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Purification and Characterization of a Lymph Node Sulfotransferase Responsible for 6-*O*-Sulfation of the Galactose Residues in 2′-Fucosyllactose and Other Sialyl Lewis^X-Related Sugars

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A microsomal galactose-6-*O*-sulfotransferase (Gal-6-*O*-Stase) from porcine lymph nodes, able to transfer the sulfate group from adenosine 3′-phosphate 5′-phosphosulphate (PAPS) onto 2′-fucosyllactose (2′-FL) and other sialyl Lewis^X (sLe^x)-related sugars, has been purified and characterized. The enzyme was purified to about 35,000-fold by a combination of conventional and affinity chromatographic steps. The purified enzyme preparation exhibited two protein bands at around 80-90 and 170 kDa on 7.5% SDS-PAGE under reducing conditions. Both of these protein bands always comigrated in the gel when peak fractions containing Gal-6-*O*-Stase activity from the 3′,5′-ADP-agarose column were subjected to 6% SDS-PAGE under reducing conditions. These protein bands also showed similar binding patterns to WGA (wheat germ agglutinin), Con A (concanvalin A), and EBA (elderberry agglutinin). Similarly, when the enzyme preparation after the hydroxylapatite step was photolabeled with 8-azido-[^{32}P]-PAPS, both 80-90 and 170 kDa protein bands were labeled in a specific manner. These results suggest a possible association of these two protein bands with the enzyme activity. The carbohydrate substrate specificity of this enzyme suggests that it is well suited to catalyze the sulphonation at the C-6 position of the galactose residues of oligosaccharides that are structurally similar to sLe^x. Furthermore, a survey of several porcine organs revealed that this enzyme was selectively expressed in lymphoid tissues such as lymph nodes (peripheral and mesenteric) and spleen. These findings suggest that this enzyme may be involved in the assembly of 3′-sialyl-6′-sulfo Lewis^x, the major capping group of HEV-ligands for L-selectin.

Leukocyte adhesion to activated endothelium is a key initial event in the trafficking and recruitment of circulating leukocytes into lymphoid tissues and at sites of inflammation (1). The first step in the recruitment process is the interaction between circulating leukocytes and endothelial cells lining the blood vessels (2-4). L-selectin, a constitutively expressed cell adhesion molecule on all classes of circulating leukocytes, participates in leukocytes binding to their cognate ligands on the endothelial cells. Two high endothelial venules (HEV)-associated ligands for L-selectin, GlyCAM-1 and CD34, have been identified from mouse lymph nodes (5-7)). These two ligands are heavily sialylated, fucosylated and sulfated glycoproteins. Detailed structural analysis of O-linked glycan chains of one of these ligands indicates sulfation to occur at two sites: 1, at Gal-6-SO_3, as in the capping structure 3′-sialyl-6′sulfo-Lewis^x (NeuAcα2-3-Gal-6-SO_3 β1-4(Fucα1-3)GlcNAc) (8,9), and 2, at the GlcNAc-6-SO_3 as in NeuAcα2-3Gal-β1-4(Fucα1-3)GlcNAc-6-SO_3 (10). We have demonstrated previously that a 120 kDa glycoform of CD34 is the major ligand for L-selectin in the porcine peripheral lymph nodes (PLN), and that the sialylation and sulfation of this ligand was critical for its binding to L-selectin as well as to MECA-79 (11-14), a monoclonal antibody that selectively recognizes HEV-ligands (11). Interestingly, the sialomucin CD34 has been shown to be widely distributed in the blood vessels of both mouse and human tissues. However, MECA-79 reactive glycoform of the CD34 is expressed only on the HEV of lymph nodes and on inflamed tissues (15), suggesting that the protein backbone of

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Abbreviations used: Galactose-6-*O*-sulfotransferase, Gal-6-*O*-Stase; 2-FL, 2-fucosyllactose; PAPS, adenosine 3′-phosphate 5′-phosphosulphate; WGA, wheat germ agglutinin; EBA, elderberry agglutinin; Con A, concanavalin A; DTT, dithiothreitol; Gal, galactose; GlcNAc, N-acetylglucosamine; Fuc, fucose; sLe^X, 3′-sialyl Lewis^x; NeuNAc, N-acetylneuraminic acid; HEV, high endothelial venule; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis and HP-TLC, high performance-thin layer chromatography.

