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Note

A new fluorescent assay for sialyltransferase

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Abstract

A new fluorescent assay for the sialyltransferase reaction was established. After incubation of the sialyltransferase reaction, the sialyloligosaccharide obtained was treated by acid hydrolysis, and then the NeuAc that was released was labeled with 1,2-diamino-4,5-methylenedioxibenzene. The fluorescent-labeled NeuAc could be estimated by HPLC (excitation: 373 nm; emission: 448 nm) and a Lineweaver–Burk plot could be plotted with the data from this analysis.

Keywords: Sialyltransferase assay; Fluorescent labeling; Sialic acid

Sialyltransferases catalyze the transfer of sialic acid residues from CMP-NeuAc to specific acceptors. These transfer reactions have been assayed by several procedures using either ^{14}C- or ^{3}H-labeled sugar residues,[1,2] continuous spectrophotometry,[3] or HPLC and fluorescence.[4–8] The most typical assay involves using CMP-[U-^{14}C or ^{3}H]-NeuAc.[1,2] Using the HPLC method, transfer reactions are monitored by detecting *p*-nitrophenyl-,[4] 2-aminopyridyl-[5,6] and 4-methylumberipheryl-labeled[7] products. The thiobarbituric acid (TBA) method[9–11] and solid-phase systems[12,13] have also been used for sialyltransferase assays.

All these methods have both merits and limitations. Methods that employ radioisotope-labeled donors are sensitive down to the pmolar range, but handling and disposal of these materials is troublesome. For methods using acceptors labeled with *p*-nitrophenyl-, 2-aminopyridyl- and 4-methylumberipheryl-groups, preparation of the labeled acceptor is time consuming. Although the TBA method is convenient for detecting NeuAc and its analogues, it is not as sensitive as radioisotope- and fluorescent-labeled compounds, and it cannot be applied to 4-modified NeuAc analogues.[9] Continuous photometric assay[3] is also feasible, but requires no commercially available nucleotide monophosphate kinase.

Recently, a new fluorescent analytical method for NeuAc and its analogues using 1,2-diamino-4,5-methylenedioxibenzene (DMB) has been reported.[14–16] This labeling method can be applied to any NeuAc analogues because DMB reacts with both the carboxyl and ketone groups at the 1- and 2-positions of NeuAc. In addition, the sensitivity for detection of NeuAc is the same as with radioisotope methods.

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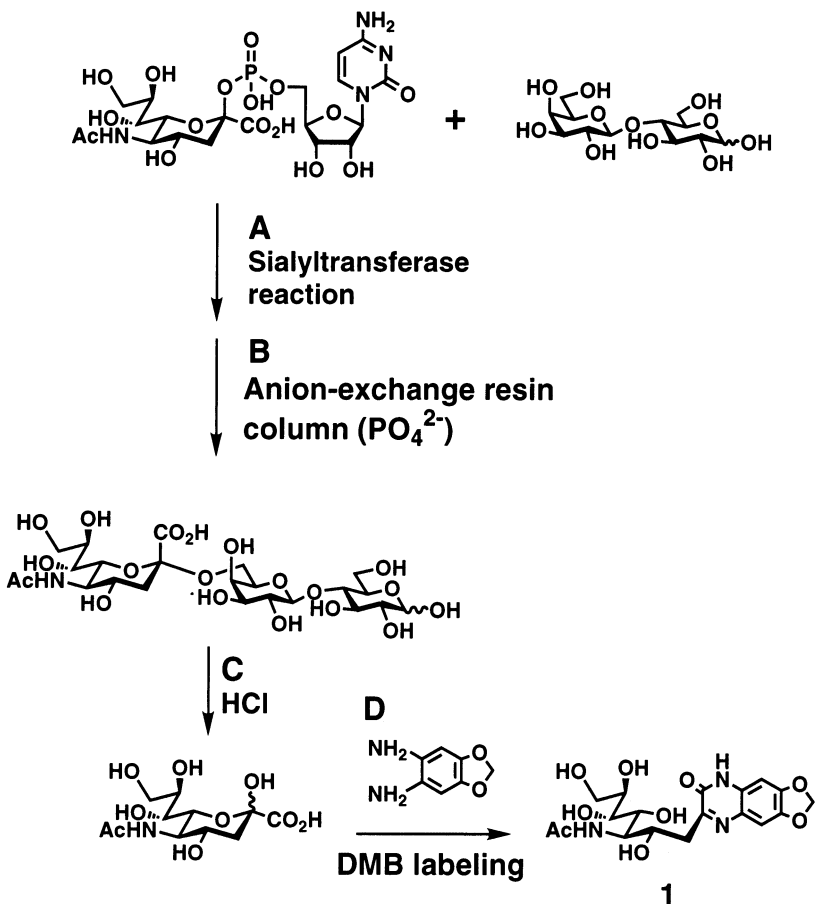
We report herein a new fluorescent assay for sialyltransferase reactions by the combined use of DMB-labeling and HPLC analysis.

