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Preparation of chondroitin sulfate libraries containing disulfated disaccharide units and inhibition of thrombin by these chondroitin sulfates

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Abstract Chondroitin sulfate (CS) containing GlcA-GalNAc(4,6-SO_4) (E unit) and CS containing GlcA ($2SO_4$)-GalNAc($6SO_4$) (D unit) have been implicated in various physiological functions. However, it has been poorly understood how the structure and contents of disulfated disaccharide units in CS contribute to these functions. We prepared CS libraries containing E unit or D unit in various proportions by *in vitro* enzymatic reactions using recombinant GalNAc 4-sulfate 6-*O*-sulfotransferase and uronosyl 2-*O*-sulfotransferase, and examined their inhibitory activity toward thrombin. The *in vitro* sulfated CSs containing disulfated disaccharide units showed concentration-dependent direct inhibition of thrombin when the proportion of E unit or D unit in the CSs was above 15–17%. The CSs containing both E unit and D unit exhibited higher inhibitory activity toward thrombin than the CSs containing either E unit or D unit alone, if the proportion of the total disulfated disaccharide units of these CSs was comparable. The thrombin-catalyzed degradation of fibrinogen, a physiological substrate for thrombin, was also inhibited by the CS containing both E unit and D unit. These observations indicate that the enzymatically prepared CS libraries containing various amounts of disulfated disaccharide units appear to be useful for elucidating the physiological function of disulfated disaccharide units in CS.

Keywords Chondroitin sulfate · Disulfated disaccharide · Sulfotransferase · Inhibition of thrombin

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Introduction

Chondroitin sulfate (CS) chains are mainly composed of repeating disaccharide unit, GlcAβ1-3GalNAc β1-4, with sulfate groups at position 4 or position 6 of the GalNAc residues. In addition to these monosulfated disaccharide units, CS chains contain disulfated disaccharide units. In vertebral CSs, mainly two kinds of disulfated disaccharide units are present; GlcA-GalNAc(4,6-SO_4) (E unit) and GlcA($2SO_4$)-GalNAc($6SO_4$) (D unit). CSs containing E unit (CS-E) was first found in squid cartilage [1, 2], and have been identified in bone marrow-derived mast cells and mucosal mast cells [3]. CS-E has been implicated in various physiological processes such as promotion of neurite outgrowth [4, 5], binding of chemokines [6, 7], midkine [8] and various heparin binding growth factors [9, 10], infection of herpes symplex virus [11, 12], metastasis of cancer cells [13, 14], migration of neuronal precursors during cortical development [15] and activation of matrilysin [16]. On the other hand, CSs containing D unit (CS-D) were reported to be involved in outgrowth of neurite [17] and binding of pleiotrophin [18], and migration of neuronal precursors during cortical development [15]. Most of the studies on the function of CS-E have been carried out using CS-E obtained from squid cartilage. Proportion of E unit in

commercial CS-E prepared from the squid cartilage was more than 60%; however, the proportion of E-unit in the vertebral CSs rarely exceed 40%. In addition, CS-E from the squid cartilage contains unique structures such as glucose branches on GalNAc residues [19], and 3-*O*-sulfated GlcA residues [20]. To estimate function of E-unit correctly, it is necessary to use CSs which contain E unit in a similar proportion to those of the vertebral CSs, and free from the unique structures.

These disulfated disaccharide units are synthesized by specific sulfotransferases. *N*-Acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase (GalNAc4S-6ST) transfers sulfate to position 6 of GalNAc($4SO_4$) residues in CS-A and generates E unit [21–23]. We have shown previously that, when CS-A was sulfated by GalNAc4S-6ST purified from squid cartilage in the presence of 2 mM PAPS, about half of GlcA-GalNAc($4SO_4$) could be converted to E unit [24]. We also found that a recombinant squid GalNAc4S-6ST expressed in COS-7 cells could sulfate not only CS-A but also CS-C and CS-D and generated E-D hybrid tetrasaccharide structure from CS-C and CS-D. On the other hand, uronosyl 2-*O*-sulfotransferase (2OST) transferred sulfate to position 2 of GlcA residues adjacent to GalNAc($6SO_4$) in CS-A or CS-C to yield D unit [25, 26].

In this report, we prepared recombinant squid GalNAc4S-6ST and human 2OST by a baculovirus expression system, and synthesized libraries of CSs containing various proportion of E unit or D-unit from vertebral CS-A or CS-D by the *in vitro* enzymatic reactions using these recombinant enzymes.

To estimate biological activity of the disulfated disaccharide units, we determined inhibitory effects of the CSs containing various amounts of the disulfated disaccharide unis toward thrombin. We found that a critical proportion of the disulfated disaccharide units in CSs is required for direct inhibition of thrombin by CSs.

Materials and methods

Materials

The following commercial materials were used: $H_2{}^{35}SO_4$ was from Perkin-Elmer Life Science; chondroitinase ABC, CS-A (whale cartilage), CS-D (shark cartilage), heparan sulfate (bovine liver), 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-0S), 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose (ΔDi-6S), 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose (ΔDi-4S), 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose (ΔDi-diS_D), 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4,6-bis-*O*-sulfo-D-galactose (ΔDi-diS_E), 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose (ΔDi-diS_B), and 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-4,6-bis-*O*-sulfo-D-galactose (ΔDi-triS) were from Seikagaku Corporation, Tokyo; Senshu Pak Docosil SP100 was from Senshu Scientific, Tokyo; Fast Desalting Column HR 10/10, and heparin-Sepharose CL-6B were from Amersham Bioscience; 3′, 5′-ADP-agarose, heparin sodium salt, bovine thrombin, bovine antithrombin, bovine plasma factor Xa, chromogenic substrate F3301 (CH_3OCO-D-CHA-Gly-Arg-pNA-AcOH) and bovine fibrinogen were from Sigma; DNA polymerase KOD FX was from TOYOBO, Osaka; chromogenic substrate L-1150 (Bz-Phe-Val-Arg-pNA) was from BACHEM; unlabeled 3′-phosphoadenosine 5′-phosphosulfate (PAPS) was from Yamasa, Choshi; Fresh squids (*Ommastrephes sloani pacificus*) were locally prepared from Choshi Gyokyo.

