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Sulfation of sialyl lactosamine oligosaccharides by chondroitin 6-sulfotransferase

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We have previously shown that chondroitin 6-sulfotransferase (C6ST) catalyzes transfer of sulfate not only to position 6 of GalNAc residue of chondroitin but also to position 6 of Gal residue of keratan sulfate. In this study, we examined the sulfation of sialyl lactosamine oligosaccharides by C6ST. C6ST catalyzed transfer of sulfate to NeuAcα2–3Galβ1–4GlcNAc (SLN), NeuAcα2–3Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc (SL1L1), NeuAcα2–3Galβ1–4(6-sulfo)GlcNAcβ1–3(6-sulfo)Galβ1–4(6-sulfo)GlcNAc (SL2L4), and their desialylated derivatives, but not to NeuAcα2–3Galβ1–4(Fucα1–3)GlcNAc (SLe^x). The sulfated product formed from SLN was degraded with neuraminidase and reduced with $NaBH_4$. The resulting sulfated disaccharide alditol showed the same retention time in SAX-HPLC as that of [^{3}H]Gal($6SO_4$)β1–4GlcNAc-ol. The sulfated product formed from SLN was also degraded by a reaction sequence of neuraminidase digestion, hydrazinolysis, deamination, and $NaBH_4$ reduction. The final product was coeluted with [^{3}H]Gal($6SO_4$)β1–4anhydromannitol in SAX-HPLC. These observations show that C6ST could transfer sulfate to position 6 of Gal residue of SLN. Incorporation of sulfate into SL2L4 was much higher than the incorporation into SL1L1, suggesting that sulfate moiety attached to adjacent GlcNAc residue may stimulate the transfer of sulfate to Gal residue. The recombinant C6ST also catalyzed sulfation of the sialyl lactosamine oligosaccharides, indicating that a single protein catalyzes sulfation of chondroitin, keratan sulfate, and sialyl lactosamine oligosaccharides. These results raised a possibility that C6ST may be one of the candidates involved in the biosynthesis of sulfated sialyl Lewis x ligand for L-selectin.

Key words: keratan sulfate/L-selectin/sialyl lactosamine/sialyl Lewis x/sulfotransferase

Introduction

Chondroitin 6-sulfotransferase (C6ST), which catalyzes transfer of sulfate to position 6 of GalNAc residue of chondroitin, was purified from the culture medium of chick chondrocytes (Habuchi *et al.*, 1993), and the cDNA of C6ST was cloned (Fukuta *et al.*, 1995). When the cloned cDNA was transfected in COS-7 cells, the expressed sulfotransferase transfers sulfate not only to position 6 of GalNAc residue of chondroitin but also to keratan sulfate (Fukuta *et al.*, 1995). We showed that position 6 of Gal residue of keratan sulfate was sulfated by the purified sulfotransferase (Habuchi *et al.*, 1996).

L-Selectin is a lectin-like cell adhesion molecule involved in the recruitment of leukocyte into lymphoid tissues and site of inflammation (Lasky, 1992, 1995; Bevilacqua and Nelson, 1993). L-Selectin recognizes wide spectrum of oligosaccharides including sialylated, fucosylated and sulfated structures (Varki, 1994; Green *et al.*, 1995; Lasky, 1995). GlyCAM-1 is one of high endothelial venule-associated ligands (Imai *et al.*, 1991; Lasky *et al.*, 1992) and was reported to contain *O*-linked sugar chains containing sulfated sialyl Lewis x structure (Hemmerich and Rosen, 1994; Hemmerich *et al.*, 1995). The presence of sulfate group is thought to be essential for high-affinity binding to L-selectin (Imai *et al.*, 1993). Structural analysis of GlyCAM-1 has identified Gal($6SO_4$) and GlcNAc($6SO_4$) as the major sulfated sugars (Hemmerich *et al.*, 1994). Sulfotransferase that is able to transfer sulfate to Gal or GlcNAc residue of sialyl Lewis x oligosaccharide, however, has not been reported so far. Since sialyl Lewis x oligosaccharide contains Galβ1–4GlcNAc structure, which is a repeating carbohydrate skeleton of keratan sulfate, we are interested in the possibility that C6ST may play a role in the sulfation of Gal residue of sialyl Lewis x oligosaccharide. To test the possibility, we investigated whether or not oligosaccharides containing sialyl Lewis x–related structure could be sulfated *in vitro* by C6ST, and found that oligosaccharides with NeuAcα2–3Galβ1–4GlcNAc moiety served as acceptor for C6ST.

Results

Incorporation of $^{35}SO_4$ into various oligosaccharide acceptors

Structure and abbreviation of oligosaccharides used in this report are shown in Table I. When these oligosaccharides were incubated with the purified C6ST (90 ng as protein), radioactive products were separated by Superdex 30 chromatography (Figure 1). The retention time of the sulfated products were 2–3 min faster than the retention time of acceptors used (which are shown by arrows in Figure 1). When SLe^x was used as acceptor, the retention time of the sulfated product was expected to be 83–84 min, but no radioactivity was detected around the retention time. When [^{35}S]PAPS used for this experiment was applied to this column, ^{35}S-radioactivity appeared at about 85 min and peaked at 102 min; therefore, ^{35}S-radioactivity observed in the presence of the boiled enzyme seemed to be the contamination of [^{35}S]PAPS or $^{35}SO_4$. The radioactive products formed from LN overlapped with the trail of the peak of $^{35}SO_4$ and [^{35}S]PAPS since significant radioactivity was detected in the boiled enzyme control. The contaminated radioactivity contained in the sulfated LN fraction was clearly removed by paper electrophoresis (data not shown). Incorporation of $^{35}SO_4$ into these oligosaccharides are shown in Table II. Incorporation of $^{35}SO_4$ into oligosaccharides with sialic acid at their nonreducing termini were slightly larger than those into the corresponding oligosaccharides without sialic acid. Among these oligosaccharides, incorporation of $^{35}SO_4$ was the highest in SL2L4; suggesting that sulfate group

Table I. Structure of sialyl lactosamine oligosaccharides used for acceptors of C6ST

Abbreviation	Structure
LN	Galβ1–4GlcNAc
SLN	NeuAcα2–3Galβ1–4GlcNAc
SLe^x	NeuAcα2–3Galβ1-4(Fucα1–3)GlcNAc
L1L1	Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc
SL1L1	NeuAcα2–3Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc
L2L4	Galβ1–4GlcNAc(6SO$_4$)β1–3Gal(6SO$_4$)β1–4GlcNAc(6SO$_4$)
SL2L4	NeuAcα2–3Galβ1–4GlcNAc(6SO$_4$)β1–3Gal(6SO$_4$)β1–4GlcNAc(6SO$_4$)

attached to adjacent GlcNAc residue may stimulate incorporation of sulfate to Gal residue. Comparison of the kinetic parameters between SL2L4 and SL1L1 could not be achieved, since a typical saturation curve was not obtained when SL1L1 was used as acceptor. The rate of incorporation of $^{35}SO_4$ into SL2L4 was about 1.5% of the rate of incorporation into keratan sulfate under the same conditions.

