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Enhancement of t-PA–Mediated Plasminogen Activation by Partially Defucosylated Glycosaminoglycans from the Sea Cucumber *Stichopus japonicus*

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Sea cucumber glycosaminoglycan (SC-GAG) was isolated from the body wall of the sea cucumber *Stichopus japonicus*. The SC-GAG consists of a chondroitin sulfate E-type core polymer with sulfated fucose branches attaching glycosidically to almost every disaccharide unit of the core polymer at the C-3 position of the GlcA or at C-4 and/or C-6 position(s) of GalNAc. SC-GAG was subjected to mild acid-hydrolysis, which cleaved selectively the glycosidic linkages between the core polymer and the fucose branches, resulting in two types of partially defucosylated SC-GAG derivatives. One type (type A), obtained by 3 h-hydrolysis, contained 33% of the fucose branches and the other type (type B), obtained by 6-h hydrolysis, contained 10% of the fucose branches. The molecular masses of types A and B were determined to be 8 and 4 kDa, respectively, by gel permeation HPLC. A chondroitinase ABC (Chase ABC)-digestion demonstrated that types A and B contained 46 and 66% of digestable disaccharide units, respectively, and both types contained 29% of E-type unsaturated disaccharide units bearing no fucose branches. Intact SC-GAG and types A and B were compared for t-PA–mediated plasminogen activation by an *in vitro* assay system. Although intact SC-GAG and type B exhibited rather weak activity at 6.25 μg/ml, type A exhibited 5 to 10-fold higher activity than intact SC-GAG and type B at the same concentration. The activity of type A was almost one-third that of purified chondroitin sulfate E (127 kDa containing 64.5% E-type disaccharide units) from squid cartilage at 6.25 μg/ml concentration. These results suggest that t-PA–mediated plasminogen activation requires the presence of E-type disaccharide units bearing no fucose branches and a molecular mass larger than 7.5 kDa in terms of the chondroitin sulfate E structure with or without fucose branching.

Key words: E-type disaccharide unit, partial removal of fucose branches, sea cucumber glycosaminoglycan, threshold of molecular weight, t-PA–mediated plasminogen activation.

Chondroitin sulfate E (CS-E)-type polysaccharides of living organisms contain linear polysaccharide structures in which the main repeating disaccharide unit is composed of glucuronic acid (GlcA) and *N*-acetylgalactosamine (GalNAc) sulfated at both the C-4 and C-6 positions (*1, 2*). This type of CS-E was isolated for the first time from squid cartilage and characterized more than thirty years ago (*1, 2*). Habuchi *et al.* (*3*) later showed that the C-6 positions of GalNAc are modified by glucose at a small percentage in the squid CS-E structure. In the case of mammals, CS-E is found in mast cells (*4–7*), monocytes (*8, 9*), and mesangial cells (*10–12*). Very recently, a key enzyme involved in the biosynthesis of human CS-E, *N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase, was cloned and characterized (*13*). On the other hand, peculiar types of CS-E derivatives have been found and characterized over the last decade (*14–16*), exclusively sourced from the connective tissues of echinoderms such as sea cucumber. Accordingly, these unusual types of CS-E are alternatively called sea cucumber glycosaminoglycans (SC-GAGs). As summarized in Fig. 1A, the structure of SC-GAG from *Stichopus japonicus* comprises a CS-E-type polysaccharide (core polymer) with sulfated fucose branches glycosidically linked to almost every disaccharide unit of the core polymer at the C-3 position of GlcA or at

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Abbreviations: Chase ABC, chondroitinase ABC; CS-C, chondroitin sulfate C; CS-E, chondroitin sulfate E; DS, dermatan sulfate; ΔDi-0S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyl uronic acid)-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-S_D, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔDi-S_E, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-4,6-bis-*O*-sulfo-D-galactose; ΔDi-TriS, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-4,6-bis-*O*-sulfo-D-galactose; ΔDi-diS, disulfated unsaturated disaccharide; ΔDi-monoS, monosulfated unsaturated disaccharide; ΔDi-zeroS, non-sulfated unsaturated disaccharide; ΔOligo, unsaturated oligosaccharide; Fuc, fucose; GAG, glycosaminoglycan; GalNAc, *N*-acetylgalactosamine; GlcA, glucuronic acid; HA, hyaluronic acid; HCII, heparin cofactor II; HPLC, high performance liquid chromatography; SC-GAG, sea cucumber GAG; t-PA, tissue-type plasminogen activator; u-PA, urinary-type plasminogen activator.

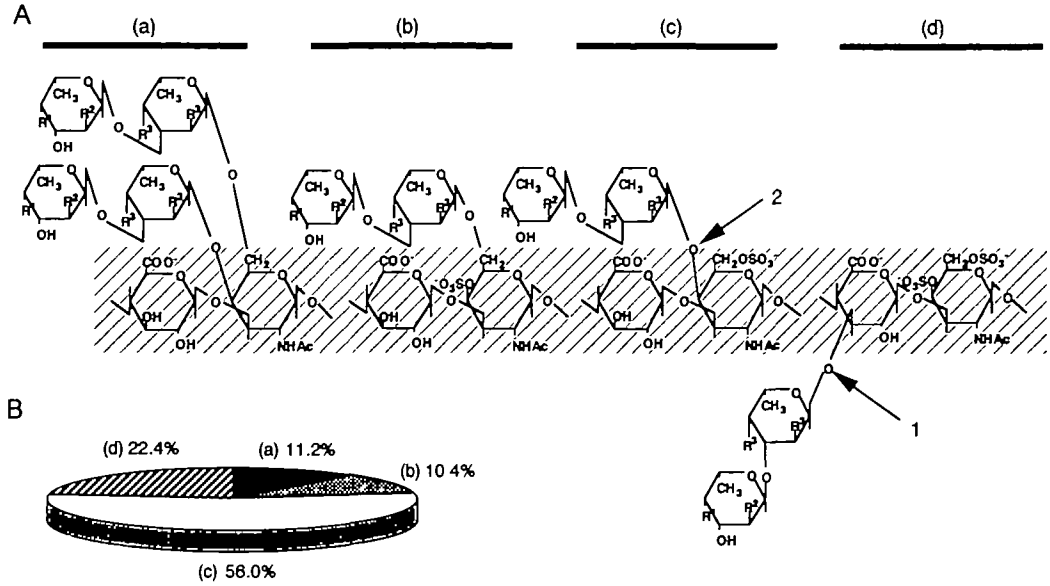

Fig. 1. **(A) Hypothetical structure of SC-GAG derived from previous studies (*14, 16*) and the present study.** Fucose branches are attached to a core chondroitin sulfate unit of the E-type (d) and to other core disaccharide units (a–c). The sequence of core disaccharide units is based on assumption. The linear backbone of core chondroitn sulfate E polymer represented by the shaded area is likely to be generated by mild acid hydrolysis. The glycosidic linkage indicated by arrow 1 should be cleaved rapidly, followed by slow cleavage at the site indicated by arrow 2 during mild acid hydrolysis. See "DISCUSSION" for details. Sulfate substitution patterns in fucose branches, whose possible localizations are represented by R^1, R^2, and R^3, are classified into six types as follows; [R^1, R^2, R^3] = [OSO_3^-, OSO_3^-, OH], [OSO_3^-, OH, OH], [OH, OSO_3^-, OH], [OSO_3^-, OSO_3^-, OSO_3^-], [OSO_3^-, OH, OSO_3^-], and [OH, OSO_3^-, OSO_3^-]. **(B) Percentage distribution of core chondroitin sulfate units comprising SC-GAG.** Core chondroitin sulfate units represented by (a–d) are those shown in (A).

the C-4 and/or C-6 position (s) of GalNAc (*14, 16*).

