4

Purification of Marine Bacterial Sialyltransferases and Sialyloligosaccharides

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1. Introduction

Sialic acids are important components of carbohydrate chains and are usually found at the terminal position of the carbohydrate moiety of glycoconjugates (Angata & Varki, 2002; Schauer, 2004). Sialyloligosaccharides of glycoconjugates play important roles in many biological processes (Gagneux & Varki, 1999; Varki, 1993). The transfer of sialic acids to carbohydrate chains is performed by specific sialyltransferases in the cell (Angata & Varki, 2002; Vimr et al., 2004). Thus, sialyltransferases are considered to be key enzymes in the biosynthesis of sialylated glycoconjugates. Detailed investigations of the biological functions of sialylated glycoconjugates require an abundant supply of the target compounds. To date, many sialyltransferases, and the genes encoding them, have been isolated from various sources including mammalian, bacterial, and viral sources (Schauer, 2004; Sujino et al., 2000; Yamamoto et al., 2006). During our research, we have isolated over 20 bacteria that produce sialyltransferase and have revealed the characteristics of these enzymes (Kajiwara et al., 2009; Yamamoto, 2010). In this chapter, we will introduce our research activities focusing on methods for (1) screening bacteria for glycosyltransferase activity; (2) purifying native sialyltransferases from marine bacteria; and (3) synthesizing and purifying sialyloligosaccharides produced by marine bacterial sialyltransferases.

Sialic acid is a family of acidic monosaccharides comprising over 50 naturally occurring derivatives of neuraminic acid (5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid or Neu) (Angata & Varki, 2002; Vimr et al., 2004). Structurally, sialic acid is one of the more complicated naturally occurring monosaccharides and is based on a skeleton of nine carbons (Schauer, 2004). *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), and 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nonulosonic acid (deaminoneuraminic acid, KDN), are the three most common members of this family (Angata & Varki, 2002; Schauer, 2004). The structure of Neu, Neu5Ac, Neu5Gc and KDN are shown in Figure 1. Although sialic acid is widely distributed in higher animals and some classes of microorganisms, only Neu5Ac is ubiquitous (Angata & Varki, 2004). Usually, sialic acid exists in the carbohydrate moiety of glycoconjugates, including glycoproteins and glycolipids, and is linked to the terminal positions of the carbohydrate chains of the glycoconjugates. Many studies have been carried out to clarify the structure-function relationship of carbohydrate chains containing sialic acid. These studies have revealed that Neu5Ac is the most common sialic acid component of carbohydrate chains and sialylated carbohydrate chains of

glycoconjugates play significant roles in many biological processes including inflammation, glycoprotein clearance from circulation, cell-cell recognition, cancer metastasis, and virus infection (Kannagi, 2002; Paulson, 1989). Sialyltransferases commonly transfer Neu5Ac from cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac) to various acceptor substrates (Angata & Varki, 2002). Thus, sialyltransferases are thought to be one of the important enzymes in the biosynthesis of sialylated glycoconjugates.

(A) Neuraminic acid (Neu), (B) *N*-acetylneuraminic acid (Neu5Ac), (C) *N*-glycolylneuraminic acid (Neu5Gc), (D) deaminoneuraminic acid (KDN).
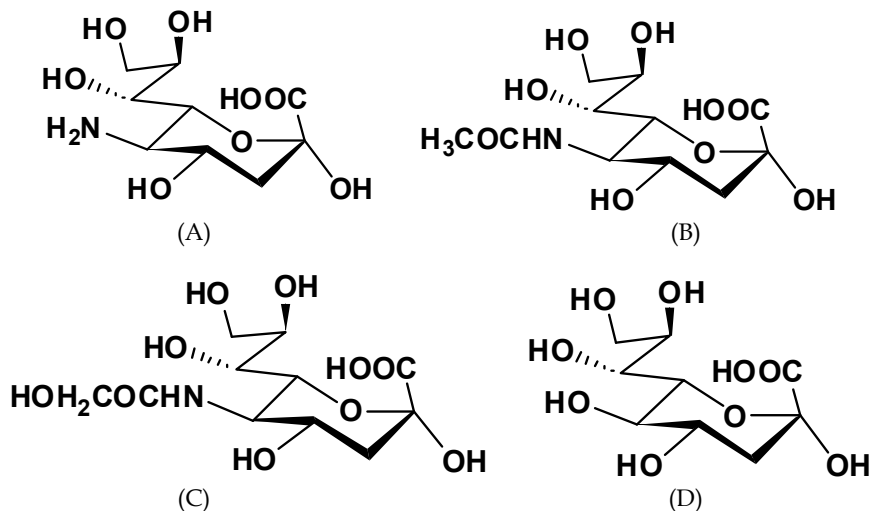
Fig. 1. Structures of sialic acids.

Among the biological phenomena described above, the relationship between the carbohydrate chain structure of the host cell and host cell recognition by influenza virus is one of the best investigated (Suzuki, 2005; Weis et al., 1988). Many reports have shown that influenza A and B viruses bind via viral hemagglutinin to host cell surface receptors that are Neu5Ac- or Neu5Gc-linked glycoproteins or glycolipids (Suzuki, 2005). Furthermore, these influenza viruses also recognize the carbohydrate chain structure of the host cell (Connor et al., 1994). Confirming evidence has shown that avian influenza viruses recognize Neu5Acα2-3Galβ1-3/4GlcNAc structures, and that human influenza viruses recognize Neu5Acα2-6Galβ1-3/4GlcNAc structures (Connor et al., 1994; Suzuki, 2005). The host cell specificities of the influenza A and B viruses are determined mainly by the linkage of Neu5Ac or Neu5Gc to the penultimate galactose residues and core structure of the host glycoproteins or glycolipids. For this reason, the distribution of Neu5Ac and Neu5Gc and their linkage patterns on the host cell surface are important determinants of host tropism.

A large variety of oligosaccharides exist in nature. For example, many kinds of sialyloligosaccharides, such as 3'-sialyllactose, 6'-sialyllactose, and sialyllacto-*N*-neotetraose, are contained in milk of various animals (Kunz et al., 2000); however, the purification and isolation of sialyloligosaccharides from natural sources is very difficult due to their structural complexity. Therefore, the research use and development of drugs that depend on

sialyloligosaccharides relies on sialyloligosaccharide synthesis by chemical or enzymatic methods. Although many methods for the synthesis of sialyloligosaccharides including chemical- and enzyme-based methods using glycosyltransferases have been developed, it is still difficult to synthesize large amounts of sialyloligosaccharides. Therefore, only a limited amount and only a few kinds of sialyloligosaccharides are currently available as research reagents. In this chapter, we introduce our results with regard to methods for screening for bacterial glycosyltransferases, purification of native sialyltransferases from bacteria, and the synthesis and purification of sialyloligosaccharides produced by marine bacterial sialyltransferases. The methodologies introduced in this chapter might to be useful for screening of bacteria that produce other types of glycosyltransferases, purification of membrane-binding proteins, and purification of oligosaccharides.