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the ligand is insufficient to mediate L-selectin adhesion. Therefore, it is likely that the post-translational modifications of oligosaccharide chains on these HEV-ligands may be responsible for their ligand-binding activity. This led to the proposal that the expression of these recognition determinants on HEV-ligands is differentially regulated by tissue specific sugar-modifying enzymes. The complete synthesis of a sulfo-sialyl Le^X from the precursor N-acetyllactosamine requires actions of a sialyltransferase, a fucosyltransferase and a sulfotransferase. Although a sialyltransferase (16) and fucosyltransferases (17,18) have been identified and their genes have been cloned, a sulfotransferase involved in the synthesis of the sulfated form of sialyl-Le^X has yet not been identified. These observations stimulated tremendous interest in the carbohydrate-specific sulfotransferases catalyzing the sulfation at the C-6 positions of the galactose and the GlcNAc residues of the oligosaccharides present on HEV-ligands. It has earlier been shown that the purified chondroitin 6-sulfotransferase (C6ST) catalyzed transfer of sulfate not only to the C-6 position of GalNAc residues of chondroitin but also to the C-6 position of galactose residue in the karatan sulfate (19,20). In addition, Spiro and Bhoyroo have recently identified a spleen sulfotransferase activity responsible for sulphonation at the C-6 position of galactose (21). We describe here the purification and characterization of this sulfotransferase activity from porcine PLN that catalyzes the transfer of sulfate from PAPS to the C-6 position of the galactose residue of 2′-FL and other sugars.

MATERIALS AND METHODS

Materials. All common chemicals and 3′,5′-ADP-agarose were obtained from Sigma Chemical Co., St. Louis, MO. Lectins and conjugated lectins were purchased from Vector laboratories, Burlingame. CA. PAP^{35}S was synthesized in our laboratory by using an enzymatic procedure (22). 8-azido-[^{32}P]-PAPS was synthesized from 8-azido-[^{32}P]-ATP by utilizing a procedure as described (22). Fresh peripheral lymph nodes were obtained from Pel-Freeze, Little Rock, AR. DEAE-TSK was purchased from Supelco Co., IL. DNA grade hydroxylapatite and Affi-gel blue were obtained from Bio-Rad laboratories, CA. Unless otherwise mentioned, all buffers contained 5 μg/ml each of the following protease inhibitors: Leupeptin, Pepstatin A, and antipain.

Enzyme assay. The enzyme assay[2] mixture contained 20 mM 2′-FL, 80 μM [^{35}S]-PAPS (~ 1 μCi), 2 mM ATP, 10 mM NaF, 10 mM $MgCl_2$, 1 mM DTT, 100 mM Hepes buffer, pH. 7.4 and the enzyme solution (1-10 μl) in a final volume of 20 μl. After an incubation of 2 to 4 h, the reaction mixture was diluted to 1 ml with 120 mM ammonium bicarbonate and passed through an ion-exchange column (Dowex AG 1-X8), which was pre-equilibrated with 120 mM ammonium bicarbonate. The column was eluted with 10 ml of 120 mM ammonium bicarbonate and the eluate was mixed with an equal volume of Ultima Gold XR scintillation cocktail for radioactive measurement using a scintillation counter (Beckmann Instruments Co.). The counts recovered in the control assay (minus enzyme) were subtracted from the total counts recovered in the experimental sets. One unit of the enzyme activity was defined as 1 pmole of the product formed/min at 26°C.

Preparation of crude extract. Fresh PLN (2 kg) were cut into small pieces and homogenized with 4 liters of homogenizing buffer (20 mM Tris-HCl, 0.25 M sucrose, 5 mM EDTA, 5 mM β-mercaptoethanol and 0.5 mM PMSF, pH 8.0). The microsomal fraction from the tissue homogenate was obtained by using a method essentially as described (23). The microsomes were resuspended in 400 ml of solubilizing buffer (20 mM Hepes/NaOH, 1 mM DTT, 10% glycerol and 1% (w/v) Triton X-100, pH. 7.2). The suspension was kept on ice with gentle shaking for an hour and centrifuged at 100,000 g for 1h. The supernatant was collected (~500 ml) and kept frozen at −80°C.

Purification procedures. Approximately, 500 ml of Affi-gel blue sepharose was washed thoroughly and equilibrated in buffer A (20 mM Hepes/NaOH, 10% glycerol, 0.1% Triton X-100, and 0.2 mM DTT, pH 7.0). The equilibrated gel was allowed to bind with 500 ml of the crude extract for about 2 h at 4°C. The gel beads were collected by centrifugation at 400 g for 5 min. After washing with 2 liters of the buffer A, the gel suspension was poured into a column (internal diameter 10 × 100 cm). The column was attached to an FPLC system (Pharmacia Biotech, Piscatsaway, NJ) and washed with 3 liters of buffer A containing 0.4 M NaCl. Proteins bound to the gel were eluted with 2 liters of buffer A containing 2M NaCl at a flow rate of 10 ml/min. Fractions (20 ml each) containing enzyme activity were pooled and concentrated to approximately 50 ml by ultrafiltration using YM-100 membranes using an Amicon apparatus.

