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Development of a Sensitive Separation and Quantification Method for Sialyl Lewis X and Lewis X Involving Anion-Exchange Chromatography: Biochemical Characterization of α1-3 Fucosyltransferase-VII

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The biosynthesis of the carbohydrate antigen sialyl Lewis X (sLe^x) in human leukocytes is mediated by α1-3 fucosyltransferase-VII (FucT-VII), which catalyzes the transfer of fucose from GDP-β-fucose to the 3-OH of α2-3 sialyl *N*-acetyllactosamine (SA-LN). We developed a simple method for quantitating the reaction product of FucT-VII involving Anion-Exchange Chromatography (AEC). The AEC assay involved the separation of a radio-labeled acceptor from the unreacted nucleotide sugars with 0–0.5 M NH_4OAc (pH9.0) on QAE-Toyopearl 550C. Furthermore, this assay enabled the separation of the fucosylated products of sialylated and non-sialylated oligosaccharides with this column. Analysis of the FucT-VI reaction mixture showed that Lewis X (Le^x) was eluted in the flow-through fraction and sLe^x was eluted with 0.1 M NH_4OAc, and these products were clearly separated from the fraction of unreacted GDP-[^{3}H]fucose. Therefore, this method could be a powerful tool for the characterization of recombinant FucT-VII and for establishing a high-throughput screening system for FucT-VII inhibitors. Beside FucT-VII, this method will be applicable to the assaying of many different glycosyltransferases, including sialyltransferases and glucosaminyltransferases, which are reactive to α2-3 SA-LN or *N*-acetyllactosamine sequences.

Key words: anion-exchange chromatography, expression, inhibitor, fucosyltransferase, glycosylation, screening system.

Abbreviations: α1-3 FucT, fucosyltransferase; sLe^x, sialyl Lewis X; SA-LN, α2-3 sialyl *N*-acetyllactosamine; LN, *N*-acetyllactosamine; AEC, anion-exchange chromatography; NEM, *N*-ethylmaleimide; ESI-MS, electrospray ionization mass spectrometry.

Cell-surface antigen sLe^x [NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc-R] is a constituent of the ligand for cell adhesion molecules, *i.e.* the selectins (lectin-EGF-complement binding–cell adhesion molecules [LEC-CAMs]). The interaction of these molecules leads to the attachment and rolling of leukocytes on blood endothelial cells during an inflammatory condition (*1*, *2*). The final step in the biosynthesis of sLe^x-epitopes is mediated by α1-3 FucT [EC 2.4.1.-], which catalyzes the transfer of fucose from GDP fucose to sialyl *N*-acetyllactosamine *via* an α1-3-linkage (*3–9*). There are two FucTs (FucT-IV and FucT-VII) expressed to a significant degree in human leukocytes, and they are critically involved in the biosynthesis of selectin ligands in these cells. In fact, FucT-VII can clearly construct the sLe^x determinant in a cell-free enzyme assay, as well as in cultured cell lines transfected with expression vectors encoding this enzyme (*4–7*). Furthermore, FucT-VII–deficient mice exhibit a leukocyte adhesion deficiency that is characterized by the absence of both leukocyte E- and P-selectin ligand activity, as well as a deficiency of L-selectin ligand activity in high endothelial venules (*2*). Therefore, the inhibition of FucT-VII activity could suppress the expression of selectin ligands, thereby reducing selectin-dependent leukocyte adhesion to endothelial cells. Thus, selective inhibitors of FucT-VII are expected to be potential therapeutics for the treatment of inflammatory diseases.

The assaying of α1-3 FucT requires a sensitive method for the separation of a radio-labeled acceptor from the unreacted nucleotide sugars and receptors. Commonly used methods such as gel filtration chromatography, HPLC and paper electrophoresis (*10–12*) are both time consuming and labor intensive. The conventional method of anion-exchange chromatography, called the Dowex assay, is rapid and sensitive, and facilitates the completion of numerous simultaneous assays (*13*). However, this column cannot precisely separate the fucosylated products of sialylated and non-sialylated oligosaccharides from the α1-3 FucT reaction mixture. These reactants were generally applied to a chloride form column for the Le^x products and a phosphate form column for the sLe^x products. Thus, the column most suitable for each structure of the fucosylated products has to be prepared with a restricted equilibrium buffer. In addition, the volume of the wash buffer for removing the non-specific product has to be restricted to raise the detection limit, because these

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reaction products are generally eluted in the flow-through fraction.

In this report, we describe a novel α1-3 FucT assay system involving anion-exchange chromatography (AEC). This method involves the highly sensitive detection of the fucosylated products of sialylated and non-sialylated oligosaccharides by high-resolution separation of each fraction on QAE-Toyopearl 550C. We also demonstrated that this method is sensitive and has a high-throughput capacity with recombinant FucT-VII.

MATERIALS AND METHODS

All radio-labeled nucleotide sugars were purchased from Amersham Pharmacia. Chemicals were purchased from Sigma unless otherwise stated. α2-3 sialyl *N*-acetyllactosamine was obtained from Dextra Laboratories. *N*-acetyllactosamine, sLe^x, Le^x, sialidase, and *N*-glycosidase F were obtained from Oxford Glycobiosystems. Soluble recombinant human FucT-VI was obtained from CALBIOCHEM. Chemiluminescent detection film (Hyperfilm-ECL film) was purchased from Kodak. A BaculoGold kit was purchased from PharMingen. Anion-exchange chromatographic resin (QAE-Toyopearl 550C) was purchased from Tosoh. All chemicals were of the highest available purity.

Cell Culture—Sf21 was obtained from the American Type Culture Collection (Rockville, MD), and maintained at 27°C in TNM-FH (Invitrogen).

Isolation of cDNAs Encoding FucT-VII—The full-length cDNA of FucT-VII was amplified from single-stranded cDNA of THP-1 by the polymerase chain reaction (PCR) using primers FT7-NE1 (5′-ATTGAATTCTCTCGGGTCTCTTGGCTGACTG-3′) and FT7-CX1 (5′-AGGGGAATTCGCCTGGTGGTTTGATTTCG-3′), digested with *Eco*RI, and inserted into the *Eco*RI site of pBluescript II SK+, and then sequence of the fragment was determined by the dideoxy-chain-termination method, using an automated DNA sequencer (model 373A, Applied Biosystems) and a Taq Dye Primer Cycle Sequencing Kit (Perkin-Elmer Cetus). The resulting plasmid was designated as pBluFucT-VII-L. A putative catalytic domain of the FucT-VII (amino acids 39–342) was amplified by PCR using primers FT7-T1 (5′-CGGTGAATTCCGCTTCCCAGACAGCACAGGTACCCCGGCACCCCAGCCCAC-3′) and FT7-T2 (5′-CCGCGGATTCTCAGGCCTGAAACCAACC-3′), and pBluFucT-VII-L as a template, digested with *Eco*RI and *Bam*HI, and then cloned into the *Eco*RI and *Bam*HI sites of pEZZ 18 (Pharmacia) to yield a plasmid, pEZZ18-FucT-VII, expressing a protein A–fused FucT-VII protein in *E. coli*.

Preparation of FucT-VII Protein from Insect Cells—Recombinant protein A-fused human FucT-VII was produced by infecting Sf21 cells with recombinant baculoviruses. The baculovirus transfer vector pAcProAFucT-VII was constructed by inserting the DNA fragment encoding the IgG-binding domain of *Staphylococcus aureus* protein A and the putative catalytic domain (amino acids 39–342) of FucT-VII from pEZZ18-FucT-VII into pAcGP67 (Pharmingen) containing a gp67 signal sequence, which is one of the most effective baculovirus-encoding signal sequences for protein secretion. The pAcProFucT-VII was co-transfected with linearized Autographa californica nuclear polyhedrosis virus (AcNPV) DNA (PharMingen) in Sf21 cells to obtain recombinant baculovirus BV ProAFucT-VII.

Sf21 cells (1×10^6 cells/ml) in serum-free EX-CELL400 (JRH Bioscience) medium were infected with the BV ProAFucT-VII (m.o.i. = 10). After incubation at 27°C for 72 h the cell supernatant was routinely harvested and stored at –80°C until use as the enzyme source.

Purification of Recombinant FucT-VII—The recombinant enzyme was purified from the cell culture medium by IgG-agarose column chromatography (Amersham). The recombinant FucT-VII was eluted from the IgG-agarose resin as follows. IgG-agarose beads were briefly suspended in 0.1 M citrate buffer, pH 4.3, and then the supernatant was immediately removed and placed in a tube containing neutralization buffer [1 M Tris-HCl (pH 8.0) and 0.1% BSA]. The protein concentration of the enzyme fraction was determined by a Micro-BCA protein assay (Pierce Chemical), and the amount of FucT-VII protein in the cell supernatant was determined from the protein recovery rate from the culture medium.

Western Blot Analysis—Aliquots of supernatants of 3×10^4 cells were subjected to 10% SDS-PAGE in minigels (ATTO). The protein was transferred from the gels to Immobilon-P membranes (Millipore). The membranes were blocked in Block ACE (Dainippon Seiyaku) at 4°C for 24 h. Membranes loaded with protein A fusions were transferred to a solution of peroxidase-conjugated dog IgG (Rockland) in buffer A (10 mM Tris containing 0.1% Tween20 and 1 mM EDTA) for 2 h at room temperature. After three washes (10 min each) in buffer A, the membranes were developed with ECL luminescent substrate (Amersham Pharmacia), as recommended by the supplier, and then exposed for a few seconds to the Hyperfilm-ECL film (Amersham Pharmacia).

Detection of the Fucosylated Product Using the Fucosyltransferase Reaction—The purchased FucT-VI (CALBIOCHEM) was used as a model enzyme for identification of the eluted fraction of the fucosylated product. The standard FucT-VI reaction was carried out in a volume of 20 μl comprising 10 mM $MnCl_2$, 5 mM ATP, 10 mM L-fucose, 20 μM GDP-fucose, 120,000 dpm of GDP-[^{3}H]fucose, 3 mM *N*-acetyllactosamine (LN) or α2-3 sialyl *N*-acetyllactosamine (SA-LN), 50 mM HEPES/NaOH (pH 6.5), and 0.1 mU of FucT-VI. In contrast, the standard FucT-VII reaction was carried out in a volume of 20 μl comprising 10 mM $MnCl_2$, 5 mM ATP, 10 mM L-fucose, 20 μM GDP-fucose, 120,000 dpm of GDP-[^{3}H]fucose, 3 mM α2-3 SA-LN, 50 mM cacodylate/NaOH (pH 6.5), and 10 μl of cell supernatant (containing 1 μg of FucT-VII protein) as enzyme.

Each reaction mixture was incubated at 30°C for 18 h before the reaction was stopped by freezing it in a liquid nitrogen bath, and diluted with 180 μl of cold water, and then the 150 μl of the mixture was added to 1 ml of QAE-Toyopearl 550C resin in a Pasteur pipette. The column was washed with 1 ml of water and then subjected to successively eluted with a stepwise elution with 0–0.5 M NH_4OAc [pH 9.0] at 1 ml/fraction. The fucosylated fractions (1 ml) were collected in 9 ml of ACSII scintillation cocktail (Amersham Pharmacia) for scintillation counting. Control reactions, without enzyme, were used to determine the background counts.

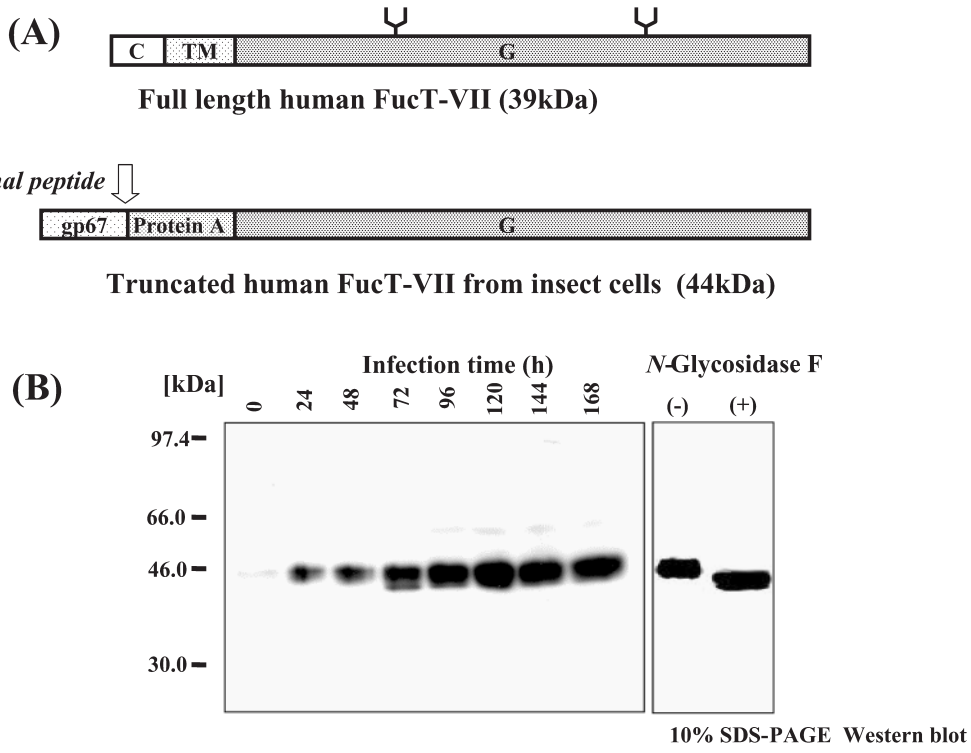

Fig. 1. **Schematic representation of FucT-VII constructs and Western blot analysis of the recombinant fusion FucT-VII from insect cells.** (A) Proposed domain structure of the predicted polypeptide of FucT-VII. The boxes indicate the cytoplasmic domain (C), transmembrane domain (TM), Golgi lumenal catalytic domain (G), *N*-glycosylation site (**Y**), gp67 signal peptide (gp67), and IgG-binding domain of *Staphylococcus aureus* protein A (Protein A). (B) Sf21 cells were infected with recombinant virus at 10 MOI. Cell supernatant fractions were harvested at various time points after infection (left), or Sf21- produced FucT-VII (72 h–infected cell) was treated with (+) or without (–) *N*-glycosidase F (right), and then analyzed by Western blotting. The migration of molecular weight standards is indicated on the left.

The results are generally expressed as Net DPM and Nonspecific breakdown of GDP-fucose was assessed by means of the enzyme assay in the absence of the acceptor saccharide. All results are representative of at least three experiments.

Identification of sLe^x Fraction on AEC Resin by ESI-MS—Authentic sLe^x (NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc; Oxford Glycobiosystems) was used for identification of the fraction by the AEC method. The sLe^x (20 pmol/ml) was added to QAE-Toyopearl 550C resin, and elution was performed as described above. In order to identify the sLe^x fractions, the eluted samples were monitored by ESI-MS (Finnigan MAT: LCQ iontrap mass spectrometer). For detection of the sLe^x in the negative ion mode, the polarity of the instrument was reversed, and a stream of MeOH/H_2O (80/20, v/v) was constantly infused into the ion source at 50 μl/min. The eluted fractions were injected into the stream via a 3 μl injection loop, and normal spectra were recorded by scanning from m/z 150 to m/z 900 (1.0 Da step size, 0.86 s/scan).

Analysis of Glycosylation—N-Glycosidase F treatment of the generated recombinant FucT-VII: A 40 μl sample of the supernatant (containing 4 μg of FucT-VII) from infected cells was mixed with 0.5% SDS and 5% β-mercaptoethanol, and then boiled for 2 min. Then 10 μl of the boiled sample was treated with 0.4 U of *N*-glycosidase F in 20 mM sodium phosphate (pH 7.5) and 50 mM EDTA at 37°C for 18 h. After the glycosidase treatment, SDS-PAGE was performed as described above.

Sialidase treatment of the reaction mixture: The reaction mixture for α2-3 SA-LN, after the FucT-VI or FucT-VII reaction, was directly treated with 0.1 U sialidase in 50 mM sodium acetate (pH 5.5) at 37°C for 18 h. Elution and monitoring of the fucosylated product after treatment with the sialidase were performed as described above.

Measurement of Apparent K_m Values of FucT-VI and FucT-VII—For measurement of the apparent K_m values of FucT-VI for GDP-fucose, 3 mM LN and 2.3–150 μM GDP-fucose were used. For LN, 20 μM GDP-fucose and 0.16–10 mM LN were used. The reaction mixtures were incubated for 30 min at 30°C.

In contrast, for measurement of the apparent K_m values of FucT-VII for GDP-fucose, 3 mM SA-LN and 2.3–150 μM GDP-fucose were used. For SA-LN, 20 μM GDP-fucose and 0.16–10 mM SA-LN were used. The reaction mixtures were incubated for 60 min at 30°C.

The fucosylated products were washed and monitored as described above. The K_m values were estimated from Linweaver-Burk plots.

Inhibitor Studies—For inhibition studies, the assays were performed as above except that the concentration of GDP-fucose used was varied. The inhibitors were prepared as 100-fold concentration stocks in DMSO, and DMSO was added to give a final concentration of 1.0% (v/v) in all samples assayed. For determination of the K_i values for GDP, the enzyme activity was measured in reaction mixtures containing different concentrations of GDP-fucose (6.3, 12.5, 25 and 50 μM), that was mixed with GDP to give a final concentration of 0–0.1 mM. The K_i value for each GDP was calculated from a Dixon plot.

Data Analysis—All kinetic data were fitted with the nonlinear regression algorithm in GraphPad Prism version 4.0 (GraphPad Software). Error bars on the graphs represent the standard deviation of three independent averaged data points.

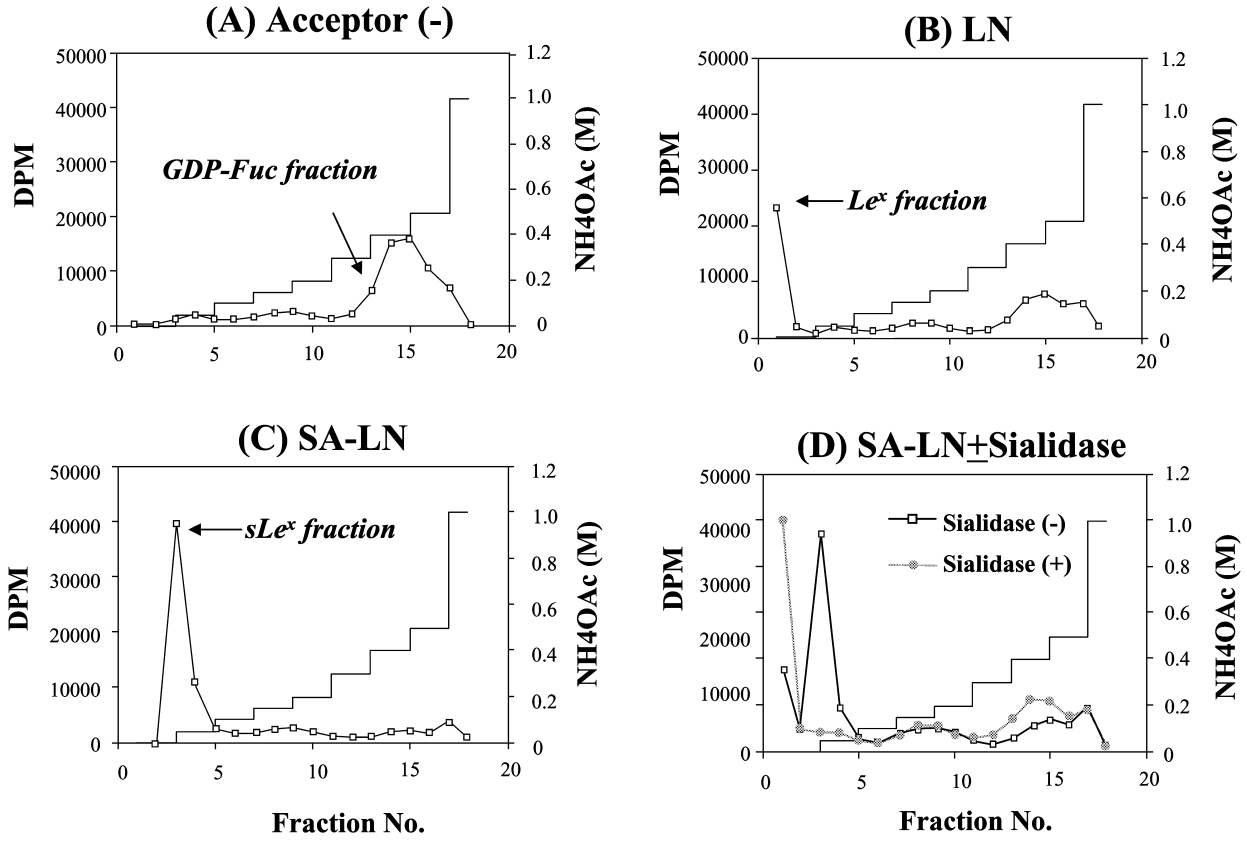

Fig. 2. **Elution profiles of the reaction products of FucT-VI with or without an acceptor on anion-exchange chromatography.** FucT-VI reactions were carried out (A) in the absence of an acceptor, and with (B) *N*-acetyllactosamine and (C, D) sialyl *N*-acetyllactosamine for 18 h at 30°C. The reaction products were treated with (solid cirlces) or without (open squares) sialidase before being applied to a QAE-Toyopearl 550C column . Then the fucosylated product was detected.

RESULTS AND DISCUSSION

Expression of the Recombinant FucT-VII—To prepare the enzyme for establishment of a screening system for the FucT-VII inhibitor, we expressed human recombinant FucT-VII in insect cells as a secretory protein fused with the IgG-binding domain of protein A (Fig. 1A). Sf21 cells were infected with the recombinant baculovirus containing the secretory FucT-VII cDNA under the control of the polyhedrin promoter, and cultured at 27°C for 0–168 h. Next, to detect the recombinant FucT-VII, the supernatant of the infected cells was subjected to SDS-PAGE followed by Western blot analysis. A 46 kDa truncated FucT-VII protein was clearly detected in the supernatant of the culture of 24 to 168 h after infection (Fig. 1B). We confirmed the correct cleavage of the signal peptide by analysis of the N-terminal amino acid sequence of the expressed protein (data not shown).

FucT-VII has two conserved glycosylation sites and FucT-VII expressed in insect cells is known to be modified with *N*-linked carbohydrate molecules (*14*). The presence of carbohydrate chains might have an influence on its biological properties, presumably because the carbohydrate chains affect protein stability, activity, and folding (*15*). To investigate the glycosylation of the generated recombinant FucT-VII from Sf21 cells, the supernatant of the cell culture at 72 h after infection was treated with glycosidases and then subjected to Western blot analysis. As expected, the molecular weight of the expressed FucT-VII was slightly reduced by the *N*-glycosidase F treatment (Fig. 1B). We confirmed that our generated FucT-VII was also modified through *N*-glycosylation as a result of eukaryotic modification.

Identification of the Fucosylated Product fraction by the AEC Method—We have determined the optimal conditions for the separation of radio-labeled oligosaccharides from nucleotide sugars. In order to select the most suitable ion-chromatographic resin for the α1-3 FucT assay, several columns were compared with one of the widely used Dowex resin. As a result, QAE-Toyopearl 550C (Tosoh) showed preferential properties, i.e: GDP-[^{3}H]fucose can be retained sufficiently and readily eluted with 0.3–0.5 M NH_4OAc (pH 9.0) (Figs. 2A and 4, C and F), and a high recovery of approximately 95% can be achieved with elution with 1 M NH_4OAc (pH 9.0). Therefore, we selected QAE-Toyopearl 550C as the AEC chromatographic resin and used a 0–0.5 M NH_4OAc (pH 9.0) gradient to analyze the fucosylated product from the α1–3 FucT reaction mixture.

In order to identify the fraction of the fucosylated product, recombinant FucT-VI was used as a model enzyme. Since FucT-VI recognizes the type II acceptor of both sialylated and non-sialylated saccharides, the elution patterns of these fucosylated products were expected to be simultaneously monitored with the QAE-Toyopearl 550C.

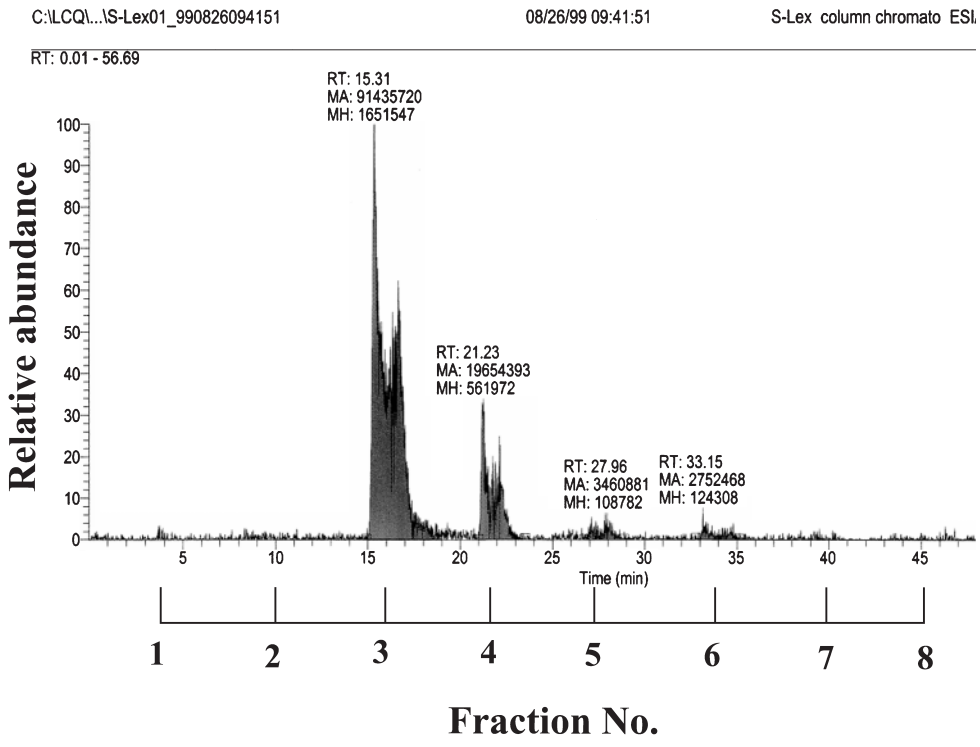

Fig. 3. **Negative ion mode ESI-MS spectra of authentic sLex after separation on a QAE-Toyopearl 550C column.** Authentic sLe^x was separated by QAE-Toyopearl 550C column, and the eluted fractions were detected by ESI-MS as described under "MATERIALS AND METHODS."

After 18 h of the FucT-VI reaction, each reaction mixture was applied to the column and fractionated as follows. The products were separated with a stepwise gradient of NH_4OAc (pH 9.0, 0–1.0 M) (Fig. 2). Using LN as the acceptor for the reaction, a single radio-labeled product was detected in the flow-through fraction (Fig. 2B). In parallel, the peak of GDP-[^{3}H]fucose simultaneously decreased as a result of consumption of the donor substrate. When α2-3 SA-LN was used as the acceptor, the radio-labeled product was also detected as a single peak eluted at fraction Nos. 2 to 3, and this product was clearly separated from the GDP-[^{3}H]fucose (Fig. 2C). Moreover, treatment of the product derived from SA-LN with α2–3 sialidase resulted in complete conversion to a new product, which was eluted in the flow-through fraction (Fig. 2D).

To confirm the details of the sLe^x fraction on QAE-Toyopearl 550C, authentic sLe^x (NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc: OGS) was applied to the column and its eluted fractions were monitored by ESI-MS analysis (Fig. 3). It was found that the sLe^x fraction was equivalent to the fraction of the fucosylated product derived from α2-3 SA-LN in the FucT-VI reaction. In contrast, Le^x could not be detected because it did not produce any detectable ions in the negative ion mode. These results are strongly supported by the fact that the main fucosylated product derived from α2-3 SA-LN by the FucT-VI should be sLe^x and therefore eluted with 0.1 M NH_4OAc (pH 9.0), whereas the fucosylated product derived from LN should be the Le^x eluted in the flow-through fraction. Thus, the AEC method is useful for the detection of the sialylated and non-sialylated products of the α1-3 FucT enzyme reaction in a one-form column.

Detection of FucT-VII Activity—FucT-VII has been reported to only fucosylated α2-3 SA-LN of the type II acceptor, *i.e.* not neutral acceptors such the LN (*5*, *6*). The acceptor specificity of recombinant FucT-VII from Sf21 insect cells is described in the legend to Fig. 4. The radio-labeled products of the reaction after 18 h were analyzed by the AEC method as described under Materials and Methods. Recombinant FucT-VII showed that α2-3 SA-LN was specifically fucosylated (Fig. 4A) but not with LN oligosaccharide as an acceptor (Fig. 4B). This is consistent with previous reports (*5*, *6*). Furthermore, when the supernatant of AcNPV-infected Sf21 culture cells was used as a control, the peak of a fucosylated product was not detected (Fig. 4, D, E, and F). From these results, we concluded that the synthesis of the fucosylated product of α2-3 SA-LN was due to the generated recombinant human FucT-VII. Additionally, other non-specific peaks containing decomposed products were not detected during the 48 h reaction time, and the background radio-label detected at 0 min in the flow-through fraction was very low (average cpm < 330), indicating that this assay method has sufficient sensitivity for the detection of FucT-VII activity.

LN and α2-3 SA-LN were used as reference acceptors for FucT-VI and FucT-VII-dependent reactions. We demonstrated that FucT-VI efficiently fucosylated LN and α2-3 SA-LN, whereas FucT-VII specifically fucosylated α2-3 SA-LN but not LN. Thus, human FucT-VII and FucT-VI showed big differences in their acceptor specificities. Further characterization of FucT-VII will be useful for understanding the difference in enzymatic properties within α1–3 FucT and for discovery of its specific inhibitors. As shown above, recombinant FucT-VII from insect cells was appropriate as an enzyme source for the elucidation of its properties and for the establishment of a screening system for FucT-VII inhibitors involving the AEC method.

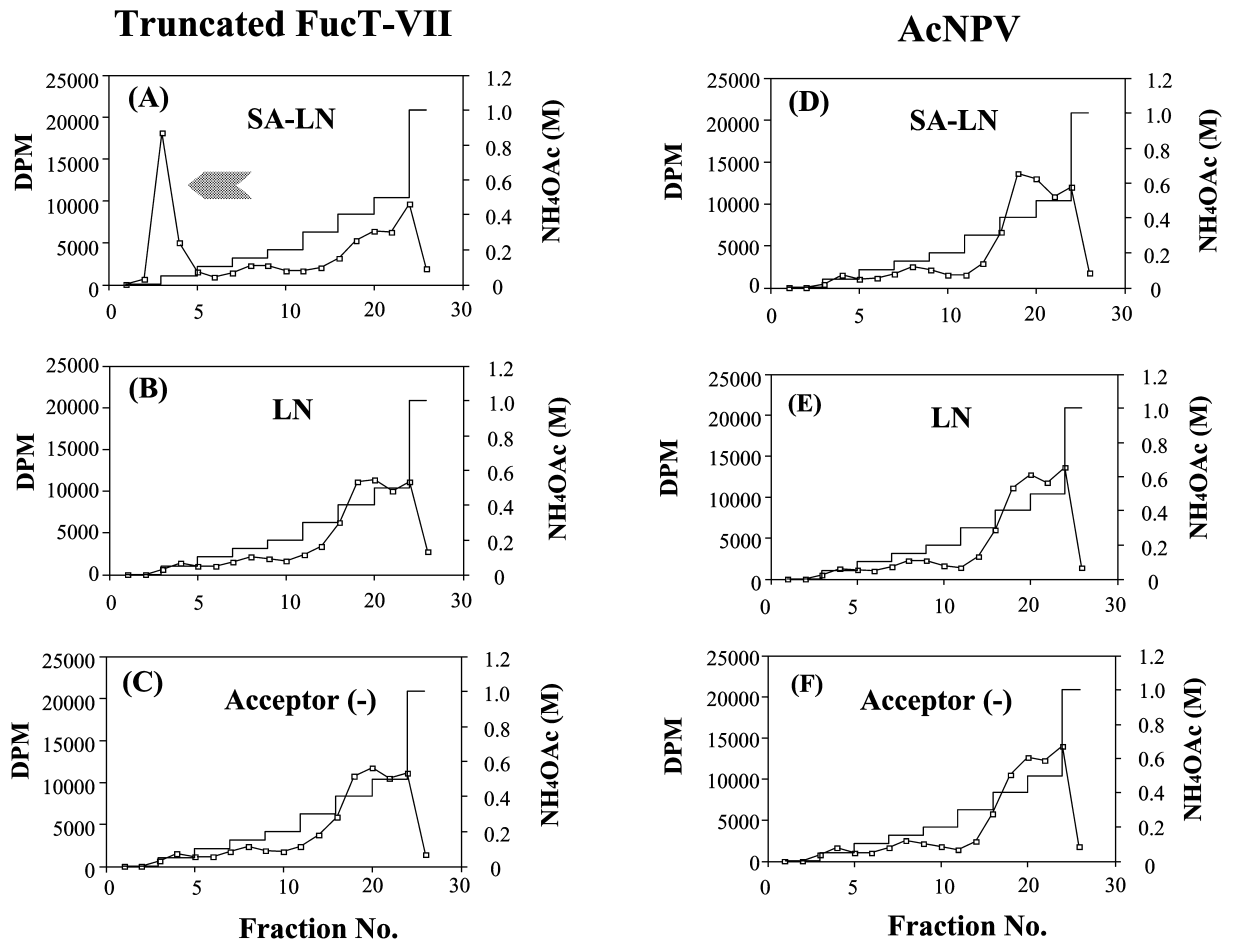

Fig. 4. **Elution profiles of the reaction products of FucT-VII with or without an acceptor on anion-exchange chromatography.** FucT-VII reactions were carried out with (A) sialyl *N*-acetyllactosamine and (B) *N*-acetyllactosamine, and (C) in the absence of an acceptor for 18 h at 30°C under in standard conditions. The wild type virus-transfected cell supernatant fraction (AcNPV) was also incubated with (D) sialyl *N*-acetyllactosamine and (E) *N*-acetyllactosamine, and (F) in the absence of an acceptor for 18 h at 30°C under the standard conditions.

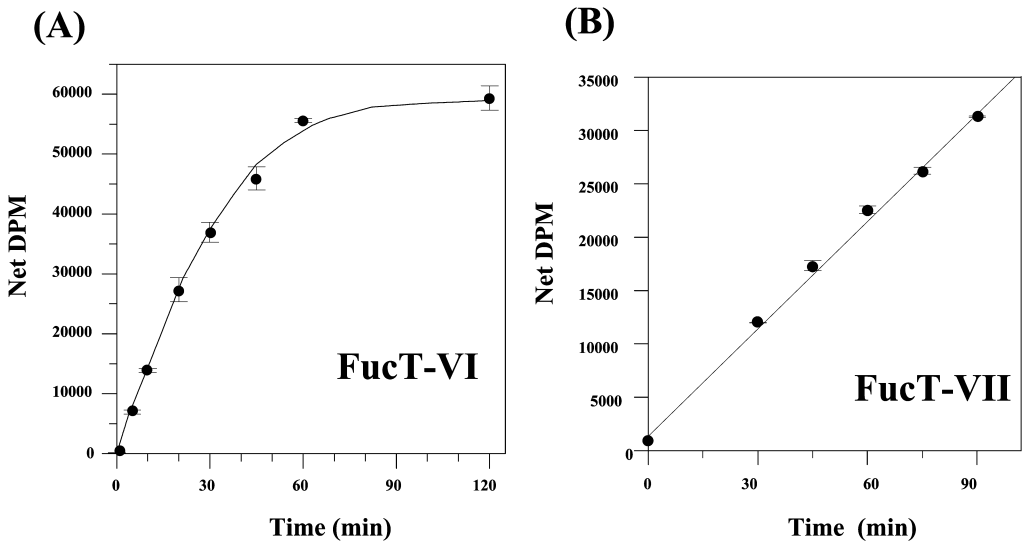

Fig. 5. **Time-dependence of [^{3}H]fucose transfer by FucT-VI and FucT-VII.** (A) The FucT-VI reaction was conducted in a volume of 20 μl comprising 10 mM $MnCl_2$, 5 mM ATP, 10 mM L-fucose, 20 μM GDP-fucose, 120,000 dpm GDP-[^{3}H]fucose, 3 mM *N*-acetyllactosamine (LN), 50 mM HEPES/NaOH (pH 6.5), and 0.1 mU of FucT-VI for up to 120 min at 30°C. (B) The FucT-VII reaction was performed in a volume of 20 μl comprising 10 mM $MnCl_2$, 5 mM ATP, 10 mM L-fucose, 20 μM GDP-fucose, 120,000 dpm GDP-[^{3}H]fucose, 3 mM α2-3 SA-LN, 50 mM cacodylate/NaOH (pH 6.5), and 10 μl cell supernatant (containing 1 μg FucT-VII protein) for up to 120 min at 30°C.