Since the SC-GAG from *S. japonicus* possesses such a unique structure, its physicochemical properties are also interesting. It has been suggested that SC-GAG is involved in the cation-dependent change in connective tissue toughness of the sea cucumber (*17, 18*). In this process, the viscosity alteration of SC-GAG by the valency and species of cation is quite different from those of common GAGs such as dermantan sulfate (DS) (*19, 20*). Ultracentrifugation analysis of SC-GAG also demonstrated a conformational change from an extended to a flexible form with increasing cation concentration, accompanied by aggregation of the SC-GAG molecule (*21*). Furthermore, SC-GAG is digested by neither chondroitinase ABC [EC 4.2.2.4] nor AC [EC 4.2.2.5] (*21*).

From a pharmaceutical point of view, SC-GAG has been examined for biological effects such as anticoagulant activity by the measurement of its interactions with heparin cofactor II (HCII), which binds to a specific structure of DS with high affinity (*22*). Mourão *et al.* (*23*) revealed that the SC-GAG from another sea cucumber, *L. grisea*, possesses anticoagulant activity as determined by measuring activated partial thromboplastin time using defucosylated, desulfated and carboxyl-reduced SC-GAG derivatives. They claimed that the activity is due to an interaction between SC-GAG and HCII or SC-GAG and antithrombin. However, they did not clarify the involvement of SC-GAG in fibrinolysis, which is another kind of antithrombotic system in animals. Very recently, we reported that squid CS-E markedly enhances plasminogen activation by tissue-type plasminogen activators (t-PAs) and urinary-type plasminogen activators (u-PAs) *in vitro* (*24*). Our SC-GAG, however, produced almost no enhancement of t-PA–mediated plasminogen activation, possibly because the presence of the sulfated fucose branch hindered the development of the activity (*24*). This explanation can also be applied to the indigestibility of intact SC-GAG by chondroitinases. After mild acid-hydrolysis of SC-GAG, the resultant partially hydrolyzed SC-GAG containing a reduced amount of sulfated fucose branches was substantially digested by the enzymes (*14*). Thus, it is possible that partially hydrolyzed SC-GAG also exhibits t-PA–mediated plasminogen activation due to the reduced content of fucose branches.

This situation prompted us to prepare partially defucosylated SC-GAG derivatives and to assess their capabilities to enhance t-PA–mediated plasminogen activation. Two kinds of partially defucosylated SC-GAG derivatives were prepared according to the preparative conditions for partially hydrolyzed SC-GAG (*14*). The physicochemical properties of the resultant two products were determined, and they were subjected to the t-PA–mediated plasminogen activation assay (*24, 25*). Analyses of the activities of partially defucosylated SC-GAGs in comparison with intact SC-GAG and squid CS-E revealed that an intermediately defucosylated SC-GAG derivative exhibits substantial activity (*ca.* 30% of that of squid CS-E at a concentration of 6.25 μg/ml), if the following two conditions are satisfied: (i) Defucosylation occurs preferentially at E-type disaccharide units within the SC-GAG derivatives; and (ii) the molecular mass of the derivative remains greater than 7.5 kDa irrespective of the presence of fucose branches.

MATERIALS AND METHODS

Materials—Live specimens of a sea cucumber *Stichopus japonicus* (about 400 g in body weight) were purchased at

the Tokyo Central Wholesale Market. The dorsal portion of the body wall was dissected, desalted under running tap water, and used in the following experiments.

Chondroitinase ABC [EC 4.2.2.4] from *Proteus vulgaris*, along with unsaturated disaccharides, ΔDi-0S, ΔDi-6S, ΔDi-4S, ΔDi-S_D, ΔDi-S_E, and ΔDi-triS, and authentic GAGs, chondroitin sulfate C (CS-C), chondroitin sulfate E (CS-E), and hyaluronic acid (HA) were obtained from Seikagaku Corporation (Tokyo). Heparin was purchased from Scientific Protein Laboratories (Waunakee, WI), and Actinase [EC 3.4.24.4] from Kakenseiyaku (Tokyo).

Glu-plasminogen, whose N-terminal amino acid is Glu (*26*), was purchased from Molecular Innovations Inc. (Southfield, MI). Single-chain tissue-type plasminogen activator (t-PA) was from American Diagnostica (Greenwich, CT), and chromogenic substrate S-2251 was a product of Chromogenix AB (Mölndal, Sweden).

Preparation of Sea Cucumber Glycosaminoglycan—Preparation of sea cucumber glycosaminoglycan (SC-GAG) was performed according to the method reported previously (*14*). Briefly, sea cucumber body wall (*ca.* 2 kg) was minced and homogenized. The homogenate was treated with chloroform/methanol (2:1, v/v) to remove lipids, then autoclaved at 120°C for 30 min. The resulting residue was digested with Actinase at 55°C for 8 h, followed by treatments with 0.4 M NaOH and 10% trichloroacetic acid. After dialysis, the solution was centrifuged and ice-cold ethanol in the presence of 2.5% sodium acetate was added to the resultant supernatant, resulting in precipitation. The pellet was recovered, dried under reduced pressure, and pulverized. The preparation (*ca.* 15 g) was used as the crude SC-GAG fraction.

A portion (500 mg) of the crude SC-GAG powder was dissolved in 5 ml of 50 mM ammonium bicarbonate (pH 8.0) and applied to a Sephadex G-100 column (ϕ3.4 × 100 cm) equilibrated with 50 mM ammonium bicarbonate. Elution was performed with the same solution, under monitoring for SC-GAG by the carbazole (*27*) and the anthrone methods (*28*) as described below. This gel-filtration chromatography was repeated twice, yielding 574 mg of SC-GAG from 1,000 mg of crude powder. A portion (500 mg) of the SC-GAG was further purified on a DEAE-cellulose column (ϕ1.8 × 18 cm). Elution was performed with a linear gradient of 200 ml each of 100 mM sodium acetate (pH 5.0) with and without 1.2 M NaCl. SC-GAG was monitored by the carbazole method (*27*) and the corresponding fractions were combined, desalted, and lyophilized. The yield of SC-GAG thus purified was approx. 210 mg.

Preparation of Partially Defucosylated SC-GAGs—We reported previously (*14*) that SC-GAG is converted to a partially hydrolyzed form containing reduced amounts of sulfate and fucose, and susceptible to enzymatic digestion with chondroitinases after hydrolysis with 0.05 M H_2SO_4 at 80°C for 6 h. A portion (40 mg) of purified SC-GAG was hydrolyzed under the same conditions as above, while another portion (40 mg) of purified SC-GAG was hydrolyzed with 0.05 M H_2SO_4 at 80°C for 3 h in order to produce another partially hydrolyzed form of SC-GAG. The reactions were terminated by cooling and neutralization with 0.1M NaOH, and the hydrolysates were evaporated to dryness. The resulting solids and purified SC-GAG (40 mg) as a control were dissolved separately in 5 ml aliquots of 0.2 M NaCl and applied successively to a Cellulofine GCL-90 column (ϕ3.4 × 100 cm) equilibrated with 0.2 M NaCl. Elutions were performed with 0.2 M NaCl at a flow rate of 60 ml/h, with monitoring for uronic acid and neutral sugars as described above. The elution profiles of purified, 3 h-hydrolyzed and 6 h-hydrolyzed SC-GAGs were compared in order to estimate the difference in the degree of fucose branch-removal. Fractions containing purified and partially hydrolyzed SC-GAGs (indicated by bold bars in Fig. 2) were applied to a Cellulofine GCL-25 column (ϕ2.0 × 25 cm) equilibrated with distilled water for desalting. Elutions were performed with distilled water, with monitoring for purified and partiallly hydrolyzed SC-GAGs by absorbance at 210 nm. Purified and partially hydrolyzed SC-GAGs thus desalted were dried under reduced pressure. The yields of purifed, 3 h-hydrolyzed (type A) and 6 h-hydrolyzed (type B) SC-GAGs were 34.7, 18.1, and 15.0 mg, respectively. The partially hydrolyzed SC-GAGs were used as partially defucosylated SC-GAGs in the following experiments.

Preparation of Low Molecular Mass CS-E Derivatives by Partial Digestion of Squid CS-E with Chondroitinase ABC—Two kinds of low molecular mass CS-E derivatives were prepared according to the previous method for the partial depolymerization of squid CS-E by chondroitinase ABC (Chase ABC)–digestion for 10 min or 1 h (*24*). Conditions for enzymatic digestion are described below. After terminating the digestion of 20 mg each of squid CS-E, each digest was applied to a Cellulofine GCL-90 column (ϕ3.4 × 100 cm) equilibrated with 0.2 M NaCl and eluted with the same solvent with monitoring for uronic acid. The fractions that eluted earlier than the unsaturated disaccharide fractions were collected, desalted and lyophilized, yielding 15.3 and 10.7 mg for 10 min- and 1 h-digested squid CS-E derivatives, respectively. The molecular masses of the resultant CS-E derivatives were measured together with their abilities to enhance t-PA–mediated plasminogen activation as described below.

Measurement of Molecular Masses of Partially Defucosylated SC-GAGs, of Squid CS-E and Its Low Molecular Mass Derivatives, and of CS-C—Five kinds of molecular mass markers (HA of 104 kDa, CS-Cs of 52.2, 39.1, and 8.05 kDa, and ΔDi-diS of 458 Da) were applied to a Tosoh CCPM HPLC equipped with a series of TSK-gel G-4,000, G-3,000, and G-2,500 PW_{XL} columns (ϕ7.5 × 300 mm each) for gel permeation chromatography using 0.2 M NaCl at 40°C and a flow rate of 0.6 ml/min, with monitoring by refractometry. The molecular masses of HA and CS-Cs were determined alternatively by light scattering (*29*). Peak-maximum retention times of GAG samples were measured and compared with the molecular mass standard curve in order to estimate their molecular masses.

Enzymatic Digestion and HPLC Analyses—Two hundred micrograms each of purified, 3 h-hydrolyzed or 6 h-hydrolyzed SC-GAG were digested with 0.5 U of Chase ABC in 30 μl of 130 mM Tris-HCl (pH 8.0) containing 130 mM sodium acetate and 0.03% bovine serum albumin at 37°C for 18 h. After the addition of 50 μl of distilled water, the mixture was heated at 100°C for 1 min to terminate the enzymatic reaction. A portion (15 μl) of the supernatant obtained by centrifugation at 3,000 ×*g* for 15 min was applied to a Tosoh CCPM HPLC equipped with the same three columns described above (ϕ7.5 × 300 mm each) and eluted with 0.2 M NaCl at 40°C and a flow rate of 0.6 ml/min, with

monitoring by refractometry. Peak areas were measured by a Shimadzu C-R4A integrator. The elution profiles of the digests of purified, 3 h-hydrolyzed and 6 h-hydrolyzed SC-GAGs were compared in order to estimate the difference in the degree of enzymatic digestion.

The digests obtained above were analyzed for unsaturated disaccharides by a Hitachi L-6,200 HPLC equipped with a YMC-PA120S5 column (ϕ4.0 × 250 mm), essentially according to a previously described method (*30*). HPLC was performed at 40°C using a linear gradient from 16 to 520 mM NaH_2PO_4 over 38 min at a flow rate of 1.0 ml/min, with monitoring by spectrophotometry at 230 nm. Peak areas were measured by a Tosoh multistation LC-8,020 integrator. The elution profiles of the digests of purified, 3 h-hydrolyzed and 6 h-hydrolyzed SC-GAGs were compared in order to estimate the difference in the percentage distributions of unsaturated disaccharides.

Cellulose-acetate Membrane Electrophoresis—Electrophoresis was performed on a Separax cellulose–acetate membrane (Fuji Film, Tokyo) in 0.1 M pyridine–formic acid (pH 3.3) at 0.6 mA/cm width for 40 min. After the run, the membrane was stained with 0.25% Alcian blue in 0.5% acetic acid, and destained with 0.5% acetic acid (*31, 32*).

Measurement of the Stimulatory Effects of SC-GAG Derivatives on the Activation of Plasminogen by t-PA and Calculation of Potentiation Factors—Assays were carried out essentially according to the previously described methods (*24, 25*), using a 96-well microtiter plate at 37°C in 50 mM Tris-HCl (pH 7.4) containing 0.05% Tween 80. Briefly, 0.2 μM Glu-plasminogen and 2.5 nM t-PA were incubated with varying amounts of SC-GAG derivatives and 0.6 mM of chromogenic substrate S-2251 (*H*-D-Val-Leu-Lys-*p*-nitroanilide). The 96-well microtiter plates were monitored every 15 s by absorbance at 405 nm with an automatic microplate reader. Progression curves were analyzed using the plots of absorbance at 405 nm *versus* $(\text{time})^2$. Initial velocity was determined from the slope of straight lines fitted to progression curves by the Microsoft Excel version 7.0 software. When the stimulation of SC-GAG derivatives was too high, the progression curves deviated appreciably from linearity. On such occasions, experimental data points more than 10% above the maximum level were omitted. The potentiation factor was calculated as the ratio of initial velocity in the presence of GAG to that in the absence of GAG (*33*). CS-E, low molecular mass CS-E derivatives and CS-C were used as controls.