Eluates from two preparations (each from 2 kg of PLN) from Affi-gel blue sepharose were pooled (~100 ml). The pooled fraction was loaded onto a Sephacryl S-300 column (10 × 120 cm), which was pre-equilibrated with buffer C (20 mM Hepes/NaOH, 0.2 mM DTT, 10% glycerol and 50 mM NaCl). The enzyme activity was eluted with buffer C at a flow rate of 2.5 ml per minute. Fractions containing the enzyme activity were pooled and immediately applied to a DEAE-TSK column (2.5 × 30 cm) that was previously equilibrated with buffer D (20 mM Hepes/NaOH, 0.25 mM DTT and 10% glycerol, pH. 7.0). The column was subsequently washed with buffer D (100 ml) and buffer D containing 250 mM NaCl (200 ml). The enzyme activity was eluted with a linear gradient of 500 ml from 250-600 mM concentration of NaCl. Activities of both Gal-6-*O*-Stase and GlcNAc-6-*O*-Stase were assayed in each fraction. Fractions containing the upper half of the Gal-6-*O*-Stase activity were pooled and concentrated by ultrafiltration using YM-100 membranes using an Amicon apparatus.

The enzyme fraction after the DEAE-TSK step was diluted with buffer E (potassium phosphate buffer 10 mM, 0.25 mM DTT, 10% glycerol, pH. 7.2) and applied onto a hydroxylapatite column (2.0 × 10 cm) that was pre-equilibrated with the same buffer. The column was subsequently washed with 60 ml each of buffer E and buffer F (potassium phosphate buffer 50 mM, 0.25 mM DTT, 10% glycerol, pH. 7.2). The bound enzyme activity was eluted with 120 ml of a linear gradient with increasing concentrations of potassium phosphate from 50-500 mM. The enzyme activity eluted as a sharp peak between 70 to 100 mM. The upper half of the enzyme activity peak was pooled and immediately concentrated to about 20 ml and 5 ml of 25 mM $MgCl_2$, 0.5% Triton X-100 and 40% glycerol was added to partially stabilized the enzyme activity.

The enzyme after hydroxylapatite was further purified by affinity chromatography on an 3′,5′-ADP-agarose column (0.5 × 5 cm). The column was packed and equilibrated in buffer G (20 mM Hepes/NaOH, 0.25 mM DTT, 20% glycerol, 0.1% Triton X-100 and 5 mM $MgCl_2$). The hydroxylapatite-purified enzyme was applied onto 3′,5′-ADP-agarose column at a flow rate of 0.25 ml/min. After washing the column with 25 ml of buffer G, the enzyme activity was eluted with buffer G containing 1 M NaCl at a flow rate of 0.5 ml/min. Since the recovery of the catalytically active enzyme from 3′,5′-ADP-agarose column was very poor in the eluate when 3′,5′-ADP was used for

[2] Details of the assay will be published elsewhere (C. C. Gorka, K. O. Broschat, Q. K. Huynh, and G. S. Jacob).

TABLE I

Purification of Gal-6-*O*-Stase from Porcine Peripheral Lymph Nodes

Purification steps	Total volume (ml)	Total units[a]	Total protein[b] (mg)	Specific activity[a] (Units/mg)	Fold purification	Yield %
Solubilized extract[c]	1000	3782	10,965	0.34	1	100
Affi-gel blue sepharose	100	1594	415	3.84	11	42.12
Sephacryl S-300	380	1190	195	6.10	18	31.44
DEAE-TSK	65	893	37	24.13	71	23.59
Hydroxylapatite	25	469	4.56	102.85	303	12.39
3′,5′-ADP-sepharose	5	71	0.006[d]	11,833.34	34,804	1.87

[a] One unit of enzyme activity was defined as the amount required to catalyze the transfer of 1 pmole of sulfate per min. Specific activity was expressed as units per mg of protein.

[b] Protein concentration was determined by using bovine serum albumin as the standard.

[c] Two preparations of solubilized extracts from 2 kg of PLN each were pooled. Hence, a total of 4 kg tissue was used for the enzyme purification.

[d] Protein concentration was estimated by amino acid analysis.

elution, we had to use 1M NaCl for elution. Fractions (1.5 ml each) were collected and the enzyme activity was assayed immediately. The enzyme activity containing fractions were concentrated to about 5 ml and stored frozen at −80°C. The enzyme preparation after 3′,5′-ADP-agarose was extremely unstable, and was not suitable for further purification.

Photoaffinity labeling. The procedure used for the photoaffinity labelling was a modification of the procedure as described previously (25). Briefly, 10 to 20 μl of the enzyme was incubated with 2 μCi 8-azido-[^{32}P]-PAPS in the presence of 1 mM $MgCl_2$ 4 mM 2′-FL and 0.25 mM DTT in a final volume of 30 μl for 2 min on ice. After the incubation, the reaction mixture was exposed to UV (short wave length 254 nm) by a hand held UV lamp for 2 min. The reaction was stopped by adding 10 μl of Laemmli buffer (4 × concentration) containing 20% β-mercaptoethanol followed by heating at 95°C for 2 min. The samples were then subjected to reducing SDS-PAGE on 4-15% linear gradient gels (Bio-Rad Laboratories) followed by autoradiography (25).