2. Screening bacteria for sialyltransferase activity

2.1 Basic screening method

Samples of seawater, sea-sand, mud, seaweed, and small animals including various kinds of fishes and shells, were collected from various coastal locations in Japan. Bacteria that grew on marine agar 2216 or nutrient agar (Becton-Dickinson, Franklin Lakes, NJ, USA) that was supplemented with 2% NaCl at 15°C, 25°C, or 30°C were isolated from the samples. Aliquots of the bacteria were suspended in 10% glycerol and stored at −80°C. For each bacterial isolate, 6 mL of marine broth 2216 (Becton-Dickinson) in a 15-mL test tube was inoculated with bacteria and cultivated at 15°C, 25°C, or 30°C for 18 h on a rotary shaker (180 rpm). After the cultivation, bacteria were harvested from 2 to 4 mL of the culture broth by centrifugation and then suspended in 200 μL of 20 mM sodium cacodylate buffer (pH 6.0) that contained 0.2% Triton X-100, lysed by sonication on ice, and measured immediately for sialyltransferase activity. Sialyltransferase activity was confirmed as follows: the reaction mixture (30 μL) consisted of the bacterial lysate as the sample of enzyme, 120 mM lactose, 2.3 mM CMP-Neu5Ac (Nakarai Tesque, Kyoto, Japan), 4620 Bq CMP-[4,5,6,7,8,9-14C]-Neu5Ac (Amersham Biosciences, Little Chalfont, UK), 100 mM Bis–Tris buffer (pH 6.0), 0.5 M NaCl, and 0.03% Triton X-100. The reaction was carried out at 25°C for 2 h. The reaction mixture was then diluted with 5 mM sodium phosphate buffer (pH 6.8) to a final volume of 2 mL, and applied to a column (0.5 × 2 cm) of Dowex-1 × 8 (phosphate form, Bio-Rad Laboratories, Hercules, CA, USA). The eluate (2 mL) was collected directly into a scintillation vial for counting. The radioactivity of [4, 5, 6, 7, 8, 9-14C]-Neu5Ac that had transferred to the acceptor substrate in the eluate was measured by using a liquid scintillation counter, and the amount of Neu5Ac transferred was calculated. Unreacted CMP-Neu5Ac was not eluted in this buffer concentration. Using this procedure, we have isolated many bacteria that possess sialyltransferase activity. Many of the marine bacteria that produced sialyltransferases were classified in genus *Photobacterium* or the closely related genus *Vibrio*. For instance, *Photobacterium phosphoreum* JT-ISH-467 that showed α2,3-sialyltransferase activity was isolated from the outer skin of Japanese common squid, *Todarodes pacificus* (Tsukamoto et al. 2007); *Photobacterium damselae* JT0160 that expressed α2,6-sialyltransferase activity was isolated from seawater (Yamamoto et al., 1998); *Photobacterium* sp. JT-ISH-224 that contained both α2,3- and α2,6-sialyltransferase activities was isolated from the gut of Japanese barracuda, *Sphyraena pinguis* (Tsukamoto et al., 2008); and *Photobacterium leiognathi* JT-SHIZ-145 that expressed α2,6-sialyltransferase activity was isolated from the outer skin of Japanese squid, *Loliolus japonica* (Yamamoto et al., 2007).

2.2 Simultaneous measurement of several glycosyltransferases activities

To assess the activities of various glycosyltransferases, not only sialyltransferases, we performed the enzyme assay using a mixture of the donor substrates of glycosyltransferases (GDP-fucose, the common donor substrate of fucosyltransferase; UDP-galactose, the common donor substrate of galactosyltransferase; and UDP-GlcNAc, the common donor substrate of *N*-acetyl-glucosaminyltransferase), and a mixture of the acceptor substrates of glycosyltransferases (4-Nitrophenyl α-D-galactopyranoside {Gal-α-pNp}; 4-Nitrophenyl β-D-galactopyranoside {Gal-β-pNp}; 4-Nitrophenyl *N*-acetyl-α-D-galactosaminide {GalNAc-α-pNp}; 4-Nitrophenyl *N*-acetyl-β-D-galactosaminide {GalNAc-β-pNp}; 4-Nitrophenyl *N*-acetyl-α-D-glucosaminide {GlcNAc-α-pNp}; 4-Nitrophenyl *N*-acetyl-β-D-glucosaminide {GlcNAc-β-pNp}; 4-Nitrophenyl α-D-glucopyranoside {Glc-α-pNp}; 4-Nitrophenyl β-D-glucopyranoside {Glc-β-pNp}; 4-Nitrophenyl α-L-fucopyranoside {Fuc-α-pNp}; 4-Nitrophenyl β-L-fucopyranoside {Fuc-β-pNp}; 4-Nitrophenyl α-D-mannopyranoside {Man-α-pNp}; and 4-Nitrophenyl β-D-mannopyranoside {Man-β-pNp}), and bacterial lysate described in 2.1 as the enzyme sample. An example of results obtained by using this method is shown in Figure 2.
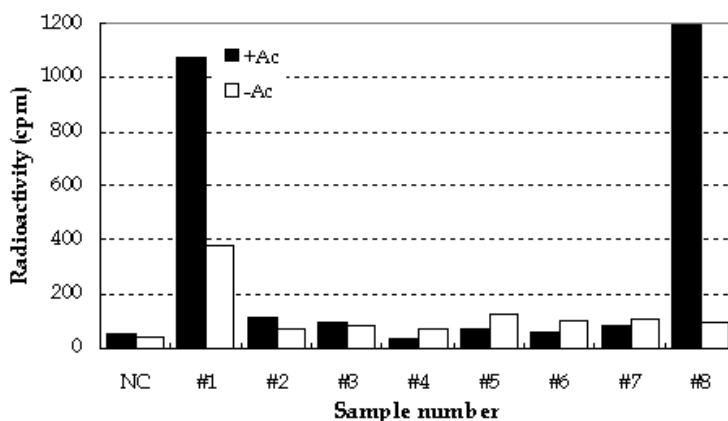

Fig. 2. Screening assay for glycosyltransferase activities.

High levels of radioactivity were observed in the eluates from the reaction mixture of samples #1 and #8, respectively, when the reaction was performed in the presence of acceptor substrate. From this result, it was strongly expected that lysates prepared from bacteria number #1 and #8 contained fucosyltransferase, galactosyltransferase and/or *N*-acetylglucosaminyltransferase. NC; negative control, +AC; containing acceptor substrate mixture in the reaction mixture, -AC; no acceptor substrate mixture in the reaction mixture.