Chemical Analyses—Sulfate: The sample was subjected to a 2 h-hydrolysis with 2 M HCl at 110°C, followed by removal of the HCl under reduced pressure at 50°C. To the residue was added 50 μl of distilled water, and an aliquot (10 μl) of the solution was applied to a TSK-gel IC-Anion PW column (ϕ4.6 × 50 mm). HPLC was performed isocratically on a Tosoh CCPM liquid chromatograph equipped with a CM-8,000 conductivity monitor, using TSK IC-A as the eluent at 40°C and a flow rate of 1.2 ml/min. Peak areas were measured by a Shimadzu C-R4A integrator.

Uronic acid: Uronic acid was determined by the carbazole method (*27*), using authentic D-glucuronolactone as the standard. Hexosamine: Hexosamine was determined essentially according to a previously described method (*34*), using authentic D-galactosamine as the standard. Neutral sugars: Neutral sugars were determined by the anthrone method (*28*), using authentic fucose as the standard.

RESULTS

Characterization of Two Types of Partially Defucosylated SC-GAG Derivatives—In the previous study (*14*), SC-GAG was treated by mild acid-hydrolysis at 80°C for 6 h, resulting in a partially hydrolyzed SC-GAG derivative (type B) susceptible to enzymatic digestion with chondroitinases. Thus, it appears that SC-GAG had lost a substantial number of sulfated fucose branches and was converted to partially hydrolyzed SC-GAG possessing a linear CS-E backbone bearing no sulfated fucose, together with a branched backbone still bearing sulfated fucose (Fig. 1A).

A sample of the purified SC-GAG was subjected to mild acid-hydrolysis at 80°C for 3 h to prepare a partially hydrolyzed SC-GAG (type A). The type A contains less linear CS-E backbone and more branched CS-E backbone, compared with type B (*14*). The time condition of 3 h was chosen from the results of a time-course study of mild acid hydrolysis as described previously (*14*).

Figure 2 shows the elution profiles of intact SC-GAG and types A and B on Cellulofine GCL-90 chromatography. Intact SC-GAG (Figure 2a) gave a single peak in a high mo-

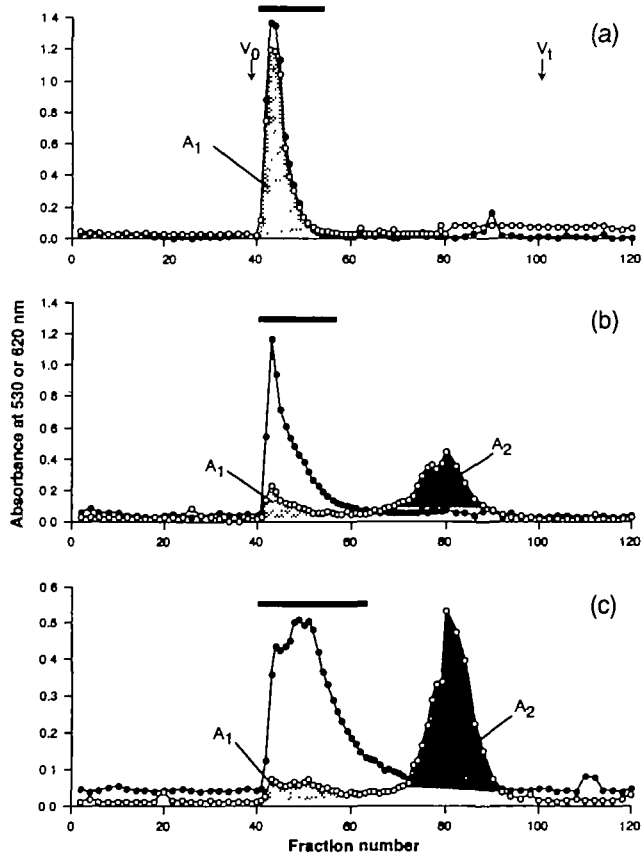

Fig. 2. **Elution profiles of intact SC-GAG (a) and 3 h- (b) and 6 h-hydrolyzates of SC-GAGs (c) from Cellulofine GCL-90 column chromatography.** Eluates were monitored for GlcA and fucose by the carbazole (●) and the anthrone methods (○), respectively. Spectrophotometries for the carbazole and the anthrone methods were performed at 530 and 620 nm, respectively. The fractions indicated by bold bars were combined and used as intact SC-GAG, type A and type B SC-GAG derivatives, respectively.

lecular mass region that was rich in both GlcA and fucose. Type A (Fig. 2b) showed two peaks, the first of which eluted in the high molecular mass region where intact SC-GAG was detected. The second peak eluted in a lower molecular mass region. The first peak was rich in GlcA but poor in fucose, whereas the second peak was rich in fucose. Type B (Fig. 2c) also contained two peaks, the first in the high molecular mass region where intact SC-GAG eluted, and the second in the lower molecular mass region where the second peak of the 3 h-hydrolyzate of SC-GAG eluted. The first peak was rich in GlcA but low in fucose, whereas the second peak was rich in fucose but low in GlcA. Furthermore, prolonged hydrolysis made the first peaks of the partially hydrolyzed SC-GAGs much broader. Overall, these results show that fucose branch–release from the core polymer is time-dependent. In order to clarify the number of fucose branches released from the core polymer, the degree of defucosylation for each of the three samples was calculated using the peak areas determined by the anthrone method. Peak areas of A_1 and A_2 (Fig. 2) were quantified with NIH Image 1.62 software. The percentages of the peak A_2 area to the sum of peak A_1 and A_2 areas are 67% for type A and 90% for type B. These values correspond to the degree of defucosylation of types A and B, as intact SC-GAG was taken as 0%.

As shown in Fig. 3, the molecular masses of intact SC-GAG, and types A and B are 21, 8, and 4 kDa, respectively, as determined by gel permeation HPLC . Using the same method, the molecular masses of squid CS-E, its low molecular mass derivatives prepared by Chase ABC-digestion for either 10 min or 1 h, and CS-C were measured as 127, 21, 7, and 45 kDa, respectively.

Figure 4 shows the cellulose-acetate membrane electrophoretic patterns of types A and B in comparison with those of intact SC-GAG and authentic GAGs. It is well known that electrophoretic mobility depends on the net of negative charge of sample molecules in the buffer system used. Although the mobility of intact SC-GAG was identical to that of heparin, the mobility of type B decreased almost as much as that of CS-C. The mobility of type A was intermediate between those of intact SC-GAG and type B. The degree of defucosylation and mobility were reversely correlated, confirming that the removal of sulfated fucose branches increases with reaction time of mild acid hydroly-

