Studies on the Mucopolysaccharides from Hagfish Skin

Fumiko Akiyama and Nobuko Seno

Department of Chemistry, Faculty of Science, Ochanomizu University, Tokyo
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Introduction

Many previous papers on the mucopolysaccharides of various animal skins have been largely limited to those of mammals[1-4], in which hyaluronic acid and dermatan sulfate are found to be the major components, with chondroitin sulfate A, C, heparin and heparan sulfate as minor components. From the skin of another vertebrates, a dermatan polysulfate has been isolated for the first time from shark skin[5]; this skin also contains hyaluronic acid. It has recently been shown that the main mucopolysaccharides of chicken skin in three age groups are hyaluronic acid and dermatan sulfate[6]. On the other hand, there are a few studies on the mucopolysaccharides of invertebrate skin. From squid skin, a large amount of non-sulfated chondroitin[7] and an over-sulfated chondroitin sulfate[8][38] have been isolated, but hyaluronic acid and dermatan sulfate were not found. These studies thus demonstrate a difference in mucopolysaccharide patterns between vertebrate and invertebrate skins.

The present paper describes the isolation and chemical structure of mucopolysaccharides from the skin of two hagfishes (*P. atami* and *E. burgeri*), which belong to the more primitive branch of cyclostomes, among the lowest vertebrates.

Experimental

Materials

The two species of hagfish, *Paramyxine atami* and *Eptatretus burgeri*, were collected, respectively, from the Japan Sea and the Pacific Ocean, off the coasts of Japan.

Potassium hyaluronate prepared from umbilical cords was a gift from Dr. T. Ohya. Sodium chondroitin sulfate A (ChS-A) was prepared from whale cartilage[9]. Crude chondroitin sulfate C from shark cartilage was fractionated by Dowex 1 column chromatography[10]; sodium chondroitin sulfate C (ChS-C) was obtained from the 1.5*M* NaCl fraction and sodium chondroitin sulfate D (ChS-D) from the 3.0*M* NaCl fraction. Sodium chondroitin sulfate E (ChS-E) was a

preparation from squid cartilage[11], chondroitin sulfate H (ChS-H) from hagfish notochord[12] and dermatan polysulfate (SChS-B) from shark skin[5]. Sodium dermatan sulfate (DS) was purchased from Seikagaku Kogyo Co. Ltd.

Pronase [EC 3.4.4] was purchased from Kaken Kagaku Co. Ltd. Hyaluronidase (AMANO)[13] from *Streptomyces hyalurolyticus* was a gift from Dr. T. Ohya. Chondroitinase-ABC from *Proteus vulgaris*, chondroitinase-AC from *Flavobacterium heparinum*, chondro-4-sulfatase and chondro-6-sulfatase from *Proteus vulgaris* were commercial preparations from Seikagaku Kogyo Co. Ltd. Cellulose acetate strips (Separax) were purchased from Jōkō Sangyo Co. Ltd.

Analytical methods

Uronic acid was determined by the carbazole reaction[14], Bitter and Muir's modification[15] of this reaction and the orcinol reaction[16] using glucuronolactone as standard. Hexosamine was determined by a modified Elson-Morgan reaction[17] after hydrolysis of 2 mg of sample in 1 ml 3*M* HCl at 100°C for 15 h in a sealed tube. Glucosamine and galactosamine were separated and determined by the method of Gardell[18] or by an amino acid analyzer. For the latter analysis, chondroitin sulfate (200 μg) was hydrolyzed in 3*M* HCl at 100°C for 10 h in a sealed tube, dermatan sulfate (200 μg) in 2*M* HCl at 100°C for 12 h and the digestion products with chondroitinase-ABC in 2*M* HCl at 100°C for 4 h[19]. After removing the hydrochloric acid *in vacuo*, the hydrolysate was applied to an amino acid analyzer (JEOL JLC-5AH) with a short column (0.8×15 cm) at 45°C, using 0.35*M* citrate buffer (pH 5.28) at a flow rate of 0.52 ml/min. Sulfate was determined by the method of Dodgson and Price[20], after hydrolysis in 1*M* HCl at 105°C for 5 h; and N-sulfated hexosamine by the method of Lagunoff *et al.*[21] using glucosamine as standard. Protein was determined by the method of Lowry *et al.*[22] using serum albumin as standard, and neutral sugar by the anthrone reaction[23] using galactose as standard. The reducing power was determined by the method of Park and Johnson[24] using glucose as standard. Amino acids were determined by the amino acid analyzer after hydrolysis with 6*M* HCl in a vacuum-sealed tube at 100°C for 22 h.

Paper chromatography of the digestion products with chondroitinases

Paper chromatography of the digestion products of mucopolysaccharides with chondroitinase-ABC or -AC was carried out by the descending method on Toyo No. 51A paper (40 cm long) essentially

according to the method of Saito *et al.*[25] using 1-butyric acid : 0.5*M* ammonia (5 : 3, v/v) for 41–69 h after development in 1-butanol : ethanol : water (52 : 32 : 16, v/v) for 24–48 h to remove interfering salts. The unsaturated compounds were detected by viewing under an ultraviolet lamp and by staining with alkaline silver nitrate[26] and also with 1% toluidine blue in 95% ethanol[27]. To determine the unsaturated compounds, each of the ultraviolet light-absorbing regions was cut into small pieces and extracted with 0.01*M* HCl at 50°C for 10 min. From the absorbance at 232 mμ of the extract, the contents were calculated by the use of the millimolar extinction coefficients of unsaturated disacharides[25,28] ($\triangle$Di-0S : 5.7, $\triangle$Di-4S : 5.1, $\triangle$Di-6S : 5.5, $\triangle$Di-diS$_B$: 6.0, and $\triangle$Di-diS$_E$: 6.0). As the millimolar coefficient of "X", 6.0 was tentatively used.

Paper chromatography of uronic acid

Paper chromatography for uronic acid was carried out on Toyo No. 50 paper by the descending method for 14 h in 2-dimethyl-1-propanol : 2-propanol : water (4 : 1 : 2, v/v)[29] or 1-butanol : acetic acid : water (4 : 1 : 2, v/v)[29], and detected by staining with alkaline silver nitrate. Before being applied to the paper, the uronic acids were liberated by hydrolysis in 1 ml of 0.05*M* HCl containing 10 mg of sample and 100 mg of Dowex 50 (H^+ form) at 100°C for 24 h[30]. The hydrolysate was neutralized and concentrated.

Column chromatography of uronic acid

The hydrolysate obtained under the conditions described above was applied to the top of a Dowex 1 column (X4, 200–400 mesh, acetate form, 1.2×10 cm)[31]. The column was washed with water and then eluted with 1.6*M* acetic acid. Uronic acids were determined by the orcinol and carbazole reactions.

Electrophoresis

Electrophoresis of mucopolysaccharides on cellulose acetate strips (Separax) was carried out in 1.0*M* acetic acid-pyridine (pH 3.5), at 0.5 mA/cm for 20 min or in 0.2*M* calcium acetate, at 1 mA/cm for 3 h[32]. After drying the strips in air, mucopolysaccharides were stained by dipping in 0.5% of toluidine blue for 5 min or 0.5% of alcian blue 8GN for 20 min, rinsed with water and dried.

High voltage electrophoresis of the digestion products of mucopolysaccharides with chondroitinase-ABC was carried out on Toyo No. 51A paper (60 cm long) in 0.1*M* acetic acid-pyridine (pH 5.0), at 40 V/cm for 1 h[33]. After drying in air, the products were detected by viewing under an ultraviolet lamp and with silver nitrate reagent.

