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Purification and Characterization of UDP-GlcNAc:Galβ1–4-GlcNAcβ1–3*Galβ1–4Glc(NAc)-R(GlcNAc to *Gal) β1,6*N*-Acetylglucosaminyltransferase from Hog Small Intestine*

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A β1,6*N*-acetylglucosaminyltransferase (β1–6GnT) responsible for the formation of the β1,6-branched poly-*N*-acetyllactosamine structure has been purified 210,000-fold in 2.4% yield from a homogenate of hog small intestine by successive column chromatographies involving CM-Sepharose FF, Ni^{2+}-chelating Sepharose FF, and UDP-hexanolamine-agarose, using an assay wherein pyridylaminated lacto-*N*-neotetraose (Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA) was used as an acceptor substrate, and the reaction product was Galβ1–4GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4Glc-PA. The apparent molecular weight of the purified enzyme was 76,000 under nonreducing conditions. The enzyme has a pH optimum at 7.0 and has no requirement for any divalent metal ions. The K_m values for pyridylaminated lacto-*N*-neotetraose and UDP-GlcNAc were 0.96 and 2.59 mM, respectively. For its activity, this enzyme was shown to have an absolute requirement of at least a complete LacNAc (LacNAc = Galβ1–4GlcNAc) residue bound to position 3 of the acceptor Gal residues, *i.e.* it is capable of acting only on the Gal residues of internal LacNAc units. The data strongly suggest that this enzyme could be involved in generating branches to central positions of preformed as well as growing polylactosamine chains, but not in synthesizing the distal branches to growing polylactosamine chains.

Linear and branched poly-*N*-acetyllactosamine chains, which consist of repeating *N*-acetyllactosamine units (LacNAc = Galβ1–4GlcNAc),[1] occur in glycoproteins, glycolipids, and proteoglycans. In linear poly-*N*-acetyllactosamine chains, the *N*-acetyllactosamine units are linked via β1,3 linkages. In branched glycans, some of the 3-*O*-substituted Gal residues in the primary chains are also substituted at position 6 by additional *N*-acetyllactosamine units. Linear and branched poly-*N*-acetyllactosaminoglycan backbones represent the blood group i and I antigens, respectively (1–3), the expression of which is known to be regulated in the development of fetal to adult erythrocytes (4–6), in the course of murine embryonic development (7), and in embryonal carcinoma cells (8).

At the present time, two types of β1–6GnT activities are known to act on linear poly-*N*-acetyllactosamine chains, leading to the generation of the blood group I antigen structure. The first type of the enzyme activity acts at the (growing) ends of poly-*N*-acetyllactosamine chains, converting GlcNAcβ1–3Galβ1–4GlcNAcβ1-R to GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAcβ1-R (9–14). We refer to this enzyme activity as dIGnT6 to emphasize the *distal* site of its action (15, 16). The second type of the enzyme activity acts at the midchain positions of the (completed or growing) poly-*N*-acetyllactosamine chains, converting Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ1-R to Galβ1–4GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAcβ1-R (13, 15–17). Accordingly, we refer to this enzyme activity as cIGnT6.

Until now, the substrate specificity of the purified forms of these enzymes has not been reported. This study reports on the purification of the cIGnT6 from hog small intestine by successive column chromatographies on CM-Sepharose FF, Ni^{2+}-chelating Sepharose FF, and UDP-hexanolamine-agarose, using pyridylaminated lacto-*N*-neotetraose (Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA) as an acceptor substrate, the product of which was Galβ1–4GlcNAcβ1–3(**GlcNAcβ1–6**)Galβ1–4Glc-PA. By using several acceptor compounds, this purified enzyme was shown to have an absolute requirement for a complete LacNAc residue bound to position 3 of the acceptor galactose residue for its activity. Consequently, it was devoid of dIGnT6 activity.

EXPERIMENTAL PROCEDURES

Materials—Materials were obtained from the following suppliers: UDP-GlcNAc, UDP-glucose, UDP-galactose, UDP-glucronic acid, UDP-hexanolamine, UDP-hexanolamine-agarose (ligand concentration: 2.4 μmol/ml), UDP, UMP, UTP, AMP, GDP, and maltohexaose from Sigma; CM-Sepharose FF, Q-Sepharose FF, chelating Sepharose FF, CNBr-activated Sepharose 4B, and Superdex Peptide HR 10/30 column from Pharmacia (Uppsala, Sweden); Tris, MES, and MOPS from Nacalai tesque (Kyoto, Japan); Triton X-100, pAPMSF, glycine, DTT, and metal chlorides from Wako (Osaka, Japan); EDTA and heptylthioglucoside from Dojindo Laboratories (Kumamoto, Japan); lacto-*N*-neotetraose, lacto-*N*-tetraose, lacto-*N*-hexaose, and jack bean β-galactosidase from Seikagaku Corp. (Tokyo, Japan); *N*-acetylgalactosaminodecamer from ICN Pharmaceuticals Inc. (Costa Mesa, CA); Bio-Gel P-4 from Bio-Rad. UDP-hexanolamine-Sepharose 4B (ligand concentration over 8 μmol/

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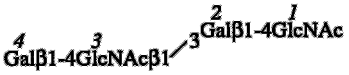
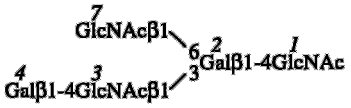
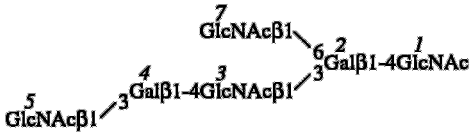
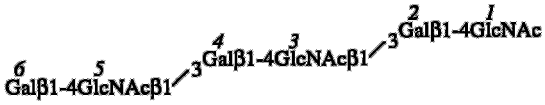
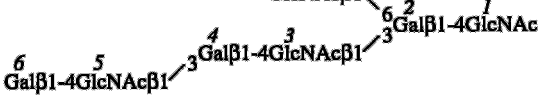
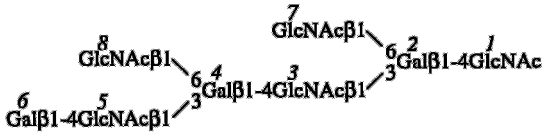
[1] The abbreviations used are: LacNAc, *N*-acetyllactosamine; β1–6GnT, β1,6-*N*-acetylglucosaminyltransferase; BSA, bovine serum albumin; cIGnT6, "centrally acting" β1,6-*N*-acetylglucosaminyltransferase; dIGnT6, "distally acting" β1,6-*N*-acetylglucosaminyltransferase; DTT, dithiothreitol; Gal and G, D-galactose; GlcNAc and Gn, *N*-acetyl-D-glucosamine; GnT, *N*-acetylglucosaminyltransferase; ^{1}H NMR, proton nuclear magnetic resonance; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PA, 2-aminopyridine; pAPMSF, (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride; PAGE, polyacrylamide gel electrophoresis.