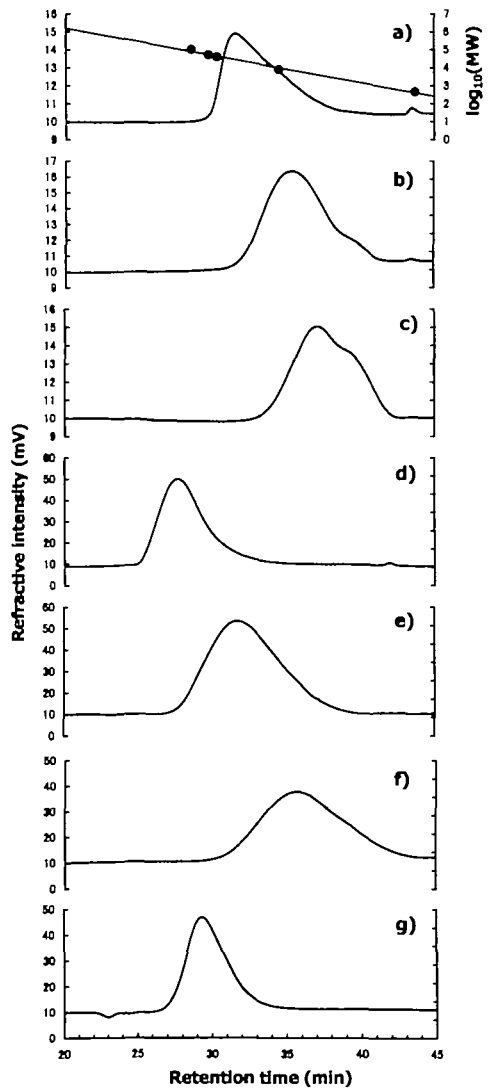

Fig. 3. **Gel permeation HPLC patterns of intact SC-GAG (a), type A (b) and type B SC-GAGs (c), squid CS-E (d) and its low molecular mass derivatives digested by Chase ABC for 10 min (e) and for 1 h (f), and CS-C (g).** Molecular mass standards used were HA (104 kDa), CS-Cs (52.2, 39.1, and 8.05 kDa), and ΔDi-diS (458 Da). Molecular masses of intact SC-GAG, type A and type B SC-GAG derivatives, CS-E and its low molecular mass derivatives and CS-C were calculated using their peak-maximum retention times together with the standard curve shown in (a).

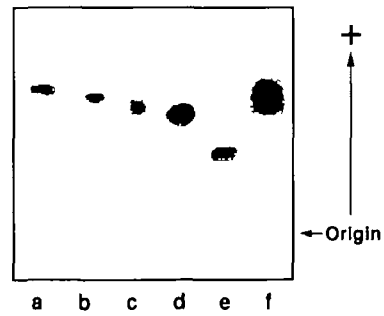

Fig. 4. **Cellulose-acetate membrane electrophoretic patterns of intact SC-GAG (a), type A (b) and type B SC-GAG derivatives (c). Authentic GAGs used were CS-C (d), HA (e) and heparin (f).** Electrophoresis was carried out in 0.1 M pyridine–formic acid (pH 3.3) at 0.6 mA/cm width for 40 min.

TABLE I. **Chemical compositions of partially defucosylated SC-GAGs in comparison with intact SC-GAG.**

Fraction	Sulfate	GalNAc	GlcA	Fucose
		(mmol/g)		
Intact SC-GAG	2.99	0.81	0.69	1.93
	(3.69)[a]	(1.00)	(0.84)	(2.38)
Type A[b]	2.53	1.49	1.31	0.87
	(1.70)	(1.00)	(0.88)	(0.58)
Type B[c]	1.44	1.37	1.07	0.49
	(1.06)	(1.00)	(0.78)	(0.36)

[a]Numbers in parentheses represent molar ratios relative to GalNAc. [b]Type A, partially defucosylated SC-GAG containing 33% fucose branches. [c]Type B, partially defucosylated SC-GAG containing 10% fucose branches.

sis of SC-GAG, as shown in Fig. 1A.

The chemical compositions of intact SC-GAG, and types A and B were determined (Table I). All samples contained almost equimolar amounts of GalNAc and GlcA, consistent with the CS-E backbone (*14*). As expected, sulfate and fucose contents were markedly lower in type B than in intact SC-GAG, while those in type A were intermediate. The chemical composition (Sulfate:GalNAc:GlcA:Fucose = 3:3:2:1) of type B is similar to that (3:2:2:1) of partially hydrolyzed SC-GAG reported previously (*14*). These results are consistent with the changes in the elution pattern from the Cellulofine GCL-90 column (Fig. 2).

The intact SC-GAG, and types A and B were analyzed by gel permeation HPLC after Chase ABC-digestion (Fig. 5). The digest of SC-GAG (Fig. 5a) gave a similar profile to that of intact SC-GAG (Fig. 3a), because of its low susceptibility to enzyme attack. On the other hand, types A and B were partially digested by Chase ABC (Fig. 5, b and c). The production of unsaturated disaccharides was determined as the ratio of the sum of the areas of unsaturated disaccharides to total peak area. The sum of the unsaturated disaccharide areas is the sum of areas of ΔDi-diS, ΔDi-monoS, and ΔDi-zeroS, while the total peak area is the sum of these areas plus that of ΔOligo (Fig. 5). Intact SC-GAG, and types A and B produced 0, 46, and 66% unsaturated disaccharides, respectively. Accordingly, the degrees of defucosyslation and the production of unsaturated disaccharides are very well correlated, *i.e.*, the presence of fucose branches hinders the Chase ABC-digestion of SC-GAG derivatives in a fucose content-dependent manner. The main unsaturated disaccharide component of the Chase ABC-digest of type A was ΔDi-diS, whereas that of type B was ΔDi-monoS. The ratios of the ΔDi-diS peak area to the total peak area were, however, almost identical irrespective of the degree of defucosylation, 28.8 and 29.7% for types A and B, respectively (See solid peaks in Fig. 5).

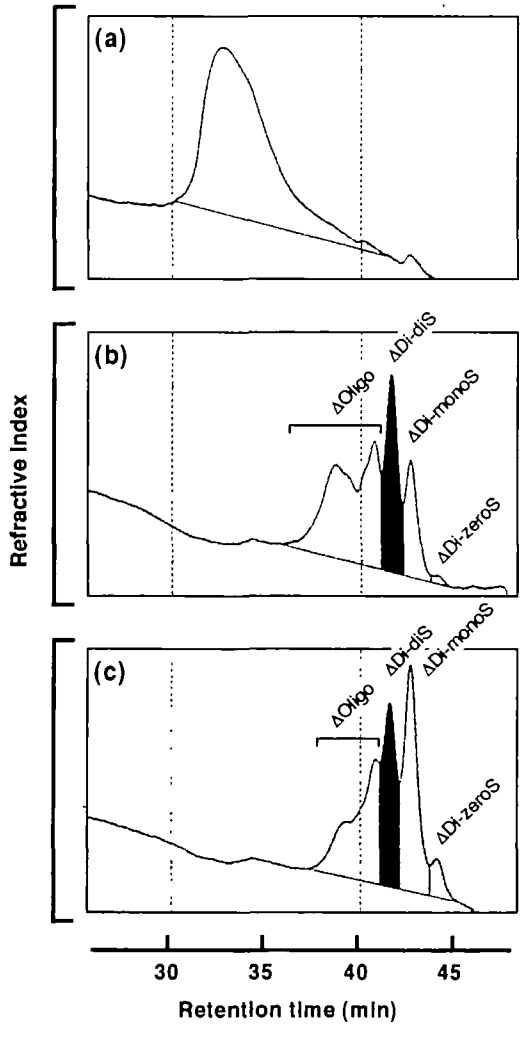

Fig. 5. **Gel permeation HPLC profiles of intact SC-GAG (a), type A (b), and type B SC-GAG derivatives (c) after chondroitinase ABC-digestion.** The solid peaks indicated by ΔDi-diS practically represent ΔDi-S_E.

