A CMP-N-acetylneuraminic Acid Synthetase Purified from a Marine Bacterium, Photobacterium leiognathi JT-SHIZ-145

Hitomi KAJIWARA,1,† Toshiki MINE,1 Tatsuo MIYAZAKI,2 and Takeshi YAMAMOTO1

1Glycotechnology Business Unit, Japan Tobacco Inc., 700 Higashibara, Iwata, Shizuoka 438-0802, Japan
2Department of Applied Life Sciences, Niigata University of Pharmacy and Applied Life Sciences, 265-1 Higashijima, Akita-ku, Niigata 956-8603, Japan

Received July 13, 2010; Accepted October 4, 2010; Online Publication, January 7, 2011

A cytidine 5'-monophospho-N-acetylneuraminic acid (CMP-Neu5Ac) synthetase was found in a crude extract prepared from Photobacterium leiognathi JT-SHIZ-145, a marine bacterium that also produces a β-galactoside α2,6-sialytransferase. The CMP-Neu5Ac synthetase was purified from the crude extract of the cells by a combination of anion-exchange and gel filtration column chromatography. The purified enzyme migrated as a single band (60 kDa) on sodium dodecylsulfate–polyacrylamide gel electrophoresis. The activity of the enzyme was maximal at 35 °C. The synthetase migrated as a single band (60 kDa) on sodium dodecylsulfate–polyacrylamide gel electrophoresis. The purified enzyme migrated as a single band (60 kDa) on sodium dodecylsulfate–polyacrylamide gel electrophoresis.

Key words: cytidine 5'-monophospho-N-acetylneuraminic acid (CMP-Neu5Ac) synthetase; N-acetylneuraminic acid; N-glycolylneuraminic acid; Photobacterium leiognathi

Sialic acids (Sias) are negatively charged 9-carbon monosaccharides that carry the systematic name 5-amino-3,5-dideoxy-d-glycero-d-galacto-non-2-ulopyranonic acid (Neu). The three major Sias are N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5GaC), and 2-keto-3-deoxy-d-glycero-d-galacto-nononic acid. Sias, especially Neu5Ac, are found predominantly in vertebrates, some bacteria, and fungal species, although Neu5Gc has not been found in bacteria. These Sias usually exist on the non-reducing terminal positions of carbohydrate chains of glycoproteins and glycolipids, although Neu5Ac has been found at internal positions of carbohydrate chains produced by some shellfish. Sias play important roles in mediating cell–cell surface adhesion and cell signaling processes, including pathological processes such as viral infection, inflammation, and tumor metastasis. In bacteria, Sias are components of capsular polysaccharides and lipopolysaccharides, which are important components of bacterial virulence factors. Various sialyltransferases have been obtained from vertebrate cells and some bacteria, mainly. All sialyltransferases, except for trans-sialidases, require cytidine 5'-monophospho-N-acetylneuraminic acid (CMP-Neu5Ac) as the donor substrate. CMP-Neu5Ac is produced by CMP-Neu5Ac synthetase (also known as N-acetylneuraminic cytidyltransferase, CMP-sialate pyrophosphorylase, and acylneuraminic cytidyltransferase, EC 2.7.7.43) in the cells, and CMP-Neu5Ac synthetases have been isolated from vertebrates and bacteria. These enzymes have many properties in common; for example, they show maximum activity under basic conditions. Recently, a temperature-resistant synthetase was cloned from Clostridium thermocellum. To date, some synthetases have been crystallized, and their structures have been investigated. Moreover, the following reaction mechanism of the enzyme has been proposed: CMP-Neu5Ac synthetases catalyze the nucleophilic attack of the anomeric oxygen of β-Neu5Ac on the α-phosphate of cytidine triphosphate (CTP) and produce CMP-Neu5Ac and pyrophosphate by an inversion mechanism. Here we report a CMP-Neu5Ac synthetase from the marine bacterium P. leiognathi JT-SHIZ-145, an organism that also possesses α2,6-sialyltransferase activity. This is the first report of a CMP-Neu5Ac synthetase from a marine bacterium.