TABLE I

Structures of the acceptor and the product oligosaccharides

Structures of the acceptor oligosaccharides and products obtained in reactions catalyzed by the β1–6GnT of hog small intestine. Denotation of monosaccharide residues is also shown.

No	Acceptor	No	Product(s)
1		**4**	
2		**5**	
3		**6**	
		7	
		8	

ml) was prepared according to a previously reported method (18).

Preparation of the Substrates and the Authentic Products—The numbering and structures of key oligosaccharides are shown in Table I. Glycans **1, 2,** and **3** were synthesized essentially as described previously (15, 17, 19). Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA (lacto-*N*-neotetraose-PA), Galβ1–3GlcNAcβ1–3Galβ1–4Glc-PA (lacto-*N*-tetraose-PA), and Galβ1–3GlcNAcβ1–3(Galβ1–4GlcNAcβ1–6)Galβ1–4Glc-PA (lacto-*N*-hexaose-PA) were prepared by pyridylamination of lacto-*N*-neotetraose, lacto-*N*-tetraose, and lacto-*N*-hexaose using the commercial pyridylamination apparatus Glyco TAG (Takara, Shiga, Japan), respectively. GlcNAcβ1–3Galβ1–4Glc-PA was prepared by digestion of lacto-*N*-neotetraose-PA with jack bean β-galactosidase. GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4Glc-PA and Galβ1–3GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4Glc-PA were prepared by complete or partial digestion of lacto-*N*-hexaose-PA with jack bean β-galactosidase.

Determination of β1–6GnT Activity—β1–6GnT activity was assayed according to the method of Gu *et al.* (13) with minor modifications. The standard incubation mixture contained the following components in a total volume of 10 μl; 200 mM MOPS buffer (pH 7.0), 10 mM UDP-GlcNAc, 0.5% Triton X-100, 5 mM EDTA, 2 mg/ml BSA, 20 μM lacto-*N*-neotetraose-PA, and enzyme fraction (3 μl). After incubation at 37 °C for 4 h, 40 μl of water was added, and the enzyme reaction was quenched by boiling for 1 min. After centrifugation at 10,000 rpm for 5 min, 10 μl of the supernatant from the reaction mixture was applied to a TSK-gel ODS-80TM column (4.6 × 250 mm; Tosoh, Tokyo, Japan). The elution was performed at 50 °C using an eluent of 20 mM ammonium acetate (pH 4.0) at a flow rate of 1 ml/min. Fluorescence was monitored with excitation and emission wavelengths of 320 and 400 nm, respectively. The specific activity of the enzyme was expressed as picomoles of product/hour/mg of protein. The protein concentration was determined with a BCA kit (Pierce) or a Bio-Rad protein assay reagent (Bio-Rad) using BSA as the standard.

Buffers Used in Purification of β1–6GnT—The buffers used in this paper were as follows, with the pH measured at 4 °C: Buffer A, 0.25 M sucrose, 100 μM pAPMSF, 10 mM Tris-HCl (pH 7.4); Buffer B, 20% glycerol, 1% Triton X-100, 100 μM pAPMSF, 10 mM MES (pH 6.5); Buffer C, 20% glycerol, 0.1% Triton X-100, 20 mM MES (pH 6.0); Buffer D, 0.8 M NaCl/Buffer C; Buffer E, 0.3 M NaCl, 20% glycerol, 0.1% Triton X-100, 20 mM Tris-HCl (pH 8.0); Buffer F, 0.2 M glycine/Buffer E; Buffer G, 20% glycerol, 0.1% Triton X-100, 0.5 mM DTT, 20 mM MOPS (pH 7.0); Buffer H, 0.5 M NaCl/Buffer G; Buffer I, 25 mM EDTA/Buffer H; Buffer J, 20% glycerol, 1% heptylthioglucoside, 0.5 M NaCl, 0.5 mM DTT, 20 mM MOPS (pH 7.0); Buffer K, 4 mM UDP/Buffer J; Buffer L, 20% glycerol, 1% heptylthioglucoside, 0.5 mM DTT, 20 mM MOPS (pH 7.0).

Homogenization and Preparation of the Microsome Fraction (Step 1)—All purification steps were carried out at 4 °C. Frozen hog small intestine (127 g) was homogenized with a Waring blendor in 4 volumes of Buffer A. After centrifugation at 900 × *g* for 10 min, the supernatant was pooled, and the pellet was subjected to two more extractions, after which all the supernatants were combined. After centrifugation at 78,000 × *g* for 2 h, the microsomal fraction was obtained as a precipitate.

Solubilization of β1–6GnT (Step 2)—The microsomal fraction was suspended in 3 volumes of Buffer B, gently stirred for 2 h, and then centrifuged at 105,000 × *g* for 1 h. The supernatant fraction was collected, and the residual pellet was subjected to another extraction followed by ultracentrifugation. The first and the second Triton extracts were combined and used for further enzyme purification.

CM-Sepharose FF Column Chromatography (Step 3)—Three volumes of Buffer C was added to the combined Triton X-100 extracts and then applied to the column of CM-Sepharose FF (5.0 × 14.5 cm), which had been equilibrated with Buffer C. The column was washed with Buffer C until the protein concentration was reduced to below 0.05 mg/ml. The elution was carried out with a linear gradient between 850 ml of Buffer C and 850 ml of Buffer D. The fractions containing β1–6GnT activity were combined.

Ni^{2+}-chelating Sepharose FF Column Chromatography (Step 4)—An equivalent volume of Buffer E was added to the pooled enzyme fraction

from the CM-Sepharose FF column chromatography step and then loaded on a column of Ni^{2+}-chelating Sepharose FF (2.5 × 8.2 cm), which had been equilibrated with Buffer E. Ni^{2+}-chelating Sepharose resin was layered on the chelating Sepharose FF resin without metal ions (2.5 × 4.1 cm), to avoid any possible leakage of Ni^{2+} into the enzyme fractions. The β1–6GnT activity was completely bound to the column. After washing the column with Buffer E until the protein concentration was reduced to below 0.02 mg/ml, β1–6GnT activity was eluted with a linear gradient between 200 ml of Buffer E and 200 ml of Buffer F. The fractions that contained β1–6GnT activity were pooled, and the buffer in this fraction was replaced by Buffer G by means of an Amicon Diaflow Ultrafiltrater using a YM 30 membrane (Amicon, Beverly, MA) (the final volume of this concentrated fraction was 42 ml).

UDP-Hexanolamine-Agarose Affinity Column Chromatography (Step 5)—After the above step, the column was siliconized with Sigmacote (Sigma) and siliconized tubes (Assist, Tokyo, Japan) were used for fractionation. The UDP-hexanolamine-agarose column (1.5 × 15 cm) had been equilibrated with Buffer G, and the concentrated enzyme fraction from the Ni^{2+}-chelating Sepharose FF column chromatography was applied to the column, followed by washing with 90 ml of Buffer G, 90 ml of Buffer H, 90 ml of Buffer I, and 90 ml of Buffer J. The elution was performed using a linear gradient between 90 ml of Buffer J and 90 ml of Buffer K. The fractions containing β1–6GnT activity were pooled. This buffer in this fraction was replaced by Buffer L by means of a Centriprep-30 (Amicon) to deplete UDP and NaCl (the final volume of this concentrated fraction was 2 ml). This fraction was used for the enzyme characterization.

UDP-Hexanolamine-Sepharose 4B Affinity Column Chromatography (Step 6)—The concentrated enzyme fraction was applied to a UDP-hexanolamine-Sepharose 4B column (1.0 × 14.5 cm), which had been equilibrated with Buffer L, followed by washing with 10 ml of Buffer L and 30 ml of Buffer J. The elution was performed with a gradient of up to 20 mM UDP-GlcNAc in Buffer J. In total, 70 fractions (fraction volume 1.2 ml) were collected and analyzed by SDS-PAGE.

Gel Electrophoresis—SDS-PAGE was performed by the method of Laemmli (20) using 10% gels. Molecular weight markers (Amersham, Little Chalfont, United Kingdom) were used for size standards. Proteins in the gels were stained by a silver staining kit (2D silver stain II "DAIICHI"; Daiichi Pure Chemicals, Tokyo, Japan).

Acceptor Substrate Specificity of the Purified β1–6GnT—The activity of β1–6GnT was assayed in the standard assay mixture when pyridylaminated sugar substrates were used. When glycans **1**, **2**, and **3** (Table I) were used as the acceptor substrates, the enzyme reactions were carried out at 37 °C for 48 h in a total volume of 25 μl containing 100 nmol of acceptor oligosaccharide, 3.6 μmol of UDP-GlcNAc, 200 mM MOPS (pH 7.0), 20 mM EDTA, 0.5 mM ATP, 2 mg/ml BSA, 0.5% Triton X-100, 8 mM NaN_3, and the enzyme fraction (12.5 μl). The reactions were terminated by heating at 100 °C for 5 min, followed by passage through a mixed bed of Dowex AG1 (AcO^-) and Dowex AG50 (H^+). The products were further purified by chromatography on a column of Superdex Peptide HR 10/30 column (10 × 300 mm, equilibrated with water or 50 mM NH_4HCO_3) with a flow rate of 0.5 or 1 ml/min or on a column of Bio-Gel P-4 (1 × 145 cm, equilibrated with water, fraction volume 1.5 ml). The progress of the elution was monitored by UV absorbance at 205 nm.

Endo-β-Galactosidase Digestion—Oligosaccharide samples (5 nmol) were digested with endo-β-galactosidase from *Bacteroides fragilis* (EC 3.2.1.103) (Boehringer Mannheim, Mannheim, Germany) essentially as described by Leppänen *et al*. (17). The digests were desalted by treatment with an ion-exchange resin and purified further by gel permeation chromatography on a Superdex Peptide HR 10/30 column by pooling together the eluate fractions corresponding to the oligosaccharide area from octasaccharide to GlcNAc. In a parallel control experiment, authentic GlcNAcβ1–3′LacNAcβ1–3′(GlcNAcβ1–6′)LacNAc was cleaved completely into GlcNAcβ1–3Gal and GlcNAcβ1–3′(GlcNAcβ1–6′)LacNAc as analyzed by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

NMR Spectroscopy—One-dimensional proton nuclear magnetic resonance (^1H NMR) spectra of the oligosaccharides were recorded at 23 °C in D_2O at 500 MHz on a Varian Unity 500 spectrometer as described by Leppänen *et al.* (15).

MALDI-TOF MS—MALDI-TOF MS was performed in the positive-ion delayed extraction mode with a BIFLEX™ mass spectrometer (Bruken-Franzen Analytik, Bremen, Germany), using a 337 nm nitrogen laser. 1 μl of aqueous sample solution (10 pmol) and 1.5 μl of 2,5-dihydroxybenzoic acid matrix (10 mg/ml in water) were mixed on the target plate and dried under a gentle stream of air. Maltohexaose and *N*-acetylgalactosaminodecamer were used for external calibration.

RESULTS

Purification of β1–6GnT—The activity of β1–6GnT was assayed using lacto-*N*-neotetraose-PA, basically according to the method of Gu *et al.* (13). BSA (2 mg/ml) was found to be effective in preserving enzyme activity at 37 °C, especially for the highly purified enzyme fraction (Step 5 and Step 6). The activity of this fraction was 3.8-fold higher than that under the assay conditions in the absence of BSA, and as a result, BSA was routinely included in the standard assay mixture.

Hog small intestine was chosen as an enzyme source, based on the following two criteria. First, a survey of various hog and rat tissues revealed that intestine showed the highest enzyme activity per tissue protein (13). Second, hog intestine, which has levels of enzyme activity comparable with rat intestine, was more convenient in terms of cost and quantity.