Squid cartilage cDNA was prepared as described [23]. [^{35}S]PAPS was prepared as described [27]. CS-E (squid cartilage), which was eluted with 1.5 M NaCl from DEAE-Sephadex A-50, was prepared as described [19].

Construction of recombinant Bacmid DNA containing squid GalNAc4S-6ST or human 2OST

A DNA fragment that codes for the full open reading frame of squid GalNAc4S-6ST was amplified by PCR. The first PCR was carried out using oligonucleotides sG46ST-F1 (GGTATGCTCAACAGTAAAAAACTTGTCCTACTCTG CATTCTCAC) and sG46ST-R1 (CCGTTCAGTT CAAAGGTTGAACCAATCAAAGTCAGGTTTATTG), which were synthesized according to the sequence of the GalNAc4S-6ST cDNA clone (GenBank accession number AB292855), as primers and the squid cartilage cDNA as a template. The second PCR was carried out using oligonucleotides sG46ST-F2 (TTGGATCCATGCTCAACAG TAAAAAACTTG) and sG46ST-R2 (TTGAATTCT CAAAGGTTGAACCAATCAAAGTCAGG) as primers and the first PCR mixture as the template. At the 5′-ends of oligonucleotides sG46ST-F2 and sG46ST-R2, restriction enzyme recognition sites were introduced; a *Bam*HI site for sG46ST-F2 and a *Eco*RI site for sG46ST-R2. The amplified cDNA was digested with *Bam*HI and *Eco*RI, and subcloned into these sites of pFastBacI plasmid (Invitrogen). The cDNA thus obtained was named GalNAc4S-6STpFastBacI. A DNA fragment that codes for the full open reading frame of 2OST was amplified by PCR. The first PCR was carried out using oligonucleotides hU2ST-F1 (AGCGGAGCAGGC GATGAAGAAGAAGCAGCA) and hU2ST-R1 (AACCCGCAAAACCGGGCATCTCCCAATGAC), which were synthesized according to the sequence of the 2OST

cDNA clone (GenBank accession number AB020316), as primers and the Human kidney cDNA (Clontech) as a template. The second PCR was carried out using oligonucleotides hU2ST-F2 (TTGAATTCATGAAGAAGAAG CAGCAGCATC) and hU2ST-R2 (AACTGCAGT CACCTCTTATAAATATCTTCCAGCCAC) as primers and the first PCR mixture as the template. At the 5′-ends of oligonucleotides hU2ST-F2 and hU2ST-R2, restriction enzyme recognition sites were introduced; a *Eco*RI site for hU2ST-F2 and a *Pst*I site for hU2ST-R2. The amplified cDNA was digested with *Eco*RI and *Pst*I, and subcloned into these sites of pFastBacI plasmid. The cDNA thus obtained was named 2OSTpFastBacI. The recombinant bacmid DNAs containing GalNAc4S-6ST or 2OST were produced by Tn7-mediated site-specific transposition when MAX EFFICIENCY DH10BAC competent cells (Invitrogen) were transformed with GalNAc4S-6STpFastBacI or 2OSTpFastBacI according to the manufacturer's instructions. The recombinant bacmid DNAs thus obtained were named GalNAc4S-6STbacmid or 2OSTbacmid.

Expression and purification of sulfotransferases

GalNAc4S-6STbacmid and 2OSTbacmid were expressed using Bac-to-Bac baculovirus expression system (Invitrogen) according to the manufacturer's instructions. Sulfotransferases produced in the infected Sf9 cells were extracted with 1.5 ml of 10 mM Tris-HCl, pH 7.2, 0.5% Triton X-100, 0.15 M NaCl, 10 mM $MgCl_2$, 2 mM $CaCl_2$, and 20% glycerol for 1 h with gentle stirring at 4°C. The extracts were centrifuged at 10,000 rpm for 30 min. The sulfotransferase activities in the supernatant fractions (crude extracts) were determined as described below. The crude sulfotransferases were purified by affinity chromatography using Heparin Sepharose CL-6B and 3′, 5′-ADP agarose [21]. For purification of the recombinant GalNAc4S-6ST, 1.5 ml of the crude extract was applied to a column of Heparin Sepharose CL-6B (0.5 ml) equilibrated with 0.15 M NaCl in buffer A (10 mM Tris-HCl, pH 7.2, 0.1% Triton X-100, 20 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM 2-mercaptoethanol). The column was washed with 5 ml of buffer A containing 0.3 M NaCl and was eluted with 2.5 ml of buffer A containing 1.0 M NaCl. To the fraction eluted by buffer A containing 1.0 M NaCl, an equal volume of buffer A was added, and the combined solution was applied to a column of 3′, 5′-ADP agarose (0.5 ml) equilibrated with buffer A containing 0.15 M NaCl. The column was washed with 5 ml of buffer A containing 1 M NaCl and then eluted with 2.0 ml of buffer A containing 5 M NaCl. The fractions eluted by buffer A containing 5 M NaCl was concentrated by ultrafiltration. The concentration of NaCl of the concentrated solution was decreased to 0.15 M by adding appropriate volume of buffer A. For purification of the recombinant 2OST, 1.5 ml of the crude extract was diluted by 3 ml of buffer A and applied to a column of Heparin Sepharose CL-6B (0.5 ml) equilibrated with 0.05 M NaCl in buffer A. The column was washed with 5 ml of buffer A containing 0.2 M NaCl and was eluted with 2 ml of buffer A containing 0.4 M NaCl. To the fractions eluted by buffer A containing 0.4 M NaCl, 2 ml of buffer B (10 mM Tris-HCl, pH 7.2, 0.1% Triton X-100, 10 mM 2-mercaptoethanol) was added and the combined solution was applied to a column of 3′, 5′-ADP agarose (0.5 ml) equilibrated with buffer B containing 0.15 M NaCl. The column was washed with 5 ml of buffer B containing 0.2 M NaCl and then eluted with 2.5 ml of buffer B containing 0.6 M NaCl. The fraction eluted by buffer B containing 0.6 M NaCl was concentrated by ultrafiltration. The concentration of NaCl of the concentrated solution was decreased to 0.15 M by adding appropriate volume of buffer B. The recombinant enzyme solutions thus obtained were stored at −80°C in the presence of 20% glycerol.

Assay of sulfotransferase activity

GalNAc4S-6ST activity was assayed by the method described previously [21]. The standard reaction mixture contained, in a final volume of 50 µl, 2.5 µmol of imidazole-HCl, pH 6.8, 1 µmol of $CaCl_2$, 1 µmol of reduced glutathione, 25 nmol (as galactosamine) of CS-A, 50 pmol of [^{35}S]PAPS (about 5.0×10^5 cpm), and enzyme. The enzymatic reaction was carried out at 20°C for 90 min. 2OST activity was assayed by the method described previously [25]. The standard reaction mixture contained, in a final volume of 50 µl, 2.5 µmol of imidazole-HCl, pH 6.8, 2.6 µg of protamine chloride, 25 nmol (as galactosamine) of CS-A, 50 pmol of [^{35}S]PAPS (about 5.0×10^5 cpm), and enzyme. The reaction was carried out at 37°C for 30 min. Both sulfotransferase reactions were stopped by immersing the reaction tubes in a boiling water bath for 1 min. After the reaction was stopped, ^{35}S-labeled glycosaminoglycans were isolated by precipitation with ethanol followed by gel chromatography with a Fast Desalting Column as described previously [28], and the radioactivity was determined. One unit of enzyme activity is defined as the amount required to catalyze the transfer of 1 pmol of sulfate per min.

Enzymatic synthesis of CS libraries containing disulfated disaccharide units

For the sulfation of CS-A and CS-D by GalNAc4S-6ST, the reaction mixtures contained, in a final volume of 50 µl, 2.5 µmol of imidazole-HCl, pH 6.8, 1 µmol of $CaCl_2$, 1 µmol of reduced glutathione, 25 nmol (as galactosamine) of CS-A or CS-D, 100 nmol PAPS and recombinant

GalNAc4S-6ST. The amount of enzyme and the conditions for incubation are shown in Table 1. For the sulfation of CS-A by 2OST, the reaction mixtures contained, in a final volume of 50 µl, 2.5 µmol of imidazole-HCl, pH 6.8, 2.6 µg of protamine chloride, 25 nmol (as galactosamine) of CS-A, 100 nmol of PAPS and recombinant 2OST. The amount of enzyme and the conditions for incubation are shown in Table 1. These sulfotransferase reactions were stopped by immersing the reaction tubes in a boiling water bath for 1 min. The reaction products were isolated by precipitation with 3-vol of ethanol containing 1.3% potassium acetate. These glycosaminoglycans thus synthesized were named on the basis of the acceptors used for the sulfotransferase reactions and the content of E unit or D unit in the products. For example, AE29 was generated from CS-A and the proportion of E unit was 29%.

Structural analysis of CS

CS members of the libraries were digested with chondroitinase ABC, and the disaccharide composition was determined by a reversed-phase ion-pair chromatography with postcolumn detection as described previously [29] with a slight modification of the elution program. Digestion with chondroitinase ABC was carried out for 4 h at 37°C in the reaction mixture containing, in a final volume of 25 µl, 1.25 µmol of Tris-acetate buffer, pH 7.5, 2.5 µg of bovine serum albumin, 30 mU of chondroitinase ABC and 500 pmol (as galactosamine) of CS. The reaction was stopped by immersing the reaction tubes in a boiling water bath for 1 min.

Assay of inhibitory activity of glycosaminoglycans on thrombin

Fifteen µl of glycosaminoglycan solutions containing 0.005–0.5 nmol (as galactosamine or glucosamine) of glycosaminoglycans and 0 or 0.07 unit of antithrombin in buffer C (50 mM Tris-HCl, pH 7.4, 0.1 M NaCl) was placed on 96-well microtiter plates. After incubation at 37°C for 5 min, 1.5 µl of thrombin solution containing 0.13 unit thrombin in buffer C was added to the glycosaminoglycan solutions, and the mixtures were further incubated for 5 min at room temperature. 100 µl of the chromogenic substrate solution (0.3 mM L-1150) was then added. Thrombin activity was measured by determining the absorbance at 405 nm after incubation for 10 min at 37°C.

Assay of inhibitory activity of glycosaminoglycans on Factor Xa

Thirteen µl of glycosaminoglycan solutions containing 0.005 to 0.5 nmol (as galactosamine or glucosamine) of glycosaminoglycans and 0 or 0.04 unit of antithrombin in buffer C was placed on 96-well microtiter plates. After incubation at 37°C for 5 min, 2 µl of factor Xa solution containing 10 ng factor Xa in buffer C was added to the glycosaminoglycan solutions, and the mixtures were further incubated for 10 min at 37°C. 100 µl of the chromogenic substrate solution (0.3 mM F3301) was then added. Thrombin activity was measured by determining the absorbance at 405 nm after incubation for 10 min at 37°C.

Assay of inhibitory activity of glycosaminoglycans on fibrinogen-degrading activity of thrombin

Five µl of glycosaminoglycan solutions containing 0.01–0.5 nmol (as galactosamine or glucosamine) of glycosaminoglycans and 0 or 2.2×10^{-3} units thrombin in buffer C was incubated for 5 min at room temperature. 5 µl of a fibrinogen solution containing 5 µg bovine fibrinogen in buffer C was added to the glycosaminoglycan solutions and the mixtures were incubated for 10 min at 37°C. After incubation, the reaction was stopped by adding 10 µl of 2 × loading buffer for SDS-polyacrylamide gel electrophoresis. After heating at 100°C for 2 min, the samples were

Table 1 Conditions of the enzymatic reactions used for the synthesis of CS libraries containing disulfated disaccharide units

Acceptors	Recombinant enzymes	Products	Amount of enzyme (units)	Incubation time (h)	Incubation temperature (°C)
CS-A	GalNAc4S-6ST	AE6	0.21	24	15
		AE11	0.42	24	15
		AE15	0.62	24	15
		AE29	0.62	96	15
CS-A	2OST	AD4	0.12	24	37
		AD9	0.35	24	37
		AD17	1.00	24	37
CS-D	GalNAc4S-6ST	DE2	0.21	24	15
		DE9	0.42	24	15
		DE17	0.62	24	15

subjected to SDS-polyacrylamide electrophoresis. The resulting gel was stained with Coomassie Brilliant Blue.

Results

Enzymatic synthesis and characterization of CS libraries containing disulfated disaccharide units

We synthesized CSs containing various amounts of disulfated disaccharide units using recombinant squid GalNAc4S-6ST and human 2OST as described under "Materials and methods". The synthesized CSs are named as indicated in Table 1. Disaccharide composition analysis of CSs formed from CS-A by GalNAc4S-6ST showed that, as the enzyme reaction proceeded, the proportion of ΔDi-diS$_E$ (ΔHexA-GalNAc(4,6-SO$_4$)) was increased up to 29%, whereas the proportions of ΔDi-4S (ΔHexA-GalNAc(4SO$_4$)) was decreased. The proportions of ΔDi-6S (ΔHexA-GalNAc(6SO$_4$)) and ΔDi-0S (ΔHexA-GalNAc) were not altered (Fig. 1a). These observations agreed well with the specificity of GalNAc4S-6ST which transferred sulfate to position 6 of GalNAc(4SO$_4$) residue. Disaccharide composition analysis of CSs formed from CS-A by

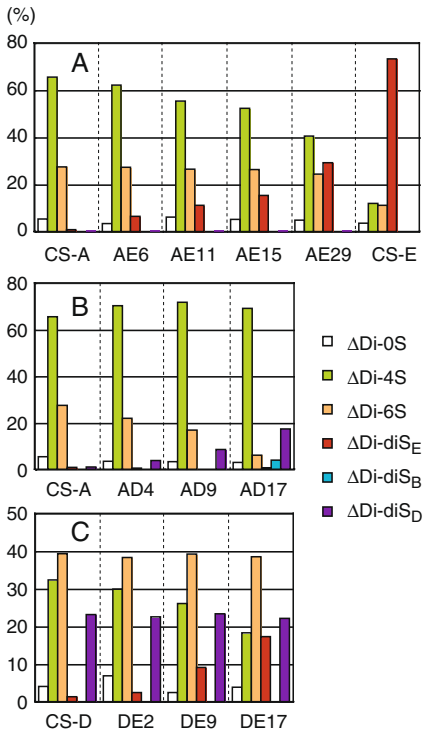

Fig. 1 Disaccharide composition of CSs containing disulfated disaccharide units. (**a**) The CS library prepared from CS-A by the reaction with GalNAc4S-6ST. (**b**) The CS library prepared from CS-A by the reaction with 2OST. (**c**) The CS library prepared from CS-D by the reaction with GalNAc4S-6ST. For nomenclature of each CS containing disulfated disaccharide units, see "Materials and methods"

2OST showed that the increase in ΔDi-diS$_D$ (ΔHexA(2SO$_4$)-GalNAc(6-SO$_4$)) was compensated by decrease in ΔDi-6S, and proportions of ΔDi-4S and ΔDi-0S were not altered (Fig. 1b). These observations appear to agree with the specificity of 2OST found by us that sulfate was transferred to position 2 of GlcA located at the center of the sequence, GlcA-GalNAc(4SO$_4$)-GlcA-GalNAc(6SO$_4$) [26]. However, unlike such specificity of 2OST, we detected a small amount of ΔDi-diS$_B$ (ΔHexA(2SO$_4$)-GalNAc(4SO$_4$)) in addition to ΔDi-diS$_D$ when AD17 was digested with chondroitinase ABC. We confirmed that ΔDi-diS$_B$ was also obtained when AD17 was digested with chondroitinase ACII (data not shown). We have shown previously that, when dermatan sulfate (DS) was sulfated by 2OST and [^{35}S]PAPS and the resulting [^{35}S]DS was digested with chondroitinase ABC, [^{35}S]ΔDi-diS$_B$ was obtained as the main disaccharide. [^{35}S]ΔDi-diS$_B$ was not obtained when the [^{35}S]DS was digested with chondroitinase ACII [26], indicating that ΔDi-diS$_B$ was released from IdoA(2SO$_4$)-GalNAc(4SO$_4$) disaccharide unit. On the other hand, ΔDi-diS$_B$ should be derived from GlcA(2SO$_4$)-GalNAc(4SO$_4$) unit in AD17, because ΔDi-diS$_B$ was obtained from AD17 by chondroitinase ACII digestion. These observations indicate that 2OST could sulfate position 2 of GlcA residues in GlcA-GalNAc(4SO$_4$) disaccharide unit when the sulfotransferase reaction was carried out in the presence of 2 mM PAPS. Disaccharide composition analysis of CSs formed from CS-D by GalNAc4S-6ST showed that the increase in ΔDi-diS$_E$ was compensated by decrease in ΔDi-4S, and proportion of ΔDi-6S, ΔDi-0S and ΔDi-diS$_D$ was not altered (Fig. 1c). DE17 contained nearly the equal amount of ΔDi-diS$_E$ and ΔDi-diS$_D$ and proportion of total disaccharide units reached 39%. These observations agreed well with our previous experiments that squid recombinant GalNAc4S-6ST could preferentially sulfate position 6 of GalNAc(4SO$_4$) residues located in a sequence included in CS-C or CS-D, GlcA-GalNAc(4SO$_4$)-GlcA(2SO$_4$)-GalNAc(6SO$_4$), to yield an E-D hybrid tetrasaccharide structure, GlcA-GalNAc(4,6-SO$_4$)-GlcA(2SO$_4$)-GalNAc(6SO$_4$).

Inhibition of thrombin by CSs containing disulfated disaccharide units

It is well known that heparin and heparan sulfate inhibit thrombin in the presence of antithrombin. We have confirmed that inhibition of thrombin by heparin and heparan sulfate was strongly enhanced by antithrombin (Fig. 2a, b). We examined whether CSs containing disulfated disaccharide units could inhibit thrombin activity. As shown in Fig. 2c and d, both CS-E and CS-D inhibited thrombin; inhibition of thrombin by CS-E and CS-D was hardly affected by antithrombin. DE17 also inhibited thrombin in a manner independent of antithrombin (data

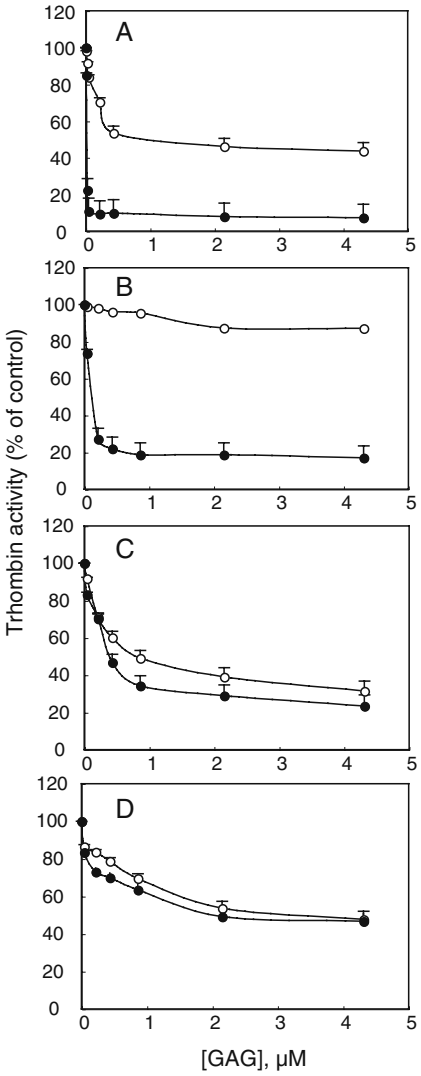

Fig. 2 Effects of various natural glycosaminoglycans on thrombin activity in the presence (closed circle) or absence (open circle) of antithrombin. (**a**) heparin, (**b**) heparan sulfate, (**c**) squid CS-E, and (**d**) shark CS-D. Thrombin activity was determined using a chromogenic substrate, L-1150, as described under "Materials and methods". Each point represents average of two independent determinations. Bars above the points represent range of values of the two determinations

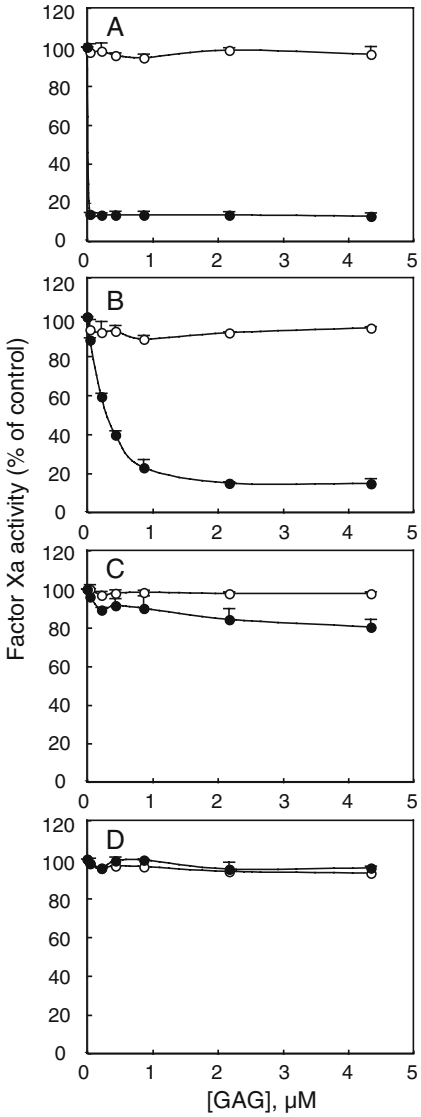

Fig. 3 Effects of various natural glycosaminoglycans on factor Xa activity in the presence (*closed circle*) or absence (*open circle*) of antithrombin. (**a**) heparin, (**b**) heparan sulfate, (**c**) squid CS-E, and (**d**) shark CS-D. Factor Xa activity was determined using a chromogenic substrate, F3301, as described under "Materials and methods". Each point represents average of two independent determinations. Bars above the points represent range of values of the two determinations

not shown). Heparin and heparan sulfate inhibited factor Xa in the presence of antithrombin, but did not inhibit factor Xa at all in the absence antithrombin (Fig. 3a, b). CS-E inhibited factor Xa weakly in the presence of antithrombin but showed no inhibitory activity in the absence of antithrombin (Fig. 3c). CS-D did not inhibit factor Xa even in the presence of antithrombin (Fig. 3d). These observations indicate that CSs containing disulfated disaccharide units are able to inhibit thrombin in a manner independent of antithrombin as observed in sulfated dehydropolymers (DHPs) of 4-hydroxycinnamic acids [30].

To examine the effect of the proportion of disulfated disaccharide units in CS chains on the inhibition of thrombin, we compared the inhibitory effects of CSs containing various amounts of disulfated disaccharide units prepared as above (Fig. 4). IC_{50} determined from Fig. 4 together with proportion of the total disulfated disaccharide units of each CS are shown in Table 2. CS-A, which barely contained disulfated disaccharide units, did not inhibit thrombin at all. Among CSs containing E units, which were derived from CS-A by the sulfation with GalNAc4S-6ST, AE15 and AE29 inhibited thrombin in a concentration-dependent manner, but AE6 and AE11 showed only a modest effect on thrombin activity (Fig. 4a). Among CSs containing D units, which were prepared from CS-A by 2OST, AD17 showed

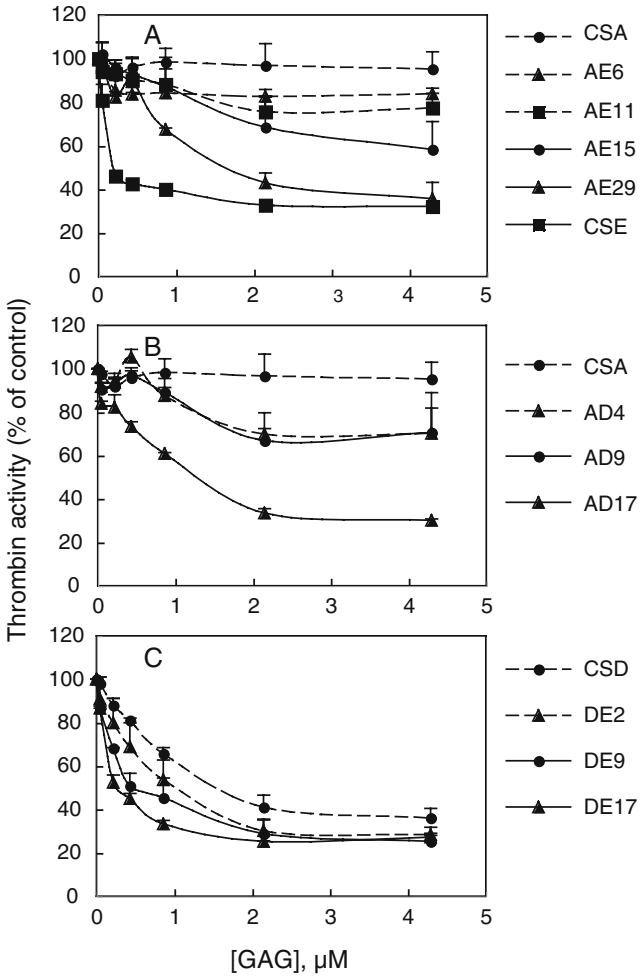

Fig. 4 Effects of the enzymatically prepared CSs containing disulfated disaccharide units on thrombin activity in the absence of antithrombin. (**a**) Effects of the CS library prepared from CS-A by the reaction with GalNAc4S-6ST. (**b**) Effects of the CS library prepared from CS-A by the reaction with 2OST. (**c**) Effects of the CS library prepared from CS-D by the reaction with GalNAc4S-6ST. Thrombin activity was determined using a chromogenic substrate, L-1150, as described under "Materials and methods". Each point represents average of two independent determinations. Bars above the points represent range of values of the two determinations

inhibitory activity toward thrombin comparable with that of CS-D, but AD4 and AD9 showed only a small inhibitory activity toward thrombin and the inhibition did not proceed beyond 40% inhibition (Fig. 4b). Next, we examined the inhibitory activity of CSs containing the E-D hybrid tetrasaccharide structure. When E units were introduced into CS-D by GalNAc4S-6ST, IC_{50} was decreased as the proportion of E unit was increased (Fig. 4c, Table 2); DE17 showed the lowest IC_{50} among the enzymatically prepared CSs containing disulfated disaccharide units. The inhibition of thrombin by any CSs containing disulfated disaccharide units examined here did not proceed to completion but became constant at near 70%, which contrasted with the observation that inhibition of thrombin by heparin in the presence of antithrombin reached near completion (Fig. 2a).

Inhibition of fibrinogen-degrading activity of thrombin by CSs containing the E-D hybrid tetrasaccharide structure

As described above, CSs containing disulfated disaccharide units inhibited chromogenic substrate-degrading activity of thrombin in the manner independent of antithrombin. To examine whether CSs containing disulfated disaccharide units could inhibit thrombin activity on physiological substrates, we determined the effects of DE17, which contained the E-D hybrid tetrasaccharide structure and showed the smallest IC_{50} among enzymatically synthesized CSs, on the degradation of fibrinogen by thrombin. When fibrinogen was digested with thrombin alone under the conditions described in "Materials and methods", α subunit (67 kDa) disappeared completely and β subunit (56 kDa) was markedly diminished (Fig. 5, lane 2). When digestion of fibrinogen by thrombin was carried out in the presence of increasing amount of heparin, β subunit remained intact and intact a subunit was still detected (Fig. 5, lane 7–10). On the other hand, when DE17 was added to the reaction mixture, intact β subunit was increased as the amount of DE17 was increased (Fig. 5, lane 3–6) and a faint intact α subunit band was also detected at 4.29 μM of DE17 (Fig. 5, lane 6). These observations indicate that DE17 containing the E-D hybrid tetrasaccharide structure is able to inhibit degradation of fibrinogen by thrombin in the manner independent of antithrombin, albeit much more weakly than heparin.

Discussion

In this paper, we synthesized CS libraries containing disulfated disaccharide units by the recombinant GalNAc4S-6ST and 2OST. One of the reasons that we used squid GalNAc4S-6ST instead of human counterpart is that squid GalNAc4S-6ST was able to sulfate CS-D to form CS containing E-D hybrid tetrasaccharide structure [23]. The degree of sulfation could be well controlled by the concentration of these enzymes and the incubation time. In the enzymatic reactions using recombinant squid GalNAc4S-6ST as the enzyme and CS-A as the substrate, the content of E unit in the product reached 29% of the total disaccharide units (AE29). When the enzymatic reaction was carried out under higher concentration of the recombinant enzyme, the proportion of E unit exceeded 50% of the total disaccharide units (data not shown). We have previously observed that such a highly sulfated CS was synthesized by the purified squid GalNAc4S-6ST when CS-A from rat cartilage was used as the substrate. On the other

Table 2 Proportion of disulfated disaccharide units and IC_{50} of various CSs containing disulfated disacharide units synthesized by GalNAc4S-6ST or 2OST. IC_{50} was determined from Fig. 4 on an assumption that inhibition of thrombin by CSs containing disulfated disacharide units reaches a plateau at 70% inhibition and the concentrations of CSs giving half maximal inhibition are obtained at 35% inhibition

CSs containing disulfated disacharide units	Proportion of the total disulfated disaccharide units (%)	IC_{50} (μM as galactosamine)
AE6	7	>4.29
AE11	12	>4.29
AE15	16	2.50
AE29	30	0.92
AD4	4	>4.29
AD9	9	>4.29
AD17	22[a]	0.70
DE2	25	0.55
DE9	32	0.25
DE17	39	0.15
CS-E	73	0.10
CS-D	24	0.95

[a] This value includes D unit and GlcA($2SO_4$)-GalNAc($4SO_4$) unit

hand, when CS-D was used as the substrate for the recombinant squid GalNAc4S-6ST, E unit in the product reached 17% of the total disaccharide units (DE17), but prolonged reaction in the presence of the newly added enzyme hardly resulted in the increase of E unit (data not shown). The content of E unit in DE17 was comparable to the content of D unit, suggesting that the introduction of E unit to CS-D may be affected by the content of the preexisting D unit. We found previously that CS-E synthesized from CS-A by the purified squid GalNAc4S-6ST showed substantial heterogeneity in Mono Q chromatography [24]. The disaccharide composition of the individual CS included in the libraries should be thus considered as an average value in differently sulfated products.

We found that, when CS-A was sulfated by 2OST, GlcA($2SO_4$)-GalNAc($4SO_4$) unit was formed in addition to D unit, indicating that 2OST is able to transfer sulfate to position 2 of GlcA residues adjacent to GalNAc($4SO_4$) in the presence of 2 mM PAPS. However, it is not clear whether this sulfation reaction occurs in animal tissues, because GlcA($2SO_4$)-GalNAc($4SO_4$) unit has rarely been found in CS extracted from animal tissues.

In preliminary experiments, we found that, when CS-A was sulfated by a crude extract of insect cells which were transfected with baculovirus containing GalNAc4S-6ST cDNA, the increase in ΔDi-diS_E was accompanied by the decrease in both ΔDi-4S and ΔDi-6S, but proportion of ΔDi-0S was unaffected. The activity responsible for the decrease in ΔDi-6S appears to be due to a protein originating from baculovirus, because this activity was neither included in the affinity-purified enzyme nor in the crude extracts of the insect cells, which were not infected with baculovirus. On the other hand, Kobayashi *et al.* reported that 2OST activity

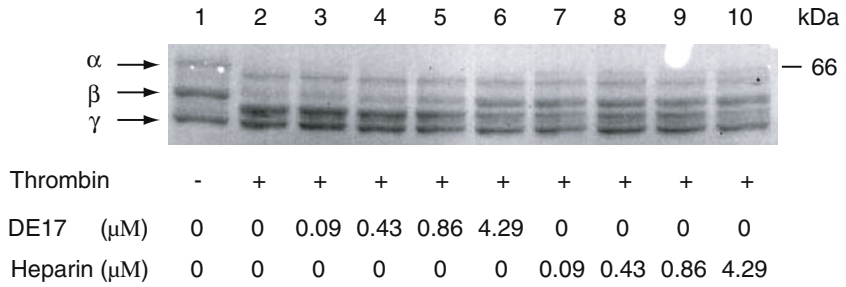

Fig. 5 Inhibition of fibrinogen-degrading activity of thrombin by DE17. Fibrinogen was digested by thrombin in the absence or presence of increasing amount of DE17 or heparin as described in "Materials and methods". After digestion, the samples were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue. Arrows indicate migration positions of α, β, and γ chain of fibrinogen

of the crude extract of insect cells which were transfected with baculovirus containing 2OST cDNA was higher toward DS than CS [25]. However, unlike their observation, we have shown previously that 2OST activity of the affinity-purified recombinant 2OST expressed in COS-7 cells was the highest toward CS-A [26]. These apparently conflicting results might be partially accounted for by the presence of a hyaluronidase, ODV-E66, in the extracts of the insect cells infected with baculovirus [31]. Because ODV-E66 is able to degrade CS but not DS, incubation of CS with the crude extract of the insect cells infected with baculovirus should result in the degradation of CS, thereby decreasing apparent 2OST activity toward CS. Relation between the hyaluronidase ODV-E66 and the activity responsible for the decrease in ΔDi-6S remains to be studied.

Thrombin plays central roles in hemostasis and is regulated through interaction with many proteins in the circulation and on cell surfaces [32, 33]. Thrombomodulin is a chondroitin sulfate (CS) proteoglycan and affects thrombin in different manners; direct inhibition of thrombin, inhibition of thrombin through acceleration of inhibitory effects of antithrombin or through activation of Protein C. CS moiety of rabbit thrombomodulin was reported to be essential for inhibiting thrombin directly and accelerating effect on the inactivation of thrombin by antithrombin, but not required for the activation of Protein C [34]. Structural analysis of the polysaccharide chain attached to rabbit thrombomodulin showed that the polysaccharide was CS containing GalNAc(4,6-SO_4) residue at the nonreducing terminal region[35]. The isolated CS from thrombomodulin as well as CS-E from squid cartilage showed weaker antithrombin-dependent anticoagulant activity than the intact thrombomodulin proteoglycan [35]. However, it has been poorly understood how the structure and contents of disulfated disaccharide units in CS contribute to the inhibition of thrombin, and whether CSs containing disulfated disaccharide units could inhibit thrombin in the manner dependent or independent of antithrombin as observed in sulfated dehydropolymers (DHPs) of 4-hydroxycinnamic acids [30]. We showed that CSs containing disulfated disaccharides are able to inhibit thrombin in the manner independent of antithrombin. Direct inhibition of thrombin was also observed in branched sulfated fucan obtained from brown algae [36] as well as sulfated DHPs [30]. CSs containing disulfated disaccharides did not show direct inhibition of factor Xa. In the presence of antithrombin, inhibition of thrombin by CS-E was somewhat increased (Fig. 2c), and mild inhibitory activity of CS-E on factor Xa was observed (Fig. 3c), suggesting that CS-E may have some activity for indirect inhibition of thrombin and factor Xa albeit at much lower efficiency than heparin and heparan sulfate. From the experiments using CSs containing different amounts of disulfated disaccharide units, it is suggested that there may be a critical content of disulfated disaccharide units for direct inhibition of thrombin. As for CSs containing E unit as the disulfated disaccharide unit, the concentration-dependent inhibition of thrombin was observed when the proportion of E-unit was more than 15%, although weak inhibitory activities toward thrombin were detected in AE6 and AE11 (Fig. 4a). On the other hand, the concentration-dependent inhibition of thrombin by CSs containing D unit was observed when proportion of D-unit reached 17%, although AD4 and AD9 showed a partial inhibitory activity (Fig. 4b). These observations suggest that a critical proportion of disulfated disaccharide units, which is required for the direct concentration-dependent inhibition of thrombin may be around 15%, and that this critical point appear to be independent of the structure of disulfated disaccharide units.

Proportion of disulfated disaccharide units of DE2 and DE9 was nearly the same as that of CS-D and AE29, respectively; however, IC_{50} of DE2 and DE9 was smaller than that of CS-D and AE29, respectively (Table 2). These results suggest that CS containing the E-D hybrid tetrasaccharide structure may have higher affinity to thrombin than CS containing E unit or D unit alone.

The extent of inhibition of thrombin activity by CSs containing disulfated disaccharides was about 70% regardless of the structure and contents of the disulfated disaccharide units. In contrast, inhibition of thrombin by heparin in the presence of antithrombin proceeded to near completion (Fig. 2a, b). At present, it is not clear why CSs containing disulfated disaccharides could not inhibit thrombin completely. One of possible reasons may be a contamination of other proteases in the thrombin preparation. Factor Xa was inhibited nearly completely by heparin in the presence of antithrombin, but was not inhibited at all by CSs containing disulfated disaccharides in the absence of antithrombin (Fig. 3). It may be possible that commercial thrombin used in this experiments would be contaminated by proteases similar to factor Xa. Alternatively, interaction of CSs containing disulfated disaccharides with thrombin might be too weak to allow total inhibition of thrombin.

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