Materials and Methods

Screening of bacteria. Marine bacteria exhibiting the sialyltransferase activity were inoculated into 6 mL of marine broth 2216 (Becton-Dickinson, Franklin Lakes, NJ) in a 15-mL test tube and cultivated at 25 °C for 18 h on a rotary shaker (180 rpm). The Bacteria were harvested from 2 mL of the culture broth by centrifugation, and the pellet was suspended in 200 μL of 50 mM Tris–HCl buffer (pH 9.0) containing 0.3% Triton X-100 and 20% glycerol. The suspension was...
lysed by sonication on ice, and the lysate was used immediately in the CMP-Neu5Ac synthetase assay.

**CMP-Neu5Ac synthetase assay.** CMP-Neu5Ac synthetase activity was assayed by the following procedure: Five µL of cell lysate was added to 45 µL of assay mix, and the mixture was incubated at 30 °C for 3 h. The final concentrations of the components in the assay mixture were as follows: 5.5 mM CTP, 2.8 mM Neu5Ac (Sigma-Aldrich Japan, Tokyo), 10 mM MgSO₄, and 100 mM Tris–HCl buffer (pH 9.0). The reaction was stopped by the addition of a 10-µL aliquot of the reaction mixture to 80 µL of 50 mM KH₂PO₄ (pH 4.65). The reaction products were analyzed by HPLC (LC10A; Shimadzu, Kyoto, Japan). An analytical anion-exchange column (Shim-Pack IC-A1; 4.6 mm × 100 mm, Shimadzu) was used to resolve CMP-Neu5Ac from the substrate CTP and any small amounts of CMP or cytidine diphosphate that may have been present. The eluent was 50 mM KH₂PO₄ (pH 4.65), first at a flow rate of 0.5 mL/min (0–8 min) and then at a flow rate of 1 mL/min (9–20 min). The CMP-Neu5Ac peak area was quantified by means of a standard curve for CMP-Neu5Ac. Detection was at 280 nm below was performed at 4°C.

**Step 1:** Preparation of crude extract. *P. leiognathi* JT-SHIZ-145 was cultivated in 6 mL of marine broth 2216 medium at 25 °C for 8 h with shaking (180 rpm). Then 300 mL of the same medium was inoculated with the seed culture, and the mixture was incubated for 16 h at 25 °C with shaking. Bacteria were harvested by centrifugation from 3.6L of culture to yield a pellet. The pellet was suspended in 50 mM Tris–HCl buffer (pH 9.0) containing 0.3% Triton X-100 and 20% glycerol (buffer A) and lysed by sonication on ice. Cellular debris was removed by centrifugation at 100,000 g for 60 min, and the supernatant was filtered through a 0.45-µm cellulose acetate membrane (450 Filter Units, 75-mm CN membrane; Thermo Fisher Scientific, Rochester, NY).

**Step 2:** Q Sepharose column chromatography. The filtrated supernatant was applied to a HiLoad 26/10 Q Sepharose HP column (0.5 cm × 10 cm; GE Healthcare, Buckinghamshire, UK) equilibrated with buffer A. After the column was washed with 2 column volumes of buffer A, the enzyme was eluted with a linear gradient of 0–1 M NaCl (1100 mL). The active fractions were pooled.

**Step 3:** Mono Q column chromatography. The pooled fractions were applied to a Mono Q 5/50 GL column (0.5 cm × 5 cm; GE Healthcare) equilibrated with buffer A. The column was washed with 2 column volumes of buffer A, and the enzyme was eluted with a linear gradient of 0–10 mM NaCl. Fractions including the enzyme activity were collected.

**Step 4:** Gel-filtration column chromatography. The collected fractions were applied to a HiLoad 16/60 Superdex 2000 prep-grade column (1.6 cm × 60 cm; GE Healthcare) equilibrated with buffer A containing 0.2 M NaCl. Elution was performed at a flow rate of 1 mL/min. The fractions exhibiting enzymatic activity were collected and pooled.

