

## Purification and Characterization of Heparan Sulfate 2-Sulfotransferase from Cultured Chinese Hamster Ovary Cells\*

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Heparan sulfate 2-sulfotransferase, which catalyzes the transfer of sulfate from adenosine 3'-phosphate 5'-phosphosulfate to position 2 of L-iduronic acid residue in heparan sulfate, was purified 51,700-fold to apparent homogeneity with a 6% yield from cultured Chinese hamster ovary cells. The isolation procedure included a combination of affinity chromatography on heparin-Sepharose CL-6B and 3',5'-ADP-agarose, which was repeated twice for each, and finally gel chromatography on Superose 12. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified enzyme showed two protein bands with molecular masses of 47 and 44 kDa. Both proteins appeared to be glycoproteins, because their molecular masses decreased after N-glycanase digestion. When completely desulfated and N-resulfated heparin and mouse Engelbreth-Holm-Swarm tumor heparan sulfate were used as acceptors, the purified enzyme transferred sulfate to position 2 of L-iduronic acid residue but did not transfer sulfate to the amino group of glucosamine residue or to position 6 of N-sulfoglucosamine residue. Heparan sulfates from pig aorta and bovine liver, however, were poor acceptors. The enzyme showed no activities toward chondroitin, chondroitin sulfate, dermatan sulfate, and keratan sulfate. The optimal pH for the enzyme activity was around 5.5. The enzyme activity was minimally affected by dithiothreitol and was stimulated strongly by protamine. The  $K_m$  value for adenosine 3'-phosphate 5'-phosphosulfate was 0.20  $\mu$ M.

Heparan sulfate and heparin bind to a variety of proteins, such as growth factors and protease inhibitors, suggesting their involvement not only in a fundamental cellular behaviors such as cell growth, differentiation, and cell adhesion, but also in the anticoagulation process and some pathological processes such as viral infections (1-3). Bindings of those ligands to heparan sulfate or heparin seem to be mediated via specific structures in heparan sulfate or heparin. For example, basic fibroblast growth factor (FGF-2)<sup>1</sup> interacts with a cluster of

GlcNSO<sub>3</sub>-IdoA(2SO<sub>4</sub>) in heparan sulfate (4-8), and, in addition, its high affinity receptor appears to interact with some specific sites containing GlcNSO<sub>3</sub>(6SO<sub>4</sub>)-IdoA(2SO<sub>4</sub>) in heparan sulfate (8). It has recently been suggested that heparan sulfate proteoglycans on cell surfaces as well as in extracellular matrix may have such specific structures and regulate the biological activity of basic fibroblast growth factor (9). In fact, the response of neural cells to either acidic or basic fibroblast growth factor (FGF-1 or FGF-2, respectively) seems to be regulated by developmentally modulated forms of heparan sulfate proteoglycans (10). Thus, microheterogeneity in the heparan sulfate structures, particularly in the sulfation positions may be an important factor to control the biological activity of basic fibroblast growth factor. In this regard, it is important to study how the microheterogeneity is caused and regulated.

Various types of sulfotransferases have been shown to be responsible for the sulfation of heparin and heparan sulfate: sulfation of 2-N (11-14), 6-O (13, 15, 16), and 3-O (17) of glucosamine residue, sulfation of 2-O (16) of L-iduronic acid residue, and sulfation of 2-O (18) of D-glucuronic acid residue. The sulfate donor in these reactions is adenosine 3'-phosphate 5'-phosphosulfate (PAPS) that is synthesized in the cytosol and transported into the lumen of the Golgi to serve as substrate (19). N-Sulfotransferases have been purified to homogeneity from rat liver and mouse mastocytoma (11, 12). Recently, molecular cloning studies have suggested that these N-sulfotransferases were closely related to but were clearly distinct from each other (20-23), suggesting that the biosynthesis of heparan sulfate and heparin may be catalyzed by different enzymes and independently regulated.

It has been suggested that O-sulfation is the final step in the modification of the structure during the biosynthesis of heparin and heparan sulfate in the lumen of the Golgi apparatus (24, 25). We recently purified heparan sulfate 6-sulfotransferase (HS6ST) that catalyzes the transfer of sulfate to position 6 of N-sulfoglucosamine residue in heparan sulfate, with a high yield from the serum-free culture medium of Chinese hamster

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<sup>1</sup> The abbreviations used are: FGF, fibroblast growth factor; GlcNSO<sub>3</sub>, N-sulfoglucosamine; IdoA, iduronic acid; HexA, hexuronic acid; GlcNAc, N-acetylglucosamine; GlcA, glucuronic acid; PAPS, adenosine 3'-phosphate 5'-phosphosulfate; HS6ST, heparan sulfate 6-sulfotrans-

ferase; HS2ST, heparan sulfate 2-sulfotransferase; CHO, Chinese hamster ovary; CDSNS-heparin, completely desulfated and N-resulfated heparin; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography;  $\Delta$ Di-6S, 2-acetamide-2-deoxy-4-O-(4-deoxy- $\alpha$ -L-threo-hex-4-enopyranosyluronic acid)-6-O-sulfo-D-glucose;  $\Delta$ Di-NS, 2-deoxy-2-sulfamino-4-O-(4-deoxy- $\alpha$ -L-threo-hex-4-enopyranosyluronic acid)-D-glucose;  $\Delta$ Di-(6)diS, 2-deoxy-2-sulfamino-4-O-(4-deoxy- $\alpha$ -L-threo-hex-4-enopyranosyluronic acid)-6-O-sulfo-D-glucose;  $\Delta$ Di-(U)diS, 2-deoxy-2-sulfamino-4-O-(4-deoxy-2-O-sulfo- $\alpha$ -L-threo-hex-4-enopyranosyluronic acid)-D-glucose;  $\Delta$ Di-(6,U)triS, 2-deoxy-2-sulfamino-4-O-(4-deoxy-2-O-sulfo- $\alpha$ -L-threo-hex-4-enopyranosyluronic acid)-6-O-sulfo-D-glucose; AMan, 2,5-anhydro-D-mannose (when a subscript R follows this abbreviation, this refers to the corresponding alditol formed by reduction of the compound with NaBH<sub>4</sub>); Mes, 4-morpholinoethanesulfonic acid; EHS, Engelbreth-Holm-Swarm; PAMN, pack polyamine.

ovary (CHO) cells (15). However, the enzyme activity to catalyze the 2-*O*-sulfation of L-iduronic acid residue of heparin and heparan sulfate was hardly detected in the culture medium of CHO cells, although heparan sulfate prepared from CHO cells contained HexA(2SO<sub>4</sub>) and GlcNSO<sub>3</sub>(6SO<sub>4</sub>) residues in a proportion of 4:3. These observations have interested us in the intracellular *O*-sulfotransferases of CHO cells. We found in the present study that in CHO cell culture more than 90% of activities of heparan sulfate *O*-sulfotransferases in the cell layer catalyzed the transfer of sulfate to position 2 of L-iduronic acid residue. In this report, we describe the purification to apparent homogeneity and some properties of this sulfotransferase from the cultured CHO cells (this enzyme was designated as heparan sulfate 2-sulfotransferase (HS2ST)).

## EXPERIMENTAL PROCEDURES

### Materials

[<sup>35</sup>S]H<sub>2</sub>SO<sub>4</sub> was purchased from the Japan Radioisotope Association (Tokyo, Japan). Adenosine 3',5'-diphosphate (3',5'-ADP), unlabeled PAPS, 3',5'-ADP-agarose, and heparin from porcine intestinal mucosa were from Sigma. Fetal bovine serum was from Cytosystems Pty. Ltd. Dulbecco's modified Eagle's medium and CHO-S-SFM II, serum-free medium for suspension culture of CHO cells were from Life Technologies, Inc. Cosmedium-001 was from Cosmo-Bio (Tokyo, Japan). Fast desalting column HR10/10, heparin-Sepharose CL-6B, Superose 12 HR10/30, and HiLoad 16/60 Superdex 30 pg were from Pharmacia Biotech Inc. (Uppsala, Sweden). Partisil-10 SAX column was from Whatman, PAMN column was from YMC (Kyoto, Japan). Heparitinase I, II, and III, chondroitin sulfate A from whale cartilage (4-sulfate unit/6-sulfate unit (80:20)), chondroitin sulfate C from shark cartilage (4-sulfate unit/6-sulfate unit (10:90)), dermatan sulfate from pig skin, keratan sulfate from bovine cornea, heparan sulfates from pig aorta and bovine liver, completely desulfated and *N*-resulfated heparin (CDSNS-heparin), and unsaturated glycosaminoglycan disaccharide kit were obtained from Seikagaku Corp. (Tokyo, Japan). Heparan sulfate from EHS tumor was a gift of T. Harada, Seikagaku Corp. Recombinant *N*-glycanase was from Genzyme Co. [<sup>35</sup>S]PAPS was prepared as described previously (26). Chondroitin (squid skin) was prepared as described previously (27).