The assay procedure using DMB-labeling is shown in Scheme 1. After the transfer reaction, five components exist in the assay mixture: sialoside, CMP-NeuAc, acceptor, CMP, and NeuAc. In order to remove CMP-NeuAc, NeuAc, and CMP, the mixture is passed through an anion-exchange column, leaving only sialoside and unreacted acceptor.[1] The eluant is treated by acid hydrolysis, and the NeuAc that is released is labeled with DMB. The fluorescent-labeled NeuAc can then be measured by HPLC using a fluorescent detector (excitation: 373 nm; emission: 448 nm).

In order to establish this assay procedure, conditions for hydrolysis of NeuAc (step C) and for DMB-labeling (step D) were optimized. Since several acid concentrations were used for DMB-labeling,[14–16] we determined the most suitable acid concentration. As shown in graph A (Fig. 1), the intensity of the fluorescent peak of **1** in the HPLC analysis showed a maximum when 20 mM of HCl solution was used for DMB-labeling.

Next the hydrolysis conditions for sialoside (sialyllactose) were optimized. Since the acid concentration for DMB-labeling have been optimized at 20 mM, the acid concentration was adjusted to 20 mM for DMB-labeling following hydrolysis of sialoside. The acid concentration for the hydrolysis assay was varied from 25 to 400 mM. For the control experiments, the same concentrations and ratios of NeuAc to sialyllactose were used under the same conditions. As shown in graph B (Fig. 1), 200 mM of HCl was found suitable for acid hydrolysis, and its peak intensity was the same level as that of NeuAc in the control experiment.

From the sialyltransferase reaction using the CMP-[^{14}C]-NeuAc at the nmolar range, the sialoside should be formed in less than 150 pmolar range because the assay is always followed to less than 15% consumption of CMP-



Scheme 1. Strategy of a new fluorescent assay for sialyltransferase.