Strong anion exchange HPLC of unsaturated disaccharides obtained by Chase ABC-treatment of types A and B detected four new peaks corresponding to unsaturated disaccharides, in addition to small amounts of unsaturated oligosaccharides (data not shown). Table II shows the percentage distributions of unsaturated disaccharides obtained from types A and B, squid CS-E, its low molecular mass derivatives, and CS-C. The unsaturated disaccharides from types A and B contained ΔDi-S_E and ΔDi-6S as the major components, whereas those from CS-E and its low molecular mass derivatives conatined ΔDi-S_E as a major component. On the other hand, unsaturated disaccharides from CS-C contained ΔDi-6S as a major component. Chase ABC-digests of types A and B and CS-E derivatives also contained small amounts of ΔDi-0S and ΔDi-4S, while ΔDi-S_D and ΔDi-triS were not detected.

Effects of Two Types of Partially Defucosylated SC-GAG Derivatives on Plasminogen Activation by t-PA—Figure 6a shows the effects of low molecular mass (21 and 7 kDa) CS-E derivatives on plasminogen activation by single-chain t-PA as compared to those of squid CS-E (127 kDa) and CS-C (45 kDa) as controls. Figure 6b shows the effects of intact SC-GAG (21 kDa), type A (8 kDa), and type B (4 kDa) on the plasminogen activation by single-chain t-PA. The profile for the enhancement by type A was bell-shaped as was that of squid CS-E. The maximum potentiation factor (49 points) of type A was almost 30% of that (162 points) of squid CS-E at 6.25 μg/ml concentration. Partially depolymerized CS-E (21 kDa) exhibited a similar enhancement

TABLE II. **Percentage distributions of unsaturated disaccharides generated from two kinds of partially defucosylated SC-GAGs with reference to those of squid CS-E and its low molecular mass derivatives in addition to that of CS-C.**

Fraction	ΔDi-					
	0S	6S	4S	S_D	S_E	triS
	(%)					
Type A (8 kDa)[a]	4.8	30.4	12.6	0.0	52.2	0.0
Type B (4 kDa)[b]	11.3	49.8	13.6	0.0	25.3	0.0
CS-E (127 kDa)[c]	6.0	9.7	19.8	0.0	64.5	0.0
CS-E (21 kDa)[d]	6.0	9.4	19.1	0.0	65.5	0.0
CS-E (7 kDa)[e]	4.7	9.2	18.2	0.0	67.9	0.0
CS-C (45 kDa)[f]	1.7	72.9	15.4	9.3	0.0	0.6

[a]Type A, partially defucosylated SC-GAG containing 33% fucose branches. [b]Type B, partially defucosylated SC-GAG containing 10% fucose branches. [c]CS-E (intact) prepared from squid cartilage. [d]CS-E partially depolymerized by Chase ABC-treatment for 10 min. [e]CS-E partially depolymerized by Chase ABC-treatment for 1 h. [f]CS-C (intact) prepared from shark cartilage.

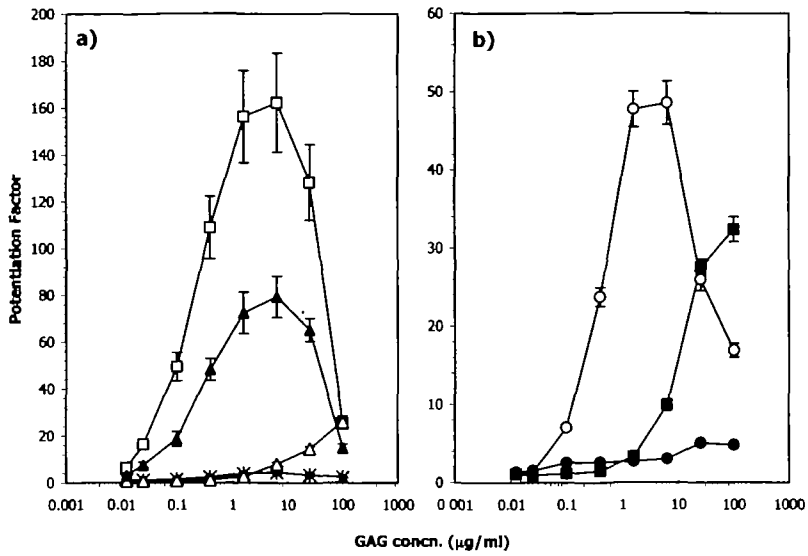

Fig. 6. **(a) Effects of various concentrations of squid CS-E (127 kDa (□)) and its low molecular mass derivatives (21 kDa (▲) and 7 kDa (△)), and CS-C (45 kDa (*)) on t-PA–mediated plasminogen activation. (b) Effects of various concentrations of intact SC-GAG (21 kDa (●)) and type A (8 kDa (○)) and type B SC-GAG derivatives (4 kDa (■)) on t-PA–mediated plasminogen activation. Data were expressed as the mean ± SE values.**

profile, and a maximum potentiation (79 points) at 6.25 μg/ml. On the other hand, type B exhibited a rather weak potentiation (10 points) at 6.25 μg/ml, which increased with concentration up to 100 μg/ml (32 points). Partially depolymerized CS-E (7 kDa) exhibited an enhancement profile similar to that of type B with a somewhat lower maximum potentiation (26 points) at 100 μg/ml. Intact SC-GAG exhibited very weak activities at every concentration examined (*24*).

DISCUSSION

For the present study, we prepared two types of SC-GAG derivatives, type A (3 h-hydrolyzed SC-GAG with 67% defucosylation and a molecular mass of 8 kDa) and type B (6 h-hydrolyzed SC-GAG with 90% defucosylation and a molecular mass of 4 kDa). These two types were further examined for their physicochemical properties by enzymatic analyses in combination with HPLC techniques.

From the elution profiles of Chase ABC-digests of types A and B on strong anion exchange HPLC, the percentage distributions of unsaturated disaccharides were calculated for both types (Table II). The unsaturated disaccharides from type A contained 52.2% ΔDi-S_E and 30.4% ΔDi-6S as the main components, whereas those from type B contained 25.3% ΔDi-S_E and 49.8% ΔDi-6S as the main components. It could be assumed that part of the ΔDi-S_E from type A would have been converted into a part of ΔDi-6S of type B due to the removal of the 4-*O*-sulfate group from ΔDi-S_E with the prolonged hydrolysis time. This assumption is, however, difficult to sustain, since the ratios of ΔDi-diS (*i.e.*, ΔDi-S_E) area to the total on gel permeation HPLC were almost identical (*ca.* 29%) regardless of type A or B (see solid peaks in Fig. 5). The productions of unsaturated disaccharides by types A and B were 46 and 66%, respectively. Accordingly, 52.4% of ΔDi-S_E in the digest of type A correspond to the 25.3% of ΔDi-S_E in the digest of type B. We reported previously (*16*) that SC-GAG comprises a CS-E-type core polymer with sulfated fucose branches attaching to almost every disaccharide core unit, and that the fucose branches are attached glycosidically to the units in four ways: (i) at the C-3 position of GlcA in GlcA β1→3GalNAc (4S, 6S) (22.4%), (ii) at the C-4 position of GalNAc in GlcA β1→3GalNAc (6S) (56.0%), (iii) at the C-6 position of GalNAc in GlcA β1→3 GalNAc (4S) (10.4%), and (iv) at the C-4 and C-6 positions of GalNAc in GlcA β1→3GalNAc (11.2%) (Fig. 1).