### Culture of CHO Cells and Preparation of the Crude Extract

CHO cells were cultured for 2 days in 10-cm culture dishes (Falcon) containing 10 ml of Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin in humidified 5% CO<sub>2</sub>, 95% air at 37 °C. The culture medium was then replaced with 10 ml of Cosmedium-001 containing antibiotics, and the culture was further continued for another 2 days. The spent medium was collected, and the cell layers were washed with phosphate-buffered saline, scraped off from the dishes in 5 ml of buffer A (10 mM Tris-HCl, pH 7.2, 0.1% (w/v) Triton X-100, 10 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 20% (v/v) glycerol) containing 0.15 M NaCl, and homogenized in a glass homogenizer. The concentration of Triton X-100 in the homogenate was then increased to 0.5% (w/v). After 1 h of gentle stirring, the homogenate was centrifuged at 4 °C for 10 min at 10,000 × *g*. The supernatant was obtained as the cell extract.

For purification of HS2ST, CHO cells were plated onto 10-cm culture dishes at a density of 2.0 × 10<sup>6</sup> cells/dish in 10 ml of CHO-S-SFM-II containing antibiotics. After 4 days the CHO cells were transferred to 500-ml spinner flasks (Techne, Cambridge, United Kingdom) at a density of 3.0 × 10<sup>5</sup> cells/ml and continued culturing with constant agitation of 90 rpm for 4 days in 500 ml of the same medium. The CHO cells were harvested and washed with phosphate-buffered saline. The cell pellet was suspended in the solution (55 ml/1 × 10<sup>9</sup> cells) of 10 mM Tris-HCl, pH 7.2, 0.5% (w/v) Triton X-100, 10 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 0.15 M NaCl, 20% (v/v) glycerol, and a mixture of protease inhibitors (5 μM *N*<sup>ε</sup>-*p*-tosyl-L-lysine chloromethyl ketone, 3 μM *N*-tosyl-L-phenylalanine chloromethyl ketone, 30 μM phenylmethylsulfonyl fluoride, and 3 μM pepstatin A). Extraction of sulfotransferases from the cells was carried out by homogenizing the cell suspension in the above buffer with a glass homogenizer on ice. After the gentle stirring at 4 °C for 1 h, the homogenate was centrifuged at 4 °C for 30 min at 10,000 × *g*. The supernatant was pooled (1.8 liters), designated as the crude extract, and stored at -20 °C until use.

### Purification of Heparan Sulfate 2-Sulfotransferase from the Crude Extract of CHO Cells

All operations were performed at 4 °C.

**Step 1: First Heparin-Sepharose CL-6B Chromatography**—One-third volume (600 ml) of the crude extract prepared as above was applied to a column of heparin-Sepharose CL-6B (30 × 70 mm, 50 ml) equilibrated with 0.15 M NaCl in buffer A containing a mixture of the protease inhibitors at a flow rate of 76 ml/h. The column was washed with 500 ml (10 volumes of a column) of 0.15 M NaCl in the above buffer and then eluted with a linear gradient from 0.15 to 1.2 M NaCl in the above buffer (total volume, 1,000 ml). Fractions of 13 ml were collected. The fractions containing the sulfotransferase activity were pooled (indicated by a horizontal bar in Fig. 1), concentrated to about 100 ml with polyethylene glycol 20,000 (molecular weight 15,000–25,000), and dialyzed against 0.05 M NaCl in buffer A for the next purification step. Triton X-100 was added to 1% (w/v) at the final concentration before dialysis.

**Step 2: First Chromatography on 3',5'-ADP-agarose**—The step 1 fraction was applied to a column of 3',5'-ADP-agarose (14 × 100 mm, 15 ml, 1.9 μmol of 3',5'-ADP/ml of gel) equilibrated with buffer A containing 0.05 M NaCl at a flow rate of 13 ml/h. The column was washed with 120 ml (8 volumes of a column) of buffer A containing 0.05 M NaCl, and then eluted with 75 ml (5 volumes of a column) of 0.2 mM 3',5'-ADP in buffer A containing 0.05 M NaCl. This fraction, containing sulfotransferase activity, was brought to 0.15 M NaCl by adding buffer A containing 1 M NaCl. The above processes (steps 1 and 2) were repeated three times, and the fractions thus obtained were combined.

**Step 3: Second Heparin-Sepharose CL-6B Chromatography**—The step 2 fraction combined was applied to a heparin-Sepharose CL-6B column (16 × 50 mm, 10 ml) equilibrated with buffer A containing 0.15 M NaCl at a flow rate of 26 ml/h. The column was washed with 30 ml (3 volumes of a column) of buffer A containing 0.15 M NaCl. The sulfotransferase activity was eluted with a linear gradient from 0.15 to 1.0 M NaCl in buffer A (total volume, 300 ml). The fractions containing sulfotransferase activity were pooled (indicated by a horizontal bar in Fig. 2), brought to 1% (w/v) Triton X-100, and dialyzed against 1 M NaCl in buffer A and then against 0.05 M NaCl in buffer A for the next purification step.

**Step 4: Second Chromatography on 3',5'-ADP-agarose**—The step 3 fraction was subjected to the second 3',5'-ADP-agarose chromatography as the first 3',5'-ADP-agarose chromatography was performed. Sulfotransferase activity was eluted with 0.2 mM 3',5'-ADP in buffer A containing 0.05 M NaCl. The fraction was brought to 0.15 M NaCl in buffer A, and concentrated to about 8 ml with polyethylene glycol. After dialysis against 0.1 M NaCl in buffer A, the fraction was applied onto a small heparin-Sepharose CL-6B column (bed volume, 0.5 ml) equilibrated with 0.1 M NaCl in buffer A. The column was washed with 5 ml of 0.1 M NaCl in buffer B (buffer A containing not 0.1% but 0.02% Triton X-100) and then eluted with 2.5 ml of 1 M NaCl in buffer B in order to remove 3',5'-ADP. The eluate was then concentrated to about 0.5 ml, and dialyzed against 1 M NaCl in buffer A for the next step.

**Step 5: Chromatography on Superose 12**—Superose 12 HR 10/30 was equilibrated with buffer A containing 2 M NaCl. The step 4 fraction was applied to the column. The column was eluted with buffer A containing 2 M NaCl at a flow rate of 0.25 ml/min. The fractions (0.25 ml each) containing sulfotransferase activity were pooled (indicated by a horizontal bar in Fig. 3A) and dialyzed against 0.15 M NaCl in buffer A. The fraction was stored at -20 °C.

### Assay for Sulfotransferase Activity

Sulfotransferase activity was determined as described previously (15). Briefly, the standard reaction mixture (50 μl) contained 2.5 μmol of imidazole HCl, pH 6.8, 3.75 μg of protamine chloride, 25 nmol (as hexosamine) of CDSNS-heparin, 50 pmol of [<sup>35</sup>S]PAPS (about 5 × 10<sup>5</sup> cpm), and enzyme. When the activity of HS2ST was monitored at each purification step, 10 mM dithiothreitol (DTT) was added to the standard reaction mixture. After incubation at 37 °C for 20 min, the reaction was terminated by heating at 100 °C for 1 min. Carrier chondroitin sulfate A (0.1 μmol as glucuronic acid) was added to the reaction mixture, and the <sup>35</sup>S-labeled polysaccharides were isolated by precipitation with ethanol containing 1.3% potassium acetate and 0.5 mM EDTA and by subsequent gel chromatography on a Fast desalting column to remove [<sup>35</sup>S]PAPS and its degradation products. The incorporation of [<sup>35</sup>S]sulfate was linear with the amounts of enzyme proteins and with the incubation time under the above conditions (up to 5.8 ng of protein and up to 30 min when the purified enzyme was used) (data not shown). One unit of enzyme activity was defined as the amount required to transfer 1 pmol of sulfate/min.