Structural analyses of ^{35}S-labeled SLN

To determine the position to which $^{35}SO_4$ was transferred to SLN, we degraded the radioactive product formed from SLN with neuraminidase and reduced with $NaBH_4$. After neuraminidase digestion, a small portion of radioactivity was remained at the position of the sulfated SLN (Figure 3B). This peak was degraded by further neuraminidase digestion with slower rate. The fractions indicated by a horizontal bar in Figure 3B were used for the next step. Molecular size of the resulting desialylated reduced material seems to be identical to that of [^{3}H]Galβ1–4GlcNAc$_R$(6SO$_4$) as judged by elution profile from Superdex 30 column (data not shown). When applied to Partisil 10-SAX HPLC, the ^{35}S-labeled desialylated reduced material was eluted at the position of [^{3}H]Gal(6SO$_4$)β1–4GlcNAc$_R$ and no ^{35}S-radioactivity was detected at the position of [^{3}H]Galβ1–4GlcNAc$_R$(6SO$_4$) (Figure 2C). After digestion with β-galactosidase, ^{3}H-radioactivity of [^{3}H]Galβ1–4GlcNAc$_R$(6SO$_4$) was disappeared and shifted to the position of [^{3}H]GlcNAc$_R$(6SO$_4$), but the elution profile of ^{35}S-radioactivity was not altered at all (Figure 2D). These results indicates that $^{35}SO_4$ was transferred to Gal residue, but not to GlcNAc residue.

The ^{35}S-labeled SLN was also degraded by a reaction sequence of neuraminidase digestion, *N*-deacetylation, deamination and $NaBH_4$ reduction, and the degradation product was identified with the HPLC. The reaction products obtained in each step were separated by Superdex 30 chromatography (Figure 3) and paper electrophoresis (Figure 4). After *N*-deacetylation, two radioactive products were detected in paper electrophoresis (Figure 4C). The slower-moving peak (indicated by a horizontal bar in Figure 4C) was thought to be deacetylated product because newly appeared free amino group should decrease negative charge, and was subjected to deamination reaction. The faster migrating peak seemed to be by-products because little ^{35}S-radioactivity remained at the position of the unreacted material in Superdex 30 chromatography (Figure 3C). The proportion of the materials was not altered when the reaction with 70% hydrazine/hydrazine sulfate was continued up to 6 h. We did not characterize the faster migrating materials further. After deamination and reduction, most of the radioactive materials moved faster (Figure 4D) as is expected from the loss of positive charge by amino group. When the material obtained after deamination and reduction was separated by the HPLC, ^{35}S-radioactivity was coeluted with [^{3}H]Gal(6SO$_4$)β1–4AMan$_R$ but no ^{35}S-radioactive peak was detected at the position of [^{3}H]Galβ1–4AMan$_R$(6SO$_4$) (Figure 5), Taken together, it is most probable that $^{35}SO_4$ was transferred to position 6 of Gal residue, but not to GlcNAc residue.

Sensitivity of ^{35}S-labeled products derived from SL1L1 and SL2L4 to β-galactosidase digestion

To obtain the information about the location of $^{35}SO_4$ transferred to SL1L1 and SL2L4, we investigated the sensitivity of the sulfated products to β-galactosidase digestion. ^{35}S-Labeled products derived from SL1L1 or SL2L4 were digested with neuraminidase to remove the terminal sialic acid, and the desialylated materials were separated by paper electrophoresis. The desialylated products derived from SL1L1 and SL2L4 were mixed with nonradioactive L1L1 and nonradioactive L2L4, respectively, and digested with β-galactosidase. The mixture of ^{35}S-labeled desialylated products and the nonradioactive oligosaccharides was applied to the Superdex 30 column before or after digestion with β-galactosidase. The eluate from the column was monitored by absorption at 210 nm and ^{35}S-radioactivity (Figures 6, 7). Before β-galactosidase digestion, the ^{35}S-labeled desialylated materials derived from SL1L1 and SL2L4 were eluted at the position of sulfated L1L1 (Figure 6A) and sulfated L2L4 (Figure 7A), respectively, indicating that desialylation proceeded completely. After β-galactosidase digestion of the mixture of ^{35}S-labeled desialylated material derived from SL1L1 and nonradioactive L1L1, the absorption at 210 nm due to nonradioactive L1L1 was completely shifted to a more retarded position (Figure 6D), whereas about one-third of the ^{35}S-radioactivity was still eluted at the original position (Figure 6B). Since β-galactosidase is unable to cleave the glycosidic bond if the galactose is sulfated, these results suggest that about one-third of $^{35}SO_4$ transferred to SL1L1 was located to nonreducing end side Gal residue. When ^{35}S-labeled sulfated L1L1 was prepared using L1L1 as sulfate acceptor and subjected to β-galactosidase digestion, the proportion of the ^{35}S-labeled materials resistant to β-galactosidase digestion was not significantly altered (data not shown). These results suggest that the nonreducing terminal sialic acid may not affect the distribution of sulfate transferred. In contrast, the ^{35}S-labeled desialylated materials derived from SL2L4 was totally insensitive to β-galactosidase (Figure 7B), while nonradioactive L2L4 was completely degraded (Figure 7D). All of the sulfate group transferred to SL2L4 thus must be located to nonreducing end Gal residue.

Sulfation of oligosaccharides with recombinant C6ST

When SL2L4, SL1L1 and SLN were incubated with the recombinant C6ST (180 μg as protein) under the conditions described under *Materials and methods*, essentially the same elution profiles as those shown in Figure 1 were obtained (Figure 8). The rate of sulfation of SL2L4 was about 1.5% of the rate of sulfation of keratan sulfate as observed in the purified C6ST. Nonsulfated oligosaccharides (SL1L1 and SLN) were also sulfated by the recombinant C6ST, but these oligosaccharides served as poorer acceptors for the recombinant C6ST than for the purified C6ST; the ratios of the incorporation of sulfate into SL1L1 and SLN to the incorporation into SL2L4 were 0.028 and 0.008, respectively.