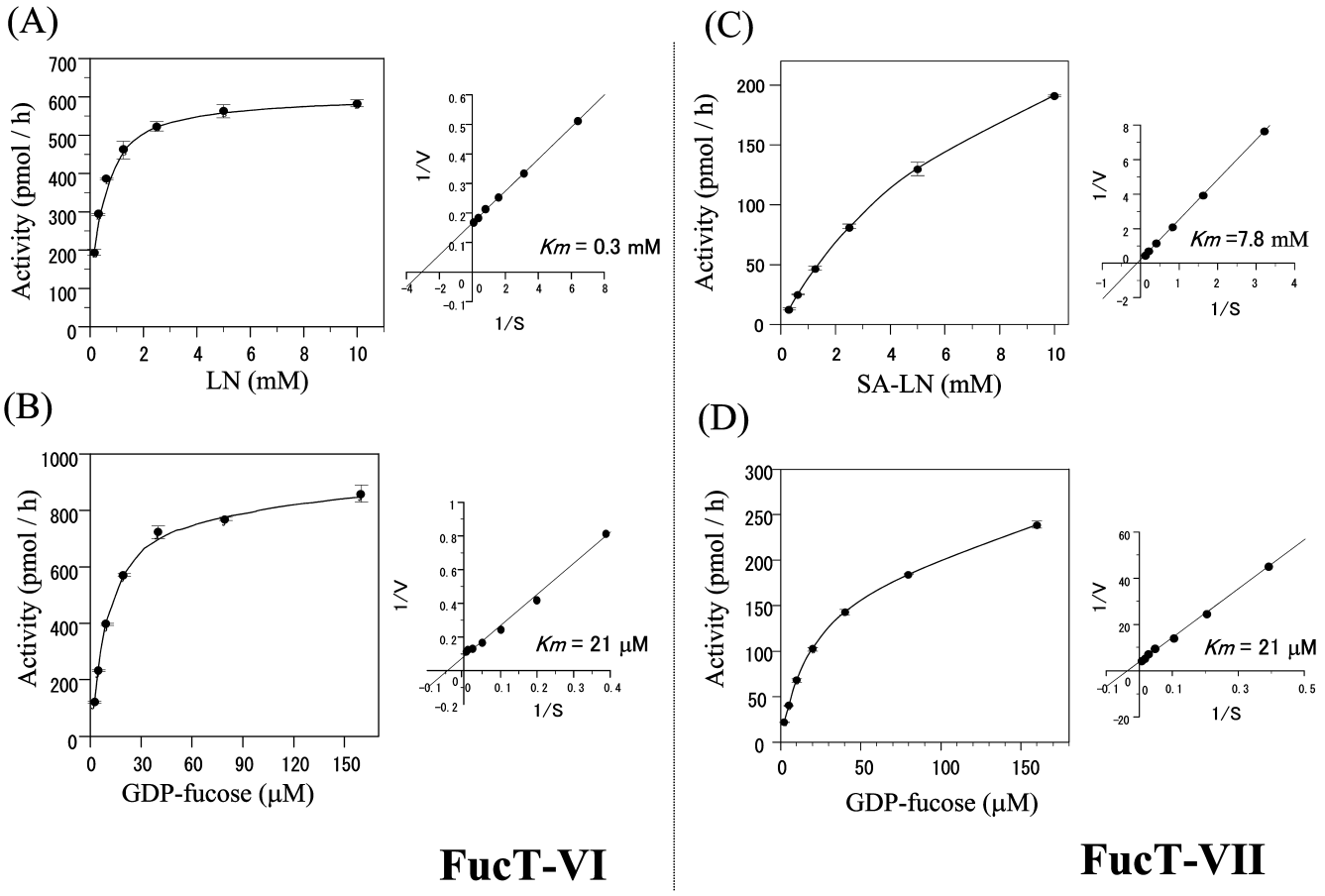

Fig. 6. **Enzyme kinetics of FucT-VI and FucT-VII.** FucT-VI activity was measured after 30 min at 30°C, and the K_m values were determined for both (A) *N*-acetyllactosamine and (B) GDP-fucose as described under "MATERIALS AND METHODS." FucT-VII activity was also measured after 1 h at 30°C, and the K_m values were determined for both (C) sialyl *N*-acetyllactosamine and (D) GDP-fucose.

Kinetic Studies of α1-3 FucT—We conducted kinetic studies on α1-3 FucT using the AEC method. Under the standard assay conditions give under "MATERIALS AND METHODS," the rate of [^{3}H]fucose incorporation mediated by FucT-VI was linear up to 45 min at 30°C (Fig. 5A). Accordingly, the FucT-VI assay was routinely performed for 30 min at 30°C. Furthermore, the enzymatic activity increased linearly with the recombinant FucT-VII protein concentration in the reaction mixture up to 10 μg and the optimal reaction temperature was 30°C (data not shown). The optimal pH was 5.5 to 6.5 in 50 mM cacodylate buffer. The FucT-VII activity was linear over the 0–90 min reaction at 30°C (Fig. 5B). We carried out subsequent experiments with a reaction time of 60 min at 30°C. These α1-3 FucT reactions can be terminated by freezing the reaction mixture in liquid nitrogen, which allows kinetic measurement of α1-3 FucT by the AEC method.

The FucT-VI activity was measured with varying concentrations of LN (0.15 to 10 mM), with a fixed GDP-fucose concentration of 20 μM, and the apparent Km value for LN was determined to be 0.3 mM from Lineweaver-Burk plots (Fig. 6A). From the plots obtained in the GDP-fucose concentration range of 2.5–160 μM with a constant LN concentration of 3.0 mM, the apparent K_m value for GDP-fucose was calculated to be 21 μM (Fig. 6B). The FucT-VII activity was also measured with varying concentrations of α2-3 SA-LN (0.15 to 10 mM), with a fixed GDP-fucose concentration of 20 μM, and the apparent K_m value for α2-3 SA-LN was 7.8 mM (Fig. 6C). From the plots obtained in the GDP-fucose concentration range of 2.5–160 μM with a constant α2-3 SA-LN concentration of 3.0 mM, the apparent Km value for GDP-fucose was calculated to be 21 μM (Fig. 6D).

These K_m values are consistent with the reported values for purified FucT-VII, which are in the range of 0.7–15 mM for sialyl LacNAc-R acceptor and 9.2–70 μM for GDP-fucose (*14*, *21–24*).

Characterization of FucT-VII—Human α1-3 FucTs have been classified based on the sensitivity to cysteine (Cys) modification as well as acceptor specificity (*15–20*). Holmes et al. previously reported that FucT-III, FucT-V, and FucT-VI were irreversibly modified at the cysteine residue involved in GDP-fucose binding by NEM, whereas FucT-IV and FucT-VII were resistant to NEM (*20*). In order to assess the NEM sensitivity of the soluble recombinant FucT-VII, the reaction mixture was preincubated with NEM at a final concentration ranging from 0.05 to 5 mM at 4°C for 1 h. As expected, measurement of the remaining FucT-VII activity revealed that it was not inactivated by NEM whereas FucT-VI was completely inactivated by 5 mM NEM (Fig. 7).

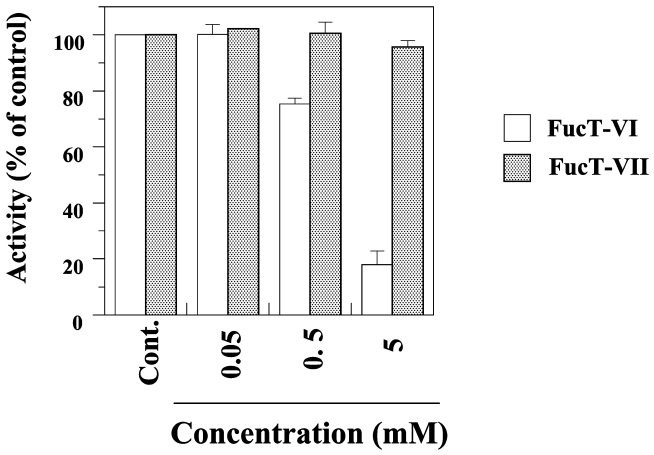

Fig. 7. **Effect of *N*-ethylmaleimide on α1-3 FucT activity.** The FucT-VI reaction was performed in a volume of 20 μl comprising 10 mM $MnCl_2$, 5 mM ATP, 10 mM L-fucose, 20 μM GDP-fucose, 120,000 dpm GDP-[^{3}H]fucose, 3 mM *N*-acetyllactosamine (LN), 50 mM HEPES/NaOH (pH 6.5), and 0.1 mU of FucT-VI with varying concentrations of NEM for 30 min at 30°C (open squares). The FucT-VII reaction was performed in a volume of 20 μl comprising 10 mM $MnCl_2$, 5 mM ATP, 10 mM L-fucose, 20 μM GDP-fucose, 120,000 dpm GDP-[^{3}H]fucose, 3 mM α2-3 SA-LN, 50 mM cacodylate/NaOH (pH 6.5), and 10 μl cell supernatant (containing 1 μg of FucT-VII protein) with varying concentrations of NEM for 1 h at 30°C (solid squares).

The inhibitory effects of various nucleotides on the enzyme activity were examined to determine the specificity of FucT-VII for the nucleotide portion of donor substrates. GDP was the most potent inhibitor among them (*9*), suggesting the availability of GDP-fucose as a donor. Figure 8 shows the inhibition curve and the Lineweaver-Burk plot for FucT-VII with various concentrations of GDP. Inhibition by GDP is concentration-dependent with an IC_{50} value of 93 μM. To assess the GDP inhibitory effect on FucT-VII, the reaction mixture was mixed with GDP to a final concentration ranging from 0 to 0.01 mM with a fixed GDP-fucose concentration of 20 μM, and the apparent Ki value for GDP was determined to be 4.5 μM from Dixon plots (Fig. 8B).

We used a truncated form of the enzyme composed of protein A fused to the catalytic domain of the α1-3 FucT. This enzyme was similar to the native, full-length one in terms of GDP-inhibitory properties (*25*). Therefore, the generated truncated FucT-VII from Sf21 cells will be useful for the screening of FucT-VII inhibitors, and the AEC method will provide the opportunity to find specific FucT-VII inhibitors.

Amino acid sequencing of five-cloned human α1-3 FucTs revealed that FucT-VII comprises a unique class in the human α1-3 FucT family, exhibiting 42–43% homology with each of the three chromosome 19p-encoded α1-3 FucTs (FucT-III, FucT-V, and FucT-VI), and 47% with FucT-IV (*5*, *6*). Since FucT-VII has been demonstrated to be involved in the biosynthesis of selectin ligands (*5*, *7*, *8*), FucT-VII-selective inhibitors might have therapeutic potential as anti-inflammatory drugs.

We described the development of a radioactive method for the measurement of FucT-VII activity involving an oligosaccharide acceptor. The non-radioactive assays that also involve transferase activity have the obvious advantage of not requiring radioactive materials, but require a number of washing steps before the final product detection with antibodies can be achieved. The Scintillation Proximity Assay (SPA), despite being isotope-based, requires no such washes nor special incubations, and could be of use for high-throughput screening without the step of separation of bound from free ligand. However, it is difficult to determine the substrate specificity and the K_m value for an acceptor by means of the SPA assay, because the glycoprotein acceptor (fetuin or asialofetuin) is pre-coated on the SPA beads. The AEC method described here is useful and advantageous for the characterization of α1-3 FucT, including the enzymatic kinetics of a donor or acceptor saccharide and its inhibitory profiling. Furthermore, this method is simple and highly sensitive, and changing of the column form to correspond to the fucosylated product of α1-3 FucT is not required.

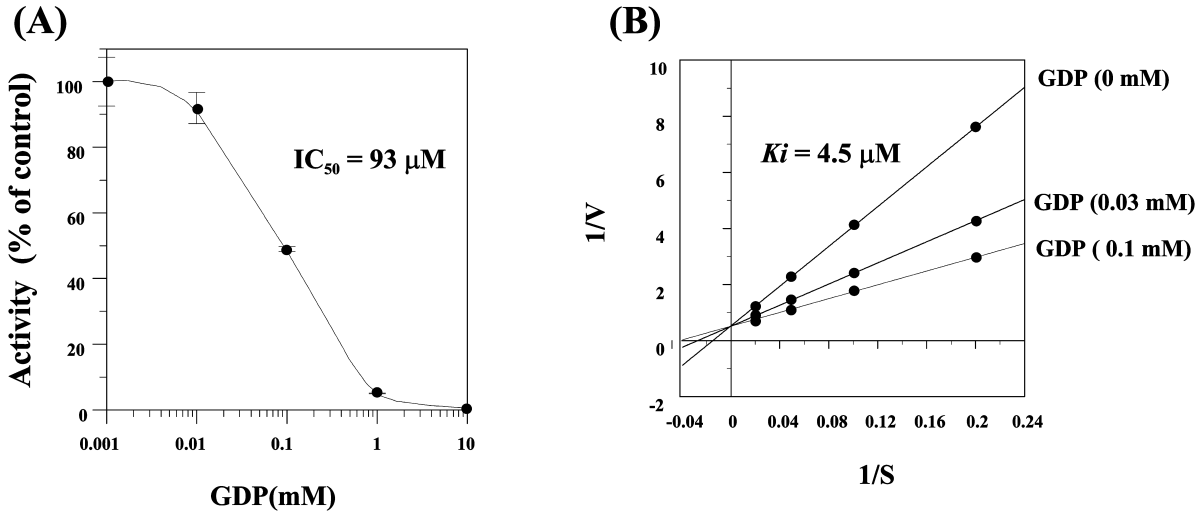

Fig. 8. **Effect of GDP on FucT-VII activity.** (A) IC_{50} values at the concentration of 20 μM GDP-fucose were determined from the inhibition curves for GDP (IC_{50} = 93 μM). (B) Lineweaver-Burk plot of FucT-VII activity in the presence of 0, 0.03, and 0.1 mM GDP with GDP-fucose concentrations ranging from 5 to 40 μM. The K_i value (4.5 μM) of GDP was determined from a Dixon plot.

Thus, this method could be applied to structure recognition analyses of oligosaccharide including glycosidase digestion by α1-3 FucTs. Furthermore, this assay could be readily adapted to other glycosyltransferases, especially sialyltransferases and glucosaminyltransferases, acting on LN or α2-3 SA-LN sequences.

Therefore, the AEC method will be a powerful method for glycobiology.

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