*SDS-Polyacrylamide Gel Electrophoresis*

For SDS-polyacrylamide gel electrophoresis (PAGE), protein in a sample was precipitated with 10% trichloroacetic acid. The precipitate was washed twice with acetone, and dissolved in sample buffer for SDS-PAGE. 2-Mercaptoethanol was removed from the sample buffer to avoid an artifact of the following silver staining. SDS-PAGE of proteins was carried out on a 10% polyacrylamide gel as described previously (28).

*Analysis of Enzymatic Reaction Products*

Analysis of enzymatic reaction products was performed by HPLC as described previously (15) with some modification. Briefly, <sup>35</sup>S-labeled products were digested with a mixture of 10 milliunits of heparitinase I, 1 milliunit of heparitinase II, and 10 milliunits of heparitinase III in 40  $\mu$ l of 50 mM Tris-HCl, pH 7.2, 1 mM CaCl<sub>2</sub>, 4  $\mu$ g of bovine serum albumin at 37 °C for 2 h. The digested products were injected together with standard unsaturated disaccharides into a column of PAMN (4.6 mm  $\times$  25 cm). Fractions of 0.6 ml were collected and mixed with 3 ml of Ready Safe Scintillator (DuPont NEN), and the radioactivity was determined. Degradation of the <sup>35</sup>S-labeled glycosaminoglycans with nitrous acid at pH 1.5 and reduction with NaBH<sub>4</sub> were carried out as described by Shively and Conrad (29). Anomalous deamination products were cleaved by the additional treatment of mild acid hydrolysis (25 mM H<sub>2</sub>SO<sub>4</sub> at 80 °C for 30 min) (16, 17). The final products were subjected to gel filtration on a Superdex 30 equilibrated with 0.5 M NH<sub>4</sub>HCO<sub>3</sub>. The fractions corresponding to <sup>35</sup>S-labeled disaccharides were collected and analyzed by HPLC on a Partisil-10 SAX column.

*Other Methods*

The galactosamine and glucosamine contents of glycosaminoglycans were determined by the Elson-Morgan method as modified by Strominger *et al.* (30) after hydrolysis of the glycosaminoglycans with 6 M HCl at 100 °C for 4 h. Uronic acid was determined by the method of Bitter and Muir (31). Protein concentration was determined by a micro-BCA protein assay reagent kit (Pierce) using bovine serum albumin as a standard. 6.6 ng of the enzyme protein was digested with 0.5 unit of *N*-glycanase at 37 °C for 16 h according to the method recommended by the manufacturer except for omitting mercaptoethanol.

## RESULTS

*Distribution of Heparan Sulfate 6-Sulfotransferase and 2-Sulfotransferase Activities in CHO Cell Culture*

In our previous study using CHO cells cultured on plates (15), HS2ST activity was hardly detected in the culture medium, although the CHO cell heparan sulfate contained HexA(2SO<sub>4</sub>) and GlcNSO<sub>3</sub>(6SO<sub>4</sub>) residues in a proportion of 4:3. To examine a possibility that HS2ST is retained in the cell layer, the activity in the cell extract was compared with that secreted into the medium as follows. The <sup>35</sup>S-labeled products formed from CDSNS-heparin and [<sup>35</sup>S]PAPS by incubation with the spent medium or the crude extract of cultured CHO cells as an enzyme source was analyzed by HPLC on a PAMN column after the digestion with a mixture of heparitinases as described under "Experimental Procedures." In the case of the products by the spent medium, major radioactivity was recovered in a peak of  $\Delta$ Di-(6)diS, and a small amount of radioactiv-

ity was in a peak of  $\Delta$ Di-(U)diS ( $\Delta$ Di-(6)diS: $\Delta$ Di-(U)diS = 96:4) as described previously (15). On the other hand, in the case of the products by the cell extract, major radioactivity was recovered in a peak of  $\Delta$ Di-(U)diS, and only a small amount of radioactivity was in a peak of  $\Delta$ Di-(6)diS ( $\Delta$ Di-(U)diS: $\Delta$ Di-(6)diS = 92:8). As is evident from the summarized results (Table I), the major sulfotransferase activity retained in the cell layer catalyzed the transfer of sulfate to position 2 of hexuronic acid residue in heparan sulfate, *i.e.* HS2ST. The similar results were also obtained when CHO cells were cultured in suspension in a spinner flask (data not shown), which enabled us to culture cells on a large scale. Therefore, spinner culture was chosen in place of adhesion culture to obtain CHO cells enough to purify HS2ST.

*Purification of Heparan Sulfate 2-Sulfotransferase*

We successfully purified HS2ST from the extract of CHO cells to an apparent homogeneity with about 51,700-fold purification. Table II shows a summary of the purification of HS2ST from 1,800 ml of the crude extract ( $3.3 \times 10^{10}$  cells). In this experiment, HS2ST activity was specifically determined using the standard assay mixtures containing 10 mM DTT as described under "Experimental Procedures." DTT at this concentration had little effect on HS2ST activity as shown later, while HS6ST activity was substantially decreased (15).

The details for each purification step are as follows.

*Step 1: First Heparin-Sepharose Chromatography*—The

TABLE I.  
*Distribution of heparan sulfate 6-sulfotransferase and 2-sulfotransferase in CHO cell culture*

After CHO cells were cultured, the spent medium and cell extract were prepared as described under "Experimental Procedures." They were both applied to a small heparin-Sepharose CL-6B column (bed volume, 0.6 ml). The column was washed with 5 ml of 0.15 M NaCl in buffer A and then eluted with 5 ml of 1.0 M NaCl in buffer A. The analysis of <sup>35</sup>S-labeled products derived from the incubation with CD-SNS-heparin, [<sup>35</sup>S]PAPS, and the eluted fractions above was performed by HPLC on a PAMN column after digestion with a mixture of heparitinases as described under "Experimental Procedures." Incorporation of [<sup>35</sup>S]sulfate was exclusively detected in  $\Delta$ Di-(U)diS and  $\Delta$ Di-(6)diS components. The radioactivity incorporated into each disaccharide component was expressed as enzyme activity according to the proportion of radioactivity into  $\Delta$ Di-(U)diS: $\Delta$ Di-(6)diS and total sulfotransferase activity.

Heparan sulfate <i>O</i> -sulfotransferase	Sulfotransferase activity	
	Cell	Medium
	<i>units/5.4 <math>\times</math> 10<sup>7</sup> cells<sup>a</sup></i>	
HS6ST	1.5	14.0
HS2ST	17.0	0.6
Total <i>O</i> -sulfotransferases	18.5	14.6

<sup>a</sup> One unit of activity is defined as 1 pmol/min sulfate transferred to CDSNS-heparin used as substrate as described under "Experimental Procedures."

TABLE II  
*Purification of heparan sulfate 2-sulfotransferase*

Step	Volume	Total activity <sup>a</sup>	Total protein	Specific activity	Purification	Recovery
	<i>ml</i>	$10^{-3} \times \text{units}^b$	<i>mg</i>	$10^{-4} \times \text{units/mg}$	<i>-fold</i>	<i>%</i>
Crude extract	1,800	20.0	4,560	0.000439	1	100
First heparin-Sepharose	300	37.5	390	0.00962	22	188
First 3',5'-ADP-agarose	225	23.6	5.60	0.421	959	118
Second heparin-Sepharose	96	12.9	1.22	1.06	2,420	65
Second 3',5'-ADP-agarose	75	4.75	0.0405	11.7	26,700	24
Superose 12	1.75	1.24	0.00546	22.7	51,700	6

<sup>a</sup> The sulfotransferase activities were determined under standard assay conditions containing 10 mM DTT as described under "Experimental Procedures."

<sup>b</sup> One unit of activity is defined as 1 pmol/min sulfate transferred to CDSNS-heparin used as substrate as described under "Experimental Procedures."

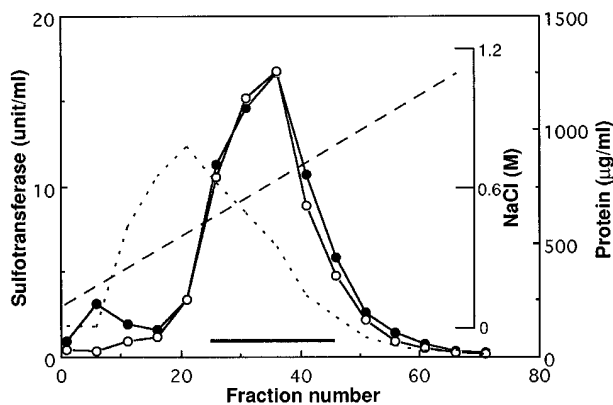


FIG. 1. First heparin-Sepharose CL-6B chromatography of the crude extract from cultured CHO cells. The crude extract from cultured CHO cells was applied to a heparin-Sepharose column as described under "Experimental Procedures." After wash with buffer A containing 0.15 M NaCl, the column was eluted with a linear gradient of NaCl. Fractions of 13 ml were collected. Sulfotransferase activity in the absence (●) or presence (○) of 10 mM DTT, and protein concentration (---) of each fraction were assayed. The broken line (---) indicates the concentration of NaCl.

crude extract of CHO cells was applied to a heparin-Sepharose column equilibrated with buffer A containing 0.15 M NaCl (Fig. 1). More than 75% of total proteins passed through the column, and most of the proteins adsorbed to the column were eluted at the low NaCl concentration earlier than HS2ST activity. The sulfotransferase activity inhibited by DTT was noted in the early fractions (fraction number approximately 6). This chromatography brought a 1.9-fold increase of the total activity of HS2ST, suggesting that some inhibitors for HS2ST or degrading enzymes for substrate PAPS might have been removed at this step.

**Step 2: First 3',5'-ADP-agarose Chromatography**—This column chromatography resulted in a 44-fold purification of HS2ST. Since the sulfotransferase activity appeared to be stable in the higher concentration of NaCl, each fraction was collected in a tube containing 1 M NaCl in buffer A to make the 0.15 M NaCl solution. HS2ST was also strongly inhibited by 3',5'-ADP, as observed with other glycosaminoglycan sulfotransferases (12, 15, 32). The concentration of 3',5'-ADP giving a 50% inhibition of this sulfotransferase activity was found to be less than 2.5  $\mu$ M (data not shown). HS2ST activity at this step, therefore, was determined after the removal of 3',5'-ADP by adsorbing HS2ST to a small heparin-Sepharose column (bed volume, 0.5 ml) and eluting it with 1 M NaCl in buffer A.

**Step 3: Second Heparin-Sepharose Chromatography**—The elution was performed with a linear gradient of NaCl concentration (Fig. 2). HS2ST activity was eluted with most of the proteins adsorbed to the column. The sulfotransferase activity inhibited by DTT was noted in the more retarded fractions on a downslope of the peak. This activity, judging from the elution position of HS6ST (around 0.8 M) as described previously (15), appeared to be brought by HS6ST. The fractions indicated by a horizontal bar in Fig. 2 were pooled. When an aliquot of this fraction was applied to a small heparin-Sepharose column and eluted with 1 M NaCl in buffer A, a 2-fold increase of HS2ST activity was detected, indicating that 3',5'-ADP still existed in the pooled fractions. Therefore, the fraction was dialyzed against 1 M NaCl in buffer A to remove 3',5'-ADP completely and then against 0.05 M NaCl in buffer A for the next step.

**Step 4: Second 3',5'-ADP-agarose Chromatography**—This column chromatography resulted in an 11-fold purification of HS2ST. Each eluate was collected in a tube containing 1 M NaCl in buffer A as described above.

**Step 5: Superose 12 Chromatography**—The second 3',5'-

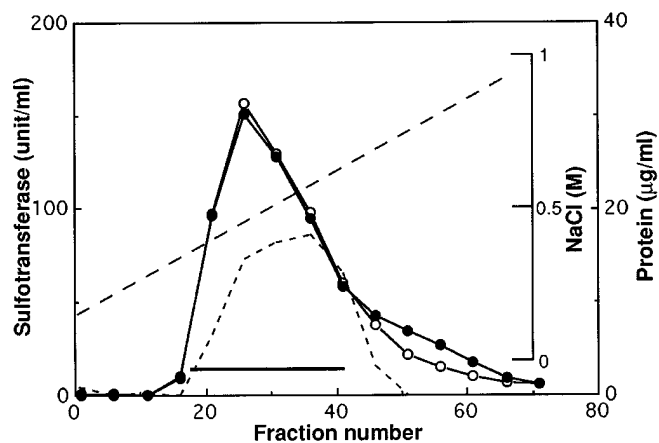


FIG. 2. Second heparin-Sepharose CL-6B chromatography of the first 3',5'-ADP-agarose fractions. The fractions eluted from the first 3',5'-ADP-agarose column with 0.2 mM 3',5'-ADP in buffer A containing 0.05 M NaCl were applied to a heparin-Sepharose column as described under "Experimental Procedures." After wash with buffer A containing 0.15 M NaCl, the column was eluted with a linear gradient of NaCl. Fractions of 4 ml were collected. Sulfotransferase activity in the absence (●) or presence (○) of 10 mM DTT, and protein concentration (---) of each fraction were assayed. The broken line (---) indicates the concentration of NaCl.

ADP-agarose fraction was applied to a Superose 12 column equilibrated with buffer A containing 2 M NaCl. A major peak of HS2ST activity was eluted in the fractions around  $M_r$  130,000 (Fig. 3A). A small peak of the sulfotransferase activity inhibited by DTT was noted in the low molecular weight fractions around  $M_r$  42,000. This elution position was nearly consistent with that of HS6ST described in our previous report (15). In addition, when digested with a mixture of heparitinases,  $^{35}$ S-labeled CDSNS-heparin produced by incubation with [ $^{35}$ S]PAPS and this DTT-inhibited fraction gave a major disaccharide of  $\Delta$ Di-(6)diS (data not shown). The fractions indicated by a horizontal bar in Fig. 3A were pooled, dialyzed as described under "Experimental Procedures," and used for the subsequent experiments as the purified HS2ST.

#### Purity of the Heparan Sulfate 2-Sulfotransferase

SDS-PAGE for the fractions (nos. 35–47) obtained by the Superose 12 chromatography showed that the fractions around a peak of HS2ST activity contained two protein bands that were migrated closely to each other with apparent molecular weights of 47,000 and 44,000, respectively (Fig. 3B). Considering the HS2ST activity in those fractions, these protein bands appeared to correspond to HS2ST. When an aliquot of the step 4 fraction was applied to chromatography on heparin-Sepharose CL-6B and every four fractions around the peak of the HS2ST activity were subjected to SDS-PAGE (Fig. 4), two protein bands of  $M_r$  47,000 and 44,000 appeared exclusively in the fractions containing the HS2ST activity. Their staining intensity with silver nitrate fairly corresponded to the activity, and their relative intensities were almost constant, which further suggested the correspondence of these two protein bands to HS2ST.

Samples at each purification step (0.47  $\mu$ g of protein for each) were also analyzed by SDS-PAGE (Fig. 5A). Both protein bands of  $M_r$  47,000 and 44,000 were stained more intensely with silver nitrate in the samples at the higher fold purification, and were the most predominantly stained in the Superose 12 fraction ("purified HS2ST") (Fig. 5A, lane 7). In addition, the fraction that passed through the second 3',5'-ADP-agarose column and had the little activity exhibited little if any of the two protein bands on SDS-PAGE (Fig. 5A, lane 6). Such a concomitancy between the two protein bands and the HS2ST activity

again suggested that the two protein bands correspond to HS2ST.

When the *N*-glycanase digest of the purified HS2ST was subjected to SDS-PAGE, the two protein bands of 47 and 44 kDa disappeared, but two sharp protein bands of 38 and 34 kDa appeared newly (Fig. 5B), indicating that both proteins are glycoproteins containing almost equal contents of carbohy-

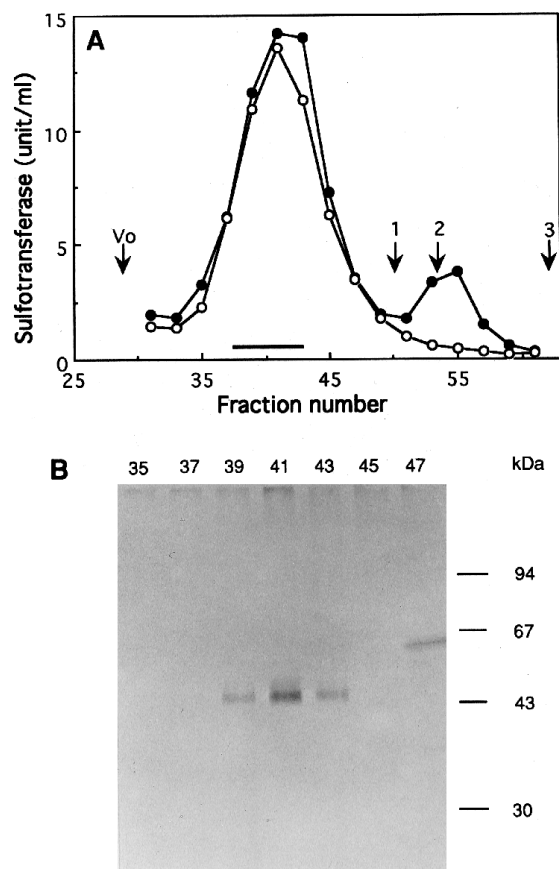


FIG. 3. Superose 12 gel chromatography of the second 3',5'-ADP-agarose fraction. *A*, the fraction eluted from the second 3',5'-ADP-agarose column with 0.2 mM 3',5'-ADP in buffer A containing 0.05 M NaCl was applied to a small heparin-Sepharose CL-6B column equilibrated with 0.1 M NaCl in buffer A. After the column was eluted with 2.5 ml of 1 M NaCl in buffer B, the eluate was concentrated and dialyzed against 1 M NaCl in buffer A. The dialysate was injected into a Superose 12 column and eluted with buffer A containing 2 M NaCl as described under "Experimental Procedures." Fractions of 250  $\mu$ l were collected. Sulfotransferase activity in the absence ( $\bullet$ ) or presence ( $\circ$ ) of 10 mM DTT of each fraction was assayed. The arrows indicate the elution positions of bovine serum albumin (67 kDa) (1), ovalbumin (43 kDa) (2), and chymotrypsinogen A (25 kDa) (3) under the same chromatographic conditions as described above. *B*, aliquots of every other fraction that showed the activity were analyzed by SDS-PAGE. Proteins were visualized with silver nitrate stain. Molecular size standards were phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and soybean trypsin inhibitor (20.1 kDa).

drates (approximately 20%). The result further suggested that both proteins are closely related to each other.

However, it was worthy of note that the molecular weights of the two protein bands were half or one-third as much as the molecular weight of the HS2ST activity determined by gel chromatography on Superose 12 using standard  $M_r$  proteins (Fig. 3A). When the purified HS2ST was again applied to the Superose 12 gel column, which was this time equilibrated with buffer A containing 4 M guanidine HCl (known to be a "dissociative" solvent), the HS2ST activity mostly disappeared in the fractions around  $M_r$  130,000 but, instead of it, a little but significant activity (<0.3% of the starting activity) was recovered in the fractions where ovalbumin (43 kDa) was eluted. Subsequent SDS-PAGE of the fractions revealed that the two proteins of  $M_r$  47,000 and 44,000 newly appeared in the fractions around 43 kDa (data not shown). The observed discrepancy between their elution position on gel chromatography and their mobilities on SDS-PAGE could be explained by the possibility that HS2ST may exist as a dimer or trimer, although other possibilities could also be considered.

**Characterization of Purified Heparan Sulfate 2-Sulfotransferase Specificity for Acceptor Substrate**—The purified fraction of HS2ST was incubated with different acceptors. The purified HS2ST was able to transfer sulfate to CDSNS-heparin and mouse EHS tumor heparan sulfate (Table III). Interestingly,

TABLE III  
Incorporation of [ $^{35}$ S]sulfate from [ $^{35}$ S]PAPS into various glycosaminoglycan acceptors by the purified heparan sulfate 2-sulfotransferase

Incorporation of [ $^{35}$ S]sulfate into the polysaccharide fraction was determined using 0.21 ng of the purified enzyme as described under "Experimental Procedures" except that the reaction mixtures contained various kinds of glycosaminoglycans (25 nmol as hexosamine) instead of CDSNS-heparin. The radioactivity incorporated was expressed as enzyme activity.

Substrate	Activity
	units/ $m^3$ <sup>a</sup>
CDSNS-heparin	7.19 (100) <sup>b</sup>
Heparin	1.44 (20)
Heparan sulfate (mouse EHS tumor)	6.59 (92)
Heparan sulfate (pig aorta)	0.81 (11)
Heparan sulfate (bovine liver)	1.12 (16)
Chondroitin <sup>c</sup>	0
Chondroitin sulfate A	0.05 (0.7)
Chondroitin sulfate C	0
Dermatan sulfate	0
Keratan sulfate	0
CDSNS-heparin + heparin <sup>d</sup>	2.66 (37)
CDSNS-heparin + chondroitin sulfate C <sup>d</sup>	6.61 (92)

<sup>a</sup> One unit of activity is defined as 1 pmol/min sulfate transferred to each glycosaminoglycan used as substrate as described under "Experimental Procedures."

<sup>b</sup> The values in parentheses indicate the percentage of the enzyme activity compared with that of CDSNS-heparin.

<sup>c</sup> The reaction mixture contained 1.25  $\mu$ g of protamine chloride instead of 3.75  $\mu$ g.

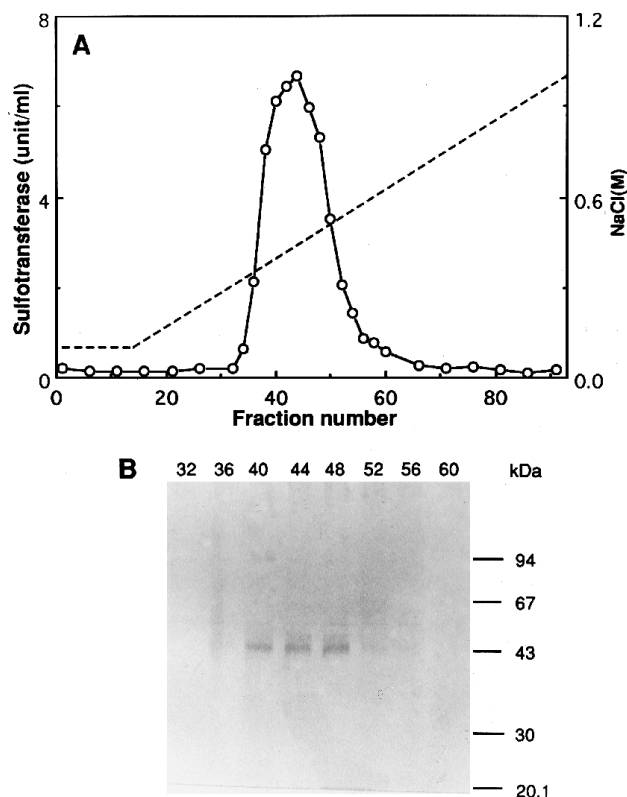
<sup>d</sup> The same amount as SDCNS-heparin of the additional glycosaminoglycan was added to the reaction mixture.

TABLE IV  
Unsaturated disaccharide compositions of heparan sulfates from EHS tumor, pig aorta, and bovine liver

The unsaturated disaccharide compositions of heparan sulfates from EHS tumor, pig aorta, and bovine liver in Table III are expressed as percent of total disaccharides, respectively.

Heparan sulfate	Unsaturated disaccharides					
	$\Delta$ Di-OS	$\Delta$ Di-NS	$\Delta$ Di-6S	$\Delta$ Di-(6)diS	$\Delta$ Di-(U)diS	$\Delta$ Di-(6,U)triS
	% of total					
Mouse EHS tumor <sup>a</sup>	33.6	59.4	0.4	2.6	2.3	0.7
Pig aorta <sup>a</sup>	63.9	18.5	6.0	2.3	4.4	3.8
Bovine liver (highly sulfated fraction)	21.9	12.7	18.2	10.0	3.3	33.9

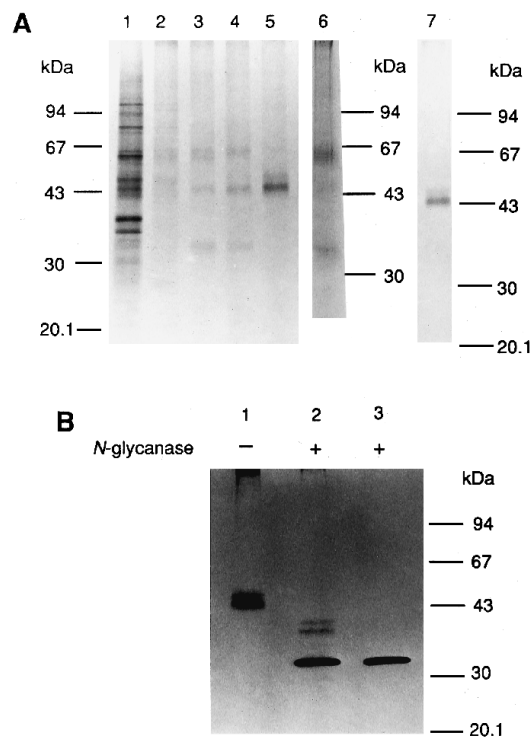
<sup>a</sup> Data were cited from the previous report (4).



**FIG. 4. Heparin-Sepharose CL-6B chromatography of the second 3',5'-ADP-agarose fraction.** *A*, an aliquot of the second 3',5'-ADP-agarose fraction in buffer A containing 0.1 M NaCl was applied to a heparin-Sepharose column (bed volume, 2.5 ml) equilibrated with 0.1 M NaCl in buffer A at a flow rate of 6 ml/h. After wash with 7.5 ml of buffer A containing 0.1 M NaCl, the column was eluted with a linear gradient from 0.1 to 1 M NaCl in buffer A (total volume, 75 ml). Fractions (1 ml) were collected. Sulfotransferase activity (○) in the presence of 10 mM DTT of each fraction was assayed. The broken line indicates the concentration of NaCl. *B*, aliquots of every fourth fraction that showed the activity were analyzed by SDS-PAGE. Proteins were visualized with silver nitrate stain. Molecular size standards were the same as described in Fig. 3*B*.

heparin, heparan sulfate from pig aorta, and heparan sulfate from bovine liver (highly sulfated fraction) were poor acceptors. The disaccharide compositions of these heparan sulfates were compared as shown in Table IV. The enzyme showed no activity toward chondroitin, chondroitin sulfate A, chondroitin sulfate C, dermatan sulfate, and keratan sulfate. Moreover, heparin, which is rich in the unit of the reaction product by HS2ST, inhibited the transfer of sulfate to CDSNS-heparin, while chondroitin sulfate C did not affect the sulfation of CDSNS-heparin.

**Characterization of the Reaction Products**—To determine the position of the sulfate group transferred to CDSNS-heparin or EHS tumor heparan sulfate, <sup>35</sup>S-labeled product derived from each glycosaminoglycan acceptor was digested with a mixture of heparitinase I, II and III, and the digest was then subjected to HPLC on a PAMN column as described under "Experimental Procedures" (Fig. 6). In either case, most of radioactivity was eluted at the position of ΔDi-(U)diS. Furthermore, HPLC on a Partisil-10 SAX column of <sup>35</sup>S-labeled disaccharides produced by nitrous acid degradation at pH 1.5 showed that most of radioactivity was eluted at the position of IdoA(2SO<sub>4</sub>)-AMan<sub>R</sub> and only a slight radioactivity was found in the fractions retarded as a shoulder (Fig. 7). The shoulder may have been in part due to side products generated by anomalous deamination/ring contraction, since it became smaller with the additional mild acid treatment than without the treatment (data not shown) (16, 17). The results indicated that the purified enzyme



**FIG. 5. SDS-PAGE of heparan sulfate 2-sulfotransferase fractions at various purification steps (A) and of the purified heparan sulfate 2-sulfotransferase before and after treatment with N-glycanase (B).** *A*, 0.47 μg of protein was loaded onto each lane. Lane 1, the crude extract; lane 2, protein eluted with a NaCl gradient from the first heparin-Sepharose CL-6B column; lane 3, protein eluted with 0.2 mM 3',5'-ADP from the first 3',5'-ADP-agarose column; lane 4, protein eluted with a NaCl gradient from the second heparin-Sepharose CL-6B column; lane 5, protein eluted with 0.2 mM 3',5'-ADP from the second 3',5'-ADP-agarose column; lane 6, protein that passed through the second 3',5'-ADP-agarose column (no sulfotransferase activity); lane 7, protein eluted with 2 M NaCl in buffer A from Superose 12 column (purified HS2ST). *B*, lane 1, 6.6 ng of protein eluted with 2 M NaCl in buffer A from Superose 12 column (purified enzyme); lane 2, digests of 6.6 ng of the purified enzyme treated with N-glycanase; lane 3, the same amount of N-glycanase alone as in lane 2. Proteins were visualized with silver nitrate stain. Molecular size standards were the same as described in Fig. 3*B*.

catalyzes sulfation of position 2 of the L-iduronic acid residue of IdoA-GlcNSO<sub>3</sub> unit in CDSNS-heparin or EHS tumor heparan sulfate (Fig. 8).

**Properties of the Heparan Sulfate 2-Sulfotransferase**—The pH dependence of HS2ST activity was shown in Fig. 9*A*. The maximal activity was observed at pH 5.4–5.8. The enzyme activity was little affected by DTT up to 10 mM (Fig. 9*B*). NaCl stimulated the enzyme activity (Fig. 9*C*). The maximal activity (approximately 2.3-fold) was observed around 100 mM NaCl. Protamine activated the enzyme activity to the maximal level (approximately 2.7-fold) around 0.05 mg/ml protamine (Fig. 9*D*). The enzyme activity increased with the increase in PAPS concentration up to 1 μM and then reached the maximal level (approximately 310 unit/μg enzyme protein) (Fig. 9*E*). The apparent *K<sub>m</sub>* value of HS2ST for PAPS was  $2.0 \times 10^{-7}$  M.

#### DISCUSSION

We have purified HS2ST to an apparent homogeneous level from the extract of the cultured CHO cells by affinity chromatography with heparin-Sepharose and 3',5'-ADP-agarose. As was also the case with heparan sulfate/heparin *N*-sulfotransferase, HS6ST and chondroitin 6-sulfotransferase (11, 12, 15, 32), affinity chromatography on those columns yielded successful purification of this enzyme. Furthermore, gel chromatogra-

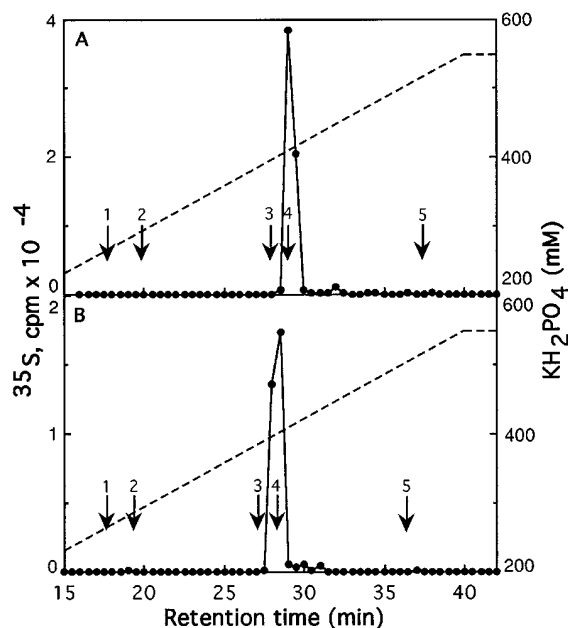


FIG. 6. HPLC on PAMN column of heparitinase-digests of  $^{35}\text{S}$ -labeled products formed from CDSNS-heparin (A) and mouse EHS tumor heparan sulfate (B) by incubation with  $[^{35}\text{S}]\text{PAPS}$  and the purified heparan sulfate 2-sulfotransferase. The products of the sulfotransferase reactions were digested with a mixture of heparinases and subjected to HPLC on a PAMN column as described under "Experimental Procedures." The broken line indicates the concentration of  $\text{KH}_2\text{PO}_4$ . The arrows indicate the elution positions of standards;  $\Delta\text{Di-6S}$  (1),  $\Delta\text{Di-NS}$  (2),  $\Delta\text{Di-(6)diS}$  (3),  $\Delta\text{Di-(U)diS}$  (4), and  $\Delta\text{Di-(6,U)triS}$  (5).

phy on Superose 12 resulted in the effective separation of the HS2ST activity from HS6ST.