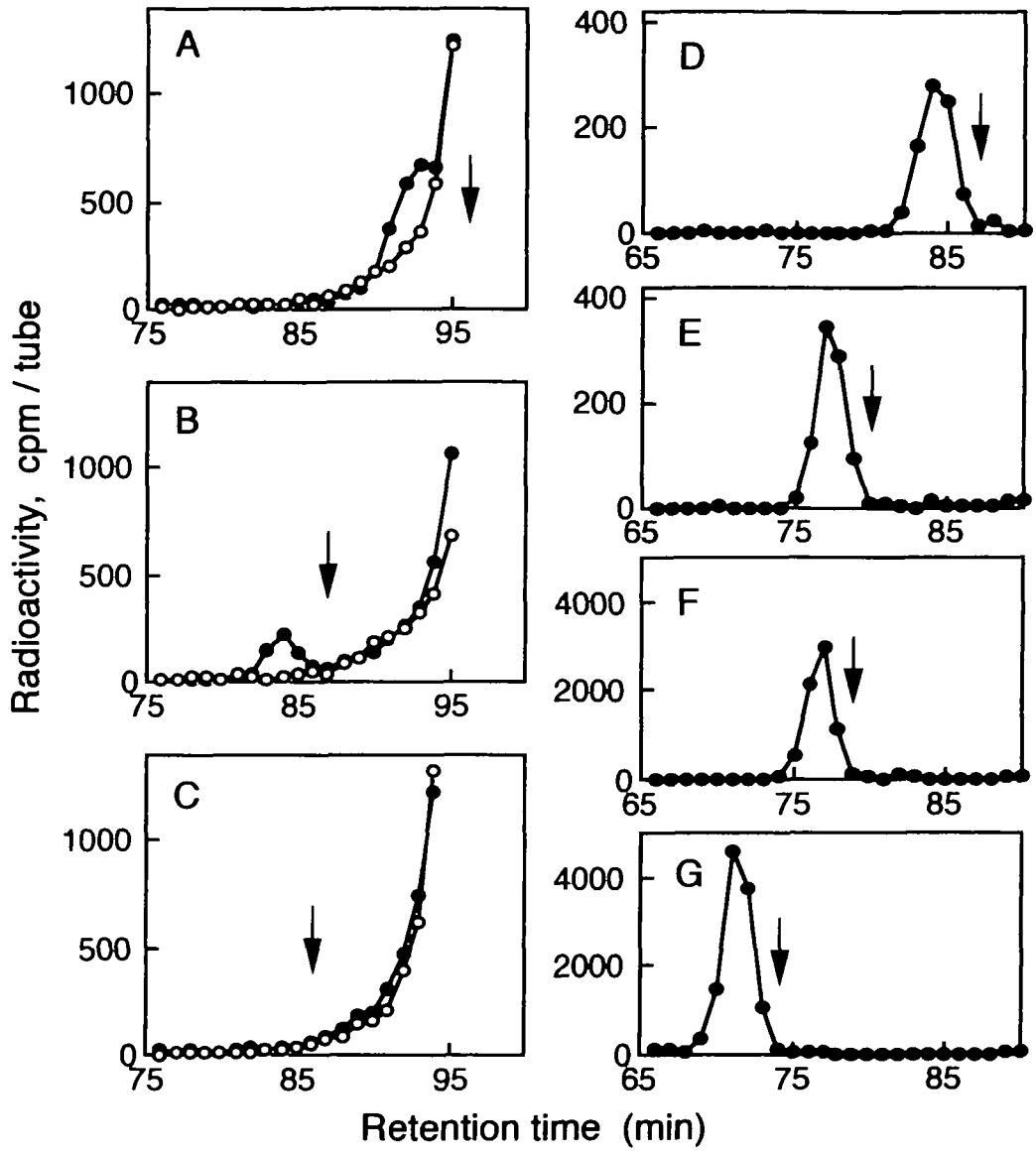

Fig. 1. Superdex 30 chromatography of ^{35}S-labeled oligosaccharides formed from LN (A), SLN (B), SLe^x (C), L1L1 (D), SL1L1 (E), L2L4 (F), and SL2L4 (G). Sulfotransferase reaction was carried out as described under *Materials and methods* using 0.09 μg of the purified C6ST. One milliliter fractions were collected, and radioactivity was determined. Arrows indicate the elution positions of oligosaccharides used as acceptors. (A–C) the purified C6ST was added to the reaction mixture before (solid circle) or after (open circle) heat inactivation for 2 min at 100°C; and (D–G) values observed in the boiled enzyme control were subtracted.

Discussion

In this report we showed evidence that oligosaccharides with NeuAcα2–3Galβ1–4GlcNAc moiety could be sulfated by C6ST *in vitro* at position 6 of Gal residue adjacent to sialic acid. Incorporation of $^{35}SO_4$ into oligosaccharides with sialic acid at their nonreducing termini were slightly larger than those into the corresponding oligosaccharides without sialic acid, suggesting that sialic acid attached at the nonreducing termini may stimulate C6ST activity. These observation seems to be consistent with the results obtained by the study of sialyltransferase (Chandrasekaran *et al.*, 1995), in which transfer of sialic acid by sialyltransferase did not occur when Gal residue was replaced by sulfate. Study of biosynthesis of GlyCAM-1 also suggests that sialic acid must be introduced before sulfation of Gal residue (Crommie and Rosen, 1995). The observations that C6ST could transfer sulfate to SLN, but not to SLe^x suggest that fucose attached to GlcNAc may inhibit the transfer of

Table II. Incorporation of $^{35}SO_4$ into sialyl lactosamine oligosaccharides; incorporation of $^{35}SO_4$ was calculated from the elution profiles shown in Figure 1

Acceptors	Incorporation of $^{35}SO_4$ (pmol/min/μg protein)
LN	0.012
SLN	0.022
SLe^x	ND[a]
L1L1	0.031
SL1L1	0.034
L2L4	0.29
SL2L4	0.48

[a]Not detected.