Substrate specificity. Purified enzyme was incubated with 4 mM oligosaccharides for 16 hrs at 25°C in 20 mM Hepes buffer, pH 7.2 containing 80 μM ^{35}S-PAPS, 1 mM DTT, 10 mM NaF, 1mM ATP, and 10 mM $MgCl_2$. A 2 μl aliquot from each sample was spotted onto a 10×10 cm HP-TLC plate (Merck 5633) and developed in ethanol: *n*-butanol: pyridine: acetic acid: H_2O = 100:10:10:3:30. Sulfonated oligosaccharides were detected by autoradiography. R_f values for the oligosaccharide acceptors were detected by the Orcinol: sulfuric acid staining (13).

Reaction product analysis. 100 μl of the enzyme after the hydroxylapatite step was incubated with 120 μM of [^{35}S]-PAPS, 20 mM of 2′-FL, 1.0 mM ATP, 10 mM NaF, 1.0 mM DTT, 10 mM $MgCl_2$ and 20 mM of HEPES/NaOH (pH 7.2) in a final volume of 200 μl. After an incubation of 16 h at 26°C, the reaction mixture was diluted to 2 ml with 120 mM ammonium bicarbonate and passed through an ion-exchange column (2.5 ml bed volume; Dowex AG 1-X8) that was pre-equilibrated with 120 mM ammonium bicarbonate. Approximately 15 ml of the eluate was collected and subjected to repeated lyophilization-thawing cycles to remove an excess of ammonium bicarbonate. Finally, the dried material was dissolved in 100 μl of double distilled water and analyzed by HP-TLC by the method as described earlier (22).

RESULTS

Purification of Gal-6-Stase. We used a number of sugars acceptors to differentiate Gal-6-*O*-Stase and GlcNAc-6-*O*-Stase activities. Based on these results, 2′-FL was selected as the substrate for Gal-6-*O*-Stase, and GlcNAc-β1,6-Gal was chosen as the substrate for GlcNAc-sulfotransferase in enzyme activity assays. These two sulfotransferase activities were found to be completely separated by the ion-exchange chromatographic step on the DEAE-TSK column. As expected, fractions containing Gal-6-*O*-Stase activity did not react with GlcNAc-β1,6-Gal as the substrate. Similarly, fractions containing GlcNAc-sulfotransferase activity were completely inactive against 2′-FL. The enzyme activity was purified to more than 35,000-fold with an overall yield of about 6 μg from 4 kg of the PLN tissue (Table I). The purified enzyme preparation showed a protein band at 170 kDa and a diffused protein band, often difficult to stain even with silver reagents, at 80-90 kDa on 7.5% SDS-PAGE under reducing condition. Both protein bands always comigrated in the gel when peak fractions containing Gal-6-*O*-Stase activity from the 3′,5′-ADP-agarose column were subjected to 6% SDS-PAGE under reducing condition. The intensities of staining of these protein bands correlated well with the enzyme activity (Fig. 1).

To identify the protein band(s) associated with Gal-6-*O*-Stase activity, the enzyme preparation after the hydroxylapatite chromatography was incubated with WGA-, EBA-, and Con A-agarose beads. After washing, the lectin-beads were directly used for the enzyme activity measurements. The enzyme activity bound to WGA-, EBA- and Con A-agarose (Fig. 2a), suggesting glycoprotein nature of the enzyme. Furthermore, when bound proteins from these lectin-beads were eluted by boiling with Lammli's buffer and subjected to SDS-PAGE under reducing conditions, both 80-90 and 170 kDa proteins bands were found to bind WGA-, EBA-, and Con A-agarose beads (Fig. 2b). The staining of 80-90 kDa protein was so faint that it could not be reproduced very well in the gel picture. An additional

