A Fluorometric Assay for Glycosyltransferase Activities Using Sugars Aminated and Tagged with 7-Hydroxycoumarin-3-carboxylic Acid as Substrates and High Performance Liquid Chromatography

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We developed a novel fluorometric assay method for the measurement of glycosyltransferase activities using mono- and di-saccharides aminated and tagged with 7-hydroxycoumarin-3-carboxylic acid (coumarin) as substrates, *N*-acetylglucosamine (GlcNAc)-coumarin for β1,4-galactosyltransferase from bovine milk and Galβ1-4GlcNAc-coumarin for α2,3- and α2,6-sialyltransferases from rat liver. Using Galβ1-3GlcNAc and Galβ1-4GlcNAc-coumarin, α1,3/4- and α1,3-fucosyltransferase activities were also determined, respectively. These enzymatic products liberated by the reactions of glycosyltransferases in the presence of sugar nucleotides, were separated by a normal phase or an ion-pair reversed phase HPLC with an isocratic elution and fluorescence detection. We applied this assay method to determine the glycosyltransferase activities in 8 kinds of human tumor cell lines, including the cell lines derived from hepatocytes (HuH-7, HepG2), colonic cells (Colo205, HT-29), myelocytes (HL-60, U-937), B-lymphocytes (Daudi) and T-lymphocytes (Jurkat). This assay method is accurate and easy compared with other isotopic and non-isotopic assay methods, and is sensitive enough to measure glycosyltransferase activities in cell homogenates.

Key words fluorometric assay; HPLC; galactosyltransferase; sialyltransferase; fucosyltransferase

Sugar chains are ubiquitously conjugated to proteins and lipids expressed on the cell surface and secreted into circulating blood. They play important roles in many biological interactions, such as cell–cell interaction,[1,2] cell–matrix interaction[3] and receptor–ligand interaction.[4] Sugar chains are synthesized and regulated by the actions of many glycosyltransferases and glycosidases.[5] Glycosyltransferase activities have been determined using radio labeled sugar nucleotides as donor substrates and either glycoproteins or oligosaccharides as acceptor substrates,[6—10] involving additional procedures to ensure the separation of enzymatic products from radio labeled sugar nucleotides. To improve these problems, solid-phase procedures have been applied.[11,12]

Non-isotopic assay methods have also been developed using fluorescence-tagged sugar chains.[13,14] Primary amine-containing fluorescence reagents such as 2-aminopyridine (2-AP) can be introduced to the reducing ends of sugar chains with a reductive amination, and have been used for sugar chain structure analysis.[15,16] These methods have also been utilized to determine glycosyltransferase activities.[17] However, using these methods, the cyclic structures of the reducing ends of the sugar chains are cleaved to change the properties of the sugar moieties through reductive amination. Therefore, we prepared aminated sugars and coupled them with 7-hydroxycoumarin-3-carboxylic acid (coumarin) instead of the reductive amination.

In this paper, we prepared GlcNAc-coumarin for β1,4-galactosyltransferase (GalT, EC 2.4.1.22) and Galβ1-3GlcNAc or Galb1-4GlcNAc-coumarin for α2,3-sialyltransferase (α2,3-ST, EC 2.4.99.6), α2,6-sialyltransferase (α2,6-ST, EC 2.4.99.1), α1,3/4-fucosyltransferase (α1,3/4-FucT, EC 2.4.1.65) and α1,3-fucosyltransferase (α1,3-FucT, EC 2.4.1.) as fluorescence acceptor substrates, and established assay methods for the measurement of glycosyltransferase activities using a normal phase or an ion-pair reversed phase HPLC for separative determination of the enzymatic products.

MATERIALS AND METHODS

Materials Coumarin was purchased from Molecular Probes, Inc. (Leiden, The Netherlands). Galβ1-4GlcNAc, β-D-galactosidase (*Streptococcus* 6646 K) and α2,3/6-neuraminidase (*Streptococcus* sp.) were obtained from Seikagaku Co. (Tokyo, Japan). Galβ1-3GlcNAc, 1-hydroxybenzotriazole (HOBT), *O*-benzotriazole-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), *N*,*N*-diisopropylethylamine, cytidine 5′-monophospho-*N*-acetylneuraminic acid (CMP-NeuAc), uridine 5′-diphospho-β-D-galactose (UDP-Gal), guanosine 5′-diphospho-α-L-fucose (GDP-Fuc) and GalT (bovine milk) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), α2,3-ST and α2,6-ST (rat liver) were from Boehringer Mannheim Co. (Heidelberg, Germany) and α2,3-neuraminidase (*Salmonella typhimurium* LT2) and α1,3/4-fucosidase (*Streptomyces* sp.142) were from Takara Co. (Otsu, Japan). Acetonitrile used was of HPLC grade and all other reagents and chemicals used were of analytical grade.

Preparation of the Coumarin-Tagged Substrates GlcNAc, Galβ1-3GlcNAc and Galβ1-4GlcNAc were reacted with saturated ammonium hydrogen carbonate at 50 °C for 16 h to form β-glycosylamines according to the previous method.[18] β-Glycosylamines thus obtained were coupled with coumarin as follows. A solution of 22.8 μmol of coumarin in 50 μl of dimethylformamide (DMF) was added to the mixture of 22.8 μmol of β-glycosylamines in 50 μl of DMF, 22.8 μmol of HBTU in 200 μl of DMF, 22.8 μmol of HOBT in 50 μl of DMF and 68.4 μmol of *N*,*N*-diisopropylethylamine. After the coupling reaction at room temperature for approximately 2 h, the reaction mixture was injected onto a preparative TSKgel ODS-80Ts column (21.5 i.d.×300 mm, 10 μm particle size, Tosoh Co., Tokyo, Japan) and the

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coumarin-tagged sugars were eluted with two solvent systems of solution A and B at a flow rate of 3 ml/min using an increasing gradient of B in A (0 min: 15% of B, 40 min: 20% of B, 42 min: 100% of B, 60 min: 100% of B, 62 min: 15% of B). Solutions A and B were composed of 50 mM ammonium acetate : acetonitrile, 95 : 5 (v/v) and 50 : 50 (v/v), respectively. The effluent was monitored at 330 nm. The yields of the reaction and purification were about 80—90%.

Preparation of Cell Homogenates We used 8 kinds of human tumor cell lines, HuH-7 and HepG2 derived from hepatocytes, Colo 205 and HT-29 from colonic cells, HL-60 and U-937 from myelocytes, Daudi from B-lymphocytes and Jurkat from T-lymphocytes. Colo 205 and HT-29 were obtained from American Type Culture Collection (ATCC, Rockville, ML), and the other cell lines, HuH-7, HepG2, HL-60, U-937, Daudi and Jurkat were from Japanese Cancer Research Resources Bank/Health Science Research Resources Bank (JCRRB/HSRRB, Tokyo, Japan). HuH-7, HepG2, Colo205 and HT-29 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Co., Tokyo, Japan) and HL-60, U-937, Daudi and Jurkat cells in RPMI1640 (Nissui Co.) supplemented with 10% heat-inactivated fetal bovine serum (JRH Biosciences, Lanex, KS) in a humidified atmosphere containing 5% CO_2 and at 37 °C. The cultured cells (1×10^7 cells) were washed 3 times with phosphate buffered saline. The washed cells were sonicated 3 times for 10 s on ice by a Model VP-5S Ultra Homogenizer (Taitec Co., Tokyo, Japan) in 100 μl of 10 mM Tris–HCl (pH 7.0) containing 1% Triton X-100. The homogenates were stored at −80 °C.

Protein concentrations were determined using a modified Lowry method with bovine serum albumin as the standard protein.[19]

Assay Conditions for Glycosyltransferase Activities The GalT assay mixture was composed of 100 mM HEPES buffer (pH 7.5), 10 mM $MnCl_2$, 1.0 mM UDP-Gal, 1.0 mM GlcNAc-coumarin and GalT from bovine milk or 30 μg protein of cell homogenates in a total volume of 20 μl. The mixture was incubated for 2 h at 37 °C.

The ST assay mixture was composed of 100 mM HEPES buffer (pH 7.0), 10 mM $MnCl_2$, 1.0 mM CMP-NeuAc, 1.0 mM Gal β1-4GlcNAc-coumarin and α2,3-ST or α2,6-ST from rat liver or 30 μg protein of cell homogenates in a total volume of 20 μl. The mixture was incubated for 6 h at 37 °C.

The FucT assay mixture was composed of 100 mM HEPES buffer (pH 7.5), 30 mM $MnCl_2$, 1.0 mM GDP-Fuc, 1.0 mM Galβ1-4GlcNAc- or Galβ1-3GlcNAc-coumarin and 30 μg protein of cell homogenates in a total volume of 20 μl. The mixture was incubated for 3 h at 37 °C.

After the enzyme reaction was stopped by adding 100 μl of acetonitrile and the mixture was centrifuged at 16000 ***g*** for 5 min, the resulting supernatant was diluted with a mobile phase. An aliquot was subjected to HPLC and the amount of enzymatic product was determined using a peak area method.To determine Michaelis constants (K_m) for donor and acceptor substrates, enzyme activities in 8 different concentrations of substrates around K_m values were measured in triplicate, and K_m values were determined by Lineweaver-Burk plot analysis. A good linear regression (r>0.989) was obtained in each regression analysis.

Normal Phase HPLC Condition The diluted mixture obtained after GalT, FucT or ST reaction was injected onto a TSKgel Amide-80 column (4.6 i.d.×250 mm, 5 μm of a particle size, Tosoh Co.) and the enzymatic product was separated from excess substrate using an isocratic elution of 100 mM ammonium acetate (pH 6.0) : acetonitrile (1 : 3, v/v) as a mobile phase at a flow rate of 1.0 ml/min. The eluent was monitored by a Model FS-8020 fluorescence detector (Tosoh Co.) at λ_{EX} 330 nm and λ_{EM} 450 nm, and the peak areas of the enzymatic products were calculated by a Model LC-8020 Multi Station (Tosoh Co.).

Ion-Pair Reversed Phase HPLC Condition The diluted mixture of ST reaction was also injected onto a TSKgel ODS-80TM column (4.6 i.d.×150 mm, 5 μm of a particle size, Tosoh Co.) and the α2,3- and α2,6-sialylated products were separated from the excess substrate using 10 mM ammonium acetate (pH 6.0) : acetonitrile (9 : 1, v/v) containing 0.05% tetrabutylammonium chloride (TBA, Sigma) as a mobile phase at a flow rate of 1.0 ml/min. The peak areas of the enzymatic products were obtained as described above.

Glycosidase Treatment To ascertain the products liberated by the glycosyltransferase reactions, the GalT, ST and FucT products purified on the normal phase HPLC were treated with glycosidases. An aliquot of GalT product purified on HPLC was treated with 10 mU of β-galactosidase from *Streptococcus* 6646 K in 20 μl of 50 mM acetate buffer, pH 6.0, at 37 °C for 1 h. Neuraminidase treatment was performed using 25 mU of α2,3-neuraminidase (*Salmonella typhimurium* LT2) or 10 mU of α2,3/6-neuraminidase (*Streptomyces* sp.) in 20 μl of 100 mM acetate buffer, pH 5.5, at 37 °C for 1 h. The FucT products were also incubated with 100 μU of α1,3/4-fucosidase (*Streptomyces* sp. 142) in 20 μl of 100 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer, pH 6.5, at 37 °C for 1 h.

After the reactions were stopped by adding 100 μl of acetonitrile and the mixtures were centrifuged at 16000 ***g*** for 5 min, the resulting supernatants were diluted with 75% (v/v) acetonitrile and the glycosidase-digested products were identified on the normal phase HPLC as described above.

RESULTS AND DISCUSSION

Properties of Coumarin-Tagged Sugars A schematic diagram for the preparation of coumarin-tagged GlcNAc and a GalT reaction is shown in Fig. 1. A cyclic structure of the reducing end of sugar should be preserved through an amination and coupling reaction with coumarin. To confirm whether the glycosyltransferase reaction occurred directly on coumarin-tagged monosaccharide, we incubated GlcNAc-coumarin with GalT from bovine milk and in HL-60 cell homogenate. After GalT reactions in the presence or absence of UDP-Gal, we analyzed the reaction mixture using a normal phase HPLC.

Typical elution chromatograms of the reaction mixtures are shown in Fig. 2. Another peak at 10.5 min of retention time (peak 2) was detected in the reaction mixture in the presence of UDP-Gal as the donor substrate, although only the peak of GlcNAc-coumarin (peak 1) was obtained in the reaction mixture without UDP-Gal.

Then, we purified this enzymatic product using a normal phase HPLC and estimated the GalT product by treatment of the purified product with β-galactosidase from *Streptococcus*

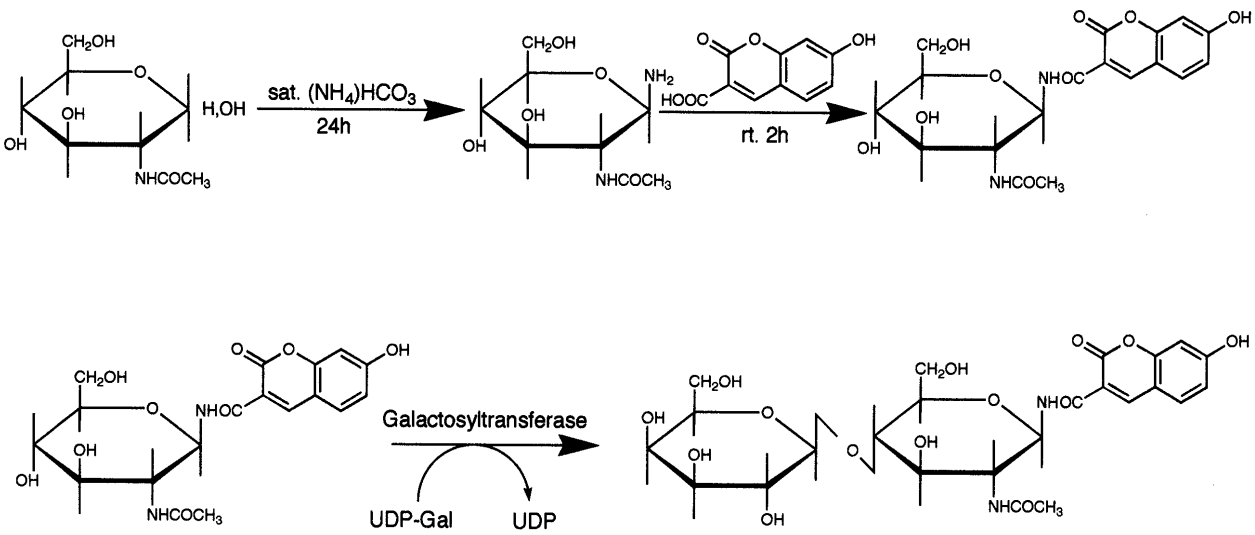

Fig. 1. A Schematic Diagram for Preparation of Coumarin-Tagged GlcNAc and GalT Reaction on Coumarin-Tagged GlcNAc

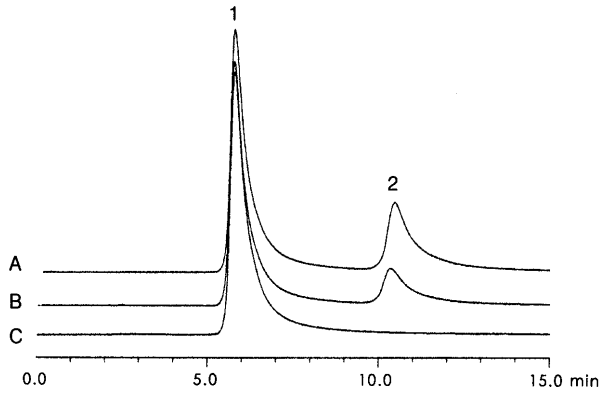

Fig. 2. Typical Elution Chromatograms of the Reaction Mixtures of GalT

A, GalT from bovine milk in the presence of UDP-Gal; B, HL-60 cell homogenate in the presence of UDP-Gal; C, HL-60 cell homogenate without UDP-Gal. Peak 1, GlcNAc-coumarin; peak 2, Galβ1-4GlcNAc-coumarin.

6646K. The galactosidase-treated product was eluted at the same retention time as that of GlcNAc-coumarin.

Coumarin derivatives were easily dissolved in water. The fluorescence intensities of coumarin derivatives were about ten times higher than those of 2-AP at a neutral pH range and were not influenced by organic solvents such as acetonitrile or methanol. Coumarin derivatives could be determined at the level of 18 fmol/injection (SN=5) and were stable under the mild acidic conditions. About 10% of the coumarin derivatives were degraded after being left at pH 10 and room temperature overnight.

Assay Conditions for GalT Activities Assay conditions for GalT activities were optimized using GalT from bovine milk. The optimum pH for GalT activities was measured in 100 mM MES buffer ranged from pH 5.0 to 7.0, in 100 mM HEPES buffer ranged from pH 6.0 to 8.0, and in 100 mM Tris–HCl buffer ranged from pH 7.0 to 9.0. The maximum GalT activities were obtained at pH 7.5 of the 100 mM HEPES buffer.

The effects of divalent metal ions such as Ba^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+} and Fe^{2+} (each 10 mM, chloride form) and EDTA (10 mM) on the enzyme activities were determined. The GalT activities were activated by Ba^{2+}, Mn^{2+}, Ca^{2+} and Co^{2+} and inhibited by Mg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+} and EDTA.

The apparent Michaelis constants (K_m) of GalT determined by Lineweaver-Burk plot analysis were 38 μM for UDP-Gal and 0.52 mM for GlcNAc-coumarin, respectively. Previously reported, K_m values[20] 8.3 mM for GlcNAc and 0.62 mM for GlcNAcβ1-4GlcNAc, indicated that the reducing end of GlcNAc on which GalT transfers a Gal residue will decrease the affinity of GalT. Coumarin-derivatized GlcNAc increased the affinity of GalT at a level similar to GlcNAcβ1-4GlcNAc.The amounts of GalT products (<30% of the substrates) correlated well with the GalT activities ranging from 1 to 35 μU/20 μl with r=0.998, and sufficient coefficients of variations in low GalT activities (1 μU/20 μl, coefficients of variation (C.V.)=7.5%, n=3) and high activities (35 μU/20 μl, C.V.=0.3%, n=3) were obtained.

Assay Conditions for Sialyltransferases Activities Using CMP-NeuAc as a donor and Galβ1-4GlcNAc-coumarin as an acceptor substrate for α2,3-ST and α2,6-ST from rat liver and HL-60 cell homogenate, enzymatic products were detected at the different retention times shown in Fig. 3, 19.5 min for α2,3-ST (peak 3) and 26.2 min for α2,6-ST (peak 4) in the normal phase HPLC system (Fig. 3a) and 11.5 min for α2,3-ST (peak 3) and 9.5 min for α2,6-ST (peak 4) in the ion-pair reversed phase HPLC system (Fig. 3 b), respectively. These enzymatic products purified on normal phase HPLC, NeuAca2-3 and NeuAca2-6Galβ1-4GlcNAc, were ascertained by treatment with α2,3-neuraminidase from *Salmonella typhimurium* LT2 and α2,3/6-neuraminidase from *Streptomyces* sp.

The peak at 6.5 min of retention time (peak 1) in normal phase HPLC after incubation with HL-60 cell homogenate was coincident with GlcNAc-coumarin, a product of β-galactosidase in the cell homogenate.

Maximum α2,3-ST and α2,6-ST activities were obtained at pH 7.0 of 100 mM HEPES buffer and 10 mM $MnCl_2$. The α2,3-ST and α2,6-ST activities were activated by the divalent metals, Ba^{2+} and Mg^{2+}, while EDTA inhibited both α2,3-ST and α2,6-ST activities.The apparent K_m of α2,3-ST and α2,6-ST determined by Lineweaver-Burk plot analysis were 63 and 140 μM for CMP-NeuAc and 2.0 and 1.8 mM for Galβ1-4GlcNAc-coumarin, respectively. The K_m value of

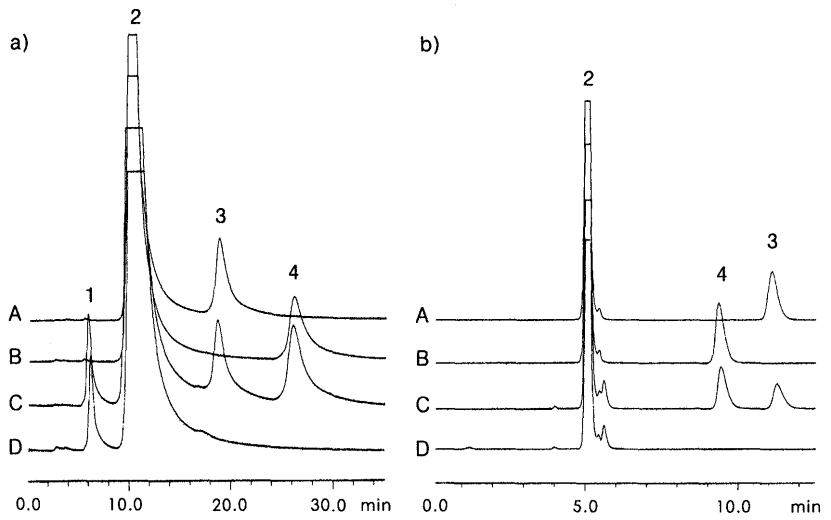

Fig. 3. Typical Elution Chromatograms of the Mixtures of ST Reactions Using Normal Phase (a) and Ion-Pair Reversed Phase HPLC (b)

A, B, α2,3-ST (A) and α2,6-ST (B) from rat liver; C, D, HL-60 cell homogenate in the presence (C) or absence (D) of CMP-NeuAc. Peak 1, degraded product (GlcNAc-coumarin); peak 2, substrate (Galβ1-4GlcNAc-coumarin); peak 3, NeuAcα2-3Galβ1-4GlcNAc-coumarin; peak 4, NeuAcα2-6Galβ1-4GlcNAc-coumarin.

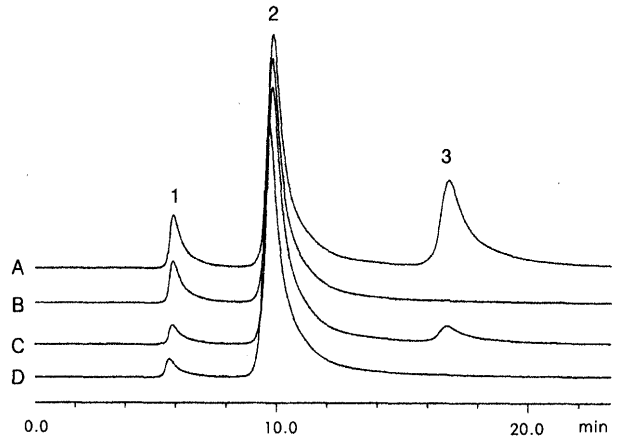

Fig. 4. Typical Elution Chromatograms of Fucosylated Products Using Normal Phase HPLC

A,B, α1,4-FucT in Colo205 cell homogenate in the presence (A) or absence (B) of GDP-Fuc; C, D, α1,3-FucT in HT-29 cell homogenate in the presence (C) or absence (D) of GDP-Fuc. Peak 1, degraded product (GlcNAc-coumarin); peak 2, substrate (Galβ1-3/4GlcNAc-coumarin); peak 3, Galb1-3/4(Fucα1-4/3)GlcNAc-coumarin.

α2,6-ST for CMP-NeuAc was reported to be 45 μM using Galβ1-4GlcNAc,[21] which was a rather high affinity compared withour result (140 μM) obtained using Galβ1-4GlcNAc-coumarin as the acceptor substrate, although the values were not obtained under the same experimental conditions.

Assay Conditions for Fucosyltransferase Activities α1,3/4- and α1,3-FucTs have been reported to be several isoenzymes,[22,23] and the preparations of these FucTs were not commercially available. Then, we tried to determine the FucT activities in human colon cancer cell lines; Colo-205 cells expressed mainly α1,3/4-FucT, FucT III, and HT-29 cells mainly expressed α1,3-FucT, FucT IV (our unpublished data). Colo-205 and HT-29 cell homogenates were incubated with Galβ1-4GlcNAc- and Galβ1-3GlcNAc-coumarin as acceptor substrates in the presence of guanosine 5′-diphosphate (GDP)-Fuc as the donor, and the reaction mixtures were subjected to normal phase HPLC (Fig. 4).

Using isotopic methods, α1,3/4-FucT has been reported to react on Galβ1-3GlcNAc but not on Galβ1-4GlcNAc,[24] and on both Galβ1-3GlcNAc and Galβ1-4GlcNAc.[25] In the case of Colo 205 cell homogenates, the enzymatic products were detected by incubation with both Galβ1-4GlcNAc- and Galβ1-3GlcNAc-coumarin, whereas HT-29 homogenates reacted only on Galβ1-4GlcNAc but not on Galβ1-3GlcNAc-coumarin.

We purified the FucT products by HPLC and treated the purified products with α1-3/4 specific fucosidase from *Streptomyces* sp. 142. Since the fucosidase-treated products were eluted at the same retention times as those of Galβ1-4GlcNAc- and Galβ1-3GlcNAc-coumarin, the FucT products should be Galβ1-4(Fucα1-3)GlcNAc- and Galβ1-3(Fucα1-4)GlcNAc-coumarin. Therefore, α1,2-FucTs would not react on both coumarin derivatives in this assay condition, although α1,2-FucTs have been reported to be expressed in colon cancer cell lines[26] and to react on both Galβ1-4GlcNAc and Galβ1-4GlcNAc-OCH_3 in the presence of 0.37 mM $MnCl_2$.[27]

The maximum α1,3/4-FucT and α1,3-FucT activities were obtained at pH 7.5 of 100 mM HEPES buffer and 30 mM $MnCl_2$. FucTs were activated by Ca^{2+}, Mg^{2+} and Ba^{2+} and inhibited by Cu^{2+}, Zn^{2+} and EDTA.

The apparent Michaelis constants of α1,3/4-FucT were 19 μM for GDP-Fuc using Galβ1-3GlcNAc-coumarin, and 3.0 and 2.2 mM for Galβ1-3GlcNAc- and Galβ1-4GlcNAc-coumarin, respectively. Those of α1,3-FucT were 15 μM for GDP-Fuc and 0.7 mM for Galβ1-4GlcNAc-coumarin. Legault D. J. *et al.*[25] reported that the K_m values of α1,3/4-FucT were 12.7 and 8.1 mM for Galβ1-3GlcNAc and Galβ1-4GlcNAc, respectively. The results indicated that the reducing end of GlcNAc on which FucT transfers a Fuc residue decreased the affinities of α1,3/4-FucT. A similar effect was observed in the case of the GalT reaction described above.

The results of this study indicated that the reducing ends of sugars on which glycosyltransferases will act might affect the affinities of these enzymes to acceptor substrates. Using coumarin-tagged substrates for GalT and α1,3/4-FucT, the affinities of the enzymes increased by estimation of their K_m values, although the experimental conditions were not controlled and the available results were limited. Furthermore, in

Table 1. The Optimized Assay Conditions of Each Glycosyltransferase

Enzymes	Buffers	Metals	Donors	Acceptors
β1,4-GalT	100 mM HEPES (pH 7.5)	10 mM $MnCl_2$	1.0 mM UDP-Gal	1.0 mM GlcNAc-R
α2,3-ST	100 mM HEPES (pH 7.0)	10 mM $MnCl_2$	1.0 mM CMP-NeuAc	1.0 mM Galβ1-4GlcNAc-R
α2,6-ST	100 mM HEPES (pH 7.0)	10 mM $MnCl_2$	1.0 mM CMP-NeuAc	1.0 mM Galβ1-4GlcNAc-R
α1,3/4-FucT	100 mM HEPES (pH 7.5)	30 mM $MnCl_2$	1.0 mM GDP-Fuc	1.0 mM Galβ1-3GlcNAc-R
α1,3-FucT	100 mM HEPES (pH 7.5)	30 mM$MnCl_2$	1.0 mM GDP-Fuc	1.0 mM Galβ1-4GlcNAc-R

R : coumarin.

Table 2. Glycosyltransferase Activities in Several Human Tumor Cell Lines

	GalT (nmol/h/mg)	α2,3-ST (pmol/h/mg)	α2,6-ST (pmol/h/mg)	α1,4-FucT (nmol/h/mg)	α1,3-FucT (nmol/h/mg)
Colo205	72.7±2.3	0.14±0.007	5.85±0.81	25.3±0.83	8.53±0.16
HT-29	42.2±1.5	1.72±0.24	4.10±0.36	n.d.	16.13±1.19
Jurkat	12.1±1.1	0.99±0.25	2.78±0.31	n.d.	0.41±0.10
Daudi	21.8±0.9	0.88±1.22	82.83±10.8	n.d.	0.27±0.04
HL-60	22.7±1.1	1.60±0.33	7.11±0.52	n.d.	4.56±0.65
U-937	10.3±0.5	0.49±0.16	2.15±0.27	n.d.	1.14±0.08
HepG2	52.8±1.7	1.95±0.23	53.73±3.85	n.d.	0.34±0.05
HuH-7	129.8±8.5	5.44±0.80	92.30±6.43	n.d.	0.87±0.14

GalT, α2,3-ST, α2,6-ST, α1,4-FucT, α1,3-FucT activities were determined in colonic cells (colo205 and HT-29), T-lymphocytes (Jurkat), B-lymphocytes (Daudi), myelocytes (HL-60 and U-937) and hepatocytes (HepG2 and HuH-7). n.d.: not detected. $n=3$.

the case of α1,3/4-FucT, modification of the reducing ends with not only *N*-coumarin, but also *O*-Octyl, and *O*-β1-3Galβ1-4Glucose,[28] affected the substrate specificities, especially by increasing reactivities on Galβ1-4GlcNAc to Galβ1-3GlcNAc. Since these enzymes were generally thought to act on the substrates whose reducing ends will be occupied by other sugar moieties or lipids and proteins, substrates to estimate the roles of these enzymes should be under further consideration. In respect to the optimum pH and dependencies of divalent metal ions for these glycosyltransferase activities, significant differences were not observed among coumarin-tagged substrates and others.

The assay conditions for each glycosyltransferase established in this study are summarized in Table 1.

Glycosyltransferases Activities in Various Cell Lines Using the assay conditions for the measurement of the glycosyltransferase activities established in this study, we assayed the glycosyltransferase activities in 8 kinds of human tumor cell lines, including myelocyte cell lines HL-60 and U-937, the B-lymphocyte cell line Daudi, the T-lymphocyte cell line Jurkat, colonic cell lines Colo205 and HT-29, and hepatocyte cell lines HepG2 and HuH-7. The results are shown in Table 2.

GalT activities were expressed ubiquitously in every cell line used, with higher activities in hepatocytes and colonic cells than in myelocytes and lymphocytes.

α2,3-ST and α2,6-ST activities were also expressed ubiquitously in every cell line, with the highest in hepatocytes (HepG2 and HuH-7) and B-lymphocytes (Daudi). In each cell line studied, α2,6-ST activities were about 10 times higher than those of α2,3-ST. α2,6-ST activities measured in this study represent hST6Gal I, considering the mRNA expression level in these cell lines.[29] α2,3-ST activities corresponded to hST3Gal IV[30,31] and hST3Gal III,[32] which prefer to react on Galβ1-4GlcNAc, compared with hST3Gal I[33] and hST3Gal II.[34,35]

The α1,3/4-FucT activities using Galβ1-3GlcNAc were determined only in Colo 205 cells, which coincided well with the results of FucT III mRNA expression.[36,37] On the other hand, α1,3-FucT activities were also determined in each cell line with high activities in colonic cells Colo 205 and HT-29, however the most of the α1,3-FucT activities in Colo 205 were derived from α1,3/4-FucT. Although FucT IV, V and VI could react on Galβ1-4GlcNAc-coumarin in greater or lesser degrees,[25] the high α1,3-FucT activities in HT-29, HL-60 and U-937 would be mainly derived from FucT IV, considering its mRNA expression levels.

This assay method for the measurement of glycosyltransferase activities was accurate using coumarin-tagged sugar as the fluorescence substrate and HPLC for separation of the enzymatic product and was sensitive enough to assay cell homogenates. This method is easy and allows specific identification of the enzymatic products liberated by the reaction of glycosyltransferases and also, the degradative products by the reaction of glycosidases in the homogenate. Furthermore, this method is applicable to determine the GalT activities using a monosaccharide as an acceptor substrate: coumarin labeled-GlcNAc, which could not react with 2-AP-GlcNAc. Coumarin-tagging acceptor substrates are useful and widely applicable to measure glycosyltransferase activities.

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