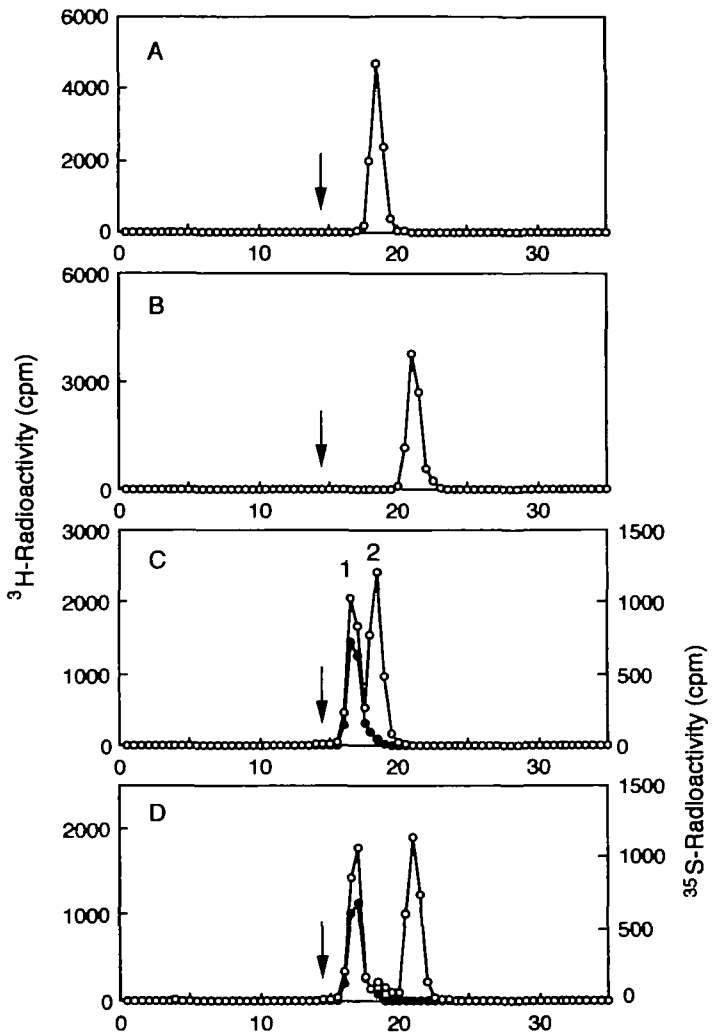

Fig. 2. HPLC separation of the desialylated and reduced product derived from ^{35}S-labeled SLN. ^{35}S-labeled SLN was digested with a neuraminidase and reduced with $NaBH_4$ as described under *Materials and methods* and subjected to HPLC using a Partisil 10-SAX column. Radioactivity of ^{3}H (open circle) and ^{35}S (solid circle) of each fraction was determined. (A) [^{3}H] Galβ1–4GlcNAc$_R$(6SO$_4$); (B) GlcNAc$_R$(6SO$_4$); (C) desialylated and reduced product derived from ^{35}S-labeled SLN mixed with a mixture of [^{3}H]Gal(6SO$_4$)β1–4GlcNAc$_R$ and [^{3}H]Galβ1–4GlcNAc$_R$(6SO$_4$), which was prepared from [^{3}H]Gal(6SO$_4$)β1–4GlcNAc$_R$(6SO$_4$) by the partial acid hydrolysis; and (D) the same as (C) except that the sample was applied to HPLC after β-galactosidase digestion. The arrows indicate the elution position of ΔDi-OS$_R$ used as an internal standard. Peak 1 and peak 2 in (C) were assigned as [^{3}H] Gal(6SO$_4$)β1–4GlcNAc$_R$ and [^{3}H] Galβ1–4GlcNAc$_R$(6SO$_4$), respectively.

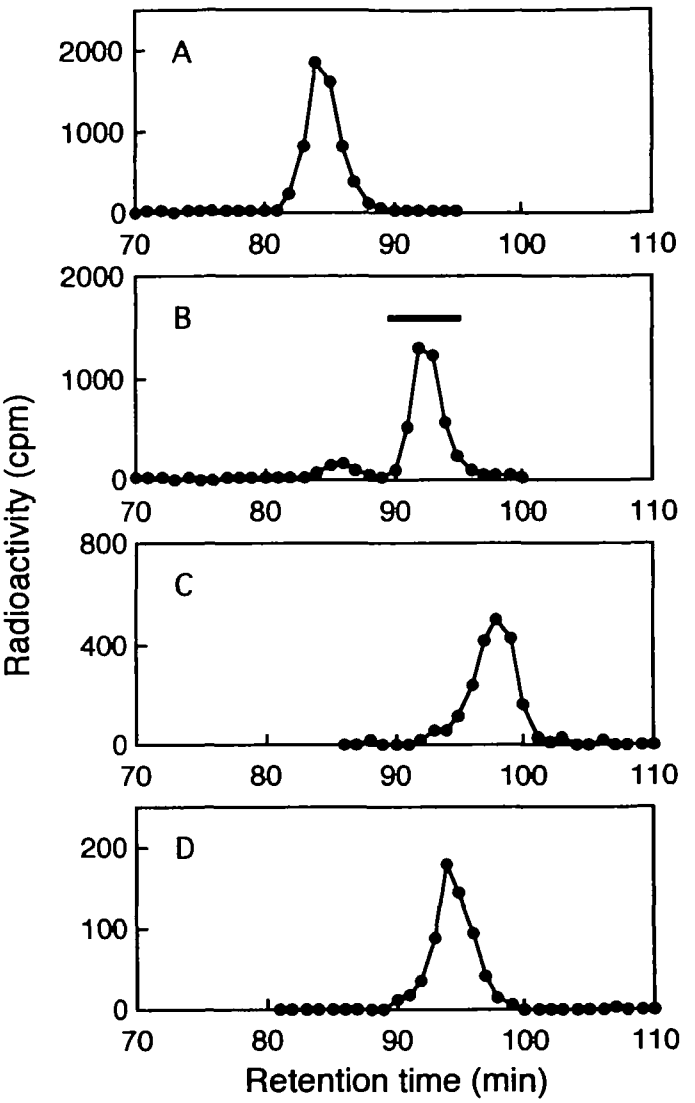

Fig. 3. Separation by Superdex 30 chromatography of the products formed from ^{35}S-labeled SLN after sequential reactions. ^{35}S-Labeled SLN was prepared as described under *Materials and methods* and sequentially degraded. (A) Intact ^{35}S-labeled SLN; (B) after digestion with neuraminidase; (C) after hydrazinolysis; and (D) after deamination and $NaBH_4$ reduction. After neuraminidase digestion, most radioactivity was shifted to a more retarded position but a small peak was remained around 86 min. The major peak (indicated by a horizontal bar in B) was used for the next step.

sulfate to adjacent Gal. Crommie and Rosen reported that transfer of fucose and sulfate occurred after transfer of sialic acid, but the order of fucosylation and sulfation remained obscure (Crommie and Rosen, 1995). Recombinant α1–3 fucosyltransferase V was reported to transfer fucose to GlcNAc(6SO$_4$) residue in NeuAcα2–3Galβ1–4GlcNAc-(6SO$_4$)β1–3Gal (Scudder *et al.*, 1994). Maly *et al.* (Maly *et al.*, 1996) showed that recombinant α1–3 fucosyltransferase VII transferred fucose *in vitro* to NeuAcα2–3Galβ1–4GlcNAc-(6SO$_4$) but not to NeuAcα2–3Gal(6SO$_4$)β1–4GlcNAc. Considering the two independent observations that C6ST could not transfer sulfate to fucosylated oligosaccharide and that NeuAcα2–3Gal(6SO$_4$)β1–4GlcNAc was inactive as a substrate for fucosyltransferase VII, a sulfotransferase whose substrate specificity is different from that of C6ST may be present and involve in the sulfation of Gal residue of sialyl Lewis x. Alternatively, C6ST or C6ST-like sulfotransferase may involve in the sulfation of Gal residue of sialyl Lewis x after GlcNAc residue was sulfated because oligosaccharides bearing sulfate on adjacent GlcNAc residue, such as SL2L4, were much better acceptors for C6ST than nonsulfated oligosaccharides. It remains to be determined whether Gal residue neighboring sulfated fucosylated GlcNAc residue is active as a substrate for C6ST. Since it is not examined whether C6ST is able to transfer sulfate to oligosaccharides attached to an intact N- or O-linked glycoprotein or glycolipid, involvement of C6ST in the biosynthesis of sulfated sialyl Lewis x at present remains hypothetical. More studies will be required to clear the function of C6ST.