If fucose branches from the C-3 positions of GlcA in GlcA β1→3GalNAc (4S, 6S) (indicated by arrow 1 in Fig. 1A) are more susceptible to hydrolytic removal than those of fucose branches from the C-4 positions of GalNAc in GlcA β1→3GalNAc (6S) (indicated by arrow 2 in Fig. 1A), the early production of ΔDi-S_E and the hydrolysis time-dependent increase in ΔDi-6S content in the Chase ABC-digest, are both reasonably explained. Mourão *et al.* (*23*) reported that hydrolysis-resistant fucose branches of SC-GAG are localized preferentially at the non-reducing end. Thus, it is possible that type A possesses a linear backbone composed mainly of GlcA β1→3GalNAc(4S, 6S) (E-type disaccharide unit) without fucose branching at the reducing end (Fig. 1A) due to preferential release of fucose branches from the C-3 position of GlcA.

Our SC-GAG from *S. japonicus* possesses a CS-E-type core polymer and fucose branches composed of only Fuc α1→3Fuc (*16*), whereas Vieira *et al.* (*15*) reported that their SC-GAG from *L. grisea* possesses a CS-E-type core polymer and different fucose branches composed of Fuc α1→2Fuc and/or Fuc α1→4Fuc. Since there is a possibility that this difference in the fucose branch structures may differently affect t-PA–mediated plasminogen activation by SC-GAGs, such a comparative trial is of importance.

In order to elucidate the possible mechanism (s) underlying t-PA–mediated plasminogen activation, the potentiation activities of types A and B were compared. Type A exhibited the maximum potentiation (49 points) at 6.25 μg/ml, whereas type B exhibited maximum potentiation (32 points) at 100 μg/ml (Fig. 6b), indicating that type B possesses weaker activity than type A. On the other hand, intact SC-GAG exhibited almost no activity, suggesting the importance, for development of t-PA–mediated plasminogen activation, of the presence of E-type disaccharide units exposed on the surface of the GAG molecule by the removal

of fucose branches.

As shown in Fig. 6, squid CS-E (127 kDa), a low molecular mass CS-E derivative (21 kDa) and type A (8 kDa) all exhibited maximum potentiation factors at 6.25 μg/ml, whereas both type B (4 kDa) and another low molecular mass CS-E derivative (7 kDa) exhibited maximum potentiation factors at 100 μg/ml. In terms of the GAG concentration at which maximum potentiation developed, the concentration was either 6.25 or 100 μg/ml in the tested range. At the former concentration, the higher the molecular mass of the active GAG, the higher the maximum potentiation factor. These results suggest that a threshold of molecular mass exists between 7 and 8 kDa (presumably 7.5 kDa), determining whether the maximum t-PA–enhancing activities are achieved at 6.25 or 100 μg/ml by those GAGs, including both linear and branched CS-E derivatives.

Kinoshita *et al.* (*35, 36*) showed that squid CS-E has a small percentage of GlcA residues additionally sulfated at the C-3 positions. Since there is a possibility that these sulfate groups are involved in the development of t-PA–mediated plasminogen activation by CS-E derivatives, their influence should be evaluated.

In conclusion, we propose that the following two conditions be fulfilled simultaneously for the development of potent t-PA–enhancing activity by CS-E derivatives. (i) CS-E derivatives without fucosylation are preferable. In the case of SC-GAG, fucose branches should be removed preferentially from E-type disaccharide units. (ii) The molecular mass of the CS-E derivative should remain above the threshold value (presumably 7.5 kDa), regardless of the presence of fucose branching.

The structure–activity relationship of partially defucosylated SC-GAGs (*e.g.*, types A and B) and low molecular mass CS-E derivatives described in this paper provide a clue to the design of suitable CS-E derivatives for the production of antithrombotic drugs based on t-PA–mediated plasminogen activation.

