Demonstration of a novel sulfotransferase in fetal bovine serum, which transfers sulfate to the C6 position of the GalNAc residue in the sequence iduronic acidα1-3GalNAcβ1-4iduronic acid in dermatan sulfate

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Abstract A novel sulfotransferase activity was discovered in fetal bovine serum using pig skin dermatan sulfate as an acceptor and [^{35}S]3′-phosphoadenosine 5′-phosphosulfate as a sulfate donor. The enzyme was separated from chondroitin:GalNAc 6-*O*-sulfotransferase by chromatographic techniques. Enzymatic analysis of the reaction products demonstrated that the enzyme transferred sulfate to the C6 position of the GalNAc residue in the sequence -iduronic acidα1-3GalNAcβ1-4iduronic acid-. Thus, the enzyme has been identified as a hitherto unreported dermatan sulfate:GalNAc 6-*O*-sulfotransferase. The finding is in sharp contrast to the current concept that in dermatan sulfate biosynthesis GalNAc 4-*O*-sulfation is a prerequisite for iduronic acid formation by C5 epimerase.

Key words: Dermatan sulfate; Sulfotransferase; Glycosaminoglycan; Proteoglycan

1. Introduction

Dermatan sulfate proteoglycans (DS-PGs) are widely distributed among various mammalian tissues including skin, tendon, sclera, cartilage, bone and blood vessel walls (see references in [1]). They are components of cell surfaces and extracellular matrices, and are also found in intracellular granules of mast cells and macrophages [2]. There is increasing evidence that DS-PGs have various biological activities including anticoagulant activity of endothelial cell surfaces [3,4], regulation of cell adhesion, migration and proliferation [5–9], and interaction with growth factors [7,10]. Although the molecular mechanisms of the expression of these activities of DS-PGs are not fully understood, at least some activities are expressed through DS side chains [3–5,9,10].

DS is a stereoisoform of chondroitin sulfate (CS), differing at C5 of the hexuronic acid moieties; CS contains D-glucuronic acid (GlcA), whereas DS contains L-iduronic acid (IdoA) as the major hexuronic acid. The disaccharide repeating units of DS are largely represented by the monosulfated unit, IdoAα1-3GalNAc(4-*O*-sulfate). However, DS is intrinsically heterogeneous with some hexuronic acid residues being GlcA as in the monosulfated unit, GlcAβ1-3GalNAc(4-*O*-sulfate) and GlcAβ1-3GalNAc(6-*O*-sulfate), and some IdoA as in the mono- or disulfated units, IdoAα1-3GalNAc(6-*O*-sulfate), IdoA(2-*O*-sulfate)α1-3GalNAc(4-*O*-sulfate) and IdoAα1-3GalNAc(4,6-*O*-disulfate) (see [11] for a review). Chang et al. [12] reported tissue-specific patterns of epimerization and sulfation observed in DS chains from various bovine tissues. Microheterogeneity in the DS structure, particularly in sulfation positions, may play important roles in various cellular phenomena. Thus, it is inevitable for functional studies of DS to investigate the biosynthetic mechanism by which the microheterogeneity is generated and regulated.

CS/DS biosynthesis is initiated by the addition of Xyl to specific Ser residues in the core protein, followed by the sequential addition of two Gal residues and a GlcA residue to form the tetrasaccharide linkage structure, GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-*O*-Ser [13]. Alternating additions of GalNAc and GlcA to the linkage tetrasaccharide region then take place, forming the repeating disaccharide region. Concomitant with elongation, or slightly after, the polymer can be modified to various degrees by the actions of C5 epimerase converting GlcA to IdoA, GalNAc 4-*O*-sulfotransferase and GalNAc 6-*O*-sulfotransferase [14]. Little information concerning the biosynthetic mechanism of DS is available although some information about CS biosynthesis has accumulated. Chondroitin:GalNAc 6-*O*-sulfotransferase (C6ST), which transfers sulfate to the C6 position of GalNAc residues in CS, has been purified, cloned and characterized [15–17]. It showed only slight activity toward DS, and had no ability to transfer sulfate to the C6 position of a GalNAc(4-*O*-sulfate) residue in CS to form a GalNAc(4,6-*O*-disulfate) structure. Chondroitin:GalNAc 4-*O*-sulfotransferase (C4ST) has been recently purified and characterized [18]. Although it showed activity toward chondroitin and chemically desulfated DS, it remains unclear whether it is involved in the DS biosynthesis. GalNAc:4-*O*-sulfotransferase acting on DS has been demonstrated in skin fibroblast microsomes [19], and 4-*O*-sulfation is a prerequisite for IdoA formation from GlcA by the action of C5 epimerase, which has also been demonstrated in various porcine tissues [19]. However, these enzymes have not been well characterized yet.

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Abbreviations: D6ST, dermatan sulfate:GalNAc 6-*O*-sulfotransferase; C4ST, chondroitin:GalNAc 4-*O*-sulfotransferase; C6ST, chondroitin:GalNAc 6-*O*-sulfotransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; CS, chondroitin sulfate; HS, heparan sulfate; GAG, glycosaminoglycan; PG, proteoglycan; GlcA, D-glucuronic acid; IdoA, L-iduronic acid; ΔHexA or $\Delta^{4,5}$HexA, 4-deoxy-α-*threo*-hex-4-enepyranosyluronic acid; Buffer A, 20 mM Tris-HCl, pH 8.0; ΔDi-0S, $\Delta^{4,5}$HexAα1-3GalNAc; ΔDi-4S, $\Delta^{4,5}$HexAα1-3GalNAc(4-*O*-sulfate); ΔDi-6S, $\Delta^{4,5}$HexAα1-3GalNAc(6-*O*-sulfate); ΔDi-diS$_B$, $\Delta^{4,5}$HexA(2-*O*-sulfate)α1-3GalNAc(4-*O*-sulfate); ΔDi-diS$_D$, $\Delta^{4,5}$HexA(2-*O*-sulfate)α1-3GalNAc(6-*O*-sulfate); ΔDi-diS$_E$, $\Delta^{4,5}$HexAα1-3GalNAc(4,6-*O*-disulfate); ΔDi-triS, $\Delta^{4,5}$HexA(2-*O*-sulfate)α1-3GalNAc(4,6-*O*-disulfate); Δhexuronate-2-, CS-4- and CS-6-sulfatase stand for $\Delta^{4,5}$hexuronate-2-*O*-, chondro-4-*O*- and -6-*O*-sulfatase, respectively; ΔU, G, U, 2S, 4S and 6S denote $\Delta^{4,5}$HexA, GalNAc, IdoA, 2-*O*-, 4-*O*- and 6-*O*-sulfate, respectively

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During the course of searching for sulfotransferases involved in CS and DS biosynthesis, we discovered a novel sulfotransferase activity that transferred sulfate to the C6 position of a GalNAc residue flanked by adjacent IdoA residues, creating an IdoAα1-3GalNAc(6-*O*-sulfate)β1-4IdoA sequence. The enzyme was hence designated DS:GalNAc 6-*O*-sulfotransferase (D6ST). Preliminary results have been presented in abstract form [20].

2. Materials and methods

2.1. Materials

[^{35}S]3′-Phosphoadenosine 5′-phosphosulfate (PAPS) (2.0 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). Unlabeled PAPS was obtained from Sigma Chemicals (St. Louis, MO, USA). The following materials and enzymes were purchased from Seikagaku Corp., Tokyo, Japan: pig skin DS, six unsaturated CS-disaccharide standards, chondroitinase B (EC 4.2.2) from *Flavobacterium heparinum*, chondroitinase AC-I (EC 4.2.2.5) from *F. heparinum*, chondroitinase ABC (EC 4.2.2.4) from *Proteus vulgaris*, chondro-4-*O*-sulfatase (EC 3.1.6.9) and chondro-6-*O*-sulfatase (EC 3.1.6.10) from *P. vulgaris*. Heparin-Sepharose CL-6B and phenyl-Sepharose CL-4B were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). All other reagents and chemicals were of the highest quality available.

2.2. Purification of D6ST in fetal bovine serum

All operations were performed at 4°C. Fetal bovine serum (10 ml) was applied to a column (5 ml) of heparin-Sepharose, which had been equilibrated with 20 mM Tris-HCl, pH 8.0 (buffer A), containing 0.15 M NaCl, washed with 50 ml of the starting buffer and then eluted stepwise with 50 ml each of buffer A containing 0.5, 1.0 or 2.0 M NaCl. These fractions were pooled, concentrated and then dialyzed against buffer A containing 0.15 M NaCl using a Centricon-30 concentrator (Amicon Inc., Beverly, MA, USA).

The fraction from the heparin-Sepharose chromatography was applied to a column of phenyl-Sepharose (1 ml) equilibrated with buffer A containing 1.0 M NaCl. The column was washed with 5 ml of buffer A containing 1.0 M NaCl, and eluted stepwise with 5 ml of buffer A containing 0.4 or 0.1 M NaCl and then buffer A without NaCl. The fraction containing the activity was pooled and dialyzed twice against buffer A containing 0.15 M NaCl as described above.

2.3. Assays for D6ST and C6ST activities

Assays for C6ST were carried out as described [21,22]. D6ST reactions were conducted in incubation mixtures containing the following constituents in a total volume of 60 μl of 10 mM Tris-HCl buffer, pH 8.0: 10 μl of the enzyme preparation, 100 μg of pig skin DS, 60 μM [^{35}S]PAPS (2.0×10^5 dpm), 0.4 mg/ml poly-L-lysine, 10 mM $MgCl_2$, 5 mM EDTA, 5 mM 2,3-dimercapto-1-propanol, and 10 μl of heat-inactivated fetal bovine serum which had been treated at 60°C for 1 h. The mixtures were incubated at 37°C for 1 or 2 h and [^{35}S]sulfate incorporation into DS chains was determined using the paper disk assay method described for C6ST [21]. Zero time blank values were subtracted from the values obtained for each time point. One unit of enzyme was defined as the amount that catalyzed the incorporation of 1 μmol of sulfate per min.

2.4. Analysis of ^{35}S-labeled DS

Enzyme reaction mixtures were treated with 5% trichloroacetic acid and centrifuged. The ^{35}S-labeled products were isolated from the supernatant fraction by gel filtration chromatography on a Sephadex G-25 column (0.8×51 cm) using 0.25 M NH_4HCO_3 containing 7% 1-propanol as a solvent. The isolated products ($2.0–3.0\times10^4$ dpm corresponding to 60–90 pmol of sulfate) were digested with chondroitinase ABC, AC-I or B essentially according to the instructions provided by the manufacturer or as reported [23]. To further identify the resultant radiolabeled unsaturated oligosaccharides, chondro-4- or 6-sulfatase digestion of chondroitinase ABC digestion products was carried out as described [24]. Each enzyme digest was analyzed by HPLC on an amine-bound silica PA03 column (YMC Co., Kyoto, Japan), which was developed using a linear gradient of NaH_2PO_4 at a flow rate of 1 ml/min at room temperature as described [25].

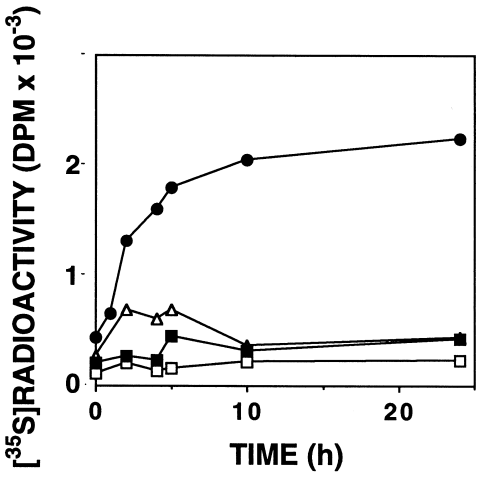

Fig. 1. Incorporation of [^{35}S]sulfate from [^{35}S]PAPS into exogenous CS and DS acceptors using fetal bovine serum as an enzyme source. CS and DS sulfotransferase reactions were carried out using 100 μg each of DS or CS isoforms as described in Section 2 and enzymatic incorporation of [^{35}S]sulfate into each acceptor was determined using the paper disk assay method. The acceptors included pig skin DS (●), whale cartilage CS-A (△), shark cartilage CS-C (■) and shark cartilage CS-D (□).

3. Results and discussion

3.1. Serum sulfotransferase activity toward DS

Fetal bovine serum was tested as an enzyme source for searching for sulfotransferases since several biosynthetic enzymes for CS and heparin/heparan sulfate occur at high concentrations in serum [22]. Fetal bovine serum was incubated with a sulfate donor, [^{35}S]PAPS, and exogenous DS or various CS isoforms, and [^{35}S]sulfate incorporation into each acceptor polysaccharide was determined by the paper disk assay method [21]. Interestingly, pig skin DS served as a good sulfate acceptor, whereas CS-A from whale cartilage, and CS-C and CS-D from shark cartilage showed negligible activity (Fig. 1). Since no sulfotransferase that transfers sulfate to native DS has been reported so far, we investigated whether the [^{35}S]sulfate incorporation into DS was attributable to an as yet unidentified sulfotransferase by enzyme purification and product identification as described below. Notably, the C6ST preparation purified from fetal bovine serum [22] did not utilize DS as a sulfate acceptor (data not shown). Furthermore, C6ST purified from chick chondrocytes shows only slight activity toward DS [15].

3.2. Purification of the DS sulfotransferase

We attempted to separate the activities of the DS sulfotransferase and C6ST using chromatographic techniques. When fetal bovine serum was subjected to heparin-Sepharose chromatography, most proteins were washed off the column with Buffer A containing 0.15 M NaCl, whereas approximately 80 and 70% of the DS sulfotransferase and C6ST activities were eluted in buffer A containing 0.5 M NaCl. Although the two enzymes were inseparable, the specific activities of both enzymes increased 32–38-fold (Table 1). This enzyme preparation was then chromatographed on a column of phenyl-Sepharose. The DS sulfotransferase activity was eluted in buffer A containing 1.0 M NaCl, being separated from the C6ST activity (Table 1), which was eluted at lower

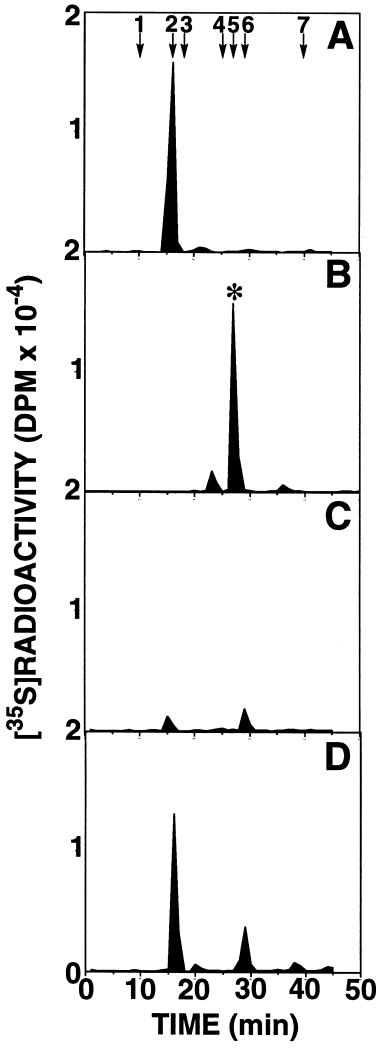

Fig. 2. Identification of the sulfotransferase reaction products. Enzymatic reactions were carried out using DS as an acceptor and [^{35}S]PAPS as a substrate donor, and the products were isolated by gel filtration as described in Section 2. The isolated products were digested by chondroitinase ABC (A), chondroitinase ABC and chondro-6-sulfatase (B), chondroitinase AC-I (C) or chondroitinase B (D), and each digest was analyzed by anion exchange HPLC on an amine-bound silica column (see Section 2). The peaks marked by asterisks were inorganic [^{35}S]sulfate. Arrows indicate the elution positions of authentic unsaturated disaccharides or inorganic sulfate: 1, ΔHexAα1-3GalNAc; 2, ΔHexAα1-3GalNAc(6-*O*-sulfate); 3, ΔHexAα1-3GalNAc(4-*O*-sulfate); 4, ΔHexA(2-*O*-sulfate)α1-3GalNAc(6-*O*-sulfate); 5, inorganic [^{35}S]sulfate; 6, ΔHexAα1-3GalNAc-(4,6-*O*-disulfate); 7, ΔHexA(2-*O*-sulfate)α1-3GalNAc(4,6-*O*-disulfate).

NaCl concentrations (data not shown). The results suggest that the DS sulfotransferase is a distinct enzyme from C6ST.

3.3. Identification of the sulfotransferase reaction products

^{35}S-Labeled reaction products were prepared by incubating the above enzyme preparation with DS and [^{35}S]PAPS. The reaction products were isolated by gel filtration, and digested with chondroitinases ABC, AC-I or B. Chondroitinase AC-I acts in an eliminative fashion on a GalNAcβ1-4GlcA linkage but not on a GalNAcβ1-4IdoA linkage, and chondroitinase B acts the other way around. Chondroitinase ABC catalyzes the eliminative cleavage of both linkages. On anion exchange HPLC, the chondroitinase ABC digest yielded a single major ^{35}S-labeled peak at the elution position of ΔDi-6S (Fig. 2A), which was shifted to the position of inorganic sulfate by the subsequent digestion with chondro-6-*O*-sulfatase (Fig. 2B). The chondroitinase AC-I digest yielded only a trace amount of materials (Fig. 2C). In contrast, the chondroitinase B digest gave a major ^{35}S-labeled peak at the position of ΔDi-6S with a minor peak at the position of inorganic sulfate. These results indicate that the sulfate had been incorporated exclusively into the GalNAc C6 position in the sequence -IdoAα1-3GalNAcβ1-4IdoA-, producing the sequence -IdoAα1-3GalNAc-(6-*O*-sulfate)β1-4IdoA-. Hence, the enzyme has been identified as DS:GalNAc 6-*O*-sulfotransferase (D6ST).

Notably, no disulfated disaccharides were detected, indicating that the enzyme did not transfer sulfate to the C6 position of a GalNAc residue in the sequence -(GlcAβ or IdoAα)1-3GalNAc(4-*O*-sulfate)-, which is rich in DS. Moreover, di- or tetrasaccharides were hardly observed for the chondroitinase AC-I digest of the products, indicating that the enzyme did not transfer sulfate to the GalNAc residue in the sequen-

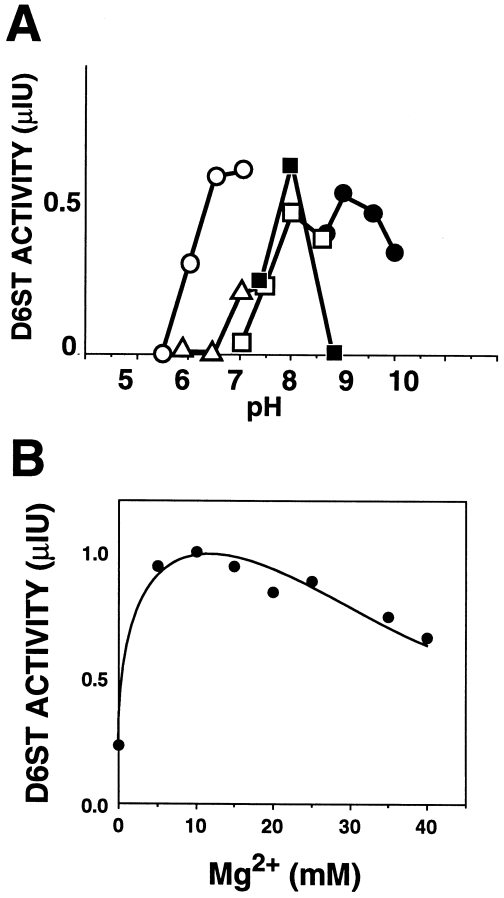

Fig. 3. Effects of buffers, pHs (A) and divalent cations (B) on the activity of the D6ST. A: The effects of pHs on the sulfate transfer to DS were determined under standard assay conditions except for the use of different buffers at a final concentration of 20 mM. The buffers used were MES-NaOH (○), imidazole-HCl (△), HEPES-NaOH (□), Tris-HCl (■), and glycine-NaOH (●). Assays proceeded as described in Section 2. B: The effects of Mg^{2+} concentrations on the sulfate transfer to DS were determined under standard assay conditions, except that the $MgCl_2$ concentration was varied.

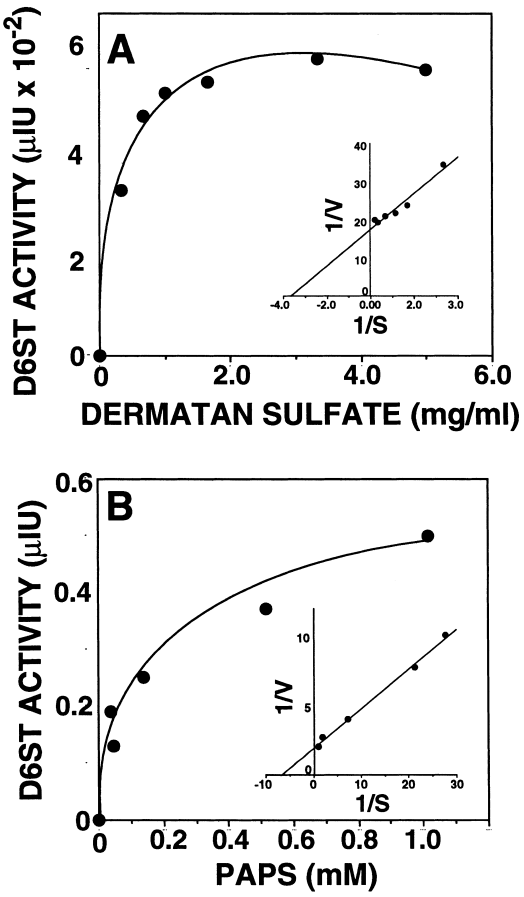

Fig. 4. Effects of DS and PAPS concentrations on the D6ST activity. DS (A) or PAPS (B) was added to the established assay mixture at different final concentrations, and assays proceeded as described in Section 2. The insets show Lineweaver-Burk plots of the same data. Data represent one of two series of independent experiments, where the two series of experiments gave essentially identical results.