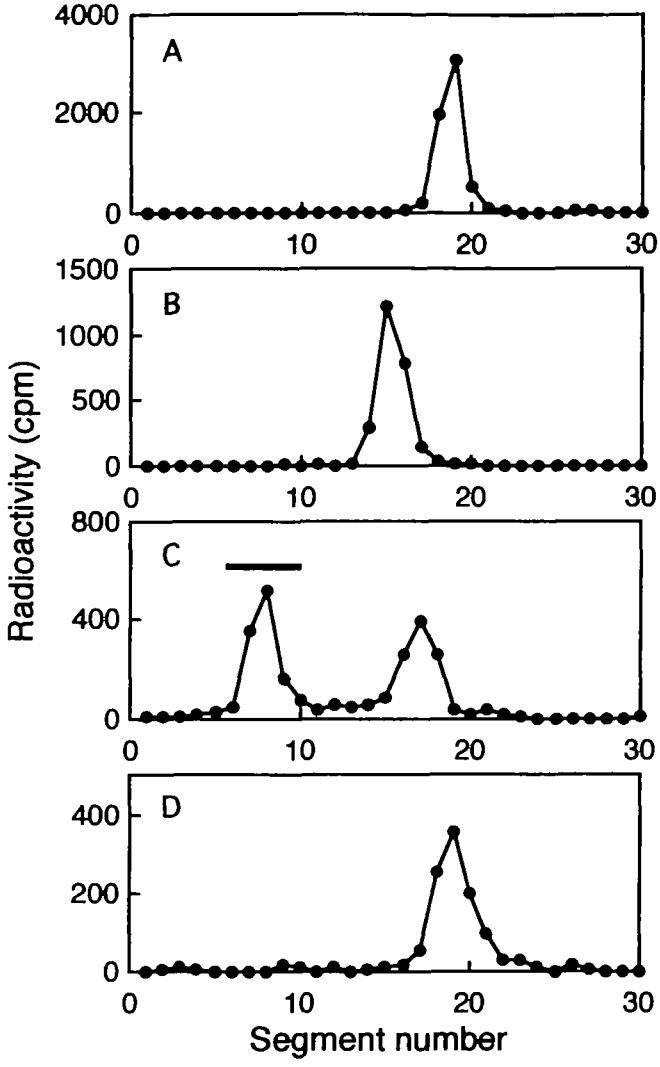

Fig. 4. Separation by paper electrophoresis of the products formed from ^{35}S-labeled SLN after sequential reactions. Samples obtained after each reaction step were separated with Superdex 30 chromatography as shown in Figure 3 and then separated with paper electrophoresis for 80 min. (A) Intact ^{35}S-labeled SLN; (B) after digestion with neuraminidase; (C) after hydrazinolysis; and (D) after deamination and $NaBH_4$ reduction. The slower migrating material in (C), indicated by a horizontal bar, was used for the next reaction step.

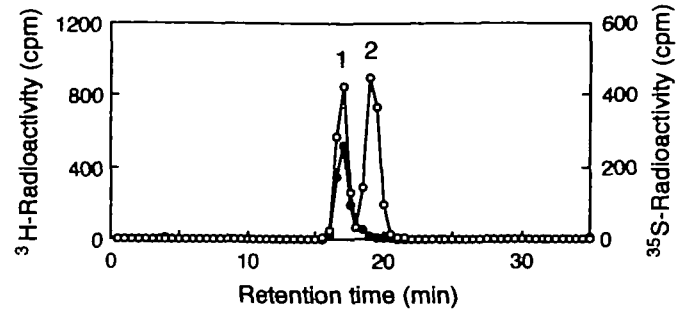

Fig. 5. HPLC separation of the sequentially degraded product derived from ^{35}S-labeled SLN. The final degradation product separated by paper electrophoresis (Figure 4D) was mixed with [^{3}H] Gal(6SO$_4$)β1–4AMan$_R$ and [^{3}H] Galβ1–4AMan$_R$(6SO$_4$), and subjected to HPLC using a Partisil 10-SAX column. Radioactivity of ^{3}H (open circle) and ^{35}S (solid circle) of each fraction was determined. Peak 1 and peak 2 were assigned as [^{3}H] Gal(6SO$_4$)β1–4AMan$_R$ and [^{3}H] Galβ1–4AMan$_R$(6SO$_4$), respectively, according to Shaklee and Conrad (Shaklee and Conrad, 1986).

The recombinant C6ST was shown to catalyze sulfation of sialyl lactosamine oligosaccharides, indicating that a single protein catalyzes sulfation of chondroitin, keratan sulfate, and sialyl lactosamine oligosaccharides. However, some differences in the acceptor specificity were observed between the purified C6ST and the recombinant C6ST; nonsulfated oligosaccharides were sulfated less efficiently by the recombinant C6ST than by the purified C6ST. The reason for the observed difference is not clear. The purified C6ST was obtained from the culture medium of chondrocytes and was thought to be cleaved at the transmembrane domain (Fukuta *et al.*, 1995). On the other hand, the recombinant C6ST was extracted from cell layer of COS-7 cells and may retain a full stretch of the transmembrane domain. Difference in posttranslational modification such as glycosylation may be present between chondrocytes and COS-7 cells. These possible structural differences between the purified C6ST and the recombinant C6ST may affect a preference for acceptor substrates. Alternatively, the purified C6ST may be composed of two enzyme proteins with different substrate specificity, since the purified C6ST gave two protein bands with the same amino terminal sequences after *N*-glycanase digestion (Fukuta *et al.*, 1995). We found that oligosaccharides with NeuAcα2–3Galβ1–4GlcNAc moiety were sulfated by C6ST; however, it remains possible that

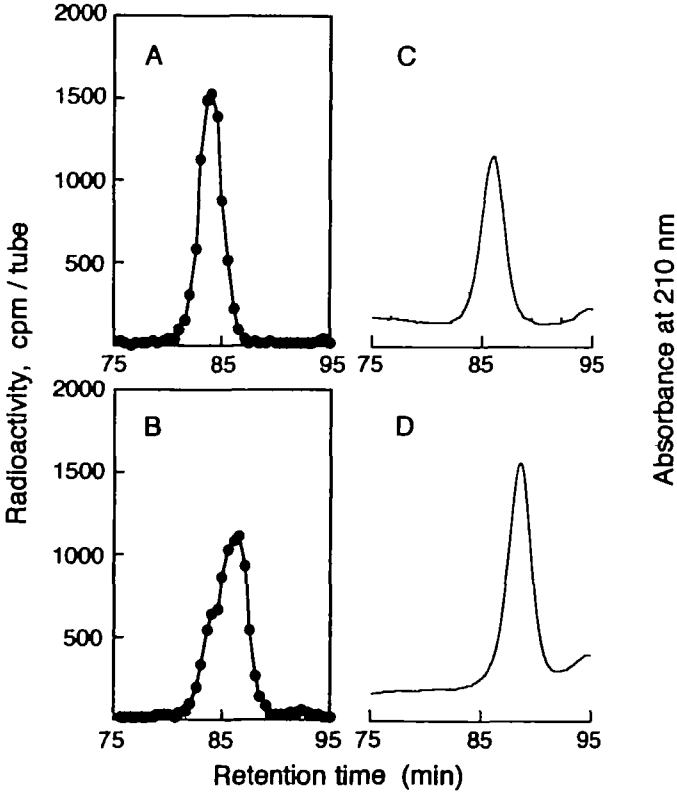

Fig. 6. β-Galactosidase digestion of the desialylated ^{35}S-labeled SL1L1. ^{35}S-Labeled SL1L1 was digested with neuraminidase, and the desialylated product was separated with paper electrophoresis. The ^{35}S-labeled material eluted from the paper was lyophilized, mixed with L1L1, and applied to Superdex 30 chromatography before (A, C) or after (B, D) β-galactosidase digestion. The eluate was monitored by absorption at 210 nm (C, D), and radioactivity of each 0.5 ml fraction was determined (A, B).

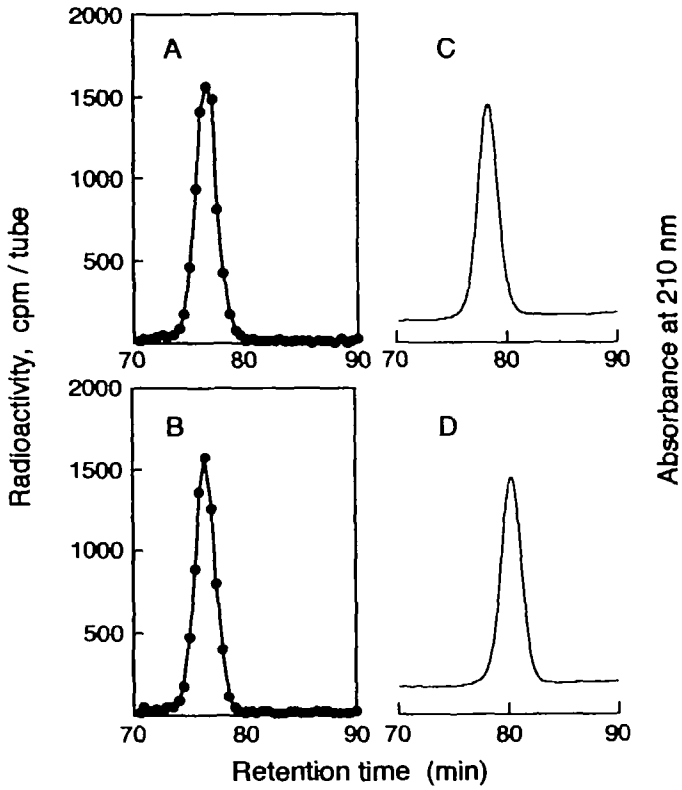

Fig. 7. β-Galactosidase digestion of the desialylated ^{35}S-labeled SL2L4. ^{35}S-Labeled SL2L4 was digested with neuraminidase, and the desialylated product was separated with paper electrophoresis. The ^{35}S-labeled material eluted from the paper was lyophilized, mixed with L2L4, and applied to Superdex 30 chromatography before (**A, C**) or after (**B, D**) β-galactosidase digestion. The eluate was monitored by absorption at 210 nm (**C, D**), and radioactivity of each 0.5 ml fraction was determined (**A, B**).

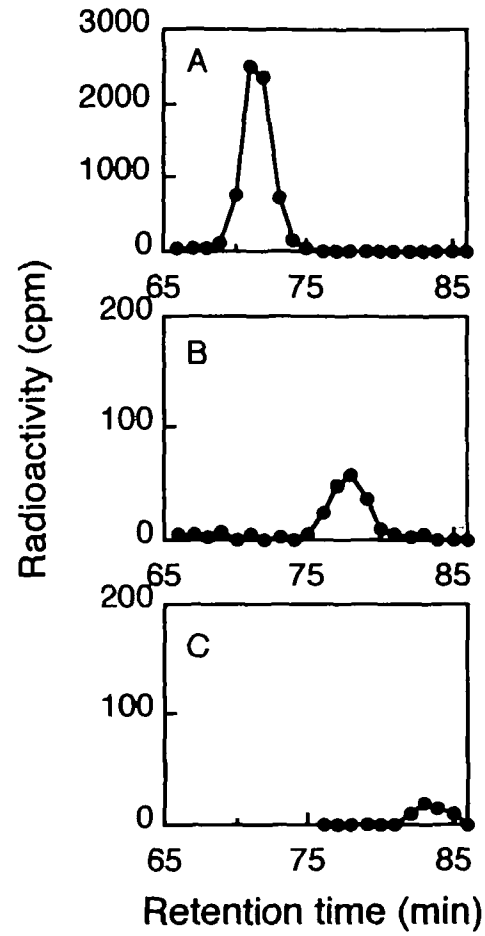

Fig. 8. Superdex 30 chromatography of ^{35}S-labeled oligosaccharides formed from SL2L4 (**A**), SL1L1 (**B**), and SLN (**C**) with the recombinant C6ST. Sulfotransferase reaction was carried out as described under *Materials and methods* using 18.8 μg of the recombinant C6ST. 1-ml fractions were collected and radioactivity was determined. Values observed in the boiled enzyme control were subtracted.

another sulfotransferase, which might catalyze sulfation of NeuAcα2–3Galβ1–4GlcNAc moiety more efficiently than C6ST, may participate in the biosynthesis of sialyl Lewis x oligosaccharide.

^{35}S-Labeled SL2L4 was completely resistant to the digestion with β-galactosidase even after desialylation; indicating that SL2L4 must be sulfated at Gal residue adjacent to the nonreducing terminal sialic acid. Various capping oligosaccharides were isolated from keratan sulfate after keratanase II digestion (Brown *et al.*, 1994, 1995; Lauder *et al.*, 1995). One of the oligosaccharides, NeuAcα2–3Gal(6SO$_4$)β1–4GlcNAc (6SO$_4$)β1–3Gal(6SO$_4$)β1–4GlcNAc(6SO$_4$), seems to be identical to the product formed from SL2L4 by C6ST; therefore, C6ST might also participate in the biosynthesis of the capping structure of keratan sulfate.

Materials and methods

The following commercial materials were used: $H_2{}^{35}SO_4$ was from Dupont/NEN; [^{3}H]$NaBH_4$ (16.3 GBq/mmol) was from Amersham Japan, Tokyo. Unlabeled PAPS and GalNAc(6SO$_4$) were from Sigma, St. Louis, MO; Fast Desalting Column HR 10/10 and Hiload Superdex 30 16/60 were from Pharmacia, Biotech, Tokyo; chondroitinase ACII, *Streptococcus* neuraminidase, *Streptococcus* β-galactosidase, keratanase II, and NeuAcα2–3Galβ1–4GlcNAc (SLN) were from Seikagaku Corp., Tokyo; Partisil 10-SAX was from Whatman, Clifton, NJ; NeuAcα2–3Galβ1–4 (Fucα1–3)GlcNAc (SLe^x) and Galβ1–4GlcNAc (LN) were from Funakoshi, Tokyo. Keratan sulfate from bovine cornea and NeuAcα2–3Galβ1–4GlcNAc(6SO$_4$)β1–3Gal(6SO$_4$)β1–4GlcNAc(6SO$_4$) (SL2L4), which was prepared from keratan sulfate by keratanase II digestion (Nakazawa *et al.*, 1989; Hashimoto *et al.*, 1990), were products of Seikagaku Corp. and generous gifts from the company. NeuAcα2–3Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc (SL1L1) and Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc (L1L1), which were prepared by desulfation of the corresponding oligosaccharides, were generous gifts from Dr. Yutaka Kariya, Tokyo Research Institute of Seikagaku Corp. The data about the structural analysis of these keratan sulfate-derived oligosaccharides will be described elsewhere. [^{35}S]PAPS was prepared as described previously (Delfert and Conrad, 1985).

Chondroitin 6-sulfotransferase was purified from the serum-free culture medium of chick chondrocytes as previously described (Habuchi *et al.*, 1993). Briefly, the culture medium was applied to heparin–Sepharose CL-6B and the absorbed materials were eluted with buffer A (Habuchi *et al.*, 1993) containing 0.45 M NaCl. The 0.45 M NaCl fraction from heparin–Sepharose CL-6B was applied to WGA–agarose, and the absorbed materials were eluted with a buffer containing 0.3 M *N*-acetylglucosamine. Finally, the WGA–agarose fraction was applied to 3′,5′-ADP-agarose and eluted with 0.2 mM 3′,5′-ADP. Chondroitin 6-sulfotransferase was purified 2400-fold and specific activity was 9.2 × 10^5 unit/mg protein. These values were the same magnitude of order as those previously reported (Habuchi *et al.*, 1993). The purified C6ST showed a single protein band with the molecular mass of 75 kDa on SDS–PAGE.

A mixture of [^{3}H] Gal(6SO$_4$)β1–4AMan$_R$ and [^{3}H] Galβ1–4AMan$_R$(6SO$_4$) used for the standard materials in HPLC was obtained by partial acid hydrolysis of [^{3}H] Gal(6SO$_4$)β1–4AMan$_R$(6SO$_4$) as described previously (Habuchi *et al.*, 1996; Shaklee and Conrad, 1986). [^{3}H]GalNAc$_R$(6SO$_4$) was prepared from GalNAc(6SO$_4$) by reduction with NaB^3H_4 as described previously (Habuchi *et al.*, 1996). Galβ1–4GlcNAc(6SO$_4$)β1–3Gal(6SO$_4$)β1–4GlcNAc(6SO$_4$) (L2L4) was prepared from SL2L4 by neuraminidase digestion. The neuraminidase digests were applied to a Partisil 10-SAX column, and

eluted by a gradient of KH_2PO_4 from 25 mM to 500 mM. The peak fractions eluted from the column were further purified with Superdex 30 chromatography and lyophilized.

Recombinant C6ST was expressed transiently in COS-7 cells as described previously (Fukuta *et al.*, 1995), and partially purified with heparin–Sepharose CL-6B and ammonium sulfate precipitation as follows. The extract from the cell layer of 45 10-cm dishes (83 mg as protein) was applied to a heparin–Sepharose CL-6B column (2.0 × 7.5 cm) equilibrated with buffer A (Habuchi *et al.*, 1993) containing 0.15 M NaCl. The absorbed materials were eluted with 0.45 M NaCl in buffer A. To the pooled fraction with C6ST activity, ammonium sulfate (0.56 g/ml) was added. The precipitate was collected with centrifugation (12000 × g, 30 min), dissolved in buffer A, and dialyzed against buffer A. During purification, recovery of chondroitin 6-sulfotransferase activity was 25% and specific activity increased 7.3-fold.

Preparation of a mixture of [^{3}H]Gal(6SO$_4$)β1–4GlcNAc$_R$ and [^{3}H]Galβ1–4GlcNAc$_R$(6SO$_4$)

Corneal keratan sulfate was digested with keratanase II. The reaction mixture for keratanase II digestion contained, in a final volume of 300 μl, 3 mg of keratan sulfate, 0.02 U of keratanase II and 15 μmol of acetate buffer, pH 6.5. After incubation at 37°C for 24 h, the digest was applied on a Partisil 10-SAX column and eluted with 5 mM KH_2PO_4 for 5 min followed by a 20 min gradient from 5 mM to 250 mM of KH_2PO_4. The flow rate was 1 ml/min. Galβ1–4GlcNAc(6SO$_4$) and Gal(6SO$_4$)β1–4GlcNAc(6SO$_4$) were eluted at 14 min and 22 min, respectively. Each peak was collected, lyophilized, and applied to a Superdex 30 column equilibrated with 0.2 M NH_4HCO_3. The eluate from the Superdex 30 column was desalted by lyophilization. The lyophilized disaccharides were reduced with NaB^3H_4 as described previously (Habuchi *et al.*, 1996). ^{3}H-Labeled disaccharide alditols were purified with paper chromatography and paper electrophoresis. The purified [^{3}H]Gal(6SO$_4$)β1–4GlcNAc$_R$(6SO$_4$) was hydrolyzed in 0.1 M HCl at 100°C for 40 min. After the partial acid hydrolysis, the sample was dried in a vacuum desiccator and dissolved in 100 μl of saturated $NaHCO_3$. For *N*-reacetylation, 2 μl of acetic anhydride was added twice to the sample at an interval of 5 min and then 6 μl of acetic anhydride was added. After 15 min at room temperature, the sample was passed through a 0.2 ml column of Dowex 50H$^+$ and washed with 1 ml water. The eluate and washing were combined, dried, and subjected to paper chromatography. After developing for 40 h, a ^{3}H-labeled peak migrating to the position of [^{3}H]Galβ1–4GlcNAc$_R$(6SO$_4$) (the second peak from the paper origin) was collected and purified with paper electrophoresis. The ^{3}H-labeled materials thus obtained were eluted as a single peak at the retention time of [^{3}H]Galβ1–4GlcNAc$_R$(6SO$_4$) in Superdex 30 chromatography (data not shown), and were eluted from the Partisil 10-SAX column in two peaks; the retention time of the second peak was 18.5 min, which is exactly the same as that of [^{3}H]Galβ1–4GlcNAc$_R$(6SO$_4$), whereas the first peak appeared 2 min earlier than [^{3}H]Galβ1–4GlcNAc$_R$(6SO$_4$) (Figure 2C). The first peak is thought to be [^{3}H]Gal(6SO$_4$)β1–4GlcNAc$_R$ because molecular size of the material in the first peak was the same as that of [^{3}H]Galβ1–4GlcNAc$_R$(6SO$_4$) as judged from the elution profile in Superdex 30 chromatography and was not degraded with β-galactosidase (Figure 2D).

Incorporation of sulfate into oligosaccharides and keratan sulfate by sulfotransferase reaction

The reaction mixture contained 2.5 μmol of imidazole-HCl, pH 6.8, 0.25 μmol of $CaCl_2$, 0.1 μmol dithiothreitol, 0.025 μmol of oligosaccharides or 0.025 μmol (as glucosamine) of keratan sulfate, 50 pmol [^{35}S]PAPS (about 5 × 10^5 c.p.m.), and the purified C6ST in a final volume of 50 μl. When the recombinant C6ST was used, 250 pmol [^{35}S]PAPS (about 2.5 × 10^6 c.p.m.) was added. The reaction mixtures were incubated at 37°C for 60 min, and the reaction was stopped by immersing the reaction tubes in a boiling water bath for 1 min. After the reaction was stopped, ^{35}S-labeled oligosaccharides and ^{35}S-labeled keratan sulfate were separated from $^{35}SO_4$ and [^{35}S]PAPS by Superdex 30 gel chromatography, and the radioactivity was determined. When SL2L4 was used as acceptor, sulfotransferase reaction proceeded linearly up to 90 ng of the purified chondroitin 6-sulfotransferase and up to 18 μg of the recombinant enzyme under the conditions described above.

Neuraminidase digestion and NaBH$_4$ reduction of ^{35}S-labeled SLN

^{35}S-Labeled SLN was prepared using the purified C6ST (0.09 μg as protein) as described above except that concentration of [^{35}S]PAPS was increased to 4-fold and incubation was carried out for 16 h. The ^{35}S-labeled SLN eluted from the Superdex 30 column was lyophilized, purified by paper electrophoresis, and digested with neuraminidase as described below. After neuraminidase digestion and aliquot of the sample was dried and dissolved in 10 μl of 0.5 M $NaBH_4$/0.2 M Na_2CO_3, pH 10.2. After the reduction with $NaBH_4$ was carried out at 0°C for 2 h, excess $NaBH_4$ was destroyed by addition of 10 μl of 3 M acetic acid. The reaction mixtures were dried under N_2 stream, dissolved in a small volume of water, and purified with Superdex 30 chromatography and paper electrophoresis.

N-deacetylation, deamination, and NaBH$_4$ reduction of ^{35}S-labeled SLN

The neuraminidase-digested sample obtained as above was deacetylated with 70% hydrazine containing 0.2% hydrazine sulfate at 95°C for 3 h (Guo and Conrad, 1989). The deacetylated materials were purified by Superdex 30 chromatography and paper electrophoresis. The slower migrating material in paper electrophoresis (indicated by a horizontal bar in Figure 4C) was subjected to deamination with pH 4 nitrous acid and reduced with $NaBH_4$ (Shaklee and Conrad, 1986). Finally, the sample was dissolved in 60 μl of water and purified by Superdex 30 chromatography and paper electrophoresis.

Digestion with neuraminidase and β-galactosidase

Reaction mixture for neuraminidase digestion contained ^{35}S-labeled oligosaccharide, 2.5 μmol of potassium acetate buffer, pH 6.5, 0.25 μmol of $CaCl_2$, and 10 mU of neuraminidase in a final volume of 25 μl (Kiyohara *et al.*, 1974). The reaction mixtures were incubated at 37°C for 60 min. Reaction mixture for β-galactosidase digestion contained desialylated ^{35}S-labeled oligosaccharide, 50 nmol of L1L1 or L2L4, 2.5 μmol of sodium acetate buffer, pH 5.5, and 10 mU enzyme in a final volume of 50 μl (Kiyohara *et al.*, 1976). The reaction mixtures were incubated at 37°C for 60 min.

Superdex 30 chromatography, paper electrophoresis, paper chromatography, and HPLC

Hiload Superdex 30 16/60 column was equilibrated with 0.2 M NH_4HCO_3. The flow rate was 1 ml/min; 1 ml or 0.5 ml fractions were collected and mixed with 4 ml Clearsol (Nakarai Tesque, Kyoto), and the radioactivity was determined. Oligosaccharides were monitored by absorption at 210 nm. Paper electrophoresis was carried out on Whatman No. 3 paper (2.5 cm × 57 cm) in pyridine/acetic acid/water (1:10:400, by volume, pH 4) at 30 V/cm for 40 min or 80 min. Samples for paper chromatography was spotted on a Whatman No. 3 paper (2.5 cm × 57 cm) and developed with 1-butanol/acetic acid/1 M NH_3 (3:2:1, by volume). The dried paper strips after paper electrophoresis or paper chromatography were cut into 1.25 cm segments and radioactivity was determined by liquid scintillation counting. HPLC separation of ^{35}S-labeled disaccharide alditols was carried out on a Whatman Partisil 10-SAX column (4.5 × 25 cm) equilibrated with 5 mM KH_2PO_4. The column was developed with 5 mM KH_2PO_4. The flow rate was 1 ml/min, and the column temperature was 40°C; 0.5 ml fractions were collected and mixed with 4 ml Clearsol, and the radioactivity was determined.

Determination of glucosamine and sialic acid

The glucosamine contents of oligosaccharides were determined by the Elson–Morgan method as modified by Strominger *et al.* (Strominger *et al.*, 1959) after hydrolysis of the glycosaminoglycans with 6 M HCl at 100°C for 4 h. Sialic acid was determined by thiobarbituric acid method (Aminoff, 1961) after hydrolysis with 0.1 M H_2SO_4 at 80°C for 60 min.

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Abbreviations

C6ST, chondroitin 6-sulfotransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; HPLC, high performance liquid chromatography; Gal(6SO$_4$), 6-*O*-sulfo-D-galactose; GlcNAc(6SO$_4$), 6-*O*-sulfo-*N*-acetyl-D-glucosamine; GlcNAc(6SO$_4$)$_R$, 6-*O*-sulfo-*N*-acetyl-D-glucosaminitol; AMan$_R$, 2,5-anhydro-D-mannitol; AMan$_R$(6SO$_4$), 6-*O*-sulfo-2,5-anhydro-D-mannitol; LN, Galβ1–4GlcNAc; SLN, NeuAcα2–3Galβ1–4GlcNAc; SLe^x, NeuAcα2–3Galβ1–4

(Fucα1–3)GlcNAc; L1L1, Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc; SL1L1, NeuAcα2–3Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc; L2L4, Galβ1–4GlcNAc(6SO$_4$)β1–3Gal(6SO$_4$)β1–4GlcNAc(6SO$_4$); and SL2L4, NeuAcα2–3Galβ1–4GlcNAc(6SO$_4$)β1–3Gal(6SO$_4$)β1–4GlcNAc(6SO$_4$)

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