Electrophoresis for uronic acid was carried out on Toyo No. 50 (40 cm long) paper using 0.15*M* HCOOH-pyridine buffer (pH 3.2), at 400 V for 3.5 h; the spots were detected with silver nitrate reagent.

Infrared spectroscopy

The infrared spectrum of a 2 mg sample was obtained with a KBr (200 mg) disk on a Jasco model IR-G spectrophotometer.

Enzymic digestion

Hyaluronidase (AMANO) digestion

Mucopolysaccharide (2 mg) was dissolved in 2 ml of 0.02*M* acetate buffer (pH 5.0) and digested with hyaluronidase (AMANO) from *Streptomyces hyalurolyticus* at a concentration of 40 units/0.2 ml at 60°C. The increase in reducing power with time was measured by the method of Park and Johnson.

Chondroitinase-AC and chondroitinase-ABC digestion

The digestion of mucopolysaccharides with chondroitinase-AC or chondroitinase-ABC was carried out at 37°C under the following conditions. The incubation mixture consisted of 300–500 μg of mucopolysaccharides in 20 μl, 10 μl of Tris-HCl buffer (pH 8.0) and 0.1 unit of chondroitinase-ABC or -AC in 20 μl. At the time intervals, 2 ml of 0.02*M* KCl-HCl buffer (pH 1.8) was added and the mixture was centrifuged at 10,800×g for 10 min and then the increase in the absorbance at 232 mμ of the supernatant was measured[28]. The digestion products after 16 h were subjected to paper chromatography.

For large scale preparations, 10 mg of mucopolysaccharides dissolved in 0.2 ml of Tris-HCl buffer (pH 8.0) was digested with 1.2 unit of chondroitinase-ABC in 120 μl for 5 h and then 1.0 unit of chondroitinase-ABC was further added to the incubation mixture. After the incubation was continued for 20 h, the products were subjected to paper chromatography.

Chondro-4-sulfatase or chondro-6-sulfatase digestion.

Unsaturated disaccharides (150 μg) in 20 μl of water and 10 μl of Tris-HCl buffer (pH 8.0) were incubated with 0.1 unit of chondro-4-sulfatase or chondro-6-sulfatase in 10 μl at 37°C for 2.5 h[25]. The digestion products were subjected to paper chromatography and detected with alkaline silver nitrate.

Extraction of mucopolysaccharides from hagfish skin

The skins of hagfish were carefully freed from extraneous tissues and mucus substances, cut into small pieces and defatted with acetone

and ether. The dried skin (80 g) was suspended in 800 ml of water and heated for 1 h in a boiling water bath. After cooling, the suspension was covered with toluene and digested with pronase (800 mg) at pH 7.8, 37°C. Four hundred mg of pronase was added after one day, and the incubation was continued for one more day. The digestion mixture was centrifuged, and the supernatant was concentrated and dialyzed. Additional protein was removed by precipitation with 10% trichloroacetic acid in an ice bath. The clear supernatant was dialyzed to remove trichloroacetic acid and the whole mucopolysaccharide was precipitated by addition of excess cetylpyridinium chloride (CPC) in the presence of 0.03*M* NaCl. The CP-mucopolysaccharide formed was collected by centrifugation, washed with 0.03*M* NaCl to remove CPC, dissolved in 10 ml of 3*M* NaCl and the dissociated mucopolysaccharide was precipitated with 2 vol. of ethanol. The precipitate was washed with ethanol to remove CPC, redissolved in 20 ml of water and reprecipitated with 3 vol. of ethanol in the presence of 1% sodium acetate. The precipitate was collected by centrifugation, washed with ethanol and dried in a vacuum desiccator over conc. H_2SO_4.

Fractionation of mucopolysaccharides

The whole mucopolysaccharide (200 mg) in 20 ml of water was applied to a column (2.8×40 cm) of Dowex 1 (X2, 200–400 mesh, Cl^- form). The column was washed with water and eluted with increasing concentrations of NaCl (0.5*M*, 1.0*M*, 1.5*M*, 2.0*M*, 3.0*M*, 4.0*M* NaCl) at a flow rate of 60 ml/h. Fractions of 10 ml were collected and uronic acid and hexose were determined by the modified carbazole reaction[15] and the anthrone reaction[23]. The fractions corresponding to each peak were collected, concentrated, dialyzed against tap water and distilled water untill chloride ion was not detected, and then lyophilized.

Gel filtration of mucopolysaccharide

Gel filtration of mucopolysacccharide (1 mg/0.7 ml) was carried out on columns (0.6×100 cm) of Sephadex G-50 and Sephadex G-75. After each column was washed with 1.0*M* NaCl until the anthrone test of the effluent become negative, the sample was applied to the column and eluted with 1.0*M* NaCl at a flow rate of 1 ml/30 min (Sephadex G-50) or 1 ml/15 min (Sephadex G-75). Fractions of 1 ml were collected and uronic acid was determined by the orcinol reaction[16]. The void volume (V_0) of each column was calculated experimentally using blue dextran-2000.

Results

Preparation of mucopolysaccharides

The yields of mucopolysaccharides from the skin of *P. atami* and *E. burgeri* and their analytical values are summarized in Table I.

Table I. Analysis of whole mucopolysaccharide.

	P. atami	*E. burgeri*
Yield (% of dry skin)	0.32	0.19
Hexosamine (%)	24.6	25.9
Glucosamine/Galactosamine	2.0/1.0	1.7/1.0
Uronic acid (%)		
Bitter & Muir	21.0	18.2
Carbazole*	15.4	15.1
Orcinol	25.5	23.6
C/O**	0.60	0.64
Sulfate (%)	7.2	6.2
Neutral sugar (%)		
(as Galactose)	10.1	15.4
Protein (Lowry) (%)	11.5	7.1

* Original carbazole reaction (Dische).
** Ratio of carbazole to orcinol.

The mucopolysaccharides of both species contained glucosamine and galactosamine and considerable amounts of hexose and protein, indicating that they were mixtures of mucopolysaccharides and contained contaminating glycoprotein.

Fractionation of mucopolysaccharides on a Dowex 1 column

Fractionation of each whole mucopolysaccharide sample was carried out on a Dowex 1 column and their elution patterns are shown in Fig. 1. In both skins, large amounts of mucopolysaccharides were eluted with 0.5*M* NaCl and 3.0*M* NaCl, and small amounts with the other NaCl concentrations. The yields and analytical values of each fraction are summarized in Table IIa and Table IIb.

Characterization of each fraction

1. 0.5*M* NaCl fraction and 1.0*M* NaCl fraction

The sulfate content of the 0.5*M* NaCl fraction was only a trace, the ratio of uronic acid determined by the carbazole reaction to that determined by the orcinol reaction (C/O) was nearly 1 and the hexosamine was mainly glucosamine. On cellulose acetate electrophoresis,

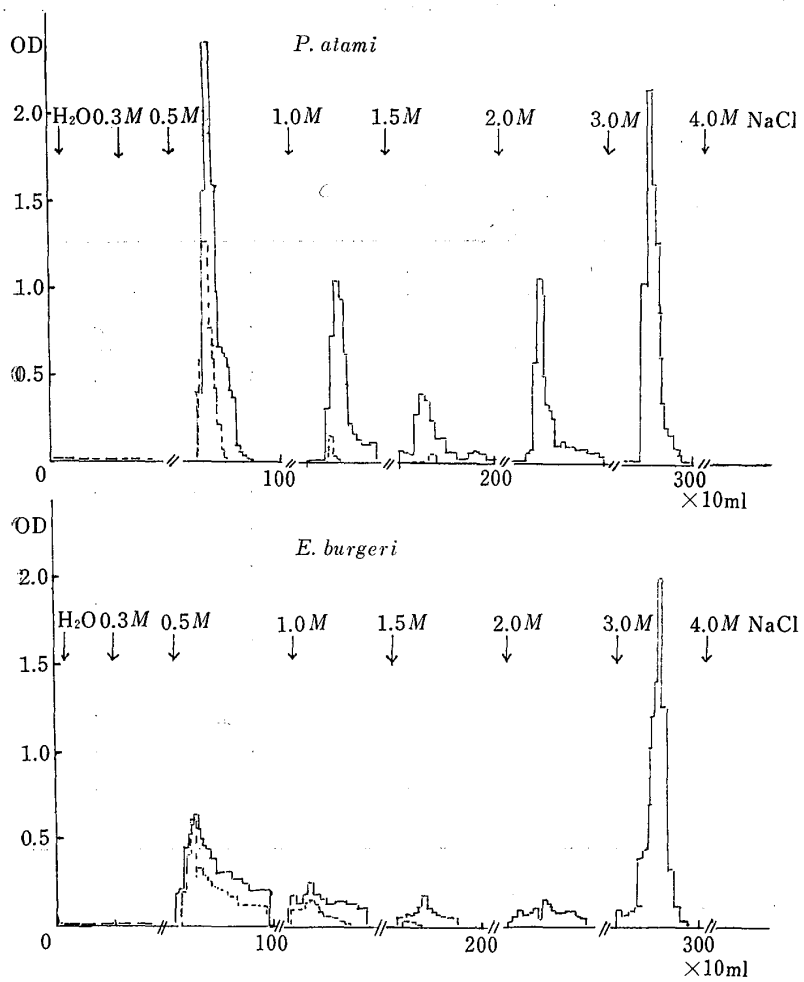

Fig. 1. Fractionation of mucopolysaccharide by Dowex 1 chromatography.
——— Uronic acid, ----- Neutral sugar

Table IIa. Analysis of mucopolysaccharide fractions obtained by Dowex 1 column chromatography.

P. atami

Fraction	0.5*M*	1.0*M*	2.0*M*	3.0*M*
Yield* (%)	32.0	17.7	14.3	26.6
Hexosamine (%)	27.1	22.5	26.9	25.9
Glucosamine/Galactosamine	9.6/1.0	—	1.0/5.6	1.0/43.3
Uronic acid (%)				
Bitter & Muir	15.5	18.1	25.1	18.7
Carbazole	16.3	19.5	23.4	9.1
Orcinol	16.3	14.8	21.8	30.0
C/O	1.01	1.30	1.07	0.30
Sulfate (%)	0.8	0.4	23.3	29.0
N-Sulfated hexosamine				<0.2
HexN/UA/SO_4^{2-}**	1.0/0.5	1.0/0.8	1.0/0.9/1.6	1.0/1.1/2.1
Neutral sugar (as Gal) (%)	18.1	7.4	6.3	4.1
Protein (Lowry) (%)	11.6	10.4	3.1	3.0
$[\alpha]_D$				−69.4°

* % of dry skin.
** Molar ratio of uronic acid and sulfate to hexosamine.

Table IIb. Analysis of mucopolysaccharide fractions obtained by Dowex 1 column chromatography.

E. burgeri

Fraction	0.5*M*	1.0*M*	1.5*M*	3.0*M*
Yield* (%)	32.6	9.2	4.1	45.0
Hexosamine (%)	22.4	18.9	19.0	22.3
Glucosamine/Galactosamine	GlcN only		—	1.0/12.1
Uronic acid (%)				
Bitter & Muir	21.3	16.3	12.6	15.6
Carbazole	21.1	16.4	10.7	8.4
Orcinol	23.3	18.2	11.1	29.4
C/O	0.91	0.90	0.96	0.29
Sulfate (%)	0.54	0.42	12.4	25.8
N-Sulfated hexosamine				<0.3
HexN/UA/SO_4^{2-}**	1.0/0.9	1.0/0.9	1.0/0.6/1.2	1.0/1.2/2.1
Neutral sugar (as Gal) (%)	21.0	13.0	13.5	5.9
Protein (Lowry) (%)	5.2	7.8	5.6	4.6
$[\alpha]_D$				−69.0°

* % of dry skin.

** Molar ratio of uronic acid and sulfate to hexosamine.

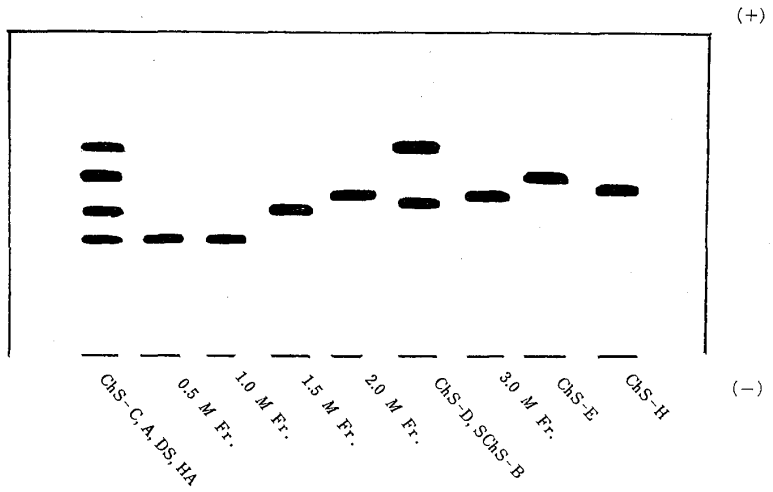

Fig. 2. Electrophoretogram on a cellulose acetate strip of each fraction obtained by Dowex 1 column chromatography.

this fraction gave a single band corresponding to the standard hyaluronic acid as shown in Fig. 2. With hyaluronidase (AMANO) which attacks only hyaluronic acid, this fraction of *P. atami* and *E. burgeri* was digested at the same velocity as hyaluronic acid as shown in Fig. 3. From these results, the mucopolysaccharide of the

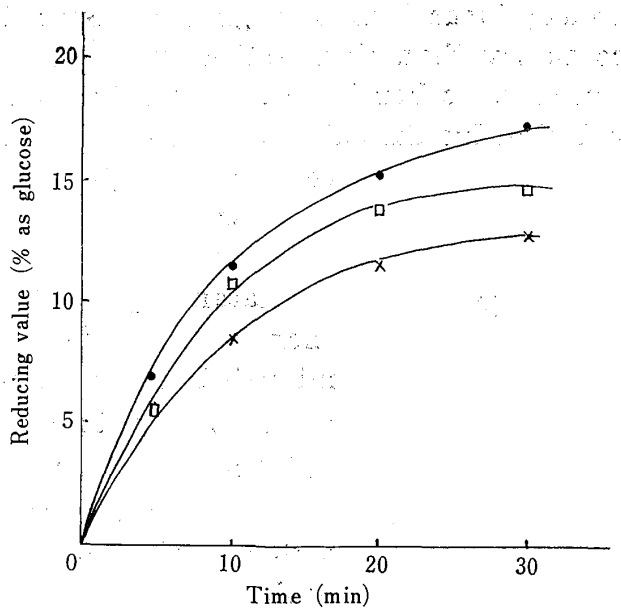

Fig. 3. Hyaluronidase digestion of 0.5*M* NaCl fractions. Incubation mixtures and conditions are described in the text. Reducing value was measured at indicated intervals.
—●—●— Hyaluronic acid
—□—□— 0.5*M* NaCl fraction of *P. atami*.
—×—×— 0.5*M* NaCl fraction of *E. burgeri*.

0.5*M* NaCl fraction was identified as hyaluronic acid.

The sulfate content of the 1.0*M* NaCl fraction was very low and the only hexosamine present was glucosamine. On electrophoresis this fraction gave a single band corresponding to hyaluronic acid as shown in Fig. 3. Accordingly, this 1.0*M* NaCl fraction also seems to be mainly hyaluronic acid.

2. 3.0*M* NaCl fraction and 2.0*M* NaCl fraction

The hexosamine of the 3.0*M* fraction of both species was galactosamine. The ratio of carbazole to orcinol was 0.30 and 0.29, respectively, indicating that the uronic acid is mainly L-iduronic acid. By paper chromatography and paper electrophoresis of the mild-acid hydrolysate of this fraction, a main spot corresponding to iduronic acid and a trace of glucuronic acid were detected. Furthermore, Dowex 1 column chromatography in 1.6*M* acetic acid gave a large peak of iduronic acid with a small peak of glucuronic acid.

Accordingly, the uronic acid of this fraction was indicated to be mainly L-iduronic acid. The sulfate content of this fraction of both species were extremely high; its molar ratio to galactosamine was 2.1. The N-sulfated hexosamine content was less than 0.3%. The optical rotation was −69.4° and −69.0°, respectively.

On cellulose acetate electrophoresis in 1.0*M* acetic acid-pyridine (pH 3.5), this mucopolysaccharide migrated faster than dermatan sulfate and more slowly than chondroitin sulfate A or C. As shown in Fig. 2, the mucopolysaccharide gave a single spot between dermatan sulfate and chondroitin sulfate A in 0.2*M* calcium acetate, in comparison with the known polysulfates [chondroitin sulfate E (ChS-E), chondroitin sulfate D (ChS-D), dermatan polysulfate (SChS-B), chondroitin sulfate H (ChS-H)], it migrated faster than SChS-B and more slowly than ChS-H, ChS-D and ChS-E (Fig. 2).

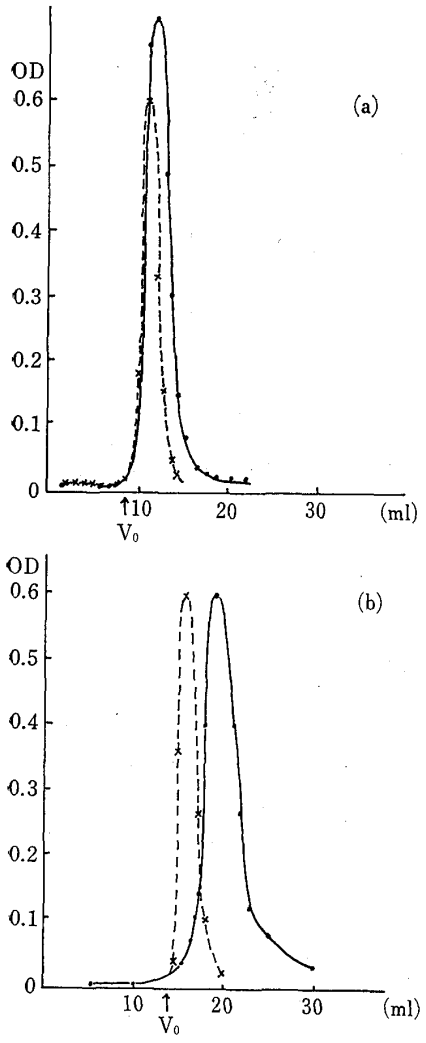

Fig. 4. Gel filtration of 3.0*M* NaCl fraction.
(a) Sephadex G-50 in 1.0*M* NaCl (0.6×100 cm)
(b) Sephadex G-75 in 1.0*M* NaCl (0.6×100 cm)
—●—●— Uronic acid
--×--×-- Blue dextran 2000

The mucopolysaccharide was eluted with the void volume of a Sephadex G-50 column in 1.0*M* NaCl, whereas it was retarded as a sharp single peak on a Sephadex G-75 column in 1.0*M* NaCl (Fig. 4), suggesting that the molecular weight of this mucopolysaccharide is about ten thousand.

As shown in Fig. 5, the infrared spectra of both the 3.0*M* NaCl fractions gave absorption bands at 928 cm^{-1} and 850 cm^{-1} for axial sulfate groups and at 820 cm^{-1} for equatorial sulfate groups binding at the C-6 of galactosamine. In addition, bands were observed at 800 cm^{-1} and 1000 cm^{-1}.

In order to determine the positions of the ester sulfate groups, the mucopolysaccharide was subjected to digestion with chondroitinase-ABC and -AC. As shown in Table III, the 3.0*M* NaCl fraction was resistant to chondroitinase-AC,

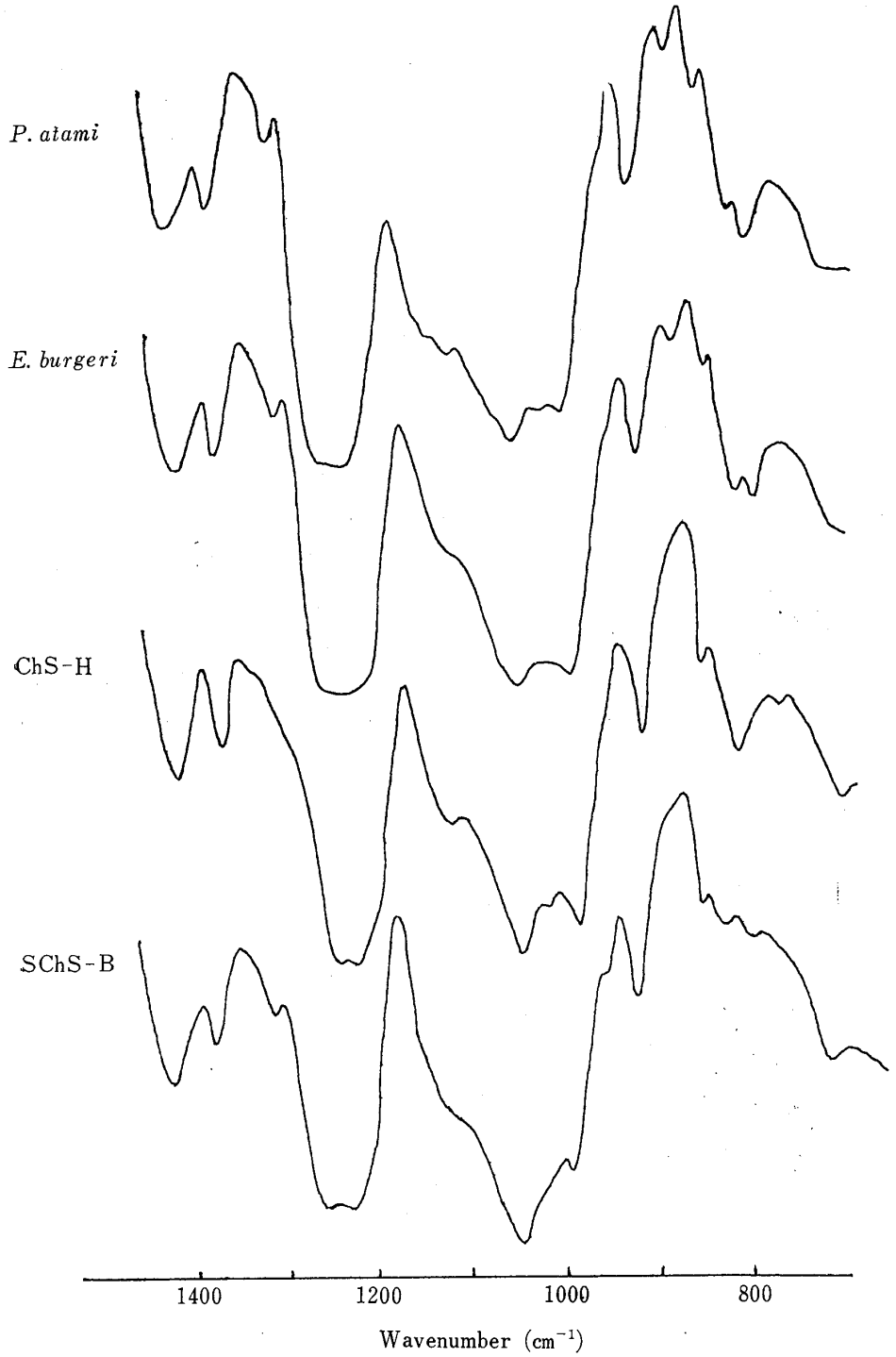

Fig. 5. Infrared spectra of 3.0*M* NaCl fractions from hagfish skin (*P. atami* and *E. burgeri*), chondroitin sulfate H (ChS-H) from hagfish notochord and sulfated chondroitin sulfate B (SChS-B) from shark skin. The spectra were obtained with a KBr disk containing 2 mg of mucopolysaccharide on a Jasco model IR-G spectrophotometer.

Table III. Chondroitinase-AC digestion products of 3.0*M* NaCl fractions.

Sample	*P. atami*	*E. burgeri*
μmole as GlcUA	0.585	0.595
Digestion products	μmole	μmole
△Di-0S	0	0
△Di-4S	0	0
△Di-6S	0	0
△Di-diS$_E$	0	0.002
△Di-diS$_B$	0	0
△Di-diS$_D$	0	0
"X"	0	0.010

in spite of the use of excess enzyme.

The increases in absorption at 232 mμ with time of digestion of this mucopolysaccharide by chondroitinase-ABC are shown in Fig. 6. This mucopolysaccharide was degraded more slowly than chondroitin sulfate A and that of *P. atami* was digested less than that of *E. burgeri*. After exhaustive digestion, however, the absorption value rose to nearly 80% of the total uronic acid in the both polysac-

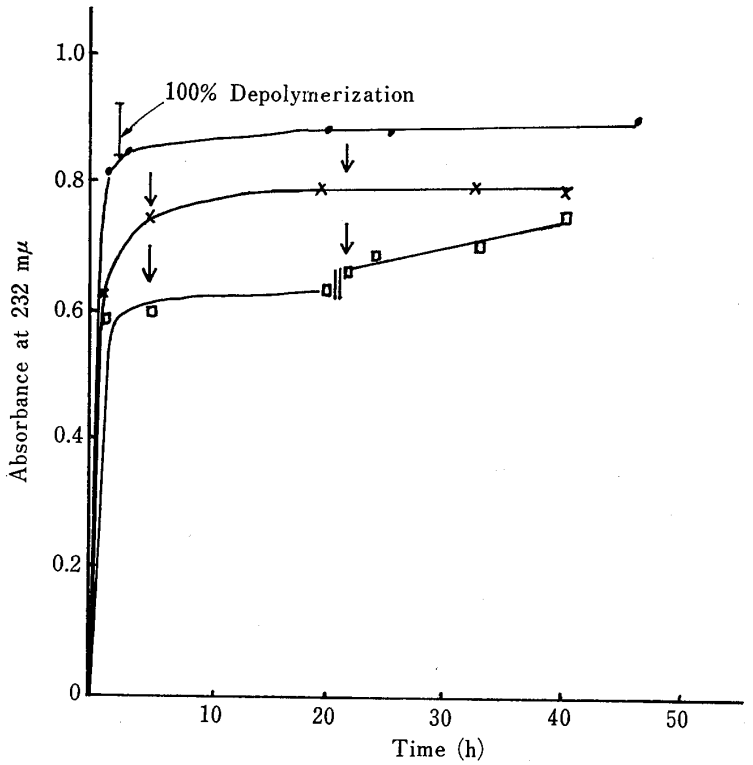

Fig. 6. Degradation of 3.0*M* NaCl fractions of hagfish skin (*P. atami* and *E. burgeri*) with chondroitinase-ABC. Incubation mixtures and conditions are described in the text. At the time indicated by arrow, 0.1 unit of enzyme was added to the incubation mixture.
—●—●— ChS-A, —□—□— *P. atami*, —×—×— *E. burgeri*

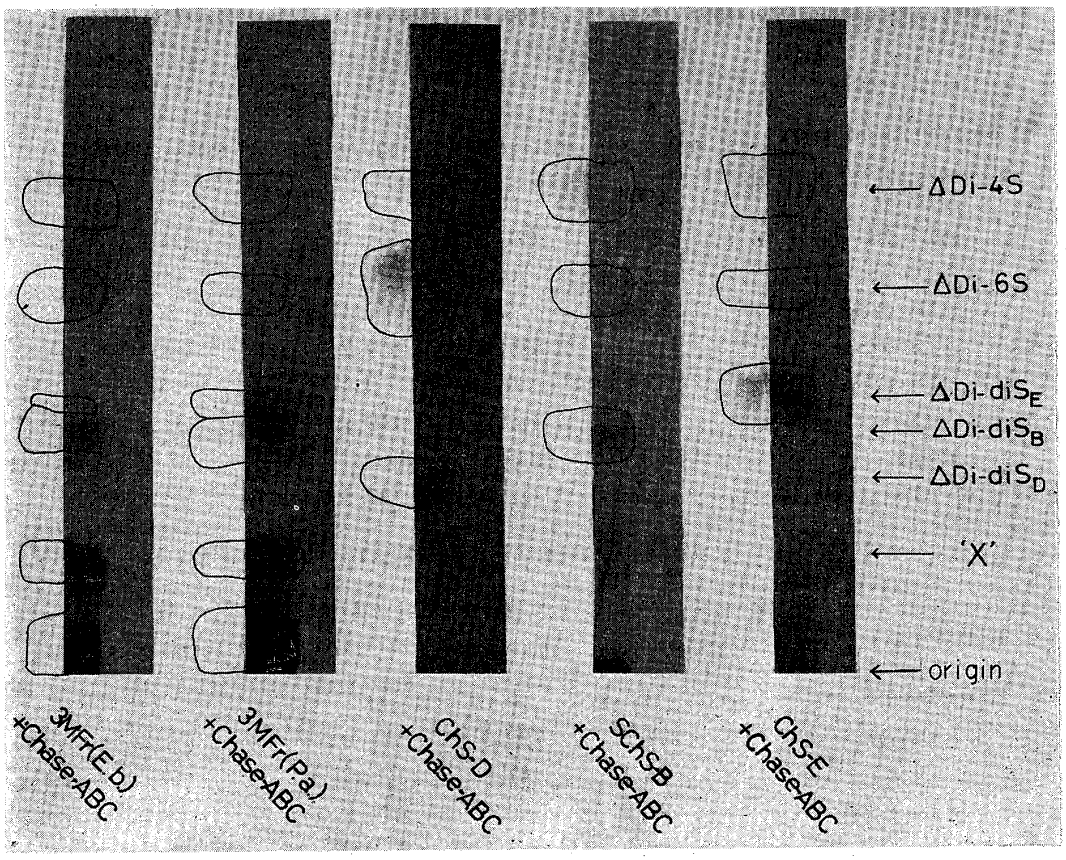

Fig. 7. Paper chromatogram of the products obtained by digesting 3.0*M* NaCl fractions of *P. atami* and *E. burgeri* with chondroitinase-ABC. The spots were detected by viewing under an ultraviolet lamp (tracing), by staining with alkaline silver nitrate (left) and by staining with toluidine blue (right).

charides.

The paper chromatogram of the digestion products of the 3.0*M* NaCl fraction with chondroitinase-ABC is shown in Fig. 7, in which each open symbol indicates the absorption regions detected by viewing under an ultraviolet lamp (left halves were stained with alkaline silver nitrate and right halves with toluidine blue). Large amounts of △Di-4S and △Di-diS$_B$, and small amounts of △Di-6S and △Di-diS$_E$ were detected, but spots corresponding to △Di-0S and △Di-diS$_D$ were not observed. Other than these known products, an unknown clear spot moving more slowly than △Di-diS$_D$, which was tentatively designated as "X", could be detected by alkaline silver nitrate and viewing under an ultraviolet lamp, and showed strong metachromatism. In addition, some products remaining at the region of origin on the paper chromatogram were detected.

The results of measurement of the digestion products are shown in Table IV. The 3.0*M* NaCl fraction of *P. atami* (3.54 μmole as UA) and *E. burgeri* (4.35 μmole as UA) were each digested with 0.55 unit of chondroitinase-ABC for 23 h, and the respective yields of unsatur-

Table IV. Chondroitinase-ABC digestion products of 3.0*M* NaCl fractions.

P. atami (3.54 μmole UA)

Digestion products	1 mμmole	2 mμmole	1+2 mμmole	% of total products
△Di-0S	0	0	0	0
△Di-4S	156.9	231.5	388.4	22.9
△Di-6S	64.1	76.4	140.5	8.3
△Di-diS$_E$	65.1	2.3	67.4	3.9
△Di-diS$_B$	114.2	525.0	639.2	37.7
△Di-diS$_D$	0	0	0	0
"X"	51.7	407.5	459.2	27.1
Yield	452.0	1242.7	1694.2	
(% of total UA)	12.6	34.8	47.5	

E. burgeri (4.35 μmole UA)

Digestion products	1 mμmole	2 mμmole	1+2 mμmole	% of total products
△Di-0S	0	0	0	0
△Di-4S	292.5	129.0	421.5	21.8
△Di-6S	94.1	43.6	137.7	7.1
△Di-diS$_E$	96.9	80.0	176.9	9.1
△Di-diS$_B$	510.0	202.0	712.0	36.4
△Di-diS$_D$	0	0	0	0
"X"	158.5	343.0	501.5	25.6
Yield	1152.0	797.6	1949.6	
(% of total UA)	26.5	18.3	44.9	

1: Digestion products obtained by the first enzyme reaction.
2: Redigestion products of the region of origin on paper chromatogram obtained from the first reaction products.

ated disaccharides produced were 12.6% of the 3.0*M* NaCl fraction from *P. atami* and 26.5% of that from *E. burgeri*. The proportions of these products were different in *P. atami* and *E. burgeri*; *i. e.* in *P. atami*, △Di-4S was predominant, while in *E. burgeri*, △Di-diS$_B$ predominated, comprising over 40% of the total products.

The region of origin on the paper chromatogram was extracted with water and redigested with 0.4 unit of chondroitinase-ABC. As shown in Table IV, "X" and △Di-diS$_B$ were the main products of this redigestion. The yield of unsaturated disaccharides was 34.8% of the starting mucopolysaccharide from *P. atami* and 18.3% of that from *E. burgeri*. Thus, the sum of the products after repeated digestion was 47.5% of the 3.0*M* NaCl fraction from *P. atami* and 45% from *E. burgeri*, and no significant difference could be found between the products of the two species.

Some products still remaining after redigestion at the region of origin on the paper chromatogram were re-extracted and digested with chondroitinase-ABC, but did not produce any identifiable products.

The analytical values for the amino acids of the 3.0*M* NaCl fraction are summarized in Table V. The predominant amino acids

Table V. Amino acid analysis of 3.0*M* NaCl fraction.
(*P. atami*)

Amino acid	Before alkali	After alkali*
	mg/g	
Aspartic acid	1.7	1.7
Threonine	0.67	0.66
Serine	4.7	2.4
Glutamic acid	6.5	6.4
Proline	2.4	—
Glycine	5.6	5.5
Alanine	0.75	0.73

* 0.5*M* NaOH at 4°C for 19 h.

were found to be glutamic acid, glycine, serine and proline; only serine was destroyed, to about 50% of the amount originally present, by treatment with 0.5*M* NaOH at 4°C for 19 h. Consequently, the mucopolysaccharide appears to be linked to peptide through the hydroxyl group of serine, like the other sulfated mucopolysaccharides (chondroitin sulfate A, C, dermatan sulfate, heparan sulfate and heparin) except keratan sulfate.

The yields of the 2.0*M* NaCl fraction were very small in both species. On cellulose acetate electrophoresis, this fraction migrated faster than dermatan sulfate, as shown in Fig. 2. The products obtained from the 2.0*M* NaCl fraction by digestion with chondroitinase-ABC were mainly △Di-4S, △Di-diS_B and "X". As shown in Table IIa, the sulfate content was high, with a ratio of sulfate to galactosamine of 1.6. These results suggest that the mucopolysaccharide is similar to that of the 3.0*M* NaCl fraction.

3. Characterization of "X" material

The "X" fraction obtained from the 3.0*M* NaCl fraction by digestion with chondroitinase-ABC, as shown in the paper chromatogram in Fig. 7, was clearly different from the known products. On high voltage paper electrophoresis in 0.1*M* acetic acid-pyridine (pH 5.0) at 40 V/cm for 1 h, as shown in Fig. 8, "X" gave a single spot that migrated to the anode more rapidly than △Di-diS_E, which contains

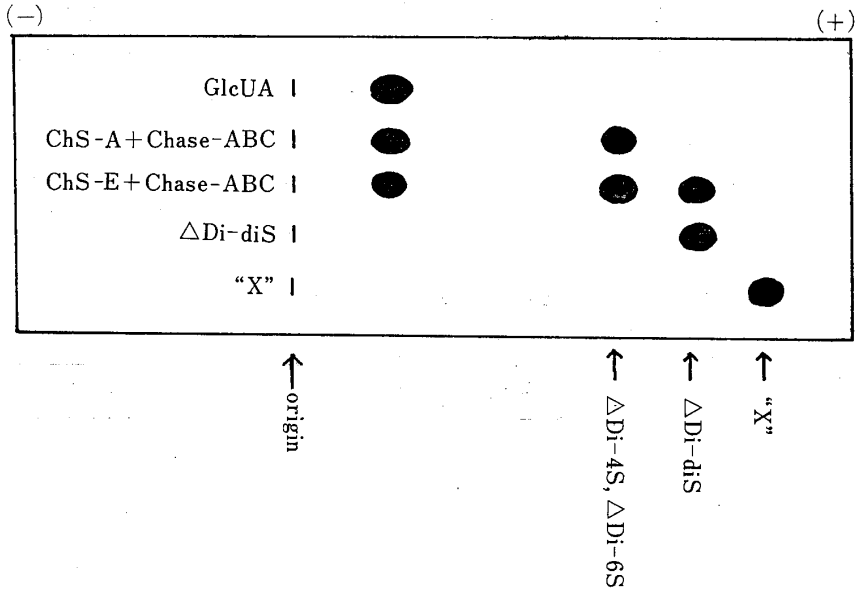

Fig. 8. Electrophoretogram of "X".

Table VI. Analysis of "X".

	Molar ratio
Hexosamine	1.00
Uronic acid	
Carbazole	1.04
Orcinol	0.81
Sulfate	2.70
$\overline{DP}$*	1.0

* as disaccharide.

two sulfate groups per disaccharide. The analytical values for "X" are shown in Table VI. The molar ratio of sulfate to galactosamine is about 3; the value for uronic acid determined by the carbazole reaction is equal to that by the orcinol reaction; and molar ratio of uronic acid to galactosamine is nearly 1, suggesting that "X" is a trisulfated disaccharide. The degree of polymerization ($\overline{DP}$) of "X" as disaccharide found to be almost 1, as calculated from the ratio of the absorbance at 232 mμ to the uronic acid value obtained by the carbazole or orcinol reaction. From these results, the "X" material was considered to be an unsaturated disaccharide trisulfate. In order to determine the position of the ester sulfate, "X" was digested with chondro-6-sulfatase. As shown in Fig. 9, paper chromatography gave a single spot corresponding to $\triangle$Di-diS$_B$, which is an unsaturated disaccharide containing one ester sulfate on the C-4 of galactosamine and the other on C-2 or -3 of uronic acid. Consequently, it is concluded that the "X" material is an unsaturated disaccharide trisulfate containing the third sulfate group on the C-6 of galactosamine of $\triangle$Di-diS$_B$, and that its structure (Fig. 10) is 2-acetamido-2-deoxy-3-*O*-(2 or 3-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-4, 6-di-*O*-sulfo-D-galactose.

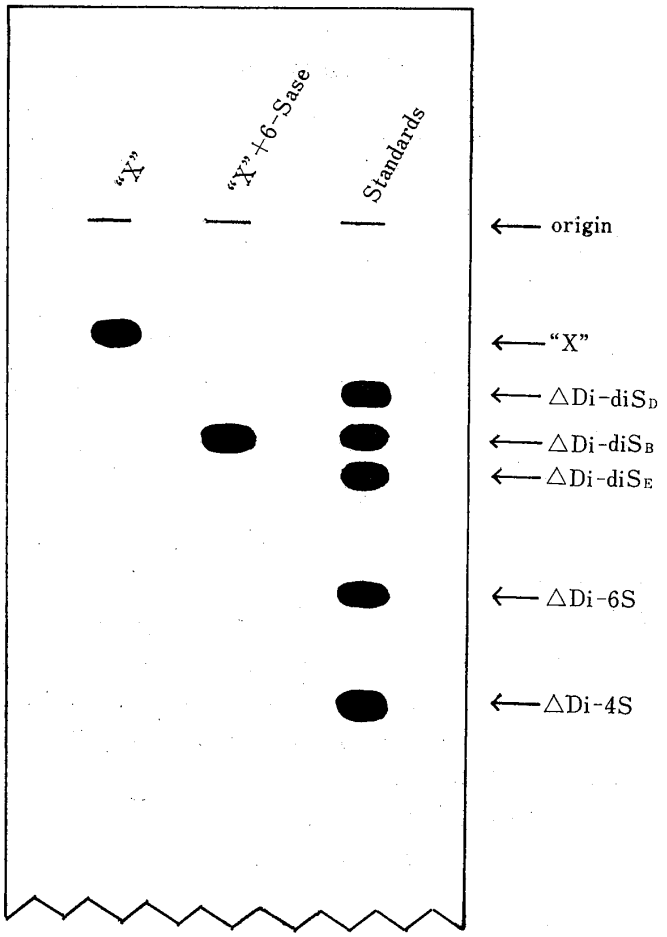

Fig. 9. Paper chromatogram of the digestion product of "X" with chondro-6-sulfatase.

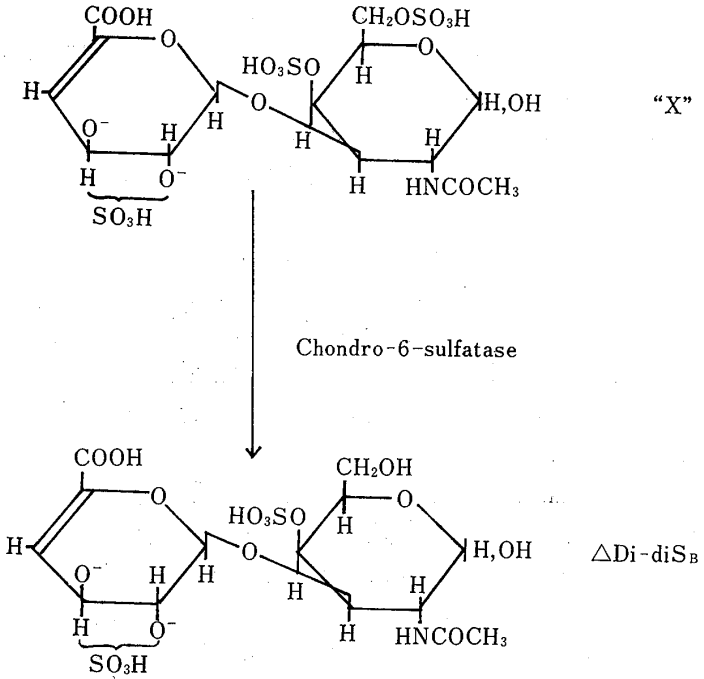

Fig. 10. Structure of "X" and its degradation with chondro-6-sulfatase.

Discussion

The yields of whole mucopolysaccharides obtained from the skin of the hagfishes, *P. atami* and *E. burgeri*, which belong to the most primitive vertebrates, were about 0.2–0.3% of the dry skin in both species, indicating that the yields did not differ from those obtained from the skins of other adult vertebrates[4–6)8)34)].

Fractionation of this mucopolysaccharide on a Dowex 1 column showed that the mucopolysaccharides were mainly eluted with 0.5*M* NaCl and 3.0*M* NaCl.

The mucopolysaccharide of the 0.5*M* NaCl fraction was identified as hyaluronic acid from the results of chemical analyses, electrophoresis and digestion with hyaluronidase (AMANO) which degrades only hyaluronic acid.

The other main fraction from the two species, *i. e.*, the 3.0*M* NaCl fraction, gave a single spot different from those of any other known mucopolysaccharide polysulfates on electrophoresis. On gel filtration it gave a single peak, indicating that the mucopolysaccharide is apparently homogeneous.

The sulfate content of this fraction was extremely high, the molar ratio of hexosamine : uronic acid : sulfate being 1.0 : 1.1 : 2.1. The ratio of carbazole to orcinol (C/O) was 0.3, and the results of paper chromatography and paper electrophoresis showed that the uronic acid was mainly L-iduronic acid. The molar ratio of sulfate to galactosamine (2.1) was the highest among the ratios of known polysulfates: chondroitin sulfate D (1.45), chondroitin sulfate E (1.55), dermatan polysulfate from shark skin (1.6) and chondroitin sulfate H from hagfish notochord (1.89).

The infrared spectrum of this mucopolysaccharide indicated that both axial and equatorial sulfate groups are present; in this property it resembles chondroitin sulfate H, suggesting that the sulfate groups are linked at C-4 and C-6 of galactosamine. Moreover, an absorption band at 800 cm^{-1} is like that of a dermatan polysulfate from shark skin, in other respects its spectrum did not coincide with any others. The absorption band at 800 cm^{-1}, as reported in connection with heparin[36)], appears to be responsible for the ester sulfate binding at the uronic acid moiety.