The purified HS2ST fraction was found to transfer sulfate exclusively to position 2 of the L-iduronic acid residue of IdoA-GlcNSO<sub>3</sub> unit in CDSNS-heparin or EHS tumor heparan sulfate (Fig. 8), and none of the activity was observed to transfer sulfate to position 2 of D-glucuronic acid residue, position 6 of N-sulfoglucosamine residue or amino group of glucosamine residue. Wlad *et al.* (33) have recently purified ~60-kDa enzyme capable of catalyzing both the 2-O- and 6-O-sulfotransferase reactions from mouse mastocytoma tissue. A proteolytic fragment (~20 kDa) of this original enzyme remains capable of promoting 2-O-sulfation but has lost the 6-O-sulfotransferase activity, indicating that the two reactions are catalyzed by separate active sites derived from a single protein. We previously showed that HS6ST from the culture medium of CHO cells transfers sulfate exclusively to position 6 of N-sulfoglucosamine residue (15). The difference between the reports by Wlad *et al.* and us suggests that our HS6ST and HS2ST from CHO cells are distinct from their enzyme from mouse mastocytoma tissue capable of catalyzing both the 2-O- and 6-O-sulfotransferase reactions. Supposing that HS2ST and HS6ST be derived from a composite enzyme containing distinct domains, each committed to a specific O-sulfotransferase reaction, the composite enzyme would be expected to have a molecular mass of 90–100 kDa. This again suggests that our sulfotransferases may be genetically different from the sulfotransferase purified by Wlad *et al.* It is very likely that sulfotransferases prepared from CHO cells are engaged in the biosynthesis of heparan sulfate and the enzyme from mouse mastocytoma tissue is mainly involved in the biosynthesis of heparin. This is also the case with N-sulfotransferases (20–23). Considering these results, the molecular organization of the O-sulfation process may differ between heparin and heparan sulfate.

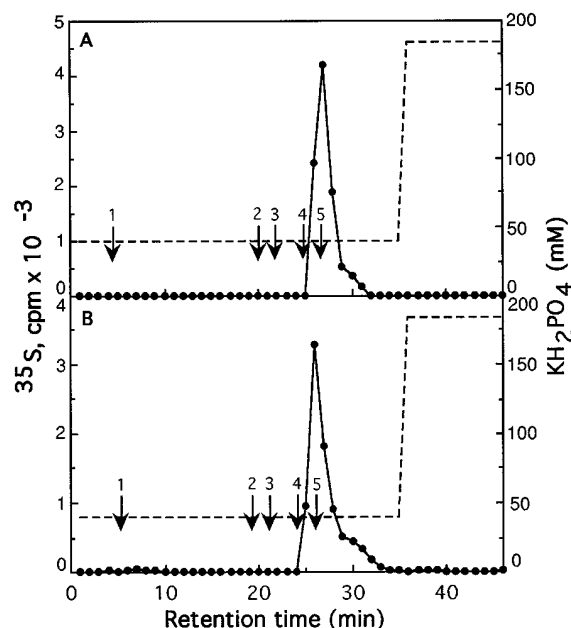


FIG. 7. HPLC on Partisil-10 SAX column of disaccharides produced by nitrous acid degradation at pH 1.5 of  $^{35}\text{S}$ -labeled products formed from CDSNS-heparin (A) and mouse EHS tumor heparan sulfate (B) by incubation with  $[^{35}\text{S}]\text{PAPS}$  and the purified heparan sulfate 2-sulfotransferase. The products of sulfotransferase reaction were degraded by nitrous acid at pH 1.5 and treated with mild acid, and subjected to gel filtration on a Superdex 30. Then the disaccharide fraction was applied to a Partisil-10 SAX column as described under "Experimental Procedures." The broken line indicates the concentration of  $\text{KH}_2\text{PO}_4$ . The arrows indicate the elution positions of HexA-AMan<sub>R</sub> (1), GlcA(2SO<sub>4</sub>)-AMan<sub>R</sub> (2), GlcA-AMan<sub>R</sub>(6SO<sub>4</sub>) (3), IdoA-AMan<sub>R</sub>(6SO<sub>4</sub>) (4), and IdoA(2SO<sub>4</sub>)-AMan<sub>R</sub> (5).

Modification reactions in the biosynthesis of heparin/heparan sulfate are thought to occur in the following sequences: N-deacetylation/N-sulfation of glucosamine, epimerization of D-glucuronic to L-iduronic acid, 2-O-sulfation of L-iduronic acid, and finally, 6-O- and 3-O-sulfation of glucosamine residue (1). In these sequential reactions, each product in the respective reaction becomes the substrate for the subsequent reaction and may be controlled by the substrate specificities of the enzymes involved. HS6ST has a capacity to catalyze 6-O-sulfation of N-sulfoglucosamine residue irrespective of the 2-O-sulfation of the neighboring L-iduronic acid residue (15), while HS2ST is unable to catalyze 2-O-sulfation of L-iduronic acid residue adjacent to GlcNSO<sub>3</sub>(6SO<sub>4</sub>) residue. This difference strongly supports the above modification reaction sequence. In relation to this control mechanism, it should be of note that HS2ST was much less active toward heparan sulfates from pig aorta and bovine liver (Table III). There seem to be some differences in the acceptor efficiency between EHS tumor heparan sulfate and the above two heparan sulfates as the good and poor acceptor substrates, respectively. The content of HexA-GlcNSO<sub>3</sub> unit in heparan sulfate may be one of factors affecting the substrate efficiency, because the content of this unit in EHS tumor heparan sulfate is the highest (Table IV). However, heparan sulfates from pig aorta and bovine liver contain significant amount of HexA-GlcNSO<sub>3</sub> unit, although they are very poor acceptors, suggesting that other factors such as the higher content of IdoA-GlcNSO<sub>3</sub> unit or a longer oligosaccharide sequence containing IdoA-GlcNSO<sub>3</sub> unit may be required for the recognition by HS2ST. Furthermore, HS2ST may be negatively controlled by its reaction products, IdoA(2SO<sub>4</sub>)-GlcNSO<sub>3</sub> and IdoA(2SO<sub>4</sub>)-GlcNSO<sub>3</sub>(6SO<sub>4</sub>) units, because heparin, a form of heparan sulfate containing the highest contents of these units, appeared to act as an inhibitor and, in addition, heparan sul-

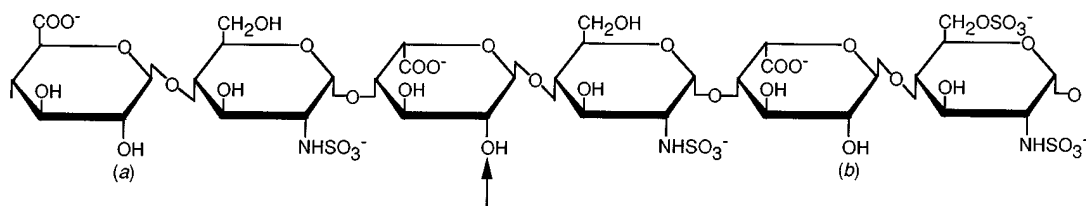


FIG. 8. Sulfation site in heparan sulfate by heparan sulfate 2-sulfotransferase. An arrow indicates the sulfation site by purified HS2ST. Sulfations of position 2 of GlcA (a) and of position 2 of IdoA adjacent to GlcNSO<sub>3</sub>(6SO<sub>4</sub>) (b) were not observed.