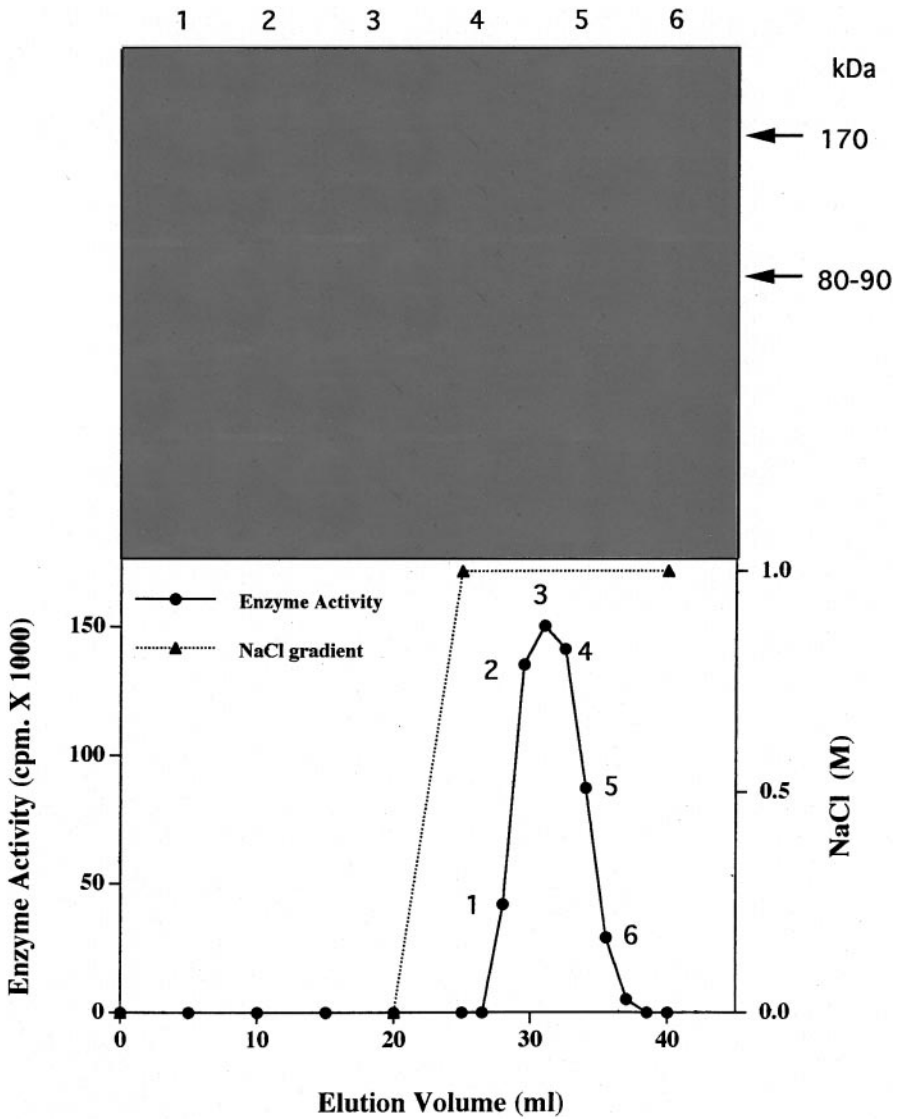

FIG. 1. Purification of the Gal-6-*O*-Stase activity on 3′,5′-ADP-agarose column. The enzyme preparation after hydroxylapatite was further purified by the affinity chromatography on 3′,5′-ADP-agarose column (see Materials and Methods). 250 μl of the samples from each fraction containing the enzyme activity, marked as 1-6, was desalted by gel filtration and concentrated to about 25 μl. The concentrated samples were mixed with the Laemmli's buffer and subjected to 7% SDS-PAGE under reducing conditions. The gel was stained with silver reagents. Arrows indicate the positions of 80-90 and 170 kDa protein bands.

protein band of 120 kDa also showed a similar lectin binding pattern. However, the amino acid analysis of this 120 kDa protein band revealed a sequence LSV-FSGGENSVHQIQYR, which was not present in any known sulfotransferases. Furthermore, this sequence was found to be homologous to amino acid sequence from a protein known as M-110 macrophage differentiation marker in mice (26). Hence, the 120 kDa protein band was excluded as the potential candidate for the Gal-6-*O*-Stase enzyme.

As a further attempt to identify the protein band(s) associated with Gal-6-*O*-Stase activity, we performed photoaffinity labeling with 8-azido-[^{32}P]-PAPS. The desalted enzyme after the 3′,5′-ADP-agarose step was extremely unstable and was not found to be suitable for the photoaffinity labeling. Hence, the enzyme after the hydroxylapatite step was used for the photoaffinity labeling (Fig. 3), which revealed sharp protein band at 170 kDa and a diffused protein band at 80-90 kDa, as indicated by arrows (lanes 2-4). The labeling of these two protein bands increased with increasing amounts of the enzyme preparation (lanes 2-4) and was UV-dependent, as the control reaction done in the absence of UV did not show radiolabeling of these protein bands (lane 5). Furthermore, UV-dependent labeling of these protein bands was competitively inhibited by 1 mM

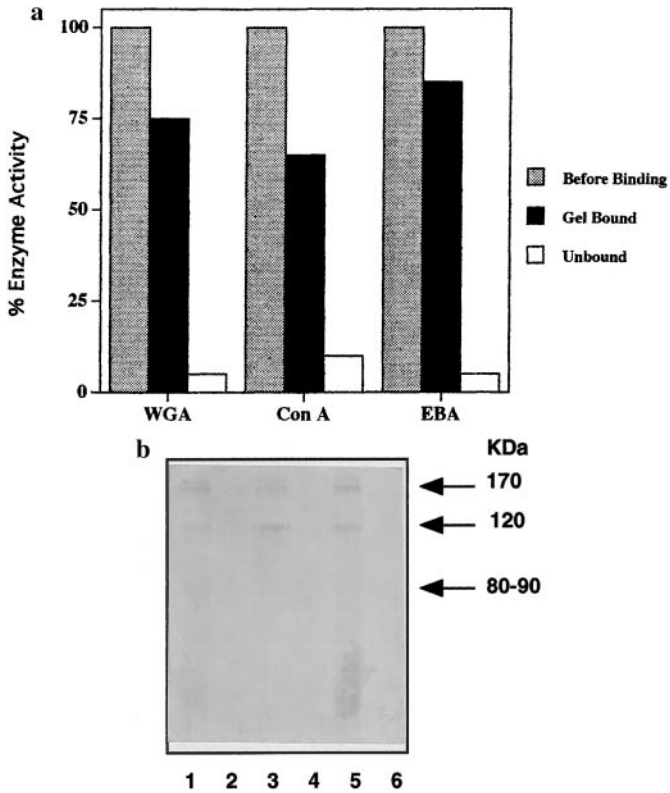

FIG. 2. Lectin binding properties of the enzyme preparation after the hydroxylapatite chromatography. (**a**) 0.5 ml of the enzyme after hydroxylapatite was allowed to bind with 200 μl of WGA-, EBA-, and Con A-agarose beads by gentle shaking for 1 h at 4°C. The beads were washed five times with buffer D. Gel beads were divided into two aliquots of 25 μl each. One was used directly for the measurement of the enzyme activity and the other was used for the electrophoresis. (**b**) 25 μl aliquots of the enzyme-bound WGA-agarose (lane 1), Con A-agarose (lane 3), and EBA-agarose (lane 5) beads were boiled for 2 min with 25 μl of 2× Laemmli's buffer containing 10% β-mercaptoethanol and subjected to 7.5% SDS-PAGE. Agarose beads of WGA- (lane 2), Con A- (lane 4), and EBA-agarose (lane 6) were used as controls. Arrows indicate the positions of 120 kDa and 170 kDa protein bands. The 80-90 kDa protein band was very faint and could not be photographically reproduced very well in the figure.

PAPS (lane 1), suggesting substrate specific photolabeling. In addition, a few other protein bands were also photolabeled, as indicated by NS. However, photoaffinity labeling of these protein bands was neither dependent on UV-light nor was inhibited by 1 mM of PAPS, indicating non-specific labeling.

Properties of the Gal-6-O-Stase. The enzyme preparation after the hydroxylapatite step was used for most of the experiments related to the characterization of this sulfotransferase. The enzyme activity towards 2′-FL increased linearly with increase in the time of incubation up to 6 hr at 26°C. The pH dependence of the purified enzyme was investigated at a pH range from 5.0 to 9.0. Similar to other sulfotransferases (27,28), the purified enzyme showed a sharp pH optimum around 7.2 (data not shown). The isoelectric pH of the enzyme was determined by a preparative isoelectric focusing with ampholytes in the pH range 3 to 10. The enzyme activity focussed sharply between pH 4.5-5.0 (data not shown). The isoelectric pH between 4.5-5.0 is consistent with its localization in the Golgi apparatus. Kinetic measurements with various PAPS concentrations yielded a Km value of 9.8 μM, a value similar to those published for other sulfotransferases (27,28).

Substrate specificity. The substrate specificity of the enzyme was evaluated with various sugar acceptors and the production of sulfated reaction products was analyzed by the HP-TLC (Fig. 4). As can be seen, a single sulfated product was produced when 2′-FL was used as the substrate (lanes 5 & 6). Similarly, 3′-sialyllactose (lane 1) and 3′-sialyllactosamine (lane 2) were efficient acceptors of the sulfation and also produced a single sulfated product. However, the enzyme was completely inactive against 6′-sialyllactose (lane 3) and 6′-sialyllactosamine (lane 4), indicating that the enzyme was not able to transfer the sulfate group if the C-6 position on the galactose residue was already occupied. These results suggested that the purified sulfotransferase may be selective for sulfation at the C-6 position of the galactose residue.

Reaction product analysis. Analysis of the reaction product (Fig. 5) was performed by incubating 2′-FL with the enzyme preparations after the Affi-gel blue