ces -GlcAβ1-3GalNAcβ1-4GlcA-, non-sulfated -GlcAβ1-3GalNAcβ1-4IdoAα1-3GalNAcβ1-4GlcA- or monosulfated -GlcAβ1-3GalNAcβ1-4IdoAα1-3GalNAcβ1-4GlcA- where one of the two GalNAc residues was sulfated at its C4 position. It should be noted that C6ST transfers sulfate to the GalNAc residue in the sequence -GlcAβ1-3GalNAcβ1-4GlcA- but not in -IdoAα1-3GalNAcβ1-4IdoA-. The sequence -IdoAα1-3GalNAc(6-*O*-sulfate)β1-4IdoA-, which is the product of the D6ST reaction, has been demonstrated in human umbilical cord DS [27]. Takeuchi et al. reported that small proteoglycans from human yellow ligament bore glycosaminoglycan chains containing IdoAα1-3GalNAc(6-*O*-sulfate), whose content increased with aging or with ossification of the yellow ligaments [26]. These results in turn suggest that the pig skin DS preparation used in the assay contained the sequence where a non-sulfated GalNAc residue was flanked by IdoA residues. The present finding is in contrast to the current concept that 4-*O*-sulfation of a neighboring GalNAc residue is a prerequisite for IdoA formation from GlcA by the action of C5 epimerase [12]. Consistent with the present finding is the report by Malmström and Fransson that the IdoAα1-3GalNAc sequence was formed in DS synthesized by incubation of a fibroblast particulate fraction with UDP-GlcA and UDP-GalNAc in the presence of PAPS [27].

3.4. Properties of D6ST

Fig. 3 shows the effects of buffers and pHs on the partially purified D6ST. The activity was maximal between pH 6.5 and 9.0. The D6ST activity was somewhat affected by the buffers used. For example, MES buffer gave over three times the activity obtained using imidazole buffer at pH 6.8. Although divalent cations were not essential for the enzymatic reaction, D6ST activity was markedly enhanced by Mg^{2+}, the optimal concentration of which was approximately 10 mM (Fig. 3B). To investigate the effects of concentrations of a sulfate acceptor DS and a sulfate donor PAPS, Michaelis constants (K_m) of D6ST for these substrates were determined. The Lineweaver-Burk plots of the data showed that the apparent K_m values for DS and PAPS were 0.32 mg/ml and 0.148 mM, respectively (Fig. 4). The content of IdoAα1-3GalNAc units in DS used as an acceptor was calculated to be 0.1% (w/w) by subtracting the amount of ΔDi-0S produced by chondroitinase AC-I from that produced by chondroitinase ABC. Since 1 mg of DS contains 1.92 μmol of disaccharide units, the K_m value for the specific acceptor sites in DS was estimated to be 0.614 μM, suggesting that DS can be sulfated by this enzyme under physiological conditions.

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Table 1
Separation of the dermatan sulfate sulfotransferase from chondroitin 6-*O*-sulfotransferase

Purification step	Total protein[a] (mg)	DS sulfotransferase				C6ST		
		Activity[b] (μIU[c])	Specific activity (μIU/mg)	Recovery (%)	Purification (-fold)	Activity[b] (mIU[c])	Specific activity (mIU/mg)	Recovery (%)
Fetal bovine serum	44.4	310	6.98	100	1	14.8	0.33	100
Heparin-Sepharose 0.5 M NaCl	0.96	252	262.5	81.3	37.6	10.5	10.9	71.1
Phenyl-Sepharose 1.0 M NaCl	0.24	52.2	217.5	16.8	31.2	N.D.[d]	–	–

[a]Protein concentration was determined with the BCA (bicinchoninic acid) kit (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard.
[b]DS sulfotransferase and C6ST activities were measured using pig skin DS or chondroitin as an acceptor substrate as described in Section 2.
[c]One unit of enzyme was defined as the amount that catalyzed the incorporation of 1 μmol of sulfate per min.
[d]N.D., not detected ($< 0.2 \times 10^{-7}$ IU).

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