Like other GnTs, β1–6GnT was concentrated in the microsome fraction. However, 20% of the β1–6GnT activity in the homogenate was found in the cytosolic fraction after ultracentrifugation, suggesting the occurrence of some proteolysis. β1–6GnT from the microsome fraction was solubilized by Triton X-100 more effectively at pH 6.5 than at pH 9.0. More than half of the β1–6GnT activity failed to bind to CM-Sepharose FF (Fig. 1*A*), and this flow-through fraction also failed to bind to CM-Sepharose FF (pH 6.0), Ni^{2+}-chelating Sepharose FF, and Q-Sepharose FF (pH 9.0), none of which was found to be effective for purification. As a result, we used the NaCl eluted fraction for further experiments. The majority of proteins were separated from the β1–6GnT activity by the CM-Sepharose FF and Ni^{2+}-chelating Sepharose FF column chromatography (Fig. 1*B*). After the Ni^{2+}-chelating Sepharose FF column chromatography (Step 4), β1–6GnT activity was more stable than in Step 2 and Step 3. Different from GnT III and GnT IV (21, 22), no β1–6GnT activity was detected after Cu^{2+}-chelating column chromatography. The most effective step for purification was affinity column chromatography (Step 5, Fig. 1*C*). The majority of the enzyme activity bound to the UDP-hexanolamine-agarose column and was eluted very sharply by UDP (about 1 mM) as a competitive ligand. The addition of DTT (0.5 mM) was about 3.3 times more effective on the activity of the eluted enzyme fraction in Step 5 compared with that without DTT, while it was without effect on the enzyme activity in Step 4. The eluted enzyme fraction in Step 5 showed two bands with molecular weights of 76,000 and 60,000 on SDS-PAGE under nonreducing conditions, followed by silver staining (Fig. 2*A*). No activity of GnT III, IV, V, and iGnT (β1–3GnT) was detected in this fraction. All experiments for enzyme characterization described below were performed using this enzyme fraction. Table II summarizes the purification of β1–6GnT, which was purified by 210,000-fold by Steps 1–5. The second affinity column chromatography (Step 6) was used to determine which band was β1–6GnT, although a complete separation of these two bands was not achieved. Two fractions (fraction 57 and 63), which contained almost the same enzyme activity (24 pmol/h/ml), were analyzed by SDS-PAGE and stained with silver (Fig. 2*B*). Only the upper band (76 kDa) showed the same intensity between the two fractions, indicating that the 76 kDa band was β1–6GnT.

pH Optimum—The activity of β1–6GnT was highest between pH 6.5 and 7.5 and displayed an optimum at pH 7.0.

Effect of Divalent Cations on β1–6GnT Activity—The effects of divalent cations on β1–6GnT activity were examined in a reaction mixture composed of 200 mM MOPS buffer (pH 7.0), 10 mM UDP-GlcNAc, 0.5% Triton X-100, 2 mg/ml BSA, 20 μM lacto-*N*-neotetraose-PA, and 10 mM metal chlorides or EDTA. The activity of β1–6GnT was not dependent on metal ions. $MgCl_2$, $CaCl_2$, and $MnCl_2$ had no effect on β1–6GnT activity,

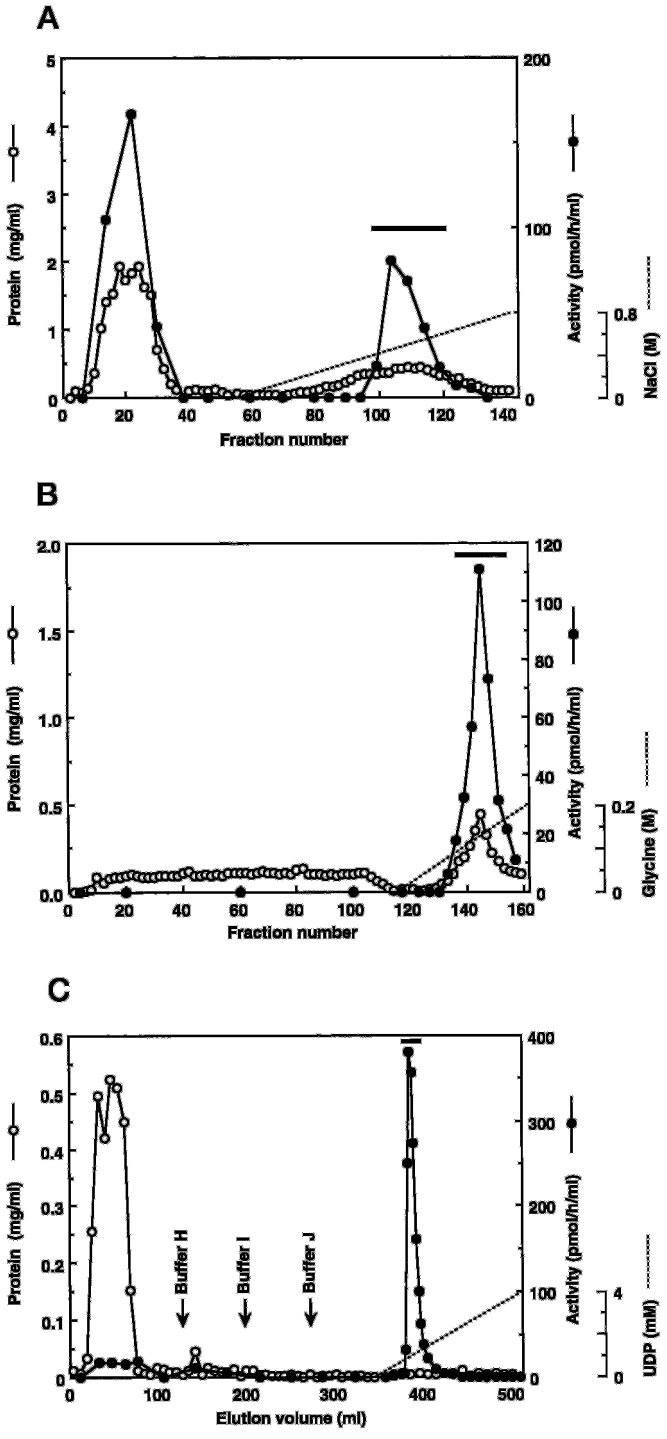


FIG. 1. **Elution patterns for the chromatographic steps in the purification of β1–6GnT.** *A*, CM-Sepharose FF column chromatography. *B*, Ni^{2+}-chelating Sepharose FF column chromatography. *C*, UDP-hexanolamine-agarose column chromatography. After loading the sample, the column was washed with Buffers G, H, I, and J. The enzyme was eluted using a linear gradient of UDP in Buffer J. Fractions indicated by *bars* were pooled.

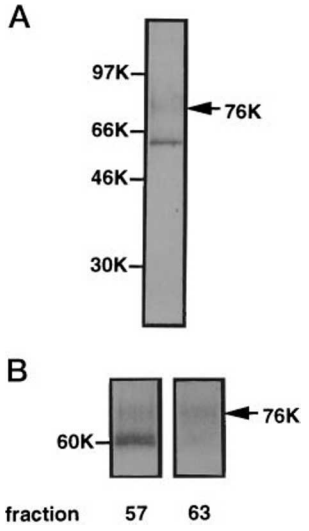


FIG. 2. **SDS-PAGE of β1–6GnT.** A 10% gel was used for electrophoresis and stained with silver. *A*, enzyme fraction after the first affinity column chromatography (Step 5). *B*, enzyme fractions at the second affinity column chromatography (Step 6).

and $NiCl_2$, $ZnCl_2$, and $CuCl_2$ inhibited enzyme activity.

Effect of UDP-GlcNAc Analogues—To investigate which portion of UDP-GlcNAc was important for enzyme activity, several UDP-GlcNAc analogues (2 mM) were added to the assay mixture containing 0.5 mM UDP-GlcNAc. These results are shown in Table III. UDP and UTP were the most potent inhibitors. The uracil moiety appeared to be essential for the enzyme activity, as evidenced by a comparison of UMP and AMP and of UDP and GDP. The comparison of UMP, UDP, and UTP suggests that the number of phosphodiesters might be important for inhibition. Sugar nucleotides having a UDP moiety also inhibited the enzyme activity, including UDP-hexanolamine which was used as an affinity ligand in Steps 5 and 6.

Acceptor Substrate and Site Specificity—Acceptor substrate and site specificity of the β1–6GnT was examined by using a set of oligo-*N*-acetyllactosamines as shown in Table I.

The Branching Reaction of Tetrasaccharide 1—Tetrasaccharide **1** (100 nmol) and UDP-GlcNAc were incubated together with the purified β1–6GnT. The MALDI-TOF mass spectrum of the purified reaction mixture showed sodiated molecular ions of the $Gal_2GlcNAc_3$-product (58%) and the $Gal_2GlcNAc_2$-acceptor (42%) (Fig. 3*A*). The pentasaccharide product was separated from the acceptor by gel permeation chromatography on a Superdex Peptide HR 10/30 column. The purity of the isolated pentasaccharide fraction was 95% as estimated by MALDI-TOF MS. Endo-β-galactosidase treatment failed to cleave the product; the reaction mixture contained only the uncleaved pentasaccharide as assessed by MALDI-TOF MS, indicating that the newly linked GlcNAc residue must be attached to the midchain galactosyl residue rather than to the terminal galactose (23). The one-dimensional ^1H NMR spectrum of the pentasaccharide product (Fig. 4*A*, Table IV) is identical with the spectrum previously reported for the authentic pentasaccharide **4** (24). In particular, a doublet with the typical chemical shift and coupling constant (4.584 ppm, 8.3 Hz) of the β1,6-linked GlcNAc H1 is present in the spectrum of the pentasaccharide product, and the chemical shifts of Gal2 H1 and Gal2 H4 are characteristic to a 3,6-disubstituted Gal, while Gal4 H1 has a chemical shift typical for a terminal galactosyl residue. If the branch-forming GlcNAc had been transferred to C-2 or C-4 of Gal2 of tetrasaccharide **1,** the H1 resonances of the new GlcNAc unit and the other reporter group signals of the pentasaccharide product would have been distinctly different from those of pentasaccharide **4** (25). Taken together, these data establish that the pentasaccharide formed from tetrasaccharide **1** by β1–6GnT is the branched pentasaccharide **4**.

The Branching Reaction of Pentasaccharide 2—The linear pentasaccharide **2** (300 nmol in 100-nmol lots) and UDP-GlcNAc were incubated together with the purified β1–6GnT. The MALDI-TOF mass spectrum of the purified reaction mixture

TABLE II
Purification of β1–6GnT from hog small intestine

Step	Total protein	Total activity	Specific activity	Yield	Purification
	mg	*nmol/h*	*pmol/h/mg protein*	*%*	*-fold*
1. Homogenate	8,550	144	16.8	100	1
2. Triton extract	1,010	56.6	56.0	39	3.3
3. CM Sepharose FF	132	14.4	109	10	6.5
4. Ni^{2+} -chelating Sepharose FF	35.7	7.86	220	5.5	13.1
5. UDP-hexanolamine-agarose	0.001	3.48	3,500,000	2.4	210,000

TABLE III
The effects of donor substrate analogues on β1–6GnT activity

The enzyme activity was assayed in the reaction mixture of 200 mM MOPS buffer (pH 7.0) containing 0.5 % Triton X-100, 2 mg/ml BSA, 20 μM lacto-*N*-neotetraose-PA, 0.5 mM UDP-GlcNAc, and 2 mM analogues. Activity is expressed as percent of the activity observed in the absence of analogue.

Donor substrate analogues	Activity
	%
No addition	100
UMP	59
UDP	0
UTP	0
UDP-glucose	49
UDP-galactose	58
UDP-hexanolamine	34
UDP-glucronic acid	64
AMP	90
GDP	81

revealed that 29% of **2** had been converted to the hexasaccharide $Gal_2GlcNAc_4$ (Fig. 3*B*). The hexasaccharide product was separated from the acceptor by gel permeation chromatography on a Bio-Gel P-4 column. The purity of the isolated hexasaccharide fraction was 94% as assessed by MALDI-TOF MS. Endo-β-galactosidase treatment completely cleaved the hexasaccharide product, releasing the tetrasaccharide $Gal_1GlcNAc_3$ (obs. $(M + Na)^+$ *m/z* 812.4 (calc. 812.3)) (Fig. 5). The peak of GlcNAcGal, the other cleavage product, was also detected among the matrix signals (obs. $(M + Na)^+$ *m/z* 406.0; calc. 406.1). These data showed that pentasaccharide **2** had been partially converted to glycan **5** by the intestinal enzyme. No signal was detected in the MALDI-TOF spectrum of Fig. 5 at *m/z* 974.3, suggesting that glycan **2** had not been branched at Gal4. The branching at this site would have generated the hexasaccharide GlcNAcβ1–3′(GlcNAcβ1–6′)LacNAcβ1–3′-LacNAc, which, in turn, had given rise to the pentasaccharide GlcNAcβ1–3′(GlcNAcβ1–6′)LacNAcβ1–3Gal in the endo-β-galactosidase experiment and a sodiated molecular ion at *m/z* 974.3 in the ensuing MALDI-TOF experiment. The ^1H NMR spectrum of the hexasaccharide formed from glycan **2** confirms that the hexasaccharide represents glycan **5** (Fig. 4*B*, Table IV). The doublet at 4.584 ppm arises from GlcNAc H1, which is β1,6-linked to Gal2 and not from a similar residue linked to Gal4 (14, 15). The GlcNAc H1 resonance for the isomeric hexasaccharide GlcNAcβ1–3(GlcNAcβ1–6)LacNAcβ1–3LacNAc at 4.592 ppm (14) was not detected in the spectrum of the hexasaccharide product. These results establish that the structure of the hexasaccharide formed from pentasaccharide **2** by β1–6GnT treatment is the branched hexasaccharide **5**.

The Branching Reaction of Hexasaccharide 3—Hexasaccharide **3** (300 nmol in 100-nmol lots) and UDP-GlcNAc were incubated together with the purified β1–6GnT. The MALDI-TOF mass spectrum of the purified reaction mixture showed that 31% of the original hexasaccharide had been converted to the heptasaccharide $Gal_3GlcNAc_4$ (obs. $(M + Na)^+$ *m/z* 1339.8; calc. 1339.5) and 5% to the octasaccharide $Gal_3GlcNAc_5$ (obs. $(M + Na)^+$ *m/z* 1542.8; calc. 1542.6) (Fig. 3*C*). The products

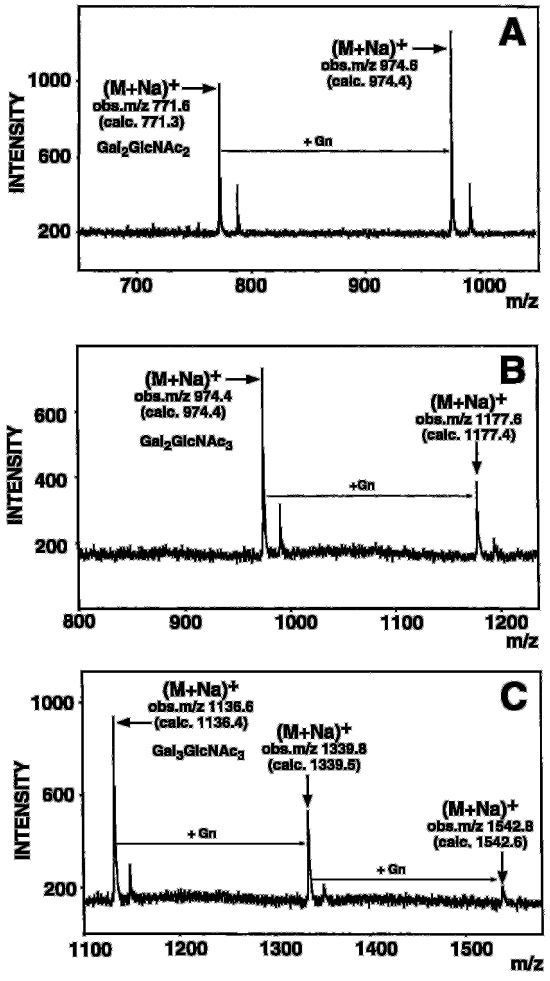


FIG. 3. **MALDI-TOF mass spectra of total oligosaccharide products from branching reactions catalyzed by β1–6GnT.** *A*, products from the reaction with tetrasaccharide **1**. *B*, products from the reaction with pentasaccharide **2**. *C*, products from the reaction with hexasaccharide **3**.

were separated from the acceptor by gel permeation chromatography on a Bio-Gel P-4 column and Peak 1 (fractions 42–45), Peak 2 (fractions 46–49), and Peak 3 (fractions 50–54) were collected. MALDI-TOF mass spectrum showed the octasaccharide $Gal_3GlcNAc_5$ was concentrated in Peak 1 (78%). Peak 2 contained largely (86%) heptasaccharides $Gal_3GlcNAc_4$ with some contaminating octa- and hexasaccharides. Peak 3 contained the hexasaccharide $Gal_3GlcNAc_3$.

Endo-β-galactosidase treatment of the heptasaccharide fraction (Peak 2) gave the tetrasaccharide $Gal_1GlcNAc_3$ (obs. $(M + Na)^+$ *m/z* 812.6; calc. 812.3) and the hexasaccharide $Gal_3GlcNAc_3$ (obs. $(M + Na)^+$ *m/z* 1136.9; calc. 1136.4) (not shown). The former represents GlcNAcβ1–3(GlcNAcβ1–6)-Galβ1–4GlcNAc, which originates from glycan **6** and the latter is LacNAcβ1–3′(GlcNAcβ1–6′)LacNAcβ1–3Gal, derived from glycan **7**. The signal sizes of these cleavage products suggests

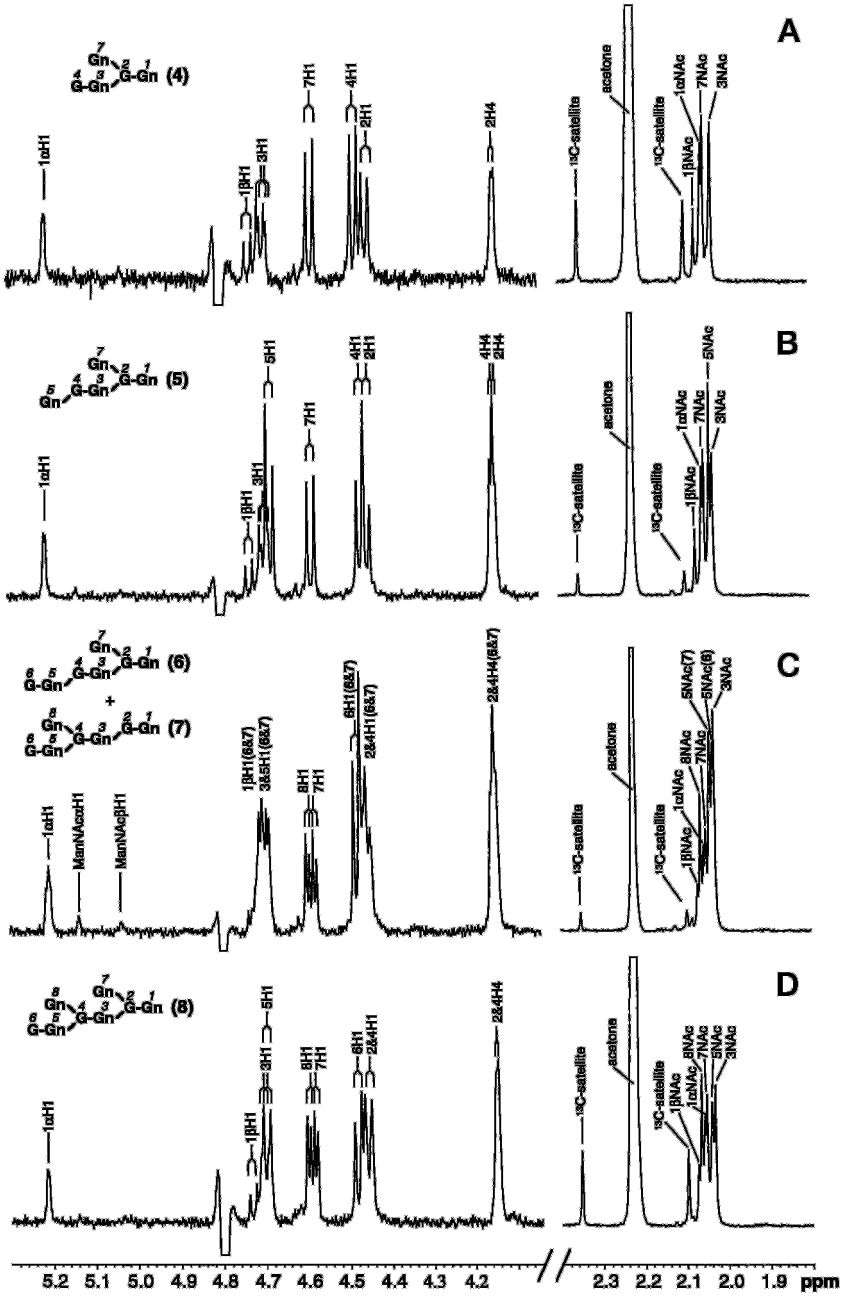


FIG. 4. **Reporter group signal area of the ^{1}H NMR spectra from branched oligosaccharides formed by the action of hog intestinal β1–6GnT.** The vertical scale at 4.1–5.3 ppm is expanded by a factor of 4. *A*, glycan **4**. *B*, glycan **5**. *C*, mixture of glycans **6** and **7**. *D*, glycan **8**.

that approximately equal amounts of glycans **6** and **7** were present in the heptasaccharide fraction. Signals representing molecular ions of $Gal_2GlcNAc$ (obs. *m/z* 568.3), derived from glycan **6** (and from contaminating acceptor glycan **3**), were also observed among the matrix peaks; small signals, which can be assigned to the octasaccharide $Gal_3GlcNAc_5$ (obs. *m/z* 1543.3) as well as uncleaved heptasaccharide $Gal_3GlcNAc_4$ (obs. *m/z* 1340.1) were also present in the MALDI-TOF spectrum. The uncleaved heptasaccharide probably contained the reducing-end ManNAc epimer of **7**, formed nonenzymatically. We have observed previously similar ManNAc-epimers as contaminants of other enzymatically synthesized polylactosamines (14, 24), and have also observed that the trisaccharide GlcNAcβ1–3Galβ1–4ManNAc is much less susceptible to endo-β-galactosidase than the corresponding GlcNAc-epimer, GlcNAcβ1–3Galβ1–4GlcNAc.[2] As estimated from the NMR spectrum of the original heptasaccharide fraction, discussed below, about 10% of the polylactosamines of this fraction is probably present as ManNAc epimers.

In the ^{1}H NMR spectrum of the heptasaccharide fraction, the partially overlapping doublets at 4.584 ppm and at 4.592 ppm can be seen (Fig. 4*C*, Table IV). These signals correspond to the β1,6-bonded GlcNAc H1 resonances reported for pure heptasaccharides **6** and **7**, respectively (15), indicating that both isomers are present also in the heptasaccharide pool in these experiments. The integrals of these peaks suggest that the heptasaccharide mixture contains 40% of **6** and 60% of **7**, which is in reasonable agreement with the results obtained from endo-β-galactosidase cleavage of the heptasaccharide fraction (see above).

The ^{1}H NMR spectrum of the octasaccharide product is very similar to that previously reported for glycan **8** (15) (Fig. 4*D*, Table IV). The doublets at 4.585 ppm and at 4.593 ppm arise from H1 of the two β1,6-linked GlcNAc at Gal2 and Gal4, respectively.

Pyridylaminated Compounds—GlcNAcβ1–3Galβ1–4Glc-PA

[2] O. Renkonen, personal communications.

TABLE IV
^{1}H chemical shifts of oligosaccharides products obtained in reactions catalyzed by the β1–6GnT of hog small intestine
Denotation of monosaccharide residues is also shown in Table I.

Reporter group	Residue	Saccharide 4	5	6	7	8
H-1	1[a]	5.212 (α)	5.211 (α)	5.208 (α)	5.208 (α)	5.212 (α)
		4.730 (β)	4.730 (β)	4.729 (β)	4.720 (β)	4.731 (β)
	2	4.453	4.452	4.451	4.465	4.456
	3[a]	4.700	4.697	4.702	ND	4.702
		4.695	4.692	4.695	4.702	4.697
	4	4.480	4.467	4.465	4.456	4.456
	5		4.680	4.702	4.695	4.697
	6			4.480	4.480	4.480
	7	4.584	4.584	4.584		4.585
	8				4.592	4.593
H-4	2	4.147	4.146	ND	4.156	4.145
	4	ND[b]	4.152	4.156	4.149	4.145
NAc	1[a]	2.055 (α)	2.055 (α)	2.055 (α)	2.055 (α)	2.056 (α)
		2.071 (β)	2.071 (β)	2.071 (β)	2.071 (β)	2.071 (β)
	3	2.032	2.029	2.030	2.030	2.031
	5		2.037	2.033	2.040	2.039
	7	2.051	2.050	2.050		2.051
	8				2.063	2.063

[a] The two values given correspond to the two anomers of the oligosaccharide
[b] ND, not determined.

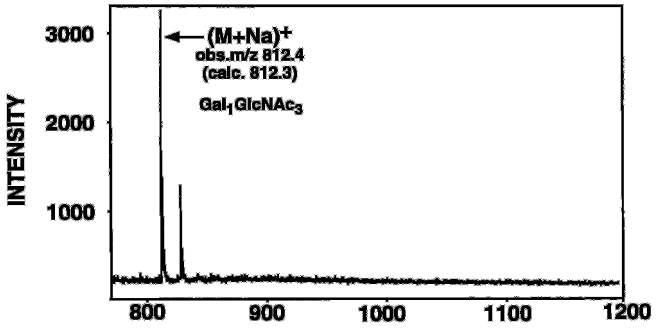


FIG. 5. **MALDI-TOF mass spectrum of the endo-β-galactosidase cleavage product of hexasaccharide 5 from the branching reaction with the linear pentasaccharide 2.**

and Galβ1–3GlcNAcβ1–3Galβ1–4Glc-PA were tested in the standard assay mixture to determine whether these could serve as acceptor compounds for β1–6GnT, but no product was obtained.

Kinetic Analysis—Lineweaver-Burk plots of the kinetic data obtained at a 40 mM UDP-GlcNAc concentration were linear, and the K_m value for lacto-*N*-neotetraose-PA was determined to be 0.96 mM. The K_m value for UDP-GlcNAc was determined to be 2.59 mM with 30 μM of lacto-*N*-neotetraose-PA present as an acceptor.

DISCUSSION

β1–6GnTs are a family of enzymes that are capable of forming four different GlcNAcβ1,6 linkages (in bold): GlcNAcβ1–3(**GlcNAcβ1–6**)Gal (blood group I-type structure), Galβ1–3(**GlcNAcβ1–6**)GalNAc (core 2 in *O*-glycans), GlcNAcβ1–3(**GlcNAcβ1–6**)GalNAc (core 4 in *O*-glycans), and GlcNAcβ1–2(**GlcNAcβ1–6**)Man (2,6-branched *N*-linked core) (26, 27). Thus, β1–6GnTs can be classified into four major groups according to their acceptor specificities: blood group I GnT6 (IGnT6), core 2 GnT6 (C2GnT6), core 4 GnT6 (C4GnT6), and GnT V. In addition, the enzyme activities which act on glycolipids and milk oligosaccharides have been shown to give rise to Galβ1–3(**GlcNAcβ1–6**)GalNAcβ1–3Galα- (28, 29) and GalNAcβ1–3(**GlcNAcβ1–6**)Galα1–4Galβ1–4Glc (30). All the β1–6GnTs characterized to date do not require Mn^{2+} ion, in contrast to other GnTs, which require divalent metal ions for full activity. Among the IGnT6s, two types of enzymes are known. The "distally acting" enzymes (dIGnT6) act on a subterminal Gal residue that is already substituted at position 3 by a GlcNAc residue. The dIGnT6s have a relaxed acceptor specificity, since enzymes from pig gastric mucosa (10), Novikoff ascites tumor cells (11), and bovine tracheal epithelium (12) can catalyze the addition of GlcNAcβ1–6 to either Gal or GalNAc residues that are presubstituted at position 3 by either GlcNAc or Gal. The second type of IGnT6 activity, referred to as cIGnT6, links a GlcNAcβ1–6 group to a midchain Gal residue of a linear poly(oligo)-*N*-acetyllactosamine structure. This type of activity has been identified in human serum (17), rat tissues (13), and PA1 cell lysates (31).

In this study, we have purified the cIGnT6 210,000-fold from hog small intestine by successive column chromatographies, using an assay where pyridylaminated lacto-*N*-neotetraose (Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA) was used as an acceptor substrate and the reaction product was Galβ1–4GlcNAcβ1–3(**GlcNAcβ1–6**)Galβ1–4Glc-PA. The purified enzyme has an apparent molecular weight of 76,000 by SDS-PAGE under nonreducing conditions (Fig. 2*A*). The purified cIGnT6 has no metal ion requirement for full activity, which is a property shared by all the other β1–6GnTs. The optimum pH is at 7.0, which is the same value as has been reported for rat intestine cIGnT6 (13). The analysis of acceptor specificity indicates that the enzyme has unique properties. The data which shows that GlcNAcβ1–3Galβ1–4Glc-PA and Galβ1–3GlcNAcβ1–3Galβ1–4Glc-PA could not be the acceptor compound revealed that this enzyme was devoid of dIGnT6 activity and that Galβ1–4 moiety, not Galβ1–3 linkage at the nonreducing terminal should be necessary for its cIGnT6 activity. A more precise analysis of the acceptor specificity of this enzyme was performed with tetrasaccharide **1**, pentasaccharide **2**, and hexasaccharide **3**. The fact that tetrasaccharide **1** was converted into glycan **4** showed that this IGnT6 was cIGnT6, consistent with the data using a pyridylaminated substrate. The pentasaccharide **2** was converted in a site-specific reaction to glycan **5** by cIGnT6 and not to the isomeric saccharide GlcNAcβ1–3′(GlcNAcβ1–6′)-LacNAcβ1–3′LacNAc. The structure of glycan **5** was established by endo-β-galactosidase treatment, which cleaved the product into the disaccharide GlcNAcβ1–3Gal and the tetrasaccharide GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAc. The ^{1}H NMR spectrum of glycan **5** confirmed its structure by showing a GlcNAc H1 resonance that is characteristic of poly-*N*-acetyllactosamines that contain a branch at the reducing-end LacNAc unit. The completely site-specific action of hog small intestinal cIGnT6 with the pentasaccharide acceptor **2** is identical to the action of cIGnT6 activities of human serum (17) and rat serum (15) as well as serum of other mammalian species (15). We note that the purified cIGnT6 from hog intestine has no dIGnT6 activity, which should convert pentasaccharide **2** into the isomeric saccharide GlcNAcβ1–3′(GlcNAcβ1–6′)LacNAcβ1–3′LacNAc. This was also confirmed in an experiment where incubation of UDP-GlcNAc donor and trisaccharide acceptor GlcNAcβ1–3Galβ1–4GlcNAc with this enzyme failed to generate detectable amounts of the expected dIGnT6 product, GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAc (data not shown). It is interesting that the membrane-bound intestinal cIGnT6 reacted more efficiently with glycan **1** than glycan **2**. This is also true for the soluble human and rat serum enzymes (15). The reaction with hexasaccharide **3** led to the production of two isomeric heptasaccharides **6** and **7** in nearly the same quantity and finally also to the doubly branched octasaccharide **8**. This clearly shows that this cIGnT6 is capable of reacting with both midchain Gal positions of the linear hexasaccahride **3** without specificity.

A human IGnT6 has been cloned by expression cloning using an antibody against I antigen (32). The cDNA for this enzyme encodes the type II transmembrane protein with 400 amino acids, but it is not known whether it represents dIGnT6 or cIGnT6 (33, 13); both have been used to generate multiply branched poly-*N*-acetyllactosamine structure. Quite recent experiments of ours suggest that this human IGnT6 may represent a cIGnT6 (31). Our current interest is whether the cIGnT6 purified from hog small intestine is the same enzyme as human cIGnT6. Protein sequencing and cloning of this hog cIGnT6 is now currently ongoing in our laboratory.

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