, isolated from human serum[21,22], is an inhibitor of hemolytic activity of C1q. In the present study, crossed immunoelectrophoresis indicated that DWA-2 could activate the later complement component, C3, in the absence of Ca^{2+}, i.e., the alternative pathway of the complement system. Although chondroitin sulfates

A–C showed anti-complementary activity lower than that of DWA-2, the enzyme-resistant fragment (B-1, which contained a protein core with chondroitin sulfate A- and C-like moieties) had the same potent activity as DWA-2. Therefore, a combination of chondroitin sulfate A- and C-like moieties attached to the protein core might play an important role in the expression of complement activation.

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