**Protein concentration determination, SDS-PAGE and IEF-PAGE.** The protein concentration was determined with Coomassie Protein Assay Reagent (Pierce Chemical, Rockford, IL) by a modification of the Lowry method with bovine serum albumin as calibration standard.23) SDS–PAGE was performed using precast polyacrylamide gels (Atto, Tokyo) and molecular mass standard samples (Bio-Rad). IEF–PAGE was performed using precast IEF gels (ID Novex IEF gel, pH 3–10; Invitrogen, Carlsbad, CA) and IEF marker samples (SERVA IEF Marker 3–10, Liquid Mix; Invitrogen), and all the gels were stained with Coomassie Brilliant Blue R-250.

**Molecular mass determination by size-exclusion chromatography.** To determine the molecular weight of the native enzyme, we calibrated the HiLoad 16/60 Superdex 2000 prep-grade column with molecular weight standards from GE Healthcare (Gel Filtration Calibration Kit LMW and HMW: mixture of thyroglobulin, 669,000; catalase, 232,000; aldolase, 158,000; albumin, 67,000; ovalbumin, 43,000; chymotrypsinogen A, 25,000; ribonuclease A, 13,700) with elution at 1 mL/min.

**Temperature, pH dependence, and properties of CMP-Neu5Ac synthetase SHIZ-145.** The pH profile for the activity of CMP-Neu5Ac synthetase from *P. leiognathi* JT-SHIZ-145 was determined at 30 °C for 1 h by the standard CMP-Neu5Ac synthetase assay described above, except that 100 mM Tris–HCl buffer (pH 9.0) was replaced with 100 mM Bis–Tris buffer (pH 6.0, 6.5, 7.0), 100 mM phosphate buffer (pH 7.0, 7.5, 8.0), 100 mM Tris–HCl buffer (pH 8.0, 8.5, 9.0), 100 mM 2-N-cyclohexylamino ethanesulfonic acid (CHES) buffer (pH 9.0, 9.5, 10.0), or 100 mM 3-(cyclohexylamino)-1-propane sulfonic acid (CAPS) buffer (pH 10.0, 10.5, 11.0). The temperature profile was evaluated at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, and 75 °C for 1 h by means of our standard CMP-Neu5Ac synthetase assay. All assays were performed in triplicate.

**Kinetic studies of CMP-Neu5Ac synthetase SHIZ-145.** To determine the kinetic parameters for CTP and Neu5Ac, we used the purified enzyme to measure enzymatic activities under various conditions. Enzyme (5 µL, 0.6 mU) was added to 45 µL of assay mix preheated to 30 °C and the mixture was incubated at 30 °C for 30 or 60 min. The concentration of Neu5Ac was varied from 0.01 to 20 mM with CTP kept at 5 mM, or the concentration of CTP was varied from 0.01 to 20 mM with Neu5Ac kept at 5 mM. The final concentrations of the other components of the assay mixture were 10 mM MgSO₄ and 100 mM Tris–HCl buffer (pH 9.0). The amount of CMP-Neu5Ac product at each time point was determined as described above. The kinetic parameters, including *Kₘ* and *Vₘₐₓ* for CTP and Neu5Ac, were calculated by Lineweaver–Burk plot.

**Substrate specificity of CMP-Neu5Ac synthetase SHIZ-145.** The substrate specificity of the CMP-Neu5Ac synthetase was confirmed by the standard CMP-Neu5Ac synthetase assay described above, except that 5 mM Neu5Ac was replaced with 5 mM Neu5Gc (Food and Bio Research Center, Kyoto, Japan), 5 mM Kdo (Funakoshi, Tokyo), or 5 mM 6-thio-neuraminic acid. 6-Thio-neuraminic acid was synthesized by the method described by Mack and Brossmer.24,25) Identification of sialyloligosaccharides produced by two-step enzymatic reactions. To investigate the formation of the sialyloligosaccharides, coupling assays using CMP-Neu5Ac synthetase and α2,3-sialyltransferase derived from *Photobacterium* sp. JT-ISH-224 were performed. The coupling reactions and HPLC analysis were carried out as follows: Purified CMP-Neu5Ac synthetase (0.65 mU) was added to 45 µL of assay mixture containing 10 mM MgSO₄, 100 mM Tris–HCl buffer (pH 9.0), and 2.8 mM Neu5Ac or Neu5Gc, and the mixture was incubated at 30 °C for 1 h. Then 5 µL of the reaction mixture was added to 10 µL of the sialyltransferase reaction mixture, along with 1.67 mM pyridylaminated lactose (FA-Sugar Chain 026; Takara Biochemicals, Shiga, Japan), 0.5 mM NaCl, 100 mM cadoxonate buffer (pH 5.0), and 10 µL α2,3-sialyltransferase derived from *Photobacterium* sp. JT-ISH-224. The mixture was incubated for 1 h at 30 °C and then applied to a PALPAK Type R analytical column (0.46 cm × 25 cm; Takara Biochemicals) that was equilibrated with 100 mM acetate–acrylic acid–triethylamine buffer (pH 5.0) containing 0.15% *n*-butanol in an HPLC. The column temperature was 40 °C, the concentration of *n*-butanol was increased linearly from 0.15 to 0.25% (0–20 min), and the flow rate was 1 mL/min. Pyridylaminated glycans were detected by fluorescence (Ex, 320 nm; Em, 400 nm).

**Production and purification of sialyloligosaccharides produced by one-pot two-enzyme coupling reactions.** Coupling assays using CMP-Neu5Ac synthetase and α2,6-sialyltransferase derived from *P. leiognathi* JT-SHIZ-145 were performed as described below. For the
The synthesis of α2,6Neu5Ac-lactose, the reaction mixture was composed of 10 mg of lactose (28 mg), 15.45 mg of Neu5Ac (50 mg), 24.2 mg of CTP (50 mg), 100 mM of Tris–HCl buffer (pH 9.0), 5 U of purified CMP-Neu5Ac synthetase SHIZ-145, and 5 U of α2,6-sialyltransferase from P. leiognathi JT-SHIZ-145. The mixture was incubated at 30 °C for 8 h. For the synthesis of α2,6Neu5Gc-lactose, the reaction mixtures consisted of 4 mg of lactose (11.2 mg), 6.5 mg of Neu5Gc (20 mg), 9.8 mg of CTP (20 mg), 100 mM of Tris–HCl buffer (pH 9.0), 2 U of purified CMP-Neu5Ac synthetase, and 2 U of α2,6-sialyltransferase from P. leiognathi JT-SHIZ-145 (α2,6-sialyltransferase SHIZ-145), were incubated at 30 °C for 12 h.

After the reaction, the reaction mixture was diluted with 40 mL of deionized water and introduced into an Econo column (1.0 cm × 10 cm; Bio-Rad Laboratories, Hercules, CA) containing AG 1-X2 ion-exchange resin (phosphate form, 200–400 mesh; Bio-Rad). The column was washed with deionized water, and then the product was eluted twice with 10 mL of 5, 10, 50, 100, 500, or 1,000 mM potassium phosphate buffer (pH 6.8). An aliquot of each eluted fraction was analyzed by TLC.

For TLC analysis, a small amount of the enzymatic reaction mixture was applied to a precoated silica gel plate (60 F254; Merck, Darmstadt, Germany) and developed with a solvent consisting of 2-propanol/acetic acid/water (3:2:1). For visualization of the organic compounds, the plate was dipped into a solution of 5% sulfuric acid in ethanol and then heated.

The fractions containing products were evaporated to dry residues. Each of the dry residues was dissolved in 2.5 mL of deionized water and then loaded onto a Sephadex G15 column (1.6 cm × 70 cm; GE Healthcare, UK). The product was eluted with deionized water. The fractions containing products were pooled and evaporated to dryness.

The purified products were analyzed by NMR spectroscopy using a Bruker DMX-500 spectrometer (Bruker, Billerica, MA) at 298 K in D2O.

### Results

#### Screening of bacteria that produced CMP-Neu5Ac synthetase

During the course of our research, we have screened many bacteria that have glycosyltransferase activity, and we have obtained several strains that show sialyltransferase activity. We prepared crude extracts from these bacteria, and then we performed a CMP-Neu5Ac synthetase assay, as described in “Materials and Methods.” CMP-Neu5Ac synthetase activity was found in the crude extract from P. leiognathi JT-SHIZ-145, which we previously reported as an α2,6-sialyltransferase producing bacterium.22)

#### Purification of CMP-Neu5Ac synthetase from P. leiognathi JT-SHIZ-145

CMP-Neu5Ac synthetase was purified from cells of P. leiognathi JT-SHIZ-145 by the procedures described in “Materials and Methods.” Because CMP-Neu5Ac synthetase activity decreased substantially upon storage in the absence of 20% glycerol (data not shown), all

### Table 1. Purification of CMP-Neu5Ac Synthetase from P. leiognathi JT-SHIZ-145

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (mL)</th>
<th>Total protein (mg)</th>
<th>Total activitya (U)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>175.3</td>
<td>1,076</td>
<td>9.19</td>
<td>0.009</td>
<td>100</td>
<td>1.00</td>
</tr>
<tr>
<td>Collection of supernatant</td>
<td>166</td>
<td>757</td>
<td>10.9</td>
<td>0.014</td>
<td>119</td>
<td>1.69</td>
</tr>
<tr>
<td>Q Sepharose chromatography</td>
<td>30</td>
<td>25.2</td>
<td>2.80</td>
<td>0.111</td>
<td>30.4</td>
<td>13.0</td>
</tr>
<tr>
<td>Mono Q chromatography</td>
<td>2</td>
<td>12.7</td>
<td>3.05</td>
<td>0.238</td>
<td>33.0</td>
<td>27.9</td>
</tr>
<tr>
<td>Superdex 200 chromatography</td>
<td>22</td>
<td>6.38</td>
<td>1.05</td>
<td>0.165</td>
<td>11.4</td>
<td>19.3</td>
</tr>
</tbody>
</table>

*aOne unit (U) was defined as the amount of enzyme that catalyzes the formation of 1 μmol of CMP-Neu5Ac per min under the conditions of the assay.

The procedures were done in 50 mM Tris–HCl buffer (pH 9.0) containing 0.3% Triton X-100 and 20% glycerol. The steps of the purification of CMP-Neu5Ac synthetase are summarized in Table 1. The enzyme was purified 19.3-fold with a yield of 11.4%. As shown in Fig. 1, it migrated as a single band with a molecular mass of 60 kDa under denaturing conditions on sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The molecular mass of the purified enzyme was estimated to be 55,800 Da by gel filtration chromatography with standard proteins. We designated the purified enzyme CMP-Neu5Ac synthetase SHIZ-145.

#### Specificity of CMP-Neu5Ac synthetase SHIZ-145

To determine the substrate specificity of the enzyme with regard to the sialic acid moiety, we carried out enzymatic reactions with Neu5Gc, 6′-thio-neuraminic acid and keto-deoxyoctulosonic acid (Kdo) as substrates instead of Neu5Ac. Analysis of the reaction mixtures by HPLC revealed the formation of the expected reaction products when Neu5Ac and Neu5Gc were used as substrates (Fig. 2). The product obtained when Neu5Ac was used as substrate (product A, Fig. 2) had the same retention time of that of CMP-Neu5Ac (retention time, 4.56), but the product obtained when Neu5Gc was used as substrate (product B, Fig. 2) had a different retention time, 4.47. In contrast, no reaction products were
observed when Kdo and 6’-thio-neuraminic acid were used as substrates (data not shown). In the presence of CMP-Neu5Ac synthetase SHIZ-145, the amount of CMP increased although it did not increase in the negative control. This might have been caused by the degradation of the CMP-Neu5Ac or Neu5Gc synthesized by the reaction.

General properties of CMP-Neu5Ac synthetase SHIZ-145

The isoelectric point of CMP-Neu5Ac synthetase SHIZ-145 was found to be about 5.0 by IEF–PAGE (Fig. 3). Enzymatic activity was maximal at 35 °C (Fig. 4A) at pH 9.0 (Fig. 4B). We also investigated the effects on enzymatic activity of five divalent cations at 10 mM. As shown in Table 2, CMP-Neu5Ac synthetase activity was highest in the presence of the Mg$^{2+}$ ion, and assay in the presence of EDTA resulted in a complete loss of activity. Maximum activity was observed at Mg$^{2+}$ concentrations greater than 5 mM (data not shown).

Kinetic properties

The apparent kinetic parameters of CMP-Neu5Ac synthetase SHIZ-145, $K_m$ and $V_{max}$ for CTP and Neu5Ac, were determined by Lineweaver-Burk plot. The apparent $K_m$ value for CTP was 1.57 mM and that for Neu5Ac was 0.867 mM. CMP-Neu5Ac synthetase SHIZ-145 exhibited a $V_{max}$ of 3.27 nmol/min/mg for CTP and 2.26 nmol/min/mg for Neu5Ac (Table 3). Under this assay condition, the amount of CMP-Neu5Ac was in proportion to the assay time, from 0 min to 60 min (data not shown).

Table 2. Effects of Divalent Cations on the Activity of CMP-Neu5Ac Synthetase SHIZ-145

<table>
<thead>
<tr>
<th>Divalent cation (10 mM)</th>
<th>CMP-Neu5Ac produced (nmol/min)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg$^{2+}$</td>
<td>0.055</td>
<td>100</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>0.003</td>
<td>5.16</td>
</tr>
<tr>
<td>Ca$^{2+}$&lt;0.001</td>
<td>0</td>
<td>0.62</td>
</tr>
<tr>
<td>Fe$^{2+}$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No divalent cation</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The HPLC profiles of the reaction products obtained with (a) Neu5Ac (retention time, 4.56 min) and (b) Neu5Gc (retention time, 4.47 min) as a substrate. (c) Negative control: reaction without sialic acid and (d) CMP-Neu5Ac as control (retention time, 4.56 min).
enzyme coupling reactions using CMP-Neu5Ac synthetase SHIZ-145 and α,2,6-sialyltransferase derived from P. leiognathi JT-SHIZ-145\(^2\) were performed. These enzymatic reactions were performed in one pot, and then the products were purified by ion-exchange column chromatography and gel-filtration column chromatography. When Neu5Ac was utilized as substrate, 6.2 mg of product was obtained, and Neu5Gc was utilized, 8.5 mg of product was obtained. The purified reaction products were analyzed by NMR spectroscopy. The production of α,2,6Neu5Ac-lactose (Supplemental Fig. 1; see *Biosci. Biotechnol. Bio sci*. Web site) and α,2,6Neu5Gc-lactose (Supplemental Fig. 2) was thus confirmed.

### Table 3. Apparent Kinetic Parameters of CMP-Neu5Ac Synthetase SHIZ-145

<table>
<thead>
<tr>
<th>Compound</th>
<th>(K_m) (mM)</th>
<th>(V_{max}) (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTP</td>
<td>1.57</td>
<td>3.27</td>
</tr>
<tr>
<td>Neu5Ac</td>
<td>0.867</td>
<td>2.26</td>
</tr>
</tbody>
</table>

**Fig. 5. HPLC Profiles of Two-Step Enzymatic Reaction Products.**

HPLC profiles of reaction products obtained using (a) Neu5Ac or (b) Neu5Gc as a substrate for CMP-Neu5Ac synthetase SHIZ-145, and pyridylaminated lactose as a substrate for α2,3-sialytransferase, (c) HPLC profiles of PA 026 (pyridylaminated lactose, retention time, 3.83 min), PA 029 (pyridylaminated N-acetylneuraminyl-α,2,3-lactose, 5.10 min), and PA 030 (pyridylaminated N-glycolyneuraminyl-α,2,3-lactose, 4.75 min). Solid line, reaction mixture; broken line, reaction mixture without sialytransferase.

**Identification of sialyloigosaccharides produced by two-step enzymatic reactions**

To confirm the enzymatic reaction products of CMP-Neu5Ac synthetase SHIZ-145, we performed a two-step enzymatic reaction sequence with α2,3-sialytransferase from *Photobacterium* sp. JT-ISH-224.\(^2\) First, the CMP-Neu5Ac synthetase reaction was performed with Neu5Ac or Neu5Gc as a substrate, and then 5 μL of the resulting product mixture was added to the α2,3-sialytransferase assay reaction mixture along with pyridylaminated lactose as the acceptor substrate. HPLC analysis of the reaction products and a comparison with pyridylaminated standards confirmed the formation of pyridylaminated N-acetylneuraminyl-α,2,3-lactose and pyridylaminated N-glycolyneuraminyl-α,2,3-lactose respectively. The retention times of pyridylaminated lactose (PA-Sugar Chain 026), pyridylaminated N-acetylneuraminyl-α,2,3-lactose (PA-Sugar Chain 029), and pyridylaminated N-glycolyneuraminyl-α,2,3-lactose (PA-Sugar Chain 030) were 3.83, 5.10, and 4.75 min respectively. As Fig. 5 shows, pyridylaminated N-acetylneuraminyl-α,2,3-lactose (Fig. 5a) and pyridylaminated N-glycolyneuraminyl-α,2,3-lactose (Fig. 5b) were produced using CMP-Neu5Ac and CMP-Neu5Gc, which were the enzymatic products of CMP-Neu5Ac synthetase SHIZ-145. These results indicate that CMP-Neu5Ac synthetase SHIZ-145 utilized not only Neu5Ac but also Neu5Gc as substrates.

**Production and purification of sialyloigosaccharides produced by one-pot two-enzyme coupling reactions**

For further confirmation of the enzymatic product of CMP-Neu5Ac synthetase SHIZ-145, one-pot two-
values are similar to those for CMP-Neu5Ac synthetases isolated from bacteria, except for the enzyme from C. thermocellum, which shows optimal activity at 50 °C. Moreover, CMP-Neu5Ac synthetase SHIZ-145 was stable. When stored at −80 °C, the purified enzyme in buffer A (described in “Materials and Methods,” 50 mM Tris–HCl buffer pH 9.0 containing 0.3% Triton X-100 and 20% glycerol) retained more than 90% of its activity for 6 months (data not shown).

Neu5Gc is known to be produced in mammals but not in the human body because humans lack the enzyme that converts CMP-Neu5Ac to CMP-Neu5Gc. Recently, it was reported that a recombinant glycoprotein drug produced in non-human mammalian cell lines contains Neu5Gc. It was also reported that this might be relevant to improving the half-life, efficacy, and immunogenicity of glycoprotein therapeutics. Thus, the investigation of Neu5Gc–containing sialosides is widely conducted. NMR spectroscopy.

To confirm this ability of sialyltransferase cloned from P. leiognathi JT-SHIZ-145, we have isolated from marine bacteria have signal sequences on their N-termini, and because mature enzymes lack these sequences, we expect that these sialyltransferases translocate across the cytoplasmic membrane to the periplasm. An α2,6-sialyltransferase from JT-SHIZ-145 is also thought to be a periplasmic membrane protein. Hence, we expect that CMP-Neu5Ac synthetase SHIZ-145 is localized in the periplasmic region and cooperates with sialyltransferase to produce sialosides on the surface of the bacterium.

In summary, we isolated and characterized a CMP-Neu5Ac synthetase from P. leiognathi JT-SHIZ-145. This enzyme is the first example of a CMP-Neu5Ac synthetase obtained from a marine bacterium that produces both CMP-Neu5Ac and CMP-Neu5Gc. One-pot two-enzyme coupling synthesis using CMP-Neu5Ac synthetase SHIZ-145 and α2,6-sialyltransferase SHIZ-145 is the simple, powerful tool for the production of various sialosides, especially including Neu5Gc, because one-pot two-enzyme coupling synthesis can produce sialosides without isolation and purification of CMP-Neu5Ac or CMP-Neu5Gc.

Acknowledgments

We thank Professor Hiroyuki Wariishi of Kyushu University and Professor Sanji Matsushima and Professor Yoshisuke Nishi of the Nagahama Institute of Bