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N-Acetylglucosamine-6-*O*-Sulfotransferase-1: Production in the Baculovirus System and Its Applications to the Synthesis of a Sulfated Oligosaccharide and to the Modification of Oligosaccharides in Fibrinogen

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***N*-Acetylglucosamine-6-*O*-sulfotransferase (GlcNAc6ST) catalyzes the transfer of sulfate from 3′-phosphoadenosine 5′-phosphosulfate to the C-6 position of non-reducing GlcNAc. Human GlcNAc6ST-1 was expressed as a fusion protein with protein A in an insect cell line (Tn 5 cells) using the baculovirus system. The recombinant enzyme was purified to homogeneity by IgG Sepharose column chromatography. The substrate specificity and the kinetic properties of the enzyme were similar to those of the enzyme expressed in the mammalian system. The purified recombinant enzyme was used to synthesize 6-sulfo GlcNAcβ1–3Galβ1–4Glc, which was identified by time of flight mass spectrometry. This sulfated trisaccharide served as a better substrate for microsomal galactosyltransferase from the mouse colon compared to 6-sulfo GlcNAc. The purified recombinant enzyme was also used to sulfate oligosaccharide chains on fibrinogen after enzymatic desialylation and degalactosylation to expose nonreducing GlcNAc residues. It is known that desialylation greatly increases the rate of clotting of fibrinogen after the addition of thrombin. Subsequent sulfation of desialylated and degalactosylated fibrinogen slightly decreased the rate of clotting. The recombinant GlcNAc6ST-1 is a useful reagent for 6-sulfate exposed GlcNAc residues both in oligosaccharides and in glycoproteins.**

Key words: glycan modification, oligosaccharide synthesis, sulfotransferases.

Abbreviations: DHB, 2,5- dihydroxy benzoic acid; GlcNAc6ST, *N*-acetylglucosamine-6-*O*-sulfotransferase; MALDI-TOF, matrix assisted laser desorption ionization time of flight; Me, methyl; nLc4, neolactotetraose; TST buffer, 50 mM Tris-HCl buffer, pH 7.6 containing 0.15 M NaCl and 0.05% Tween 20; pNP, *p*-nitrophenyl.

Sulfation of sugar residues in glycosaminoglycans as well as in glycoproteins and glycolipids renders diverse biological functions on the glycans, and the process is catalyzed by specific sulfotransferases (*1–4*). Critical roles of carbohydrate sulfation have been demonstrated in binding to growth factors, chemokines, hormones, anticoagulation factors and cell adhesion molecules (*1*). 6-Sulfation of *N*-acetylglucosamine (GlcNAc) residues occurs in a variety of oligosaccharides in glycoproteins (*5–9*). Most importantly, the 6-sulfo sialyl Lewis X structure is the ligand of L-selectin, which is involved in the regulation of lymphocyte trafficking (*9–11*). This 6-sulfation step is catalyzed by several of *N*-acetylglucosamine-6-*O*-sulfotransferases (GlcNAc6STs) (*12–22*). Generally, GlcNAc6STs cannot act on internal GlcNAc, but act only on nonreducing terminal GlcNAc (*12–14*). We cloned the first GlcNAc6ST, namely *N*-acetylglucosamine-6-*O*-sulfotransferase-1 (GlcNAc6ST-1) in both mice and humans (*14*, *15*), and elucidated its substrate specificity (*16*). The enzyme plays key roles in the reconstitution of L-selectin ligands (*23*). Since the enzyme has broad substrate specificity (*16*), GlcNAc6ST-1 is expected to be a powerful tool in the production of oligosaccharides with 6-sulfo GlcNAc and also in the modification of oligosaccharides in glycoproteins. Here, we report the production and purification of the recombinant enzyme using the baculovirus expression system, and discuss the above mentioned possibility.

MATERIALS AND METHODS

Materials—The following materials were obtained commercially from the sources indicated. [^{35}S]PAPS (2.78 Ci/mmol) and ^{14}C-UDP-Gal (278 mCi/mmol) were from PerkinElmer Life Sciences (Boston, MA); [α-^{32}P]dCTP (110 GBq/nmol) was from Amersham Pharmacia Biotech, (Buckinghamshire, UK); lacto-*N*-neotetraose (nLc4), UDP-Gal, 6-*O*-sulfated GlcNAc, β1–4 galactosidase from *Streptococcus pneumoniae* and *N*-glycanase F were from Calbiochem (La Jolla, CA); bovine thrombin, fibrinogen and GlcNAcβ1–6ManOMe were from Sigma (St. Louis, MO); neuraminidase from *Arthrobacter ureafascience* and *N*-acetyl neuraminic acid were from Nacalai Tesque (Kyoto). GlcNAcβ1–6(Galβ1–3)GalNAc-pNP (core 2) and

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GlcNAcβ1–3GalNAc-pNP (core 3) were from Toronto Research Chemicals (North York, Ontario, Canada), and GlcNAcβ1–2Man was from Dextra Laboratories (Reading, UK). GlcNAcβ1–3Galβ1–4Glc was prepared by digestion of 0.8 mg of lacto-*N*-neotetraose with 20 mU of β1–4 galactosidase in 40 μl of 20 mM of Tris-HCl buffer, pH 7.4, at 37°C for 18 h, purified by Superdex 30 chromatography, and desalted by lyophilization.

Construction of a Transfer Vector Carrying Human GlcNAc6ST-1—A cDNA fragment encoding an open reading frame without the transmembrane region of human GlcNAc6ST-1 (GenBank accession number AB014680, nucleotide number 495–1844) was amplified by PCR. pcDNA3-hGlcNAc6ST-1 (*15*) was used as the template and the primers used were: a 5′ primer containing an in-frame *Eco*RI site 5′-TGGAATTCCTGCAGCAGTGCAACCCCGAT-3′ (nt. 495–515) and a 3′ primer containing an *Eco*RI site 5′-GAGAATTCTTAGAGACGGGGCTTCCGA-3′ (nucleotide number 1826–1844). PCR was carried out with 35 cycles of 98°C for 15 s, 65°C for 20 s, and 74°C for 30 s using KOD polymerase (Toyobo Biochemicals, Tokyo). The PCR product (1,349 bp) was cut by *Eco*RI. The protein A tag sequence together with the IgM signal peptide was obtained from pcDSA vector (*24*) by cutting with *Pst*I and *Xho*I, and subcloned into pBluescript II SK- (Stratagene, La Jolla, CA). The pBluescript containing protein A tag was then cut with *Eco*RI to which the PCR product was subcloned. The insert was released by cutting with *Kpn*I and *Not*I and subcloned into pVL1392 transfer vector (Pharmingen, CA), which was cut by the same enzymes.

DNA Transfection and Selection of Positive Virus Clones—For the generation of a recombinant virus, *Spodoptera frugiperda* Sf-21 cells were cotransfected with 0.1 μg of BaculoGold DNA (Pharmingen, CA) and 1 μg of the transfer vector described above by means of lipofectAMINE PLUS (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Conditioned medium containing the recombinant baculovirus was harvested after 4 days incubation at 27°C. In order to isolate positive clones, Sf-21 cells seeded at 1×10^5/well in a 96-well plate were infected with the virus in conditioned medium serially diluted 10^2 to 10^5 times, and incubated for 5 days. Each lot of conditioned medium was collected and kept at 4°C until the next infection. Cells were lysed in 2 M NaOH in order to isolate viral DNA. The lysates were blotted onto a nitrocellulose membrane, and then hybridized with a ^{32}P-labeled human GlcNAc6ST-1 cDNA probe. The individual clones were selected and subjected to the next amplification as described previously (*25*).

Expression and Purification of Recombinant Human GlcNAc6ST-1—For the production of human GlcNAc6ST-1 recombinant protein, *Trichoplusia ni* High Five (Tn 5) cells (Invitrogen) were infected with the recombinant virus and then incubated for 72 h at 27°C in Ex-cell 405 serum-free medium (JRH Bioscience, KS). The culture medium (1,600 ml) was collected and centrifuged at 3,000 ×*g* for 10 min followed by ultracentrifugation at 100,000 ×*g* for another 30 min to remove virus particles. The supernatant obtained was applied directly to an IgG Sepharose column (Amersham Pharmacia, 0.7 × 2.5 cm) pre-equilibrated with 50 mM Tris-HCl buffer, pH 7.6, containing 0.15 M NaCl and 0.05% Tween 20 (TST buffer). The column was washed with 10 column volumes of TST buffer, and then with 5 column volumes of 5 mM ammonium acetate buffer, pH 5.0. The unabsorbed fraction was reapplied to the column to ensure binding of the protein A fused recombinant enzyme to IgG. Then the protein was eluted using 0.5 M ammonium acetate buffer, pH 3.4. The fractions were checked by SDS–PAGE using a 10% gel (*26*). The protein that was eluted in the peak fractions was pooled, concentrated with Centricon-10 (Amicon) and stored at –80°C.

Sulfotransferase Assay of Recombinant GlcNA6ST-1—To assay the sulfotransferase activity of the recombinant enzyme, we used GlcNAcβ1–6ManOMe as a sulfate acceptor. The reaction mixture comprised 2.5 μmol of Tris-HCl buffer, pH 7.2, 0.5 μmol of $MnCl_2$, 0.1 μmol of AMP, 5 μmol of NaF, 300 pmol of [^{35}S]PAPS (1.8×10^5 cpm), 0.05% Triton-X 100, 50 nmol of GlcNAcβ1–6ManOMe, and GlcNAc6ST-1 in a final volume of 50 μl. After incubation at 30°C for 1 h., the reaction was stopped by immersing the reaction tube in a boiling water bath for 1 min. The reaction products were separated by Superdex 30 column chromatography. The oligosaccharides were monitored by absorption at 210 nm and the radioactivity in the oligosaccharide fraction was determined by a liquid scintillation counter. One unit of enzyme was defined as the amount of enzyme required to catalyze the transfer of 1 μmol of sulfate per min. The following substrates were also used to determine the substrate specificity: GlcNAcβ1–6(Galβ1–3)GalNAc-pNP (core 2), GlcNAcβ1–3GalNAc-pNP (core 3), and GlcNAcβ1–2Man.

Formation of 6-Sulfo GlcNAcβ1–3Galβ1–4Glc—The sulfation reaction was performed in a reaction mixture containing 900 nmol of degalactosylated nLc4, 4.5 mU of the enzyme, 2 μmol of PAPS, 25 μmol of Tris-HCl buffer, pH 7.2, 5 μmol of $MnCl_2$, 1 μmol of AMP, 50 μmol of NaF, and 0.05% Triton-X 100 in a 500 μl reaction mixture at 30°C for 5 h. The sulfated product was subjected to Superdex 30 chromatography; the fractions around the peak were collected and desalted by repeated lyophilization. The amount of the sulfated oligosaccharide was determined by the phenol-sulfuric acid reaction (*27*).

Assay of a Galactosyltransferase Using Sulfated Sugars as Acceptors—A mouse colon microsome fraction was prepared as described previously for the preparation of microsomes from human colorectal mucosa (*28*) and used as the enzyme source for β1,4-glactosyltransferase. The reaction mixture contained 2 μM ^{14}C-UDP-galactose (6.2×10^4 cpm), 10 μM $MnCl_2$, 0.5% Triton-X100, 3 mM of 6-sulfo GlcNAcβ1–3Galβ1–4Glc or 6-sulfo GlcNAc, and 50 μg mouse colon microsome fraction in 20 μl of 50 mM Tris-HCl buffer, pH 7.5. The reaction was allowed to proceed at 37°C for 1 h. The reaction mixture was subjected to Superdex 30 gel chromatography and the radioactivity was monitored with a liquid scintillation counter. The value obtained in a control run in the absence of the acceptor was subtracted from the value obtained in the experimental run. The galactosylated product was collected, lyophylized and treated with β1–4 galactosidase in the same manner as described above, and the products were subjected again to Superdex 30 gel chromatography to determine the amount of [^{14}C]galactose released.

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Table 1. **Comparison of substrate specificities of the purified GlcNAc6ST-1 produced by the baculovirus system and the enzyme produced by transfected COS 7 cells.**

Acceptor	Relative activities[a]	
	Baculovirus-produced enzyme	COS 7 cell-produced enzyme
GlcNAcβ1-6ManOMe	100	100
GlcNAcβ1-2Man	144	128
GlcNAcβ1-6[Galb1-3]GalNAc-pNP (core2)	273	191
GlcNAcβ1-3GalNAc-pNP (core 3)	<0.1	<0.1

[a]The activity towards GlcNAcβ1-6ManOMe was taken as 100.

Sulfation of Oligosaccharides in Bovine Fibrinogen—Bovine fibrinogen (1 mg) was treated with 0.1 U neuraminidase in10 mM sodium acetate buffer, pH 5.0, containing 0.15 M NaCl at 37°C for 5 h. Untreated normal fibrinogen was incubated under the same conditions in the absence of neuraminidase. The desialylated fibrinogen was treated with 20 mU β1–4-galactosidase in 20 mM Tris-HCl, pH 7.4, at 37°C for 18 h. Then, the desialylated degalactosylated fibrinogen was sulfated in a reaction mixture containing 30 nmol PAPS as the sulfate donor in 10 mM $MnCl_2$, 100 mM NaF, 0.05% Triton-X 100, 2 mM AMP, 50 mM Tris-HCl, pH 7.2 ,and 0.3 mU of purified GlcNAc6ST-1 in a final volume of 100 μl. The reaction mixture was incubated at 30°C for 5 h. The released sialic acid or galactose was determined by the thiobarbituric acid method (*29*) or phenol-sulfuric acid reaction (*27*) after removing proteins with 70% ethanol (final concentration) at 4°C. To determine the amount of sulfate transfer, 0.3 nmol [^{35}S]PAPS was added to the reaction mixture in addition to 30 nmol of cold PAPS. After the reaction, the protein was precipitated with trichloroacetic acid (5%, w/v) and washed 5 times using the same reagent to remove excess [^{35}S]PAPS from the precipitate. The protein pellet was dissolved in 150 μl of SDS–PAGE sample buffer and mixed with the scintillation cocktail (Clear-sol I, Nacalai Tesque, Kyoto) to determine the radioactivity. The amount of radioactivity present in the precipitate upon incubation without the sulfotransferase was taken as a control, and the value was subtracted from the radioactivity incorporated after the enzymatic reaction.

Determination of Clotting Activity of Fibrinogen—Modified fibrinogens were prepared as described above, and the salts and PAPS were removed by application of the fibrinogens to Centricon 100. The buffer was replaced with 10 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl. One milligram of native or modified fibrinogen in 950 μl of 10 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl was incubated with 50 μl of 0.25 U/ml bovine thrombin. The increase in absorbance was determined at 350 nm every 10 s for 3 min at 37°C using a double beam Shimadzu UV 2200 spectrophotometer (Shimadzu, Kyoto) (*30*).

Analytical Procedures—SDS–polyacrylamide gel electrophoresis was carried out by the method of Laemmli (*26*). The gel was stained with silver reagent and /or subjected to Western blotting (*31*). The protein concentration was measured by means of the BCA assay (Pierce, Rock Ford, IL) using bovine serum albumin as the standard. Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was performed by means of Voyager Elite (Perspective Biosystems, Framingham, MA) using 2,5-dihydroxy benzoic acid (DHB) as the matrix (*32*). Superdex 30 column chromatography was performed as described previously (*14*) using 0.2 M ammonium hydrogen carbonate as the solvent.

RESULTS

Purification and Properties of Human GlcNAc6ST-1 Produced by the Baculovirus System—Human GlcNAc6ST-1 lacking the transmembrane region and fused with protein A was produced by the baculovirus system. The enzyme secreted into the culture medium was purified by IgG Sepharose affinity chromatography. From 1.6 liters of culture medium, 0.9 mg of enzyme protein was obtained with a specific activity of 15.3 milliunits per mg protein. The yield of the enzyme was 66.7% with a 428-fold increase in the specific activity. The purified enzyme appeared as a single band of about 56 kDa on SDS–PAGE (Fig. 1). The enzyme efficiently utilized GlcNAcβ1–6ManOMe, GlcNAcβ1–2Man, and core 2 oligosaccharides as acceptors, but not core 3 oligosaccharide (Table 1). Relative activities to the 3 substrates were similar to the activities of the enzyme produced in mammalian cells (*16*). The K_m values for GlcNAcβ1–6ManOMe and PAPS were 0.3 mM and 6.7 μM, respectively, and the values for the mammalian cell- produced enzyme were 0.2 mM and 1.2 μM, respectively (*16*).

Preparation of 6-sulfo GlcNAcβ1–3Galβ1–4Glc and Its Usage as a Substrate for Galactosyltransferase—We tried to synthesize 6-sulfo GlcNAcβ1–3Galβ1–4Glc with the aid of the purified recombinant enzyme. By incubating GlcNAcβ1–3Galβ1–4Glc and PAPS in the presence of the enzyme, we could 6-sulfate the trisaccharide. Starting from 0.9 μmol of GlcNAcβ1–3Galβ1–4Glc, we obtained

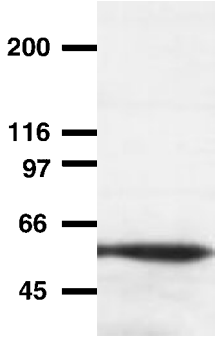

Fig. 1. **Purity of recombinant hGlcNAc6ST-1.** About 200 ng of purified hGlcNAc6ST-1 was analyzed by SDS–PAGE using a 10% running gel and silver staining. The positions to which standard substances migrated are shown with their masses in kDa.

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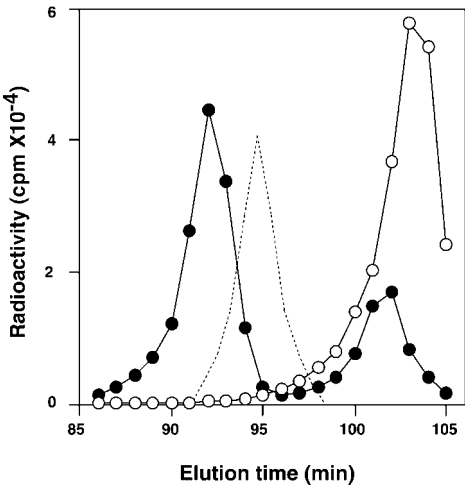

Fig. 2. **Separation of 6-sulfo GlcNAcβ1–3Galβ1–4Glc from GlcNAcβ1–3Galβ1–4Glc (degalactosylated nLc4) and PAPS by Superdex 30 gel chromatography.** In the reaction mixture, 380 pmol of [^{35}S]PAPS, 2.5 μmol Tris-HCl buffer, pH 7.2, 5 μmol of $MnCl_2$, 0.1 μM of AMP, 5 μmol of NaF, and 0.05% Triton X-100 and 0.1 mU of the recombinant GlcNAc6ST-1 were incubated in the presence (closed circles) or absence (open circles) of 90 nmol of degalactosylated nLc4. The reaction mixture was subjected to Superdex 30 gel column chromatography. The elution position of degalactosylated nLc4 (dashed line) was determined by applying the oligosaccharide to the column and monitoring the absorbance at 210 nm. Based on the result, unlabeled 6-sulfo GlcNAcβ1–3Galβ1–4Glc was purified by collecting materials eluted in fractions 88 to 92.

0.2 μmol of 6-sulfo GlcNAcβ1–3Galβ1–4Glc after Superdex 30 gel chromatography, which separated the sulfated product from GlcNAcβ1–3Galβ1–4Glc and PAPS (Fig. 2). MALDI-TOF mass spectrometry of the product gave a peak of *m*/*z* 647.06, which is compatible with 6-sulfo GlcNAcβ1–3Galβ1–4Glc, while GlcNAcβ1–3Galβ1–4Glc gave a peak of *m*/*z* 568.28 (Fig. 3). That the latter peak was not detected in the sulfated oligosaccharide indicates the high degree of purity of the product.

A microsomal fraction from human colon contained a galactosyltransferase that transferred galactose by β1–4 linkage to 6-sulfo GlcNAc (*28*, *33*). This enzymatic reaction is important for the formation of glycoconjugates with 6-sulfo-GlcNAc including L-selectin ligand. Since we were able to obtain 6-sulfo GlcNAcβ1–3Galβ1–4Glc, we compared the velocity of the enzymatic reaction to 6-sulfo GlcNAcβ1–3Galβ1–4Glc with that to 6-sulfo GlcNAc. The product of galactosylation was separated by Superdex 30 column chromatography (Fig. 4). That the enzyme transferred galactose to the acceptor by β1–4-galactosidic linkage was confirmed by the susceptibility of the product to β1-4-galactosidase: 94% of the transferred galactose was released by enzyme treatment. As shown in Fig. 5, the enzymatic reactions to the two substrates proceeded with different velocities, with a higher velocity for 6-sulfo GlcNAcβ1–3Galβ1–4Glc.

Formation of 6-sulfo Desialylated and Degalactosylated Fibrinogen and Its Clotting Activity—Bovine fibrinogen was desialylated and degalactosylated enzymatically as described under "MATERIALS AND METHODS." The molar ratios of the released sialic acid and galactose were 6.2 and 3.3 mol per mole of fibrinogen, respectively. The desialylated and degalactosylated fibrinogen was 6-sulfated using recombinant GlcNAc6ST-1 and PAPS. The amount of transferred sulfate was 0.72 moles per mole of fibrinogen. We examined the clotting rate of native and modified fibrinogen after the addition of thrombin by monitoring the absorbance at 350 nm. The clotting rate was significantly high in desialylated and degalactosylated fibrinogen (Fig. 6), as reported previously (*34–36*). 6-Sulfated, desialylated and degalactosylated fibrinogen showed a clotting rate that was slightly, but significantly, lower than that of the desialylated and degalactosylated fibrinogen. Incubation of the desialylated and degalactosylated fibrinogen with the enzyme in the absence of PAPS did not change the clotting rate (data not shown).

DISCUSSION

Since various sulfotransferases have been cloned to date, it has become possible to sulfate a specific oligosaccharide structure using a recombinant sulfotransferase, and use the sulfated oligosaccharide or glycoconjugates for functional analysis (*37*, *38*). The work presented here follows this line of research.