The reaction mixture (50 μL) consisted of the following: a sample of enzyme, a mixture of 0.5 mM acceptor substrates consisting of 4-nitrophenyl compounds, as described above, a mixture of 0.5 mM donor substrates consisting of sugar-nucleotides as described above, 4620 Bq UDP-[U-^{14}C]-galactose, 4620 Bq UDP-*N*-acetyl-D-[U-^{14}C]-glucosamine, 4620 Bq GDP-[U-^{14}C]-fucose (Amersham Biosciences, Little Chalfont, UK), 100 mM bis-Tris buffer (pH 6.0), 10 mM MnCl$_2$, and 3 mM ATP. The reaction was carried at 25°C for 16 to 18 h. After the reaction, 100 μL of water was added to the reaction mixture, and the mixture was applied to a Sep-Pac Vac 50cc column (Waters, Milford, MA, USA) that was conditioned with ethanol and equilibrated with water. The column was washed twice with 1 mL of water and the

reaction product was eluted with 1 mL of 70% ethanol. One millilitre of scintillation cocktail was added to the eluate, and the radioactivity of the mixture was measured by using a liquid scintillation counter. In this way, we could detect glycosyltransferase activities, comprising fucosyltransferase, galactosyltransferase and/or *N*-acetylglucosaminyltransferase activity, simultaneously in marine bacteria. To clarify which of the glycosyltransferase activities the bacteria displayed, the enzymatic reaction was performed independently with each of the donor substrates in turn. The two bacteria that showed glycosyltransferase activity in Figure 2 were shown to specifically produce fucosyltransferase.

2.3 Screening by lectin staining

Lectins are sugar-binding proteins that are highly specific for their sugar moieties. The lectin *Sambucus sieboldiana* agglutinin (SSA) recognizes the Neu5Acα2-6Gal or Neu5Acα2-6GalNAc structure of sialyloligosaccharides in glycoconjugates (Shibuya et al., 1989). Kajiwara et al. carried out lectin staining of the cells of *P. damselae* JT0160, *P. leiognathi* JT-SHIZ-145, *P. phosphoreum* JT-ISH-467, and *Photobacterium* sp. JT-ISH-224, by using biotin-labeled SSA, and then examined the cells by using differential interference contrast (DIC) and fluorescence microscopy (Kajiwara et al., 2010). Lectin staining was carried out as follows: the 4 bacterial species described above were cultivated in nutrient broth supplemented with 2% (w/v) NaCl at 25°C for 18 h on a rotary shaker. The bacterial cells were collected by centrifugation (8,000*g*, 15 min, 4°C) and suspended in 25 mM Tris-HCl buffer 8 (pH 7.5). The suspensions were spotted onto glass slides, fixed with a 4% (w/v) paraformaldehyde solution at room temperature for 15 min, and blocked with a 5% (w/v) bovine serum albumin (BSA) phosphate-buffered saline (PBS) solution. After the glass slides were washed, biotin-labeled SSA (5 mg mL^{-1}) was added and the cells were incubated at room temperature for 2 h. After the cells were washed 4 times with PBS, Alexa 594-labeled streptavidin (Invitrogen, Carlsbad, CA, USA) solution (5 mg mL^{-1}) was added and the incubation was continued at room temperature for 1 h. After 5 washes with PBS, the cells were mounted using Prolong Gold antifade reagent (Invitrogen) and observed by using DIC and fluorescence microscopy. The SSA bound to *Photobacteriu*m sp. JT-ISH-224, *P. damselae* JT0160, and *P. leiognathi* JT-SHIZ-145. These *Photobacterium* strains produce α2,6-sialyltransferases, so the lectin staining indirectly detected α2,6-sialyltransferase-producing bacteria (Fig. 3). SSA did not bind to *P. phosphoreum* JT-ISH-467, which produces only α2,3-sialyltransferase. Therefore, the SSA lectin might be useful to screen for not only Neu5Acα2-6Gal and/or Neu5Acα2-6GalNAc structures on the bacterial cell surface but also to screen for the production of α2,6-sialyltransferase. We consider that this method would be applicable to the screening of other glycosyltransferases by changing the type of lectin used. We have confirmed that one of the two bacteria that showed fucosyltransferase activity, described in section 2.2, was detected by biotin-labeled *Aleuria aurantia* lectin (AAL, from Seikagaku Kogyo), which recognizes the fucose residue in carbohydrate chains (Kochibe & Furukawa, 1980).

3. Purification of sialyltransferase from the native bacterium

For the purification of a protein, it is necessary and important to find the appropriate conditions for enzyme solubilization, including solubilization efficiency, and the most efficient combination of chromatography processes. Each process has a different separation mode, and it is crucial to conduct a detailed study of the conditions required for each process. Crude extracts are commonly used in such studies, but care must be taken to
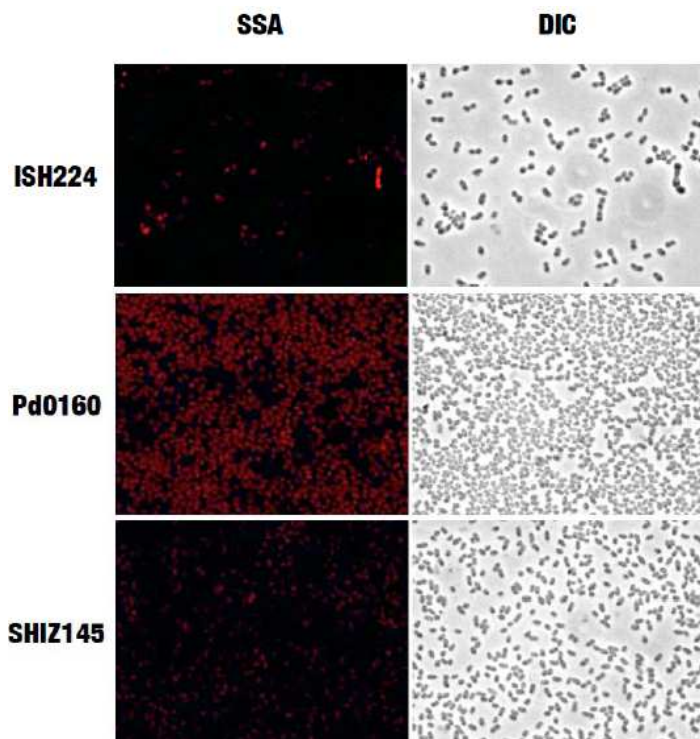
ISH224, *Photobacterium* sp. JT-ISH-224; Pd0160, *P. damselae* JT0160; SHIZ145, *P. phosphoreum* JT-SHIZ-145; SSA, fluorescence microscopy of cells stained with *Sambucus sieboldiana* agglutinin (SSA); DIC, differential interference contrast microscopy of the cells shown in the SSA panels.