This mucopolysaccharide was not affected by excess chondroitinase-AC, which further identified it as belonging to the dermatan sulfate type. The 3.0*M* NaCl fraction was digested with chondroitinase-ABC and an unknown spot of "X" was detected with ultraviolet light, in addition to four known unsaturated disaccharides ($\triangle$Di-diS_B, $\triangle$Di-4S, $\triangle$Di-diS_E, $\triangle$Di-6S). As summarized in Table IV, the first

enzymic digestion made a difference between the proportions of the products from *P. atami* and *E. burgeri*. The lower yield in *P. atami* at this stage may be explained from the time course of the digestion process, as shown in Fig. 6, since the final yields and proportions of digestion products observed after repeating the enzymic treatment two times were closely similar. The difference appearing in the first degradation is probably related to the difference in the location of sulfate groups in the two polymers.

The sulfate contents calculated from the digestion products were fairly consistent with those derived by chemical analysis of the 3.0*M* NaCl fractions.

The molar ratio of galactosamine: uronic acid: sulfate in "X" was 1:1:3, the value of uronic acid determined by the carbazole reaction was consistent with its value determined by the orcinol reaction, and the molar ratio of the value for the non-reducing terminal unsaturated uronic acid (absorbance at 232 mμ) to the value for uronic acid obtained by the carbazole or orcinol reaction was approximately 1, indicating that "X" is a disaccharide. These properties indicate that the "X" material is a trisulfated disaccharide. Furthermore, digestion with chondro-6-sulfatase, which specifically hydrolyzes the ester sulfate at the C-6 of the galactosamine moiety, yielded $\triangle$Di-diS$_B$ [2-acetamido-2-deoxy-3-*O*-(2 or 3-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose], so that "X" can be characterized as a trisulfated unsaturated disaccharide containing the third ester sulfate at the C-6 of galactosamine of $\triangle$Di-diS$_B$: 2-acetamido-2-deoxy-3-*O*-(2 or 3-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-4, 6-di-*O*-sulfo-D-galactose, as shown in Fig. 10. The "X" material was not affected by chondro-4-sulfatase, which may result from the steric hindrance of the structure of "X", because 2-acetamido-2-deoxy-D-galactose-4, 6-di-*O*-sulfate is not digested with this sulfatase, as reported by Yamagata *et al.*[35] The millimolar extinction coefficient of "X" was calculated as 7.3. It is very interesting that the disaccharide "X" showed strong metachromasy, although metachromatism has been thought to be a reaction between the acidic polymer and the basic dye.

Hitherto, only one paper has reported the isolation of a trisulfated disaccharide from mucopolysaccharide; this is 2-sulfamido-2-deoxy-4-*O*-(3-*O*-sulfo-α-D-glucopyranosyluronic acid)-6-*O*-sulfo-D-glucose obtained from heparin by digestion with heparinase[36]. It is therefore clear that "X" is an another new trisulfated disaccharide from mucopolysaccharides.

Accordingly, both the 3.0*M* NaCl fractions from the skins of these two hagfishes represent a new type of dermatan polysulfate containing the trisulfated disaccharide unit: 2-acetamido-2-deoxy-3-

O-(2 or 3-*O*-sulfo-α-L-idopyranosyluronic acid)-4, 6-di-*O*-sulfo-D-galactose unit, two types of disulfated disaccharide units: 2-acetamido-2-deoxy-3-*O*-(2 or 3-*O*-sulfo-α-L-idopyranosyluronic acid)-4-*O*-sulfo-D-galactose unit and 2-acetamido-2-deoxy-3-*O*-(α-L-idopyranosyluronic acid)-4, 6-di-*O*-sulfo-D-galactose unit, and two types of monosulfated disaccharide units: 2-acetamido-2-deoxy-3-*O*-(α-L-idopyranosyluronic acid)-4-*O*-sulfo-D-galactose unit and 2-acetamido-2-deoxy-3-*O*-(α-L-idopyranosyluronic acid)-6-*O*-sulfo-D-galactose unit.

Some material remaining at the region of the origin on the paper chromatogram after the third digestion with chondroitinase-ABC represented very small amounts of digestion products. However, the molar ratio of sulfate to uronic acid was found to be higher than 2 and C/O was less than 1, suggesting that they are higher oligosaccharides containing large amounts of "X".

The variations of dermatan sulfate which have been reported recently include higher sulfate contents in preparations from shark skin[5], Hurler spleen[37] and hagfish notochord[12]. However, trisulfated disaccharide has not hitherto been isolated from dermatan-type mucopolysaccharide, no previous report has been made of a containing three types of oversulfated disaccharide units in the molecule.

The amount of the 1.5*M* NaCl fraction was not sufficient to characterize it clearly; however, the results of electrophoresis and chemical analysis suggest that it is dermatan sulfate.

The results obtained in this study indicate that the mucopolysaccharides in both skins contain two major components, hyaluronic acid and a dermatan polysulfate. The latter does not differ basically in the backbone structure of dermatan sulfate from those of other vertebrate skins, whereas the sulfate content and the positions of sulfate groups are markedly different.

On the other hand, the mucopolysaccharide differs from that of squid skin, which contains chondroitin and chondroitin polysulfate.

Summary

The mucopolysaccharides from the skins of hagfishes were isolated after pronase digestion and fractionation on a Dowex 1 column. The main mucopolysaccharides were found to be hyaluronic acid and dermatan polysulfate, from the results of electrophoresis, chemical analyses, digestions with hyaluronidase, chondroitinase-AC, -ABC and infrared spectroscopy.

This dermatan polysulfate was homogeneous on electrophoresis and gel filtration. The molar ratio of galactosamine: uronic acid: sulfate was about 1 : 1 : 2. On cellulose acetate electrophoresis in 0.2*M*

calcium acetate, this dermatan polysulfate gave a single spot between SChS-B and ChS-H. Its infrared spectrum showed absorption bands for both axial and equatorial sulfate groups, and an absorption band at 800 cm^{-1} differing from those of SChS-B and ChS-H. This mucopolysaccharide was not attacked by chondroitinase-AC, whereas it was degraded by chondroitinase-ABC and yielded a new spot "X", which moved more slowly than $\triangle$Di-diS_D, in addition to spots corresponding to $\triangle$Di-diS_B, $\triangle$Di-4S, $\triangle$Di-diS_E and $\triangle$Di-6S.

The material "X" was characterized as a trisulfated disaccharide: 2-acetamido-2-deoxy-3-*O*-(2 or 3-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-4, 6-di-*O*-sulfo-D-galactose, on the basis of chemical analysis and chondrosulfatase digestion.

This mucopolysaccharide is a new type of dermatan polysulfate containing the trisulfated disaccharide unit: 2-acetamido-2-deoxy-3-*O*-(2 or 3-*O*-sufo-α-L-idopyranosyluronic acid)-4, 6-di-*O*-sulfo-D-galactose, two types of disulfated disaccharide units and two types of monosulfated disaccharide units.

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Foot note:

* Abbreviations used are: △Di-0S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose, △Di-4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose, △Di-6S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose, △Di-diS_B, 2-acetamido-2-deoxy-3-*O*-(2 or 3-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose, △Di-diS_E, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4,6-di-*O*-sulfo-D-galactose, △Di-diS_D, 2-acetamido-2-deoxy-3-*O*-(2 or 3-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose.