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Solvolytic depolymerization of chondroitin and dermatan sulfates

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ABSTRACT

It is essential to establish a library of glycosaminoglycan oligosaccharides from the chondroitin and dermatan sulfates to investigate their biological functions and structure–activity relationships (SARs). There are several approaches to obtain oligosaccharides using chemical and enzymatic degradation procedures; however, purification of each resulting oligosaccharide is complicated because of the diversity of sulfonation patterns present in these oligosaccharides. We have developed a new method for the solvolytic degradation for chondroitin and dermatan sulfates to obtain an oligosaccharide mixture that can be easily purified into chondro/dermato oligosaccharides for characterization by both ^{1}H NMR and MALDI-TOFMS. These oligosaccharides have a methyl-esterified uronate residue and a methyl 2-acetamido-2-deoxy-D-galactofuranoside at the nonreducing and reducing ends, respectively. All other internal repeating disaccharide units were desulfonated, but maintained their core carbohydrate structures.

1. Introduction

Chondroitin sulfate and dermatan sulfate comprise a family of galactosaminoglycans with biological and therapeutic importance and have been the subject of numerous studies.[1] There has been increased interest in chondroitin and dermatan sulfates as regulators of neurite outgrowth.[2] In our laboratory, we have been particularly interested in designing therapeutic agents for controlling inflammation, allergy, and angiogenesis.[3–5] It is necessary to establish structure–activity relationships (SARs) to study the biology and pharmacology of these polysaccharides. Because both chondroitin and dermatan sulfates are high-molecular-weight polymers (MW 20–100 kDa[6]), it is essential to partially depolymerize these molecules into oligosaccharides to establish their precise SARs.

A number of approaches have been reported for the preparation of chondroitin and dermatan sulfate-derived oligosaccharides. The mildest approach, and that typically preferred by our laboratory, is controlled enzymatic depolymerization.[7] Endolytic chondroitin/dermatan lyases ACI, B and ABC, as well as testicular hyaluronan hydrolase, have been used to prepare pure oligosaccharides ranging in size from disaccharide (degree of polymerization (dp) 2) to dodecasaccharide (dp 12).[8,9] This method requires substantial purification because structural (sequence) variability gives rise to multiple oligosaccharides of each size. Moreover, the nonreducing end of oligosaccharides prepared using lyases comprised unsaturated uronate residues that block chain extension. The removal of this unnatural nonreducing terminus requires treatment with toxic mercuric acetate.[10] Alternative chemical methods also have major limitations. Oxidative depolymerization relying on Cu^{2+}/O_2 (or

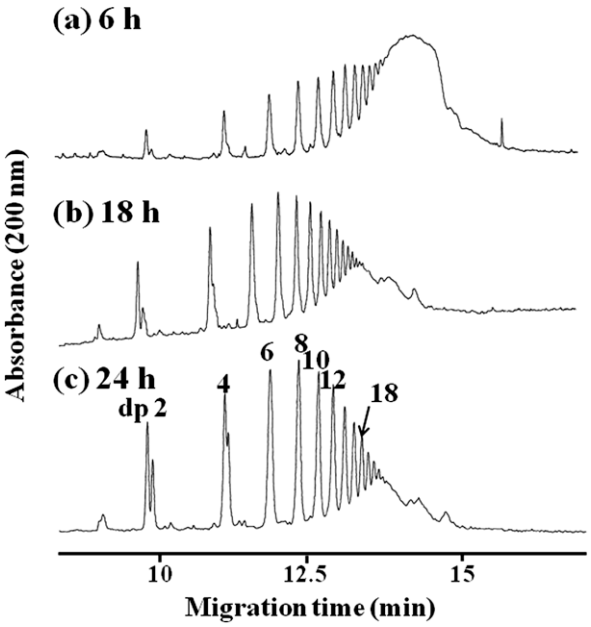

Figure 1. Capillary electropherograms of the products obtained from chondroitin sulfate by solvolysis for 6, 24 and 48 h. *Y*-axis, 0–0.001 full scale.

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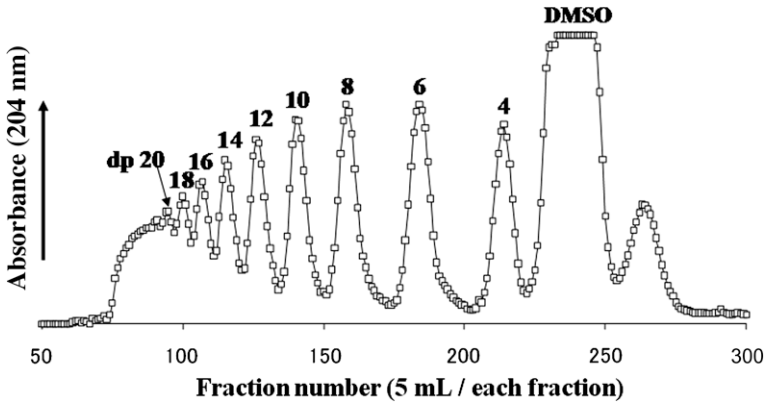

Figure 2. Elution profile on a Bio-Gel P-10 column of the products obtained from chondroitin sulfate by solvolysis. Column, Bio-Gel P-10 fine (4.4 cm I.D. × 1 m); eluent, 0.2 M NaCl; temperature, ambient; flow rate, Darcy's Law; sample loaded, 100 mg. *Y*-axis, 0.0–2.0 full scale.

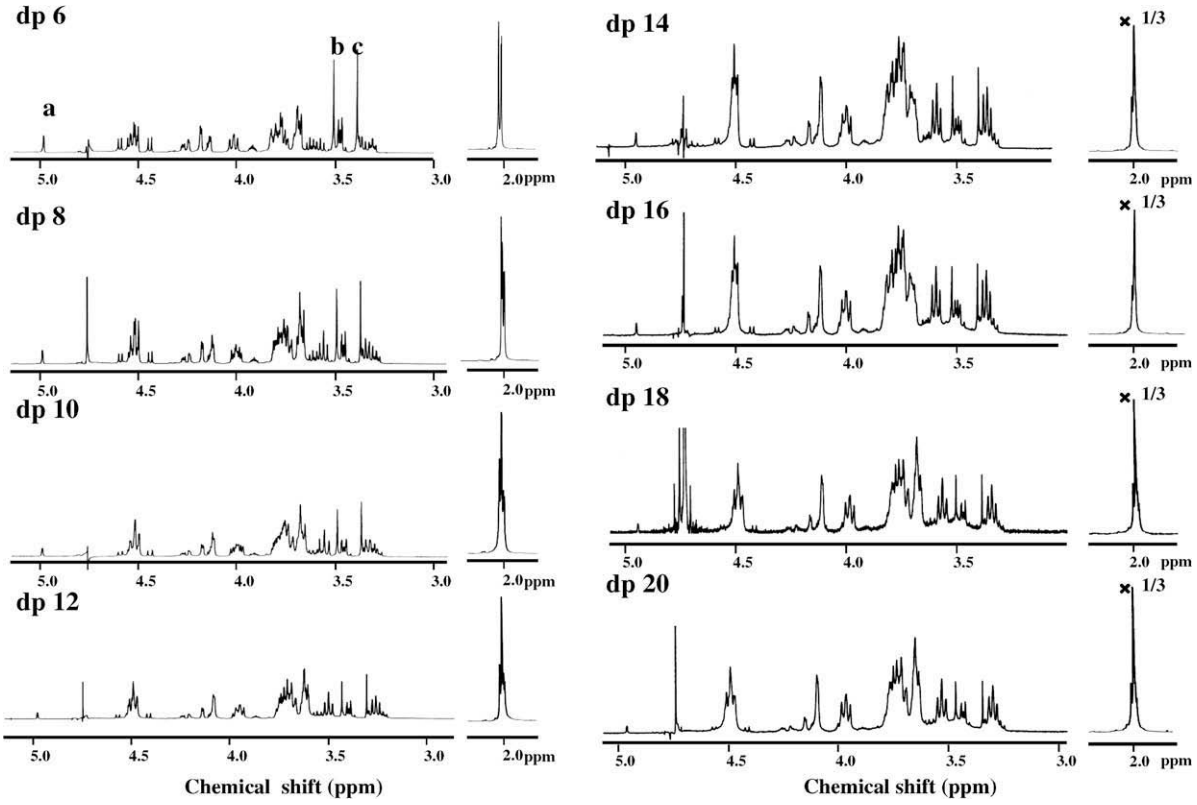

Figure 3. ^{1}H NMR spectra of the purified oligosaccharides obtained from chondroitin sulfate by solvolysis. Signals indicated a, b and c in the spectrum of fraction dp 6 are H-1 of methyl 2-acetamido-2-deoxy-D-galactofuranoside, $-COOCH_3$ of glucuronate at the reducing end, and $-OCH_3$ of methyl 2-acetamido-2-deoxy-D-galactofuranoside.