Fig. 3. Lectin staining of *Photobacterium* strains by *Sambucus sieboldiana* agglutinin.

minimize protease activity, which may decompose the target enzyme. Furthermore, it is necessary to consider the pH of the buffers used in the purification steps as well as the temperature employed during the preparation of the extracts and the purification process. For details of general procedures and methods for protein purification, we recommend that you refer to other textbooks (e.g., Deutscher, 1990; Scopes,.1982). Here, we describe examples of the purification of sialyltransferase from marine bacteria.

3.1 Purification of α2,6-sialyltransferase from *P. damselae* JT0160

3.1.1 Preparation of the crude extract from *P. damselae* cells

The first step in the purification of a protein is the preparation of an extract containing the protein in a soluble form. During the purification of sialyltransferase from *P. damselae*, we examined in detail the conditions for preparing a crude extract containing the target enzyme in a soluble form (Yamamoto et al., 1998). The method that we established was deemed appropriate for the preparation of a crude extract containing sialyltransferase because no decrease in sialyltransferase activity was detected during the procedure. We determined that the most important factor in the preparation of the crude extract was the timing of the

cell lysis after cultivation. For instance, almost no sialyltransferase activity was detected in crude extract prepared from cryopreserved cells of *P. damselae*. The procedure for crude extract preparation was as described below.

1. After cultivation, *P. damselae* JT0160 cells were harvested from the culture by centrifugation (6,000 *g*, 20 min).
2. The harvested cells were suspended in 20 mM sodium cacodylate buffer (pH 6.0) containing 0.2% Triton X-100 and 1 M NaCl, and were sonicated immediately (<4°C) until the absorbance at 660 nm reached 30% or less of that of the original cell suspension.
3. The sonicated solution was centrifuged (100,500*g*, 60 min) and the supernatant was dialyzed, using cellulose tubing, against 20 mM sodium cacodylate buffer (pH 6.0) containing 0.2% Triton X-100.
4. After dialysis, the precipitate was removed by centrifugation (100,500*g*, 60 min) to obtain the clarified extract.

3.1.2 Purification of sialyltransferase by using column chromatography

Sialyltransferase produced by *P. damselae* was then purified from the crude extract by a combination of 4 steps of column chromatography. The conditions and method used for each chromatography step are described below.

1. Q-Sepharose column chromatography. A column of Hi-Load 26/10 Q Sepharose HP (ø 2.6 × 10 cm; GE Healthcare Science, Buckinghamshire, UK) was equilibrated with 20 mM sodium cacodylate buffer (pH 6.0) containing 0.2% Triton X-100. Clarified extract was applied to the column, and the column was washed with 150 mL of the same buffer. Enzyme fractions were eluted with a linear gradient of 0 to 1 M NaCl in the buffer. The fractions exhibiting sialyltransferase activity ("active" fractions) were collected and pooled. Desalting of the "active" fractions was performed by dialysis, using cellulose tubing, against 20 mM sodium cacodylate buffer (pH 6.0) containing 0.2% Triton X-100.
2. Hydroxyapatite column chromatography. A column of hydroxyapatite (ø 2 × 10 cm; Bio-Rad Laboratories) was equilibrated with 20 mM sodium cacodylate buffer (pH 6.0) containing 0.2% Triton X-100. After the application of the enzyme solution obtained in step 1, the column was washed with the same buffer. The enzyme fraction was eluted with a gradient of 0 to 0.35 M potassium phosphate. The "active" fractions were collected and pooled, and then concentrated by ultrafiltration using Molecut L (exclusion molecular mass, 10 kDa ; Millipore, Billerica, MA, USA).
3. Gel–filtration column chromatography. A column of Hi-Load 26/60 Sephacryl S-200 HE (ø 2.6 × 60 cm; GE Healthcare Science, Buckinghamshire, UK) was equilibrated with 20 mM sodium cacodylate buffer (pH 6.0) containing 0.2% Triton X-100 and 0.1 M NaCl. The enzyme solution obtained in step 2 was applied to the column and eluted with the same buffer. The "active" fractions were collected and pooled. Desalting of these fractions was performed by dialysis, using cellulose tubing, against 20 mM sodium cacodylate buffer (pH 6.0) containing 0.2% Triton X-100.
4. CDP–hexanolamine-agarose column chromatography. A column of CDP–hexanolamine-agarose (ø 1 × 3 cm) was equilibrated with 20 mM sodium cacodylate buffer (pH 6.0) containing 0.2% Triton X-100. The enzyme solution (4 mL) obtained in step 3 was applied to the column. The column was washed with 8 mL of the same buffer. The enzyme was eluted with 6 mL of 2 M NaCl, and "active" fractions were collected and pooled.

The purity and yield of the enzyme at each step is summarized in Table 1.

Purification step	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	760	2584	21.1	0.008	100	1
Q Sepharose	240	552	12.4	0.022	59	2.8
Hydroxyapatite	120	85	8	0.094	38	11.8
Sephacryl S-200	30	20.1	6.7	0.3	32	37.5
CDP-hexanolamine-agarose	15	0.75	4.1	5.5	19	687.5

Table 1. Purification of sialyltransferase from cell lysate of *Photobacterium damselae.*

The enzyme was purified 688-fold, with a yield of 19%. The purified enzyme migrated as a single polypeptide with a molecular mass of 61 kDa by SDS-polyacrylamide gel electrophoresis under denaturing conditions.

3.2 Purification of α2,3-sialyltransferase from *P. phosphoreum* JT-ISH-467

3.2.1 Preparation of the crude extract from *P. phosphoreum* cells

The crude extract containing sialyltransferase from *P. phosphoreum* cells was prepared by the method described in section 3.1.1, with slight modifications (Tsukamoto et al., 2007), and then crude extract containing the soluble form of the enzyme was prepared.

3.2.2 Purification of sialyltransferase from *P. phosphoreum* by using column chromatography

Sialyltransferase produced by *P. phosphoreum* was purified from the crude extract by a combination of 5 steps of column chromatography. The conditions and method used for each of the column chromatography steps are described below.

1. DEAE column chromatography. The clarified crude extract was applied to a Hi-Prep 16/10 DEAE FF column (ø 1.6 × 10 cm; GE Healthcare Science) equilibrated with 20 mM bis-Tris buffer (pH 6.0) containing 0.3% Triton X-100. After the column was washed with the same buffer, the enzyme was eluted with a linear gradient of 0 to 1 M NaCl in the same buffer. The fractions with sialyltransferase activity were pooled and then diluted to three times the original volume with 20 mM potassium phosphate buffer (pH 6.0) containing 0.3% Triton X-100.
2. Hydroxyapatite column chromatography. The enzyme solution obtained in step 1 was applied to a hydroxyapatite column (ø 1.5 × 11.3 cm; Bio-scale CHT20-I; Bio-Rad Laboratories) that was equilibrated with 20 mM potassium phosphate buffer (pH 6.0) containing 0.3% Triton X-100. After the column was washed with the same buffer, the enzyme was eluted with a linear gradient of 20 to 500 mM potassium phosphate. The "active" fractions were pooled, and then diluted to two times the original volume with 20 mM potassium phosphate buffer (pH 6.0) that contained 0.3% Triton X-100.
3. Mono Q column chromatography (pH 6.0). The enzyme solution obtained in step 2 was loaded onto a column of Mono Q 10/100 GL (ø 1 × 10 cm; GE Healthcare Science) that was equilibrated with 20 mM potassium phosphate buffer (pH 6.0) containing 0.3% Triton X-100. After the column was washed with the same buffer, the enzyme was

eluted with a linear gradient of 0 to 1 M NaCl in the same buffer. The "active" fractions were pooled, and then diluted to three times the original volume with 20 mM bis-Tris buffer (pH 7.0) containing 0.3% Triton X-100.

4. Mono Q column chromatography (pH 7.0). The enzyme solution obtained in step 3 was applied to a column of Mono Q 10/100 GL equilibrated with 20 mM bis-Tris buffer (pH 7.0) containing 0.3% Triton X-100. After the column was washed with 20 mM bis-Tris buffer (pH 7.0) containing 0.3% Triton X-100, the enzyme was eluted with a linear gradient of 0 to 1 M NaCl in the same buffer. The "active" fractions were pooled.

5. Superdex 200 column chromatography. The enzyme solution obtained in step 4 was loaded onto Hi-Load 16/60 Superdex 200 pg (ø 1.6 × 60 cm; GE Healthcare Science) that was equilibrated with 20 mM bis-Tris buffer (pH 7.0) containing 0.3% Triton X-100 and 0.2 M NaCl and eluted with the same buffer. The "active" fractions were collected and pooled.

The results for the purification of the enzyme are summarized in Table 2.

Purification step	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (mU/mg)	Yield (%)	Purification (fold)
Crude extract	3155	6159	8.40	1.4	100	1
DEAE	410	932	3.10	3.4	37	3
Hydroxyapatite	264	153	1.30	8.2	15	6
Mono Q (pH 6.0)	12	24	0.96	39	11	29
Mono Q (pH 7.0)	1.5	1.7	0.52	315	6.2	29
Superdex 200	1.5	0.2	0.10	457	1.2	333

Table 2. Purification of sialyltransferase from cell lysate of *P. phosphoreum*.

The enzyme was purified 333-fold, with a yield of 1.2%. Because no affinity chromatography step was used, the yield of the protein purification in this case was very low. Therefore, preparing affinity gels with the appropriate ligand for the target enzyme is very important in the purification process.

4. Enzymatic synthesis and purification of sialyloligosaccharides

4.1 Synthesis of sialyloligosaccharides by recombinant sialyltransferases from marine bacteria

Chemoenzymatic synthesis of various sialyloligosaccharides by mammalian sialyltransferases, and the purification of the product, has been reported (Sabesan & Paulson, 1986). However, mass-production of sialyloligosaccharides by using mammalian-derived sialyltransferases remains problematic because the enzymes are unstable and difficult to produce as recombinant proteins in *Escherichia coli*. In comparison to mammalian sialyltransferases, bacterial sialyltransferases are generally more stable and productive in *E. coli* protein-expression systems (Tsukamoto et al., 2007, 2008; Yamamoto et al., 2006), and they show a broader acceptor substrate specificity (Izumi & Wong, 2001; Yu et al., 2005). Here, we report the methods that we developed to use recombinant sialyltransferases from marine bacteria to successfully produce large quantities of 6'-sialyllactose and synthesize various sialyloligosaccharides.

4.1.1 Synthesis of 6′-sialyllactose from lactose and CMP-Neu5Ac by using purified recombinant α2,6-sialyltransferase from *P. damselae* JT0160

Purified recombinant α2,6-sialyltransferase from *P. damselae* JT0160 shows broader acceptor substrate specificity than that of the mammalian enzymes. For example, it could transfer Neu5Ac to not only disaccharides but also mono- and tri-saccharides efficiently, and provided the corresponding sialosides (Fig. 4; Kajihara et al., 1996; Yamamoto et al., 1998).

Below, we describe an example of the enzymatic synthesis of 6′-sialyllactose (sialoside 1).

1. The reaction mixture was composed of 20 mg (55 μmol) of lactose (Galβ1-4Glc), 79 mg (110 μmol) of CMP-Neu5Ac, and 0.6 U of the purified enzyme in 0.5 mL of 100 mM bis-Tris buffer (pH 6.0).
2. The reaction mixture was incubated at 30°C for 2 h.
3. The product formed by the enzymatic reaction was analyzed by using thin layer chromatography (TLC) as follows: a small amount of the enzymatic reaction mixture was applied to a pre-coated silica gel plate (60 F254, Merck, Darmstadt, Germany), which was then developed with 2-propanol/acetic acid/water (3:2:1 v/v); for visualization of the organic compounds, the plate was dipped into a solution of 5% v/v sulfuric acid in ethanol and then heated.
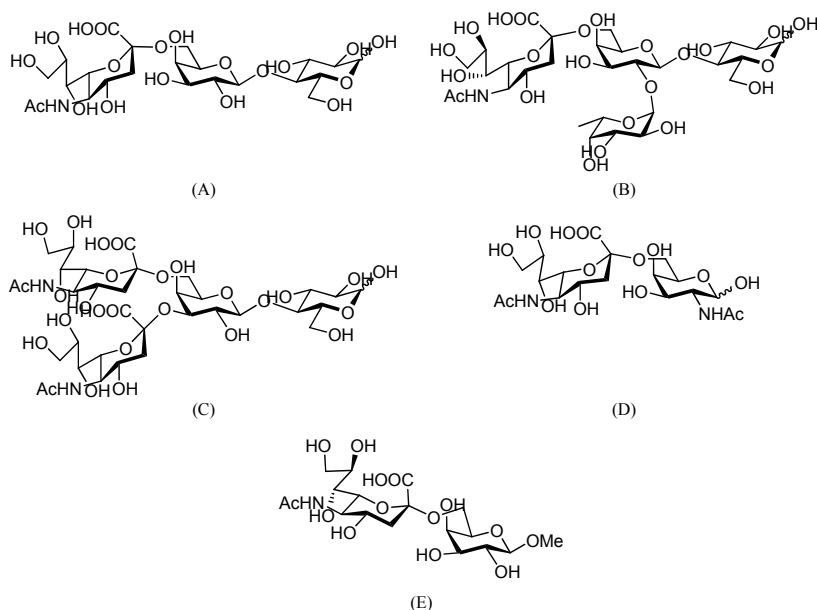
(A) 6′-sialyllactose (sialoside 1), (B) 2′-fucosyl-6′-sialyllactose (sialoside 2), (C) 3′, 6′-disialyllactose (sialoside 3), (D) 6-sialyl-*N*-acetylgalactosamine (sialoside 4), (E) 6-sialyl-methyl-β-D-galactopyranoside (sialoside 5).

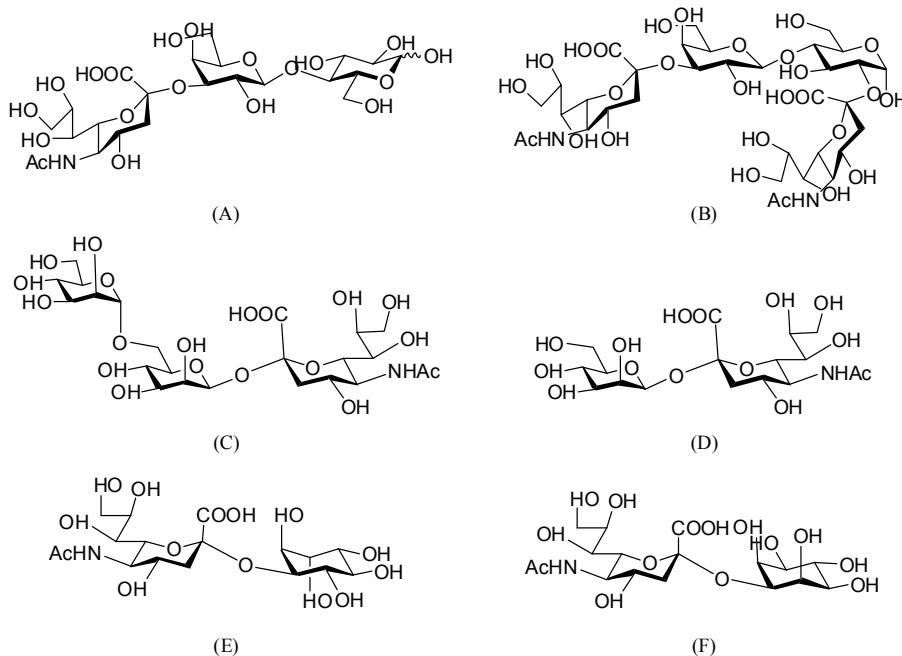Fig. 4. Structures of sialosides 1–5.

4.1.2 *In vivo* synthesis of 6′-sialyllactose by using genetically engineered *E. coli*

Recently, we succeeded in mass-producing 6′-sialyllactose by using a genetically engineered *E. coli* strain expressing the *Photobacterium* sp. JT-ISH-224 gene for α2,6-sialyltransferase

(Drouillard et al., 2010). Our method was developed from a microbiological system for the large-scale production of 3′-sialyllactose that used high cell-density cultures of a genetically engineered *E. coli* strain expressing the *Neisseria meningitidis* gene for α2,3-sialyltransferase (Fierfort & Samain, 2008). To date, we have achieved the production of 6′-sialyllactose with a final concentration greater than 30 g L⁻¹ of culture medium, by continuously feeding the culture with an excess of lactose. A detailed report of the production conditions is provided in Drouillard et al. (2010).

4.1.3 Synthesis of various sialyloligosaccharides by using purified recombinant α2,3-sialyltransferase from *Photobacterium* sp. JT-ISH-224

Using the procedure described in section 4.1.1, we could enzymatically produce 3′-sialyllactose (sialoside 6) by using α2,3-sialyltransferase instead of α2,6-sialyltransferase. While using a recombinant α2,3-sialyltransferase derived from *Photobacterium* sp. JT-ISH-224 to produce 3′-sialyllactose, we detected a by-product in the enzymatic reaction mixture and determined its structure to be 2,3′-disialyllactose (sialoside 7; Mine et al., 2010a). This recombinant α2,3-sialyltransferase can also transfer Neu5Ac from CMP-Neu5Ac to the β-anomeric hydroxyl groups of mannose and 6-mannobiose to produce sialosides 8 & 9, respectively (Mine et al., 2010b), and transfer Neu5Ac to inositols to produce sialosides 10 & 11 (Mine et al., 2010c). The structures of sialosides 6–11 are shown in Figure 5.

(A) 3′-sialyllactose (sialoside 6), (B) 2, 3′-disialyllactose (sialoside 7), (C) sialyl-6-mannobiose (sialoside 8), (D) sialyl-mannose (sialoside 9), (E) sialyl-1D-*chiro*-inositol (sialoside 10), (F) sialyl-*epi*-inositol (sialoside 11).

Fig. 5. Structures of sialosides 6–11.

4.2 Purification of sialyloligosaccharides by use of column chromatography

In general, for the separation of oligosaccharides, it is convenient to utilize high-performance liquid chromatography (HPLC), and various types of columns, such as reverse-phase columns, ion-exchange columns, and gel-filtration columns, that are commercially available. Because Neu5Ac is negatively charged, it is comparatively easy to separate sialyloligosachharide(s) from other neutral oligosaccharides by using anion–exchange column chromatography (Sabesan & Paulson, 1986). For further purification of the compound, gel–filtration column chromatography is effective.

The conditions and method used for each column chromatography step are described below.

4.2.1 Separation of the sialyloligosaccharide and unreacted substrates from the enzymatic reaction mixture

The basic procedure for anion–exchange column chromatography is as follows:

1. The reaction mixture was diluted with 10 mL of deionized water and introduced onto an Econo column (ø1.0 cm × 10 cm; Bio-Rad Laboratories) containing AG1-X2 ion–exchange resin (phosphate form; 200–400 mesh).
2. The column was washed with 3 column volumes (~ 30 mL) of deionized water.
3. Elution of the sialyloligosaccharide was performed twice with 10 mL each of 5, 10, 50, 100, 500, or 1000 mM potassium phosphate buffer (pH 6.8).
4. An aliquot of each eluted fraction was analyzed by using TLC, as described in section 4.1.1.

The column volume required for separation is dictated by the scale of the synthetic reaction. For 10 mg or less of acceptor substrate, all of the reaction product will bind to the resin described above. For more than 100 mg of acceptor substrate, it is desirable to either perform the chromatography process at least twice, or to increase the amount of resin by using a larger column (e.g., ø 2.5 cm × 10 cm).

An example of results obtained for the separation of sialyloligosaccharide by using the above procedure is shown in Figure 6.
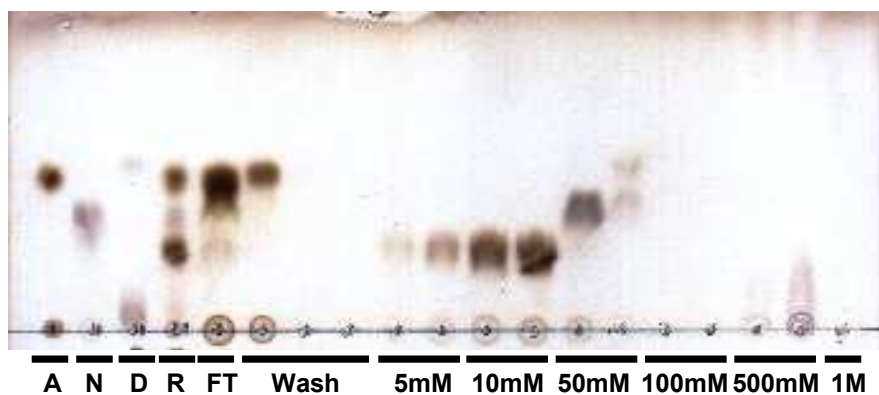

Fig. 6. TLC analysis of fractions separated by using anion–exchange column chromatography.

The reaction solution after enzymatic reaction of substrates with recombinant α2,6-sialyltransferase from *P. damselae* JT0160 strain contained unreacted lactose and CMP-Neu5Ac, free Neu5Ac as result of hydrolysis of CMP-Neu5Ac, and the product. The contents of the fractions eluted with 5, 10, 50, 100, 500, and 1000 mM potassium phosphate buffer (pH 6.8) are shown. A, lactose; N, Neu5Ac; D, CMP-Neu5Ac; R, reaction solution after enzymatic reaction; FT, flow-through.

Many mono-sialyloligosaccharides composed of di-, tri- or tetra-saccharide eluted with 5 to 10 mM potassium phosphate buffer. We also demonstrated that disialyloligosaccharides, such as sialosides 3 (Fig. 4) and 7 (Fig. 5), eluted with 100 mM potassium phosphate buffer. In contrast, many of the unreacted acceptor substrates passed through the column because of their electrically neutral property. Unreacted CMP-Neu5Ac and free Neu5Ac, resulting from the hydrolysis of CMP-Neu5Ac during the reaction, were eluted with 500 and 50 mM potassium phosphate buffer, respectively (Fig. 7). Therefore, it is easy to separate these compounds in the enzymatic reaction mixture with this column chromatography process.
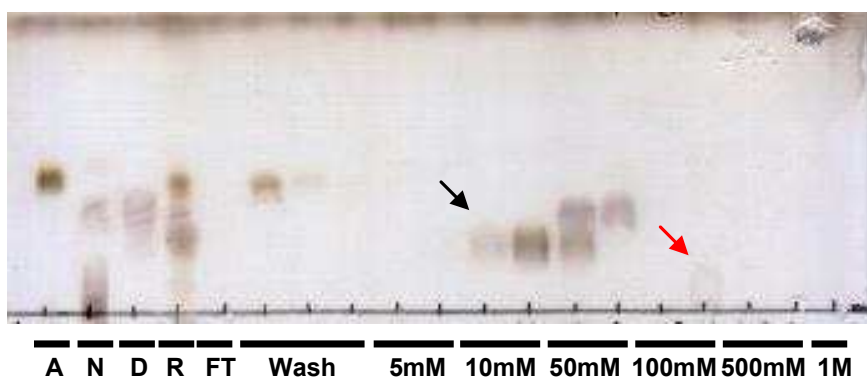

Fig. 7. Separation of mono-sialyloligosaccharide and di-sialyloligosaccharide from the reaction solution by using anion–exchange column chromatography.

The reaction solution after enzymatic reaction with recombinant α2,3-sialyltransferase from *Photobacterium*.sp. JT-ISH-224 strain contained unreacted lactose, CMP-Neu5Ac, free Neu5Ac, and both the mono-sialyloligosaccharide (sialoside 6) as main product (black arrow) and the di-sialyloligosaccharide (sialoside 7) as by-product (red arrow). The contents of the fractions eluted with 5, 10, 50, 100, 500, and 1000 mM potassium phosphate buffer (pH 6.8) are shown. A, lactose; N, Neu5Ac; D, CMP-Neu5Ac; R; reaction solution after enzymatic reaction, FT, flow-through.

During the stepwise elution described above, we sometimes observed that both the reaction product and free Neu5Ac were present in the same fraction. In this case, the separation of these compounds can be improved by increasing the volume of 10 mM potassium phosphate buffer (e.g., using 3–5 column volumes of the buffer).

This basic procedure for the separation of sialyloligosaccharide in the enzymatic reaction mixture is more effective when the enzyme reaction produces a single mono-sialyloligosaccharide. If the reaction mixture contains a variety of mono-sialyloligosaccharides, it is preferable to perform the preparative chromatography using a different column, such as TSKgel Amide-80 (Tosoh Bioscience, Tokyo, Japan) (Endo et al., 2009).

For further purification of the sialyloligosaccharide, we performed gel–filtration column chromatography. The procedure is as follows:

1. The fractions containing glycosidic Neu5Ac were evaporated to dryness.
2. The dried residue was dissolved in 2.5 mL of deionized water and then loaded onto a Sephadex G-15 column (ø 1.6 × 70 cm) and eluted with deionized water under a 2.5 mL/min flow rate and collected in increments of 1 mL.
3. The fractions containing glycosidic Neu5Ac were pooled and evaporated to dryness.

The purpose of this process is to remove salt carried from the former chromatography process. The product was eluted in the 30th to 50th fractions (Fig. 8). When Neu5Ac was mixed with the product, it could be separated from the product under a lower flow rate (e.g., 1.0 mL/min). The purity of sialyloligosaccharides obtained by using a combination of the two chromatography processes described above is usually more than 95% (data not shown).
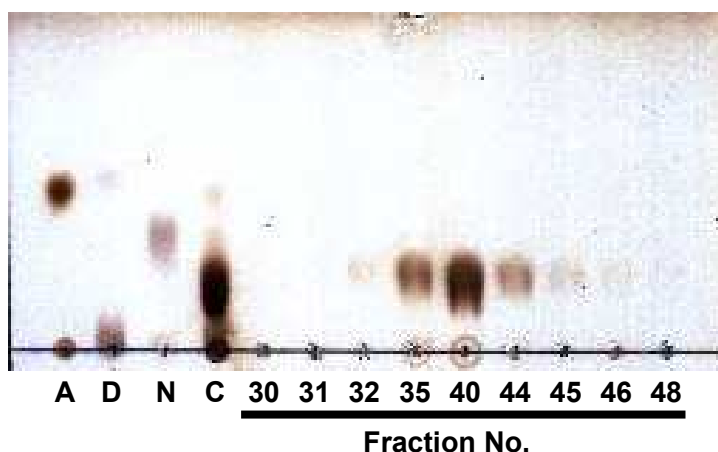
Fig. 8. TLC analysis of the fractions separated by use of gel–filtration column chromatography.

The product is usually contained in the 30th to 50th fraction eluted during the gel–filtration column chromatography; a typical example is shown. A; lactose, D; CMP-Neu5Ac, N; Neu5Ac, C; 6′-sialyllactose standard.

4.2.2 Alternative anion-exchange column chromatography method for large-scale purification of 6′-sialyllactose

As mentioned in section 4.1.2, a large volume of solution containing 6′-sialyllactose could be prepared by using high cell-density cultures of a genetically engineered *E. coli* strain. In such cases, we performed an alternative anion–exchange column chromatography process. At the end of the fermentation, the whole culture was permeabilized by autoclaving at 100°C for 50 min. The mixture was centrifuged at 7,000*g* for 30 min and the supernatant containing the oligosaccharides was removed. The pH of the extracellular fraction was lowered to 3.0 by the addition of a strong cation-exchange resin (Amberlite IR120 H+ form, Sigma-Aldrich Japan, Tokyo), and the proteins that were precipitated by this process were removed by centrifugation. The pH of the clear supernatant was then adjusted to 6.0 by the addition of a weak anion exchanger (Dowex 66 free base form; Sigma-Aldrich Japan) and, after decanting,

the supernatant was loaded onto a Dowex 1 (HCO$_3$ form, Sigma-Aldrich Japan) column (ø 5 x 20 cm). After the column was washed with distilled water, the acidic oligosaccharides retained on the Dowex 1 resin were eluted with 100 mM NaHCO$_3$. The eluted fractions containing acidic oligosaccharides were pooled and the NaHCO$_3$ was removed by treatment with Amberlite IR120 (H$^+$ form) until the pH reached 3. The pH was then adjusted to 6.0 with NaOH and the acidic oligosaccharide fraction was freeze-dried.

4.2.3 Separation of various sialyl-compounds by using HPLC

Anion–exchange column chromatography is a powerful method for separating a single mono-sialyloligosaccharide from the other components in the sample solution; however, it is impossible to separate one mono-sialyloligosaccharide from another mono-sialyloligosaccharide, such as 6'-sialyllactose or 3'-sialyllactose, by using this method. In such cases, HPLC can be used successfully for the separation, as described by Endo et al. (Endo et al., 2009). In this protocol, the HPLC system is equipped with a TSKgel Amide-80 column (particle size 5 mm, ø 4.6 × 250 mm; Tosoh Bioscience), and is run at 40°C with a flow rate of 1mLl/min, and the eluates are monitored with a UV (195 nm) detector. The elution conditions are as follows: 0 to 15 min isocratic elution with 75% acetonitrile in 15 mM potassium phosphate buffer (pH 5.2); and 15 to 45 min linear gradient elution with a gradient of 75% to 50% acetonitrile in 15 mM potassium phosphate buffer (pH 5.2).

Some examples of HPLC chromatograms produced for sialyl-compounds synthesized as described in section 4.1 are shown in Figure 9.
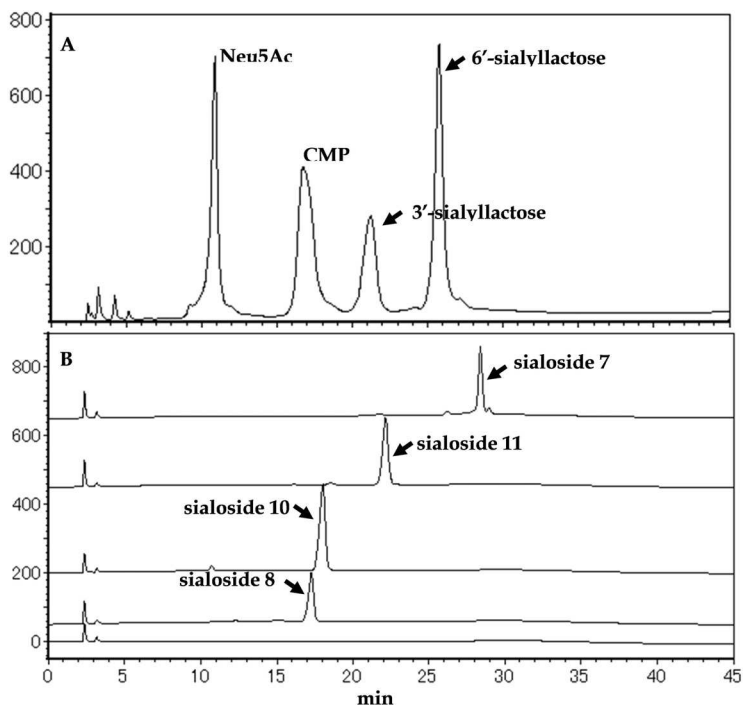

Fig. 9. Chromatograms of some sialyl-compounds.

The sample solution (150 mL) containing sialyl-compound(s) was separated as described in section 4.2.3. A; an example of the separation of two mono-sialyloligosaccharides, B; examples of the chromatogram of various mono-sialyloligosaccharides.

5. Conclusion

It is now possible to produce large amounts of sialyloligosaccharides by using newly developed methods, including chemoenzymatic methods and fermentation methods. It is also possible to produce huge quantities of sialyltransferase enzymes. However, large-scale production of other glycosyltransferases, such as *N*-acetylglucosaminyltransferase or fucosyltransferase, is still difficult. For this reason, it is of great importance to identify enzymes that could be used in the production of other glycosyltransferases and to establish mass-production methods for these enzymes.

6. Acknowledgment

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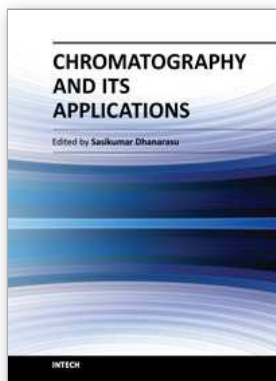
Chromatography and Its Applications

Edited by Dr. Sasikumar Dhanarasu

Chromatography is a powerful separation tool that is used in all branches of science, and is often the only means of separating components from complex mixtures. The Russian botanist Mikhail Tswett coined the term chromatography in 1906. The first analytical use of chromatography was described by James and Martin in 1952, for the use of gas chromatography for the analysis of fatty acid mixtures. A wide range of chromatographic procedures makes use of differences in size, binding affinities, charge, and other properties. Many types of chromatography have been developed. These include Column chromatography, High performance liquid chromatography (HPLC), Gas chromatography, Size exclusion chromatography, Ion exchange chromatography etc. In this book contains more details about the applications of chromatography by various research findings. Each and every topics of this book have included lists of references at the end to provide students and researchers with starting points for independent chromatography explorations. I welcome comments, criticisms, and suggestions from students, faculty and researchers.

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