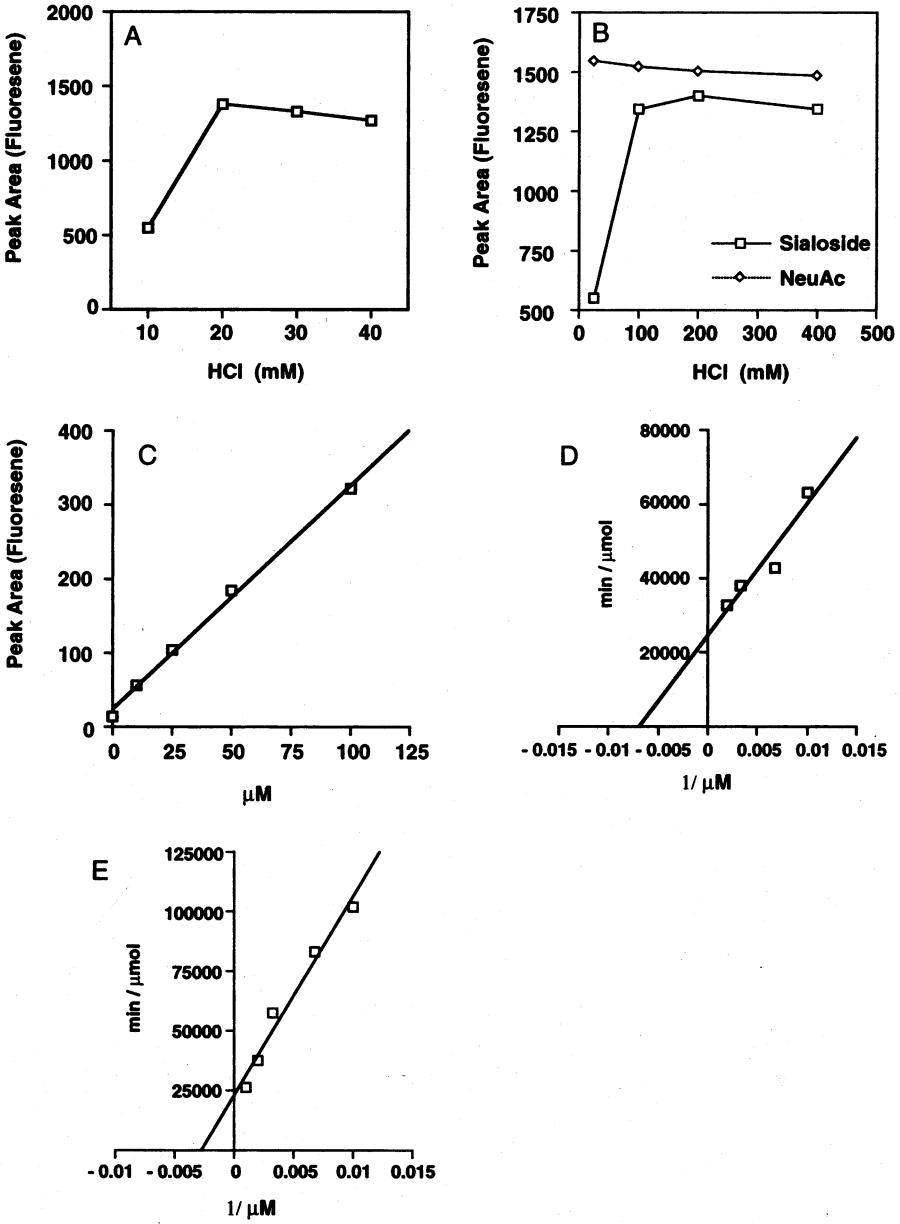


Fig. 1. (A) Optimization of DMB labeling; (B) optimization of hydrolysis condition for sialyllactose; (C) calibration of quantity of NeuAc; (D) Lineweaver–Burk plot of CMP-NeuAc; (E) Lineweaver–Burk plot of CMP-9″-amino-9″-deoxy-NeuAc.

[^{14}C]-NeuAc. Therefore, we investigated whether our assay system can detect such tiny amounts of sialoside. Four concentrations of sialyllactose (10, 25, 50, and 100 μM) were treated by acid hydrolysis and DMB-labeling following passage through an anion-exchange column.[1] As shown in graph C (Fig. 1), peak intensity of fluorescent-labeled NeuAc increased linearly as the concentration of sialyllactose increased. This experiment indicated that our fluorescent-labeled system can be used as a new sialyltransferase assay, and that this graph can be used as a calibration curve.

The sialyltransferase assay by this new procedure was performed using these optimized conditions. The enzyme used was bacterium α-(2→6)-sialyltransferase.[5] The assay was performed in duplicate, and K_m and V_{max} values were obtained by a Lineweaver–Burk plot. As shown in graph D (Fig. 1), a plot was obtained and the K_m value was found to be 140 μM. This value is two times higher than that for the assay using CMP-[^{14}C]-NeuAc (70 μM),[5] but appears in the same range. Therefore, we concluded that this assay system could be used for sialyltransferase reactions.

Since this system uses acidic conditions for hydrolysis of the sialyl linkage and for DMB labeling, assay conditions needed to be further optimized for dealing with CMP-NeuAc derivatives having basic functional groups such as CMP-9′-amino-9′-deoxy-NeuAc. Therefore, we examined an assay of CMP-9′-amino-9′-deoxy-NeuAc.[10] Prior to the enzyme assay, we determined what acid concentration is suitable for hydrolysis of the 9-amino-sialyl linkage. The intensity of the fluorescent peak during HPLC analysis was above the saturation point when 400 mM of HCl solution was used for hydrolysis of the 9-amino-sialyl linkage after transfer of CMP-9′-amino-9′-deoxy-NeuAc. In addition, 30 mM of HCl was suitable for DMB-labeling (data not shown). We then measured the K_m and V_{max} values for CMP-9′-amino-9′-deoxy-NeuAc. A transfer assay was performed under the same assay conditions as for CMP-NeuAc except for the acid concentration during hydrolysis of 9-amino-NeuAc (400 mM) and DMB-labeling (30 mM). As shown in graph E (Fig. 1), a Lineweaver–Burk plot was obtained that shows a K_m value of 370 μM and a 2% relative transfer velocity than that for CMP-NeuAc (100%). In the case of rat liver α-(2→6)-sialyltransferase, the K_m value for CMP-9′-amino-9′-deoxy-NeuAc is 14 times higher than that for CMP-NeuAc ($K_m = 50$ μM).[17]

Although the method described here requires DMB labeling and HPLC analysis, there is no need to synthesize an acceptor with fluorescent or *p*-nitrophenyl functional groups prior to starting the sialyltransferase assay. This analysis system can be used with any sialic acid analogue. Overall, we believe this method to be as good as any assay method presently in use, when time and cost are taken into account.

1. Experimental

Optimization of DMB labeling.—DMB labeling of NeuAc was performed according to the reported procedure.[14–16] A solution containing 1,2-diamino-4,5-methylenedioxybenzene: (DMB, Dojin CO. 7.0 mM), β-mercaptoethanol (1.0 M) and sodium hydrosulfite (18 mM) were freshly prepared and used immediately. To a solution (205 μL) containing NeuAc (100 μM) and HCl (concentration was varied; 10–40 mM) was added DMB solution (200 μL), and this mixture was incubated for 2.5 h at 60 °C in the dark. This labeling reaction was performed in a microcentrifuge tube (1.5 mL, black color, Treff Lab, Switzerland). The aliquot solution of this mixture was analyzed by use of HPLC. The results are summarized in a graph A (Fig. 1).

HPLC analysis.—HPLC analysis was performed with a JASCO 880-PU pump (1.0 mL/min) equipped with a fluorescensent detector (JASCO FP-210, excitation: 373 nm, emission: 448 nm). Chromatography was performed on an ODS column (YMC A314 S-5 120A, 0.8 × 28 cm) operated isocratically using 1:3:10 MeCN–MeOH–water. The retention time of NeuAc labeled by DMB was 10 min.

Optimization of acid hydrolysis of sialoside.—To a solution (205 μL) containing sialyllactose (SIGMA, 100 μM) and HCl (concentration was varied; 25, 100, 200, and 400 mM) was heated at 80 °C for 45 min. After the mixture was allowed to cool to room temperature, an aliquot of each mixture was diluted to a volume of 200 μL with an HCl concentration of 40 mM. To this mixture was added DMB solution (200 μL), and the resulting mixture was incubated for 2.5 h at 60 °C in the dark. An aliquot of this mixture was analyzed by HPLC. The peak area of DMB-labeled NeuAc was estimated to correspond to one pmol of NeuAc (summarized in a graph B, Fig. 1). A control assay was also performed using NeuAc (100 μM) instead of sialyllactose.

Enzyme assay with bacterium α-(2→6)-sialyltransferase.—The apparent kinetic parameters for bacterium α-(2→6)-sialyltransferase [EC 2.4.99.1][5] toward CMP-NeuAc was examined under the following conditions. A assay solutions containing various concentrations of CMP-NeuAc (100, 150, 300, 500 μM), lactose (50 mM), bovine serum albumin (0.1 mg), and α-(2→6)-sialyltransferase (10 μunit) in sodium cacodylate buffer (50 mM, pH 5.0, total volume 50 μL) were incubated 37 °C. The reaction was run to 15% consumption of CMP-NeuAc. The sialyllactose was isolated

by passage of the mixture diluted with 950 μL of sodium phosphate buffer (5.0 mM, pH 6.8) through a Pasteur pipette column of Dowex 1-X8 (PO_4^{2-}-form, 0.6×4 cm), and further eluted using 1 mL of sodium phosphate buffer. To an aliquot (45 μL) of the sodium phosphate buffer solution was added 2 M HCl solution (5 μL), and the mixture was incubated at 80 °C. After 1 h, the mixture was allowed to cool to rt, and to this mixture was added DMB solution (450 μL, 2-mercaptoethanol: 560 mM, sodium hydrosulfite: 10 mM, and DMB: 3.9 mM), and the mixture was incubated for 2.5 h at 60 °C in the dark. The peak area of NeuAc labeled with DMB was estimated by HPLC. In order to estimate the NeuAc transferred, a calibration curve was prepared by use of CMP-NeuAc (0, 10, 25, 50 and 100 μM) which did not contain lactose, BSA and enzyme. These solutions were treated with the same procedure as the enzyme assay. The calibration curve is shown in graph C (Fig. 1). Transferred assay and blank assay were performed in duplicate, and K_m and V_{max} values were determined using a Lineweaver–Burk plot (graph D, Fig. 1).

Assay of CMP-9′-amino-9′-deoxy-NeuAc.—Optimization of DMB labeling and acid hydrolysis were performed using the same procedure as in graphs A and B, respectively. The assay indicated that 30 and 400 mM HCl concentrations were suitable for DMB labeling and acid hydrolysis of 9‴-amino-9‴-deoxy-NeuAc-lactose, respectively. These concentrations were used for the sialyltransferase assay. The concentrations of CMP-9′-amino-9′-deoxy-NeuAc used were 100, 150, 300, 500 and 1000 μM. Enzyme used here was 100 μ unit. Concentrations of other components were the same as for the assay in graph D (Fig. 1). The Lineweaver–Burk plot for CMP-9′-amino-9′-deoxy-NeuAc is shown in graph E (Fig. 1).

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