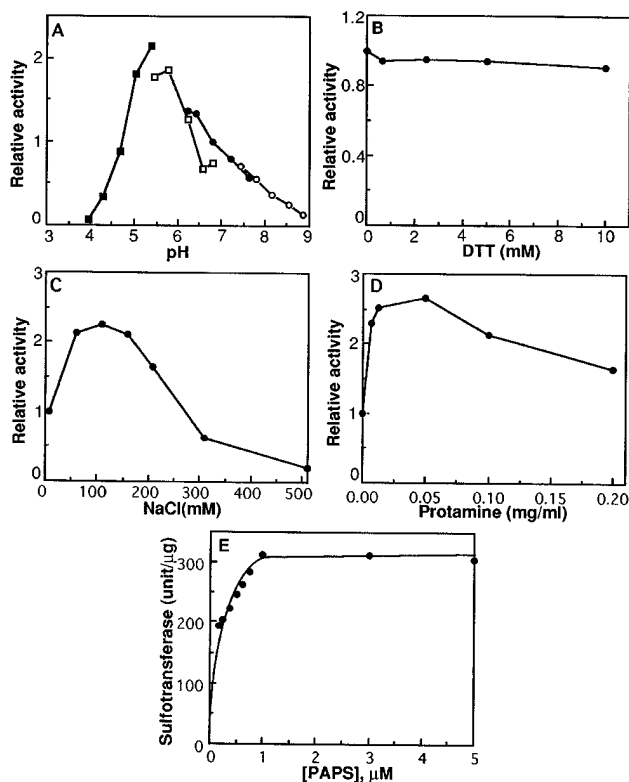


FIG. 9. Properties of the purified heparan sulfate 2-sulfotransferase. Sulfotransferase activities were determined using 0.21 ng of the purified enzyme as described under "Experimental Procedures." A, pH dependence of the sulfotransferase activity. The pH of the reaction mixtures was varied using 2.5 μmol of Tris-HCl (○), 2.5 μmol of imidazole HCl (●), 2.5 μmol of Mes (□), or 2.5 μmol of potassium acetate (■) buffer. Relative activity was expressed as a ratio to the sulfotransferase activity obtained from the standard reaction mixture. B, effect of DTT on the sulfotransferase activity. The reaction mixtures contained various amounts of DTT. Relative activity was expressed as a ratio to the sulfotransferase activity obtained from the standard reaction mixture without DTT. C, effect of NaCl on the sulfotransferase activity. The reaction mixtures contained various concentrations of NaCl. Relative activity was expressed as a ratio to the sulfotransferase activity obtained from the standard reaction mixture without NaCl. D, effect of protamine on the sulfotransferase activity. The reaction mixtures contained various amounts of protamine. Relative activity was expressed as a ratio to the sulfotransferase activity obtained from the standard reaction mixture without protamine. E, dependence of the sulfotransferase activity on PAPS concentration. Sulfotransferase activities were determined using 0.15 ng of the purified enzyme as described under "Experimental Procedures" except that the reaction mixtures contained various amounts of PAPS.

fates from pig aorta and bovine liver, poorer acceptors had the relatively higher contents of IdoA(2SO<sub>4</sub>)-GlcNSO<sub>3</sub> and IdoA(2SO<sub>4</sub>)-GlcNSO<sub>3</sub>(6SO<sub>4</sub>) units (8.2 and 37.2%, respectively) than EHS tumor heparan sulfate (3.0%) (Table IV). Since the purified HS2ST showed no activity toward dermatan sulfate, the enzyme does not appear to transfer sulfate to position 2 of L-iduronic acid residue adjacent to N-acetylgalactosamine residue.

It is interesting to compare the properties of HS2ST with

those of other purified glycosaminoglycan sulfotransferase. DTT had little effect on HS2ST, while it substantially inhibited HS6ST (15). This finding was useful to monitor activities of both sulfotransferases separately in the present study. In contrast, DTT stimulated chondroitin 4-sulfotransferase (34). HS2ST appeared to exist as a dimer or trimer, because the enzyme behaved on gel chromatography 2–3 times larger than on SDS-PAGE (Fig. 3). However, considering the effect of Triton X-100 micelles on molecular mass determinations of membrane proteins, there is another possibility that HS2ST may exist as a monomer. Mass determinations for proteins embedded in the micelles might have caused the overestimation by the mass of the detergent micelle. The guanidine treatment would have dissociated the micelles, and, therefore, the enzyme might have run as a monomer independently of the detergent. Similarly to HS2ST, chick chondrocyte chondroitin 6-sulfotransferase apparently showed a single broad protein band of  $M_r$  75,000 on SDS-PAGE although it was eluted at the position with an apparent molecular mass of 160,000 on gel chromatography (32, 35). A majority of proteins intrinsic to the Golgi apparatus membrane appear to be dimers *in situ* (36). However, this is not always the case. HS6ST from CHO cells may be a monomer (15). Rat liver heparan sulfate *N*-deacetylase/*N*-sulfotransferase is also a monomer (11, 37). The apparent  $K_m$  value for PAPS of the purified HS2ST was  $2.0 \times 10^{-7}$  M, which was in the same order of that of HS6ST ( $4.4 \times 10^{-7}$  M) (15). In contrast, that of heparan sulfate *N*-sulfotransferase of rat liver was  $1.08 \times 10^{-4}$  M (38). HS2ST as well as HS6ST appeared to have a higher affinity for PAPS than heparan sulfate *N*-sulfotransferase. The large difference in the  $K_m$  value for PAPS between our sulfotransferases and heparan sulfate *N*-sulfotransferase, however, might be due to the differences in the assay conditions used. For example, we added cationic activator, protamine, to the assay mixture in this study, while the assay mixture reported for heparan sulfate *N*-sulfotransferase (38) included Mg<sup>2+</sup> and Mn<sup>2+</sup>, instead of protamine. We previously showed that cationic proteins such as protamine and histone stimulated chondroitin 6-sulfotransferase by decreasing the  $K_m$  value for PAPS (27). The low  $K_m$  values for PAPS of HS2ST as well as HS6ST may have been caused by the presence of protamine.

SDS-PAGE of the purified HS2ST gave only two protein bands of 47 and 44 kDa, which were both sensitive to *N*-glycanase digestion to the same extent (Fig. 5), as was also observed with HS6ST (15) and chondroitin 6-sulfotransferase (32, 35). Both proteins were always comigrated in the SDS gel whenever the fractions containing the HS2ST activity were subjected to SDS-PAGE (see Figs. 3 and 4 for the Superose 12 column chromatography and the heparin-Sepharose CL-6B column chromatography, respectively). These findings strongly support that both the 47- and 44-kDa proteins bear HS2ST activity and the size difference may be due to some protein modification such as limited proteolytic cleavage as we discussed previously in the case of HS6ST (15). Several glycosyltransferases that exhibit catalytically active multiple forms with different molecular weights have been shown to be de-

rived from the single genes (39–42). However, since our several trials to detect the HS2ST activity in the gel segments after SDS-PAGE of the purified HS2ST were unsuccessful, probably owing to a difficulty of renaturation of the enzyme activity (data not shown), one could still argue that either or both of the 47- and 44-kDa proteins represent contaminants. Relating to this possibility, one could also argue that the extent of purification was rather low (51,700-fold), compared with those of other Golgi enzymes. Because HS6ST was purified 10,700-fold to apparent homogeneity from the culture medium of CHO cells, the obtained extent of purification for the HS2ST from the Triton X-100 extract of cultured CHO cells is not far from the expected range.

It should be noted that in cultured CHO cells more than 90% of the HS6ST activity was secreted into the medium, while 97% of the HS2ST activity was retained in the cells. Several reports have shown that the stem regions are cleaved off proteolytically when proteins originally present in the Golgi apparatus such as glycosyltransferases are secreted (42, 43). The molecular cloning of chondroitin 6-sulfotransferase suggests that the enzyme may be released from the Golgi apparatus by proteolytic cleavage at the specific sequence of the transmembrane domain (35). HS6ST may have such a sequence, while HS2ST may lack it. Molecular cloning of both sulfotransferases should provide us with a clue to answer these possibilities.

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