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REFERENCES

1. Kawai, Y., Seno, N., and Anno, K. (1966) Chondroitin polysulfate of squid cartilage. *J. Biochem.* **60**, 317–321
2. Suzuki, S., Saito, H., Yamagata, T., Anno, K., Seno, N., Kawai, Y., and Furuhashi, T. (1968) Formation of three types of disulfated disaccharides from chondroitin sulfates by chondroitinase digestion. *J. Biol. Chem.* **243**, 1543–1550
3. Habuchi, O., Sugiura, K., and Kawai, N. (1977) Glucose branches in chondroitin sulfate from squid cartilage. *J. Biol. Chem.* **252**, 4570–4576
4. Razin, E., Stevens, R.L., Akiyama, F., Schmid, K., and Austen, K.F. (1982) Culture from mouse bone marrow of a subclass of mast cells possessing a distinct chondroitinsulfate proteoglycan with glycosaminoglycans rich in *N*-acetylgalactosamine-4,6-disulfate. *J. Biol. Chem.* **257**, 7229–7236
5. Razin, E., Ihle, J.N., Seldin, D., Mencia-Huerta, J.M., Katz, H.R., LeBlanc, P.A., Hein, A.,Caulfield, J.P., Austen, K.F., and Stevens, R.L. (1984) Interleukin 3: A differentiation and growth factor for the mouse mast cell that contains chondroitin sulfate E proteoglycan. *J. Immunol.* **132**, 1479–1486
6. Eliakim, R., Gilead, L., Ligumsky, M., Oken, E., Rachmilewitz, D., and Razin, E. (1986) Histamine and chondroitin sulfate E proteoglycan released by cultured human colonic mucosa: indication for possible presence of E mast cell. *Proc. Natl. Acad. Sci. USA* **83**, 461–464
7. Stevens, R.L., Fox, C.C., Lichtenstein, L.M., and Austen, K.F. (1988) Identification of chondroitin sulfate E proteoglycans and heparin proteoglycans in the secretory granules of human lung mast cells. *Proc. Natl. Acad. Sci. USA* **85**, 2284–2287
8. Uhlin-Hansen, L. and Kolset, S.O. (1988) Cell density-dependent expression of chondroitin sulfate proteoglycan in cultured human monocytes. *J. Biol. Chem.* **263**, 2526–2531
9. Uhlin-Hansen, L., Eskeland, T., and Kolset, S.O. (1989) Modulation of the expression of chondroitin sulfate proteoglycan in stimulated human monocytes. *J. Biol. Chem.* **264**, 14916–14922
10. Kobayashi, S., Oguri, K., Yaoita, E., Kobayashi, K., and Okayama, M. (1985) Highly sulfated proteochondroitin sulfates synthesized *in vitro* by rat glomeruli. *Biochim. Biophys. Acta* **841**, 71–80
11. Yaoita, E., Oguri, K., Okayama, E., Kawasaki, S., Kihara, I., and Okayama, M. (1990) Isolation and characterization of proteoglycans synthesized by cultured mesangial cells. *J. Biol. Chem.* **265**, 522–531
12. Plaas, A.H., Wong-Palms, S., Roughley, P.J., Midura, R.J., and Hascall, V.C. (1997) Chemical and immunological assay of the nonreducing terminal residues of chondroitin sulfate from human aggrecan. *J. Biol. Chem.* **272**, 20603–20610
13. Ohtake, S., Ito, Y., Fukuta, M., and Habuchi, O. (2001) Human *N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase cDNA is related to human B cell RAG-associated gene. *J. Biol. Chem.* **276**, 43894–43900
14. Kariya, Y., Watabe, S., Hashimoto, K., and Yoshida, K. (1990) Occurrence of chondroitin sulfate E in glycosaminoglycan isolated from the body wall of sea cucumber *Stichopus japonicus*. *J. Biol. Chem.* **265**, 5081–5085
15. Vieira, R.P., Mulloy, B., and Mourão, P.A.S. (1991) Structure of a fucose-branched chondroitin sulfate from sea cucumber. Evidence for the presence of 3-*O*-sulfo-beta-D-glucuronyl residues. *J. Biol. Chem.* **266**, 13530–13536
16. Kariya, Y., Watabe, S., Kyogashima, M., Ishihara, M., and Ishii, T. (1997) Structure of fucose branches in the glycosaminoglycan from the body wall of the sea cucumber *Stichopus japonicus*. *Carbohydr. Res.* **297**, 273–279
17. Kariya, Y., Watabe, S., Ochiai, Y., Srikantha, S., Hashimoto, K., and Murata, K. (1986) Changes in toughness and ultrastructure of the body wall of sea cucumber *Stichopus japonicus* in association with glycosaminoglycan. *Connect. Tissue (Tokyo)* **18**, 312–313
18. Kariya, Y., Watabe, S., Ochiai, Y., Murata, K., and Hashimoto, K. (1990) Glycosaminoglycan involved in the cation-induced change of body wall structure of sea cucumber *Stichopus japonicus*. *Connect. Tissue Res.* **25**, 149–159
19. Mathews, M.B. and Dorfman, A. (1953) The molecular weight and viscosity of chondroitinsulfuric acid. *Arch. Biochem. Biophys.* **42**, 41–53
20. Mathews, M.B. (1953) Chondroitinsulfuric acid-a linear polyelectrolyte. *Arch. Biochem. Biophys.* **43**, 181–193
21. Kariya, Y., Watabe, S., Ochiai, Y., and Hashimoto, K. (1990) Glycosaminoglycan from the body wall of the sea cucumber *Stichopus japonicus*. *Comp. Biochem. Physiol.* **95B**, 387–392
22. Maimone, M.M. and Tollefsen, D.M. (1990) Structure of a dermatan sulfate hexasaccharide that binds to heparin cofactor II with high affinity. *J. Biol. Chem.* **265**, 18263–18271
23. Mourão, P.A.S., Pereira, M.S., Pavao, M.S.G., Mulloy, B., Tollefsen, D.M., Monwinckel, M.C., and Abildgaard, U. (1996) Structure and anticoagulant activity of a fucosylated chondroitin sulfate from echinoderm. *J. Biol. Chem.* **271**, 23973–23984
24. Sakai, T., Kyogashima, M., Kariya, Y., Urano, T., Takada, Y., and Takada, A. (2000) Importance of GlcUA β1-3GalNAc(4S, 6S) in chondroitin sulfate E for t-PA- and u-PA-mediated Glu-plasminogen activation. *Thromb. Res.* **100**, 557–565
25. Kyogashima, M., Onaya, J., Miyauchi, S., Arai, M., Shibata, Y., Suda, A., Sakai, T., Takada, Y., and Takada, A. (1999) Antithrombin activity of avian crown dermatan sulfate. *Thromb. Res.* **96**, 459–465

26. Bachmann, F. (1994) Molecular aspects of plasminogen, plasminogen activators and plasmin in *Haematostasis and Thrombosis* (Bloom, A.L., Forbes, C.D., Thomas, D.P., and Tuddenham, E.G.D., eds.) Vol. 1, pp. 575–613, Churchill Livingstone, New York
27. Bitter, T. and Muir, H. (1962) A modified uronic acid reaction. *Anal. Biochem.* **4**, 330–334
28. Tsiganos, C.P. and Muir, H. (1966) Estimation of pentoses and methylpentoses in biopolymers, in paticular fucose and xylose. *Anal. Biochem.* **17**, 495–501
29. Arai, M., Yada, T., Suzuki, S., and Kimata, K. (1992) Isolation and characterization of type IX collagen-proteoglycan from the Swarm rat chondrosarcoma. *Biochim. Biophys. Acta* **1117**, 60–70
30. Yoshida, K., Miyauchi, S., Kikuchi, H., Tawada, A., and Tokuyasu, K. (1989) Analysis of unsaturated disaccharides from glycosaminoglycuronan by high-performance liquid chromatography. *Anal. Biochem.* **177**, 327–332
31. Näutö, V. (1963) On the electrophoretic separation of acid mucopolysaccharides on cellulose acetate sheets. *Acta Chem. Scand.* **17**, 857
32. Seno, N., Meyer, K., Anderson, B., and Hoffman, P. (1965) Variations in keratosulfate. *J. Biol. Chem.* **240**, 1005–1010
33. Takada, Y., Urano, T., and Takada, A. (1994) Effects of heparan sulfate analogue or other sulfated polysaccharides on the activation of plasminogen by t-PA or u-PA. *Thromb. Res.* **73**, 301–311
34. Hurst, R. and Settine, J.M. (1981) An accurate colorimetric method for measurement of sulfamino hexose in heparins and heparn sulfates. *Anal. Biochem.* **115**, 88–92
35. Kinoshita, A., Yamada, S., Haslam, S.M., Morris, H.R., Dell, A., and Sugahara, K. (1997) Novel tetrasaccharides isolated from squid cartilage chondroitin sulfate E contain unusual sulfated disaccharide units GlcA(3-*O*-sulfate)beta1-3GalNAc(6-*O*-sulfate) or GlcA(3-*O*-sulfate)beta1-3GalNAc. *J. Biol. Chem.* **272**, 19656–19665
36. Kinoshita, A., Yamada, S., Haslam, S.M., Morris, H.R., Dell, A., and Sugahara, K. (2001) Isolation and structural determination of novel sulfated hexasaccharides from squid cartilage chondroitin sulfate E that exhibits neuroregulatory activities. *Biochemistry* **40**, 12654–12665