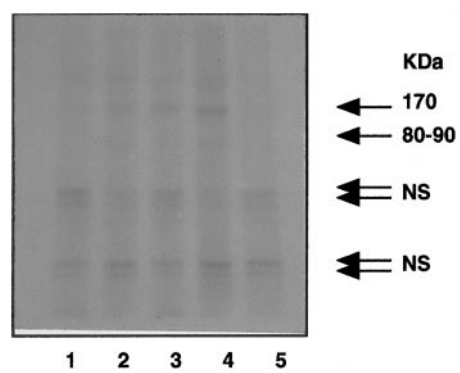

FIG. 3. Photoaffinity labeling with 8-azido-[^{32}P]-PAPS. 10 to 20 μl of the enzyme preparation after the hydroxylapatite chromatography was incubated with 2 μCi 8-azido-[^{32}P]-PAPS in the presence of 1 mM $MgCl_2$ 4 mM 2′-FL and 0.25 mM DTT in a final volume of 30 μl for 2 min on ice. After the incubation, the reaction mixture was exposed to UV light (short wave length 254 nm) by a hand held UV lamp for 2 min. The reaction was stopped by adding 10 μl of Laemmli buffer (4 × concentration) containing 20% β-mercaptoethanol followed by heating at 95°C for 2 min. The samples were then subjected to 4-15% gradient SDS-PAGE followed by autoradiography (25). Lane 1, Photolabeling of 20 μl of the enzyme was performed in the presence of 1 mM PAPS; lanes 2-4, increasing amounts of the enzyme 10, 15, and 20 μl, respectively. Control reaction without UV exposure (lane 5). Arrows indicate the positions of 80-90 and 170 kDa protein bands. NS indicates positions of non-specifically labeled proteins.

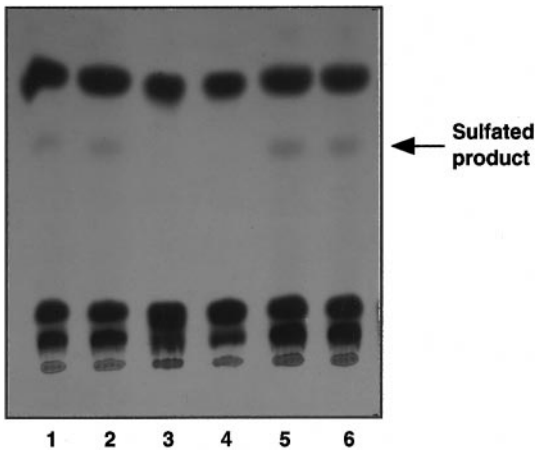

FIG. 4. Substrate specificity of Gal-6-*O*-Stase. The enzyme preparation after the hydroxylapatite step was incubated with 3′-sialyllactose (lane 1), 3′-sialyllactosamine (lane 2), 6′-sialyllactose (lane 3), 6′-sialyllactosamine (lane 4), 2′-FL purified from human milk (lane 5) and 2′-FL from Sigma Chemical Co. (lane 6) in the standard reaction system (see Materials and Methods) for 16 h at 26°C. Two μl of the reaction mixture was analyzed by the HP-TLC followed by autoradiography. The arrow indicates the position of the sulfated-sugar product. The other radioactive bands, shown in the figure, are the degradation products of [^{35}S]-PAPS.

sepharose step (lanes 1 & 2) as well as with the enzyme preparation after the hydroxylapatite step (lanes 3 & 4). In addition, two sets of control reactions were also carried out under identical conditions except that in one set of reactions the boiled crude enzyme was added (lanes 5 & 6) and in the other set 2′-FL was omitted (lanes 7 & 8). The reaction products were analyzed by the HP-TLC before (lanes 1, 3, 5 and 7) and after (lanes 2, 4, 6 and 8) their purifications by ion-exchange chromatography (see Materials and Methods). The purified reaction product exhibited a single sulfated product, as indicated by the arrow with either of the enzyme preparations (lanes 2 and 4). This radioactive band was missing in both the enzyme-minus control (lane 6) and in 2′-FL minus control (lane 8). The structure analysis by the FAB mass spectrophotometry identified this radioactive band as the Fuc(α1,2)Gal-6-*O*-sulfate (β1,4)Glc and also indicated that the sulfate substitution was at the C-6 position of the galactose residue (manuscript under preparation).[3] These results suggest that the purified sulfotransferase is specific for the sulfation at the C-6 position of the galactose residue.

DISCUSSION

It is apparent from our experiments that the PLN tissue is a rich source of a sulfotransferase that transfers sulfate from PAPS to the C-6 position of the galactose residue of 2′-FL and other sLe^X-related sugars. The enzyme was purified to about 35,000-fold by a procedure involving a combination of conventional and affinity chromatographic steps. Since we had used solubilized microsomal extract and not the tissue homogenate as the starting material, the purification fold of 35,000 is actually an underestimation. Considering a reasonable 10-fold enrichment of the enzyme activity in the solubilized microsomal extract over the tissue homogenate, the total purification fold is more likely in the range of 350,000-fold.

The most purified enzyme exhibited a sharp protein band at 170 kDa and a diffused protein band, often difficult to stain even with silver reagents, at 80-90 kDa. Both protein bands always comigrated in the gel when the peak fractions containing Gal-6-*O*-Stase activity from the 3′,5′-ADP-agarose chromatography were subjected to SDS-PAGE. In addition, both protein bands exhibited identical patterns for binding to WGA-, EBA-, and Con A-agarose beads and to photoaffinity labeling with 8-azido-[^{32}P]-PAPS. Furthermore, the native enzyme eluted in the void volume from Sephacryl S-300 gel filtration column, suggesting that the enzyme might exist as an oligomer in its native state. These observations raised the possibility that these two protein bands may be associated with the enzyme activity. Our efforts to determine the N-terminal amino acid sequences of 170 kDa remained unsuccessful because this protein was found to be N-terminally blocked and its tryptic peptides only revealed the partial amino acid sequences, which were

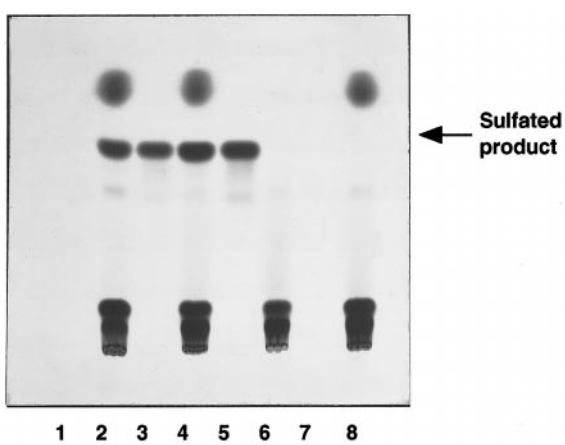

FIG. 5. Product analysis of the reaction catalyzed by Gal-6-*O*-Stase. 20 mM of 2′-FL was incubated with the enzyme preparation after the Affi-gel blue sepharose step (lanes 1 & 2) as well as with the enzyme preparation after the hydroxylapatite step (lanes 3 & 4). In addition, two sets of control reactions were also carried out under identical conditions except that in one set of reactions the boiled crude enzyme was added (lanes 5 & 6) and in the other set 2′-FL was omitted (lanes 7 & 8). Analyses of the reaction products before (lanes 1, 3, 5 and 7) and after (lanes 2, 4, 6 and 8) their purification by ion-exchange chromatography (see the Materials and Methods) were performed by the HP-TLC followed by autoradiography. Arrow indicates the position of the sulfated-2′-FL (Fuc(α1,2)Gal-6-*O*-sulfate(β1,4)Glc). The other radioactive bands, shown in the figure, are the degradation products of [^{35}S]-PAPS.

[3] Manuscript in preparation (Broschat, K.O., C. C. Gorka, S.H. Haslam, Howard R. Morris, G. S. Jacob, and Anne Dell.)

not sufficient to compare with amino acid sequences of other sulfotransferases in the databases.

The substrate specificity of the purified enzyme indicates that this enzyme is very well suited to participate in the assembly of 6′-sulfo sialyl Lewis^x. The preferred substrates for this enzyme are the sugars that are similar in structure to sLe^x and are not substituted at the C-6 position of the galactose residues, such as in 3′-sialyllactose and 3′-sialyllactosamine. Consistent with this observation, 6′-sialyllactose and 6′-sialylatosamine were not the acceptors of sulphonation by this enzyme. Our finding that the C-6 sulphonation of 3′-sialyllactose was as efficient as that of 3′-sialyllactosamine suggest that the substitution of glucose for GlcNAc does not seem to affect the enzyme activity. Coincidentally, substitution of glucose for GlcNAc does not impair the binding of sLe^x with L-selectin (29).

A survey of various porcine tissues indicated that the distribution of Gal-6-*O*-Stase is quite selective, with lymph nodes (peripheral, mesenteric) and spleen demonstrating higher level of expression, while liver and heart exhibiting lower enzyme activity. Consistent with the tissue distribution of the enzyme, we had earlier shown that a 120 kDa glycoform of the sialomucin CD-34 was the major ligand in the PLN tissue (13). In addition, the staining with MECA-79, a monoclonal antibody that selectively recognizes HEV-ligands *viz.*, GlyCAM-1 and CD-34 glycoform, was also found to be specific to HEV of this tissue (11,13). The detailed structural analysis of sugar chains on the GlyCAM-1 has demonstrated that it can have a sulfate group in the C-6 position of galactose as in 6′-sulfo sLe^x as well as in C-6 position of GlcNAc, as in 6-sulfo sLe^x (9). Subsequently, it was shown that anti-sialyl Le^x antibodies that bind to 6-sulfo sLe^x reacted to HEV in lymph nodes and also inhibited the binding of L-selectin to HEV, whereas antibodies that bind to 6′-sulfo sLe^x failed to react with HEV (30). On the other hand, a recent report demonstrated that the 6-sulfo sLe^x was not an efficient ligand for either E- or L-selectin (31). The latter report favors the view that 6′-sulfo sialyl Le^x is a much better ligand for binding to L-selectin. In view of these observations, the Gal-6-*O*-Stase is a likely candidate involved in the sulfation of the ligand for L-selectin.

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