As described herein, recombinant human GlcNAc6ST-1 was produced by the baculovirus system, and purified to homogeneity. This is the first report of the production of a pure GlcNAc6ST. The specific activity of the recombinant enzyme was 15.3 milliunits per mg protein, which is low compared to the value expected for a homogeneous enzyme. Most probably, production in the baculovirus system affected proper folding and/or glycosylation leading to lower enzymatic activity. The possibility that exposure to acidic pH during purification resulted in the loss

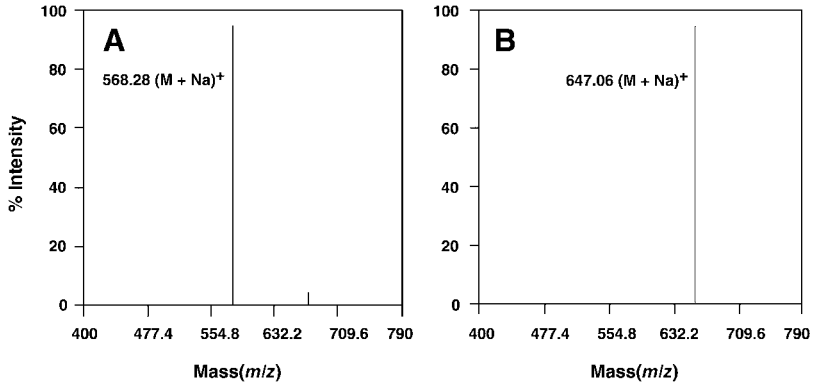

Fig. 3. **Positive-ion MALDI-TOF-mass spectra of GlcNAcβ1–3Galβ1–4Glc (A) and 6-sulfo GlcNAcβ1–3Galβ1–4Glc (B).** Fifty picomoles of each oligosaccharide was mixed with 0.3 μl DHB and then applied to a polished stainless steel target, which was dried by keeping exposed at room temperature for several min before the start of analysis.

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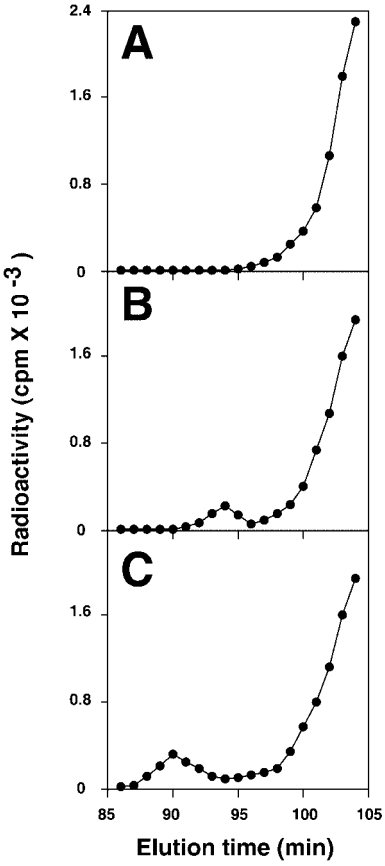

Fig. 4. **Separation of the galactosylated product by Superdex 30 column chromatography.** [^{14}C]UDP-galactose and the mouse colon microsomal fraction were incubated with no acceptor (A), 6-sulfo GlcNAc (B), or 6-sulfo GlcNAcβ1–3Galβ1–4Glc (C) for1 h as described in "MATERIALS AND METHODS." The reaction mixture was subjected to Superdex 30 gel column chromatography. The degradation products of UDP-galactose were eluted with peaks in Fraction 104–105.

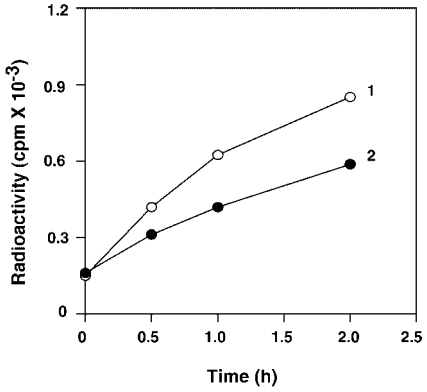

Fig. 5. **Time course of galactosylation of 6-sulfo GlcNAcβ1–3Galβ1–4Glc (1) and 6-sulfo GlcNAc (2).** Each substrate (150 nmol) was incubated with 50 μg of the microsome fraction as described in "MATERIALS AND METHODS." To calculate the radioactivity in the product, the radioactivity in the corresponding region of the control run (Fig. 4A) was subtracted from the radioactivity in fractions 91–96 (Fig. 4B) or in fractions 88–93 (Fig. 4C).