H_2O_2) or Fenton's reagent has been used to prepare low-molecular-weight glycosaminoglycans, but these have relatively large molecular weights (MWavg 5–8 kDa) making their separation and characterization quite difficult.[11] Acidic depolymerization (in HCl–CH_3OH) results in both depolymerization and desulfonation, but this reaction is quite difficult to control.[12]

In this paper, we have found a new method for the solvolytic depolymerization of chondroitin and dermatan sulfates, and have demonstrated the hyaluronidase inhibitory activity of the per-*O*-sulfonated derivatives of these oligosaccharides.

2. Results and discussion

We observed some surprising results when trying to prepare chondroitin through the solvolysis of the pyridinium salt of chondroitin sulfate. Using conditions identical to those successful for desulfonating heparin[2] without depolymerization (9:1 DMSO–H_2O, 90 °C), an unexpectedly complex mixture of products arose from chondroitin sulfate, suggesting that both desulfonation and depolymerization had taken place. We had previously also observed that milder solvolytic conditions could control heparin desulfonation.[13] Thus, we next attempted to solvolytically desulfonate chondroitin sulfate in 9:1 DMSO–MeOH at 80 °C, but again observed both depolymerization and desulfonation; however, this time the product mixture was much less complex based on analysis by capillary electrophoresis (Fig. 1).

This reaction was scaled up to prepare 100 mg of products, and the resulting mixture was fractionated on the basis of size using a Bio-Gel P-10 column (Fig. 2).

^{1}H NMR analysis of oligosaccharides (dp 4–20) showed that each fraction was remarkably pure having a single major component (Fig. 3). Each spectrum in Figure 3 shows signals expected for a chondroitin oligosaccharide as well as shows three unusual signals at 3.41, 3.50, and 5.02 ppm corresponding to $-OCH_3$ of methyl 2-acetamido-2-deoxy-D-galactofuranoside, $-COOCH_3$ of glucuronate at the nonreducing end and H-1 of methyl 2-acetamido-2-deoxy-D-galactofuranoside, respectively. The structure of the tetrasaccharide (dp 4) product was next examined by double quantum filtered-correlation spectroscopy (DQF-COSY) to assign the 1D spectra and to establish oligosaccharide structure (Fig. 4).

The structure of the tetrasaccharide showed two unusual features, the carbonyl group of the nonreducing end of glucuronate residue was methyl esterified, and the reducing-end *N*-acetyl galactosamine residue was the methyl furanosyl glycoside. These unusual features can be easily explained by the solvolytic mechanism shown in Figure 5.

Mass spectrometry showed a characteristic ion, corresponding to $[M-CH_3]^-$, for each oligosaccharide with a mass 15 *m/z* lower than that expected for each chondroitin oligosaccharide of dp 4–20 (data not shown). A similar phenomenon was also observed in the MALDI-TOF mass spectra of methylesterified hyaluronan oligosaccharides.[14] These results strongly suggest that each oligosaccharide has a methyl-esterified uronate residue and a methyl 2-acetamido-2-deoxy-D-galactofuranoside at the nonreducing and reducing ends, respectively. All other internal repeating disaccharide units were desulfonated and maintained their core carbohydrate structures.

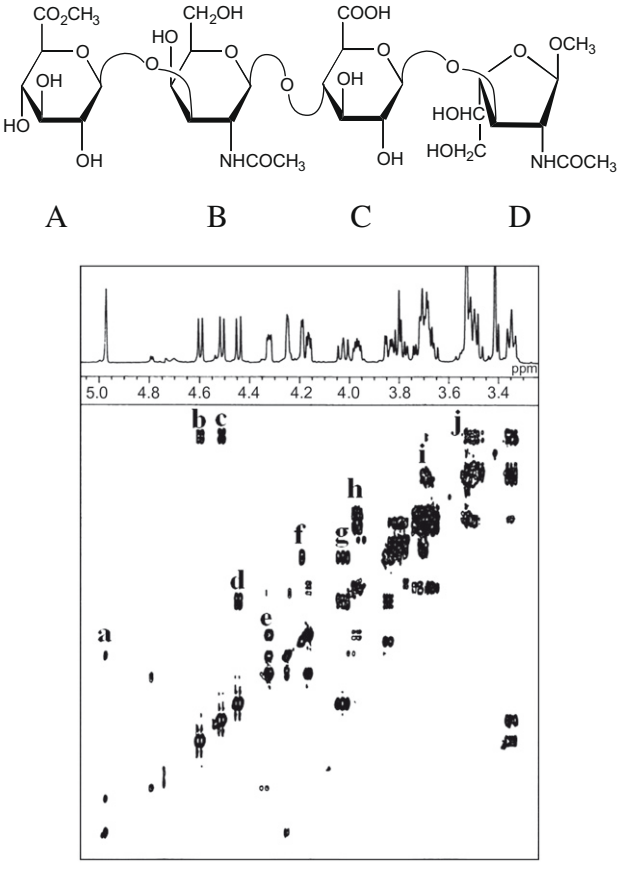

Figure 4. DQDCOSY spectrum of a purified tetrasaccharide obtained from chondroitin sulfate by solvolysis. *Cross peaks*: a, H-1/H-2 of D; b and c, H-1/H-2 of A and C; d, H-1/H-2 of B; e, H-3/H-4 of D; f, H-3/H-4 of B; g, H-2/H-3 of B; h, H-5/H-6 of D; i, H-3/H-4 of A and C; j, H-2/H-3 of A and C.

The scope of this solvolysis reaction was next examined. Dermatan sulfate gave the same type of products with iduronic acid in place of glucuronic acid throughout the oligosaccharide structure. In addition, the mixture of desulfonated dermatan oligosaccharides obtained was similarly easily purified (Fig. 6).

We next undertook their per-O-sulfonation of these oligosaccharides to confirm the utility of these oligosaccharides for the preparation of experimental therapeutic agents. The chondroitin oligosaccharides (dp 4–20) were per-O-sulfonated using pyridine–sulfur trioxide in *N*,*N*-dimethylformamide (DMF). ^{1}H NMR analysis shows that all the hydroxyl groups, with the exception of the anomeric position, were converted to *O*-sulfo groups, and the ester in the nonreducing-end uronate was hydrolyzed. (Fig. 7A). Gradient polyacrylamide gel electrophoresis (PAGE) analysis of these oligosaccharides demonstrates both their size purity and their uniform level of sulfonation (Fig. 7B).

The anti-factor IIa and hyaluronidase inhibitory activities of per-O-sulfonated chondroitin had been previously explored by our laboratory.[15,16] These per-O-sulfonated chondroitin oligosaccharides were compared to per-O-sulfonated chondroitin polymer, which has been detected as a contaminant in low-molecular-weight heparin[17] and has been shown to give rise to its serious toxicity.[18] These products were also compared to those of hyaluronan derivatives (Tables 1 and 2). Both the anti-factor IIa and hyaluronidase inhibitory activities increased as a function of oligosaccharide size. Interestingly, the hyaluronidase inhibitory activity of per-O-sulfonated oligosaccharide samples prepared from both chondroitin sulfate and hyaluronan were substantial, while the anti-factor IIa activity of these oligosaccharides was small. These observations might be explained by the mechanisms through which these per-O-sulfonated oligosaccharides exhibit each activity.[19]

In conclusion, a new reaction is described that results in the solvolytic depolymerization and desulfonation of chondroitin and dermatan sulfates. This method forms a simple distribution of oligosaccharides that can be easily purified and used to generate potential therapeutic agents.

3. Experimental

3.1. Materials

Chondroitin sulfate (CS) from bovine tracheal cartilage and dermatan sulfate (DS) from porcine skin were purchased from Shin-Nippon Yakugyo Co. (Tokyo, Japan) and Seikagaku Kogyo Co. (Tokyo, Japan), respectively. Acetonitrile was purchased from E. Merck (Darmstadt, Germany) and the other reagents were of the analytical grades available.

3.2. NMR spectroscopy[13]

^{1}H NMR spectroscopy was performed using the conditions described previously by Toida et al.[16] Three milligrams of each standard and each purified CS sample were treated repeatedly with 0.5 mL of ^2H_2O, followed by desiccation over P_2O_5 in vacuo to exchange the labile proton with deuterons. Then the thoroughly dried standards and samples were redissolved in 0.5 mL of ^2H_2O and transferred to an NMR tube (5.0 mm o.d. × 25 cm, PP-528; Willmad Glass Co., Buena, NJ, USA). All spectra were obtained by a JNM-ECP600 spectrometer (JEOL, Tokyo, Japan). The operating conditions for the one-dimensional (1D) spectra were as follows: frequency, 600 MHz; sweep width, 8 kHz; flip angle, 90 (10.8 μs); sampling points, 32,000; accumulation, 500 times; temperature, 333 K.

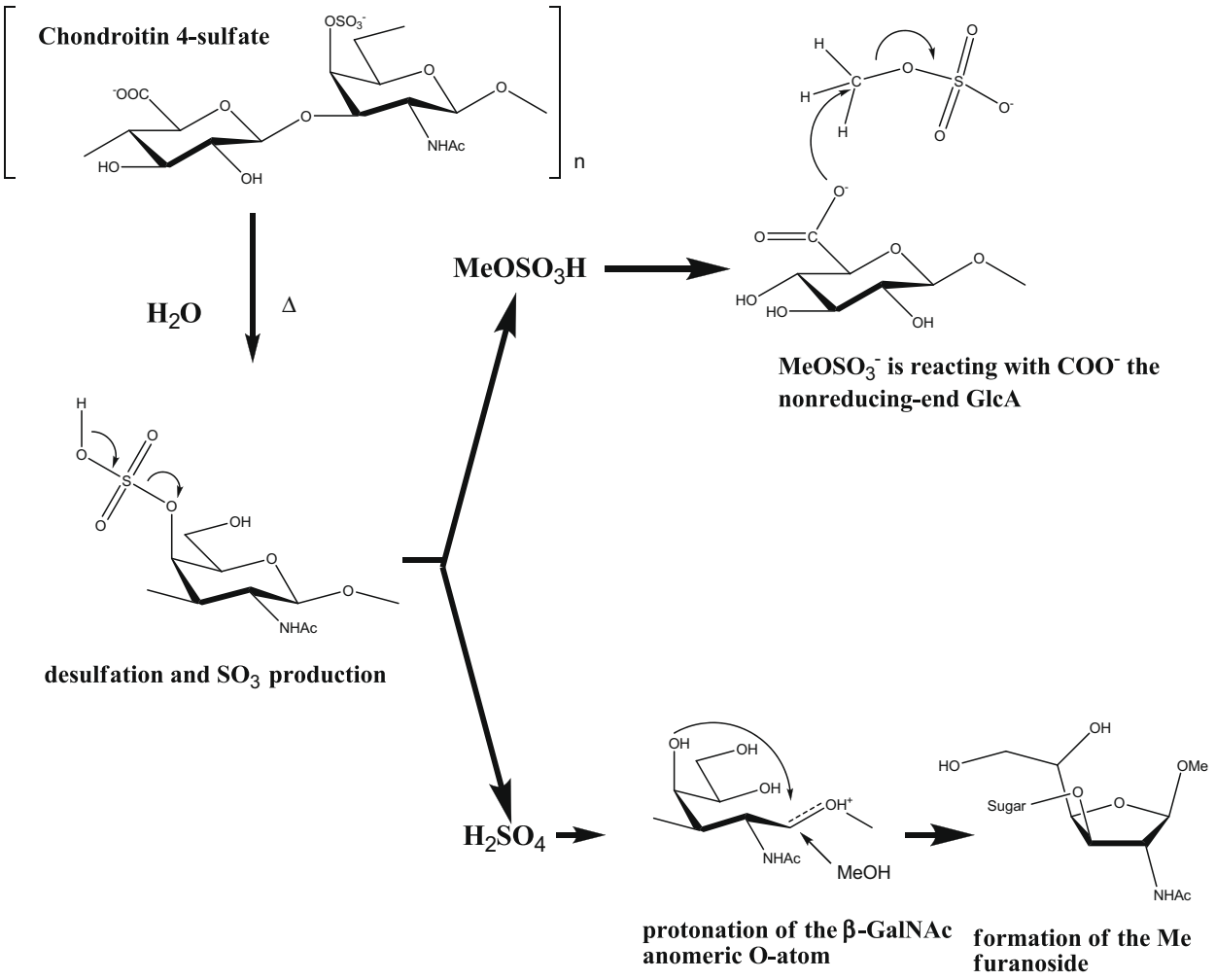

Figure 5. Depolymerization mechanism for the solvolysis of chondroitin sulfate.

3.3. Capillary electrophoresis (CE)[14]

CE was performed using a Beckman P/ACE5010 system with advanced computer interface, equipped with high-voltage power supply capable of constant or gradient voltage control using a fused-silica capillary from GL Science (Tokyo). The compositional analysis of oligosaccharide mixtures obtained from CS and DS were confirmed by CE in the normal polarity mode using a mixture of 40 mM disodium phosphate/40 mM sodium dodecylsulfate/10 mM tetraborate adjusted to pH 9.0 with 1.0 M HCl as described previously. The fused-silica capillary (75 μm I.D. × 375 μm O.D., 67 cm long) was automatically washed before use with 0.1 M NaOH, followed by nitrogen gas pressure injection (5 s) at a constant current 15 kV. The samples (1 mg mL^{-1}) were dissolved in water and loaded (7 nL) with nitrogen gas pressure injection.

3.4. MALDI-TOFMS[14]

MALDI-TOF mass spectra were collected as follows: Mass analysis was carried out in negative/positive linear and reflectron mode using an Axima™ (Shimadzu Kratos Inc., Kyoto, Japan) equipped with a 337 nm nitrogen laser. The acceleration voltage was set to 19 kV and the delay time was 450 ns. A total of 200 mass spectra were acquired and summed for each sample spot. All data were collected by searching an adequate spot on the target sample plate manually using the 'Raster mode'. Mass calibrations were performed over several *m*/*z* ranges, using commercially available protein and peptide standards. For the sample preparation, several matrices were tested and optimized (see Section 2). Briefly, 1 mg of sample was mixed with 100 μL of solvent mixture (1:2 acetonitrile–0.1% trifluoroacetic acid). One μL of sample solution was mixed with 10 μL of a 10 mg mL^{-1} solution of CHCA (α-cyano-4-hydroxycinnamic acid) in TA buffer (30% acetonitrile containing 0.1% trifluoroacetic acid). This preparation (0.2 μL) was placed onto a MALDI sample plate, and spectra were collected by raster irradiation on the sample surface. The results shown in the text were obtained by using an HA oligosaccharide sample prepared under the conditions described above.

3.5. Gradient PAGE[20]

Gradient PAGE was used to monitor the preparation and purification of per-O-sulfonated oligosaccharides of dp 10–20, as well as to check depolymerization of the oligosaccharides from CS by the O-sulfonation reaction. Polyacrylamide linear gradient resolving gels (14 × 28 cm, 10–20% acrylamide gel) were purchased and run as previously described.[19] O-Sulfonated oligosaccharides obtained from CS were visualized by Alcian Blue staining.

3.6. Small-scale depolymerization of CS

A solution containing 10 mg of CS in 1.0 mL of DMSO containing 10% MeOH was prepared to optimize the partial solvolytic depoly-

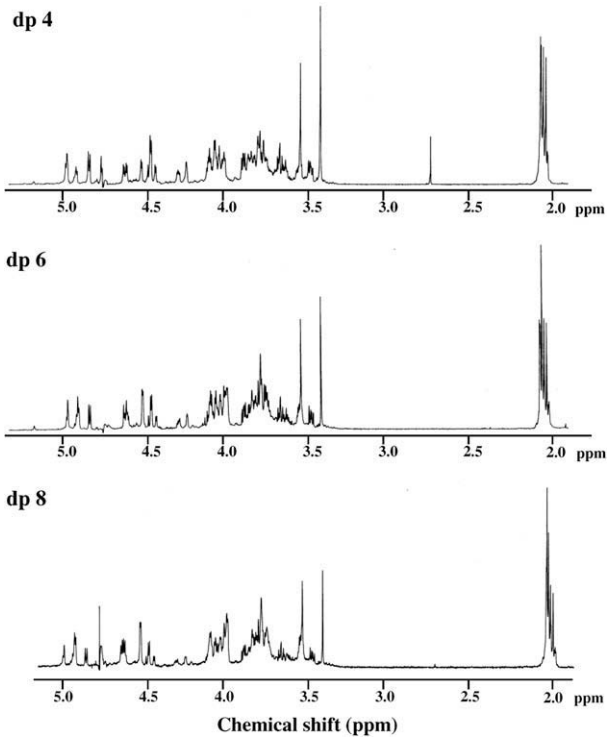

Figure 6. ^{1}H NMR spectra of the purified oligosaccharides obtained from dermatan sulfate by solvolysis.

Table 1
Anti-IIa activity of per-O-sulfonated chondroitin and hyaluronan

O-Sulfonated sample	Anti-IIa activity (units/mg)						
	dp 10	dp 12	dp 14	dp 16	dp 18	dp 20	Polymer
CS	1.00	1.22	1.64	1.88	2.22	2.60	41.4
HA	0.91	1.39	1.82	2.20	2.41	2.52	64.7

CS, chondroitin sulfate; HA, hyaluronan. Intact CS or HA do not show any activity.

Table 2
Hyaluronidase inhibitory activity of per-O-sulfonated chondroitin and hyaluronan

O-Sulfonated sample	IC_{50} (μg/mL) Inhibitation at 25 μg/mL (%)						
	dp 10	dp 12	dp 14	dp 16	dp 18	dp 20	Polymer
CS	n.d. 38.6	23.2 51.5	9.00 69.9	6.23 78.3	3.91 87.9	1.74 95.2	1.36 100
HA	2.15 75.1	1.48 81.2	1.72 87.7	0.80 100	0.71 100	0.67 100	0.56 100

n.d.; not detected. CS, chondroitin sulfate; HA, hyaluronan.

merization. The solvolytic depolymerization was carried out in a screw-capped micro tube at 80 °C. At various times intervals, a 10 μL aliquot was removed and applied to the CE by monitoring at 200 nm. From these analyses, an optimal reaction time of preparation of dp 4 to dp 20 of CS oligosaccharides was 6 h. DS polymer was also depolymerized into similar sizes.

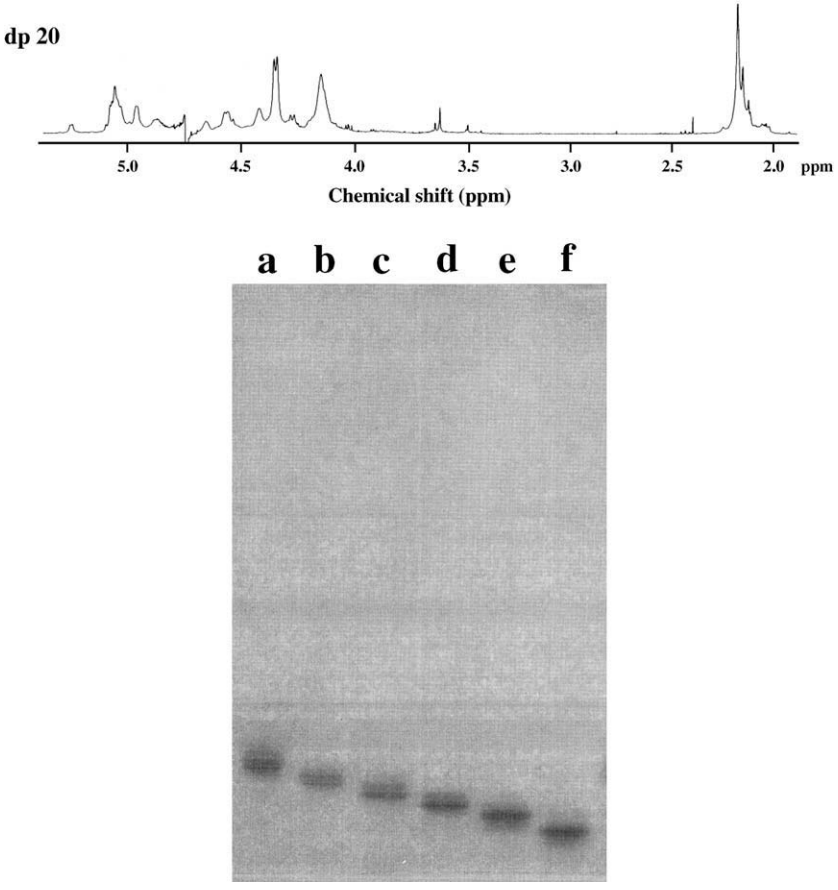

Figure 7. Gradient PAGE profile of per-O-sulfonated chondroitin oligosaccharides PAGE. *Lanes*; a, per-O-sulfonated dp 20; b, per-O-sulfonated dp 18; c, per-O-sulfonated dp 16; d, O-sulfonated dp 14; e, per-O-sulfonated dp 12; f, per-O-sulfonated dp 10. Stained by Alcian Blue.

3.7. Solvolytic depolymerization of CS and DS

The large-scale, partial depolymerization of CS or DS by solvolysis was carried out on 1.0 g of CS or DS. Briefly, a suspension containing 1.0 g of CS in 200 mL of 1:9 MeOH–DMSO was heated at 80 °C for 6 h. The sample was diluted to 500 mL by water and dialyzed for several days at 4 °C in 500 MWCO dialysis tubing against deionized and distilled water, and then the sample was freeze-dried. The resulting white powder was dissolved in 20 mL water and directly applied onto LPGC.

3.8. LPLC separation[20]

The dp 4–20 oligosaccharides, obtained from chondroitin sulfate or dermatan sulfate, were fractionated on a Bio-Gel P-10 column (4.4 cm × 1 m) eluted with 0.2 M NaCl at an optimum flow rate (0.8 mL/min) as defined by the Darcy's Law. A freeze-dried chondroitin sulfate or dermatan sulfate oligosaccharide mixture sample (100 mg) was dissolved in 20 mL of water and applied to the column, 300 fractions were collected (5 mL/tube each) and the absorbance at 200 nm of each fraction was measured. Each oligosaccharide fraction was collected and concentrated by evaporation. If necessary, this chromatographic separation was performed repeatedly. Each size-uniform oligosaccharide fraction obtained from LPLC was twice desalted by a Hi-Trap desalting column eluted with water at 1.0 mL/min, and then the fractions were freeze dried.

3.9. Preparation of chemically per-O-sulfonated dp 4–20 dp CS oligosaccharides

Preparation of per-O-sulfonated oligosaccharides from CS was based on the method described previously.[15,16] This method was slightly improved for CS oligosaccharides in this paper. To obtain the tributylamine (TBA) salt of the oligosaccharides, 50 μL of TBA was added to each size-uniformed CS oligosaccharide sample (1.0 mg) in 1.0 mL of distilled water adjusted to pH 2.8 with 0.1 M HCl. The mixture was mixed vigorously and freeze dried. The resulting salt was dissolved 0.2 mL of *N*,*N*-dimethylformamide (DMF) to which a required excess (15 mol/equiv of available hydroxyl group in CS oligosaccharides) of pyridine–sulfur trioxide complex was added. After 3 h at 40 °C, the reaction was interrupted by addition of 0.5 mL of water, and the raw product was precipitated with 4 vol. of cold EtOH saturated with anhydrous NaOAc, and then collected by centrifugation at 3000 *g* for 15 min. The resulting per-O-sulfonated CS oligosaccharide was dissolved in water. Five hundred microliters of solution was centrifuged, the supernatant was concentrated, and reagents were removed by using a MW 3000 cut-off filter device (Microcon YM-3) repeatedly until reagents were not detectable for per-O-sulfonated CS oligosaccharides of dp 10–20. The concentrated supernatant was removed and freeze dried. The small per-O-sulfonated CS oligosaccharides of dp 4–8 were prepared by an EtOH precipitation procedure, in which the final EtOH concentration was 80–90%; however, the recovery of the products was significantly lower (40–50%).

3.10. Assay for anti-factor IIa activity[15]

Normal human plasma (NHP) was collected from healthy volunteers for the assay. Anti-factor IIa activity was determined by incubating 50 μL of O-sulfonated CS oligosaccharides, 30 μL of NHP and 20 μL of human thrombin (1.2 NIH units/mL) obtained from Boehringer Mannheim (Stuttgart, Germany) in 850 μL of Tris-buffer (50 mM Tris, pH 8.3, 227 mM sodium chloride) at 25 °C for 3 min. Then 50 μL (1.9 μmol/mL) of chromozyme TH (tosyl-glycyl-prolyl-arginine-4-nitranilide acetate) from Boehringer Mannheim, was added, and the amidolytic thrombin activity was measured at 405 nm under 25 °C.

3.11. Assay for hyaluronidase inhibition by FIA[16]

Hyaluronidase (hyaluroglucosaminidase from bovine testes, Sigma–Aldrich Japan, Tokyo) inhibition by per-O-sulfonated oligosaccharides was determined by the FIA method described previously.[16] Briefly, 20 μL of per-O-sulfonated oligosaccharides solution, 10 μL of hyaluronan standard solution (as a substrate, 400 μg/mL) and 60 μL of 0.2 M NaOAc buffer (pH 5.0) containing 2.5 mM $CaCl_2$ were mixed. Ten microliters of enzyme solution (0.25 units/μL) was added to this mixture. The mixture solution was incubated at 37 °C for 2 h. The enzyme action was terminated after a fixed time by plunging the samples into a boiling water bath for 5 min. And then, 2 μL of sample solution was applied to the FIA system. The concentrations of per-O-sulfonated oligosaccharides and per-O-sulfonated intact chondroitin and hyaluronan were performed in triplicate.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2009.02.010.

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