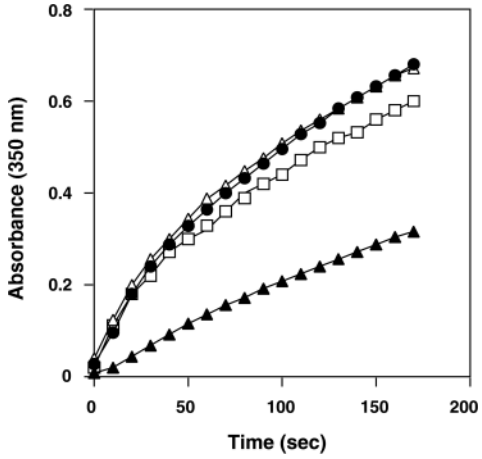

Fig. 6. **Comparison of the clotting velocity of native (solid triangle), desialylated (solid circle), degalactosylated (open triangle), and desialylated, degalactosylated, and 6-sulfated (open square) fibrinogen.** One milligram of each fibrinogen or its derivative was exposed to 0.25 U/ml of thrombin at 37°C as described in "MATERIALS AND METHODS."

of enzymatic activity is unlikely, since the yield of enzymatic activity during purification was high. The higher K_m value of the baculovirus-made enzyme to PAPS compared to that produced in the mammalian system suggests that the PAPS binding region might have an altered three-dimensional structure.

The substrate specificity of the baculovirus enzyme is broad, and similar to that of the enzyme produced in the mammalian system. Therefore, the enzyme preparation reported herein is expected to be valuable in the formation of 6-sulfated oligosaccharides and glycoproteins. Indeed, we were able to produce 6-sulfo GlcNAcβ1–3Galβ1–4Glc and 6-sulfated, desialylated and degalactosylated fibrinogen.

6-Sulfo GlcNAcβ1–3Galβ1–4Glc was used as a substrate for a galactosyl transferase, and found to be a better sulfate acceptor than 6-sulfo GlcNAc. An obvious aim of such oligosaccharide synthesis is the formation of a 6-sulfo sialyl Lewis X structure. So far, a galactosyltransferase that adds galactose efficiently to the 6-sulfo GlcNAc terminus has not been purified or cloned, although β1,4-galactosyltransferase I can transfer galactosyl residue to the 6-sulfo GlcNAc terminus (*39*). Once such an enzyme is obtained, a 6-sulfo sialyl Lewis X structure can be formed *in vitro* by combining the present enzyme with the galactosyltransferase, an α 1–3 fucosyltransferase and an α 2–3 sialyltransferase.

It has been reported that desialylation increases the clotting velocity of fibrinogen, and further degalactosylation does not have additional effects (*34–36*). Our observation is consistent with the previous findings. Although the amount of sulfate enzymatically added to desialylated and degalactosylated fibrinogen was less than a mole per mole of protein, the addition of sulfate significantly decreased the clotting velocity. This is most probably due to the addition of a negative charge to the molecule. In other words, the effect of desialylation on clotting velocity can be explained by the loss of negative charges (*34*).

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Finally, the availability of a pure GlcNAc6ST-1 opens the way for determining the three dimensional structure of GlcNAc6ST-1. Although the specific activity of the enzyme is low, the K_m value to the acceptor is similar to that of the enzyme produced in the mammalian system (*16*). Therefore, the structure of the acceptor recognition site of the recombinant enzyme is expected to be similar to that of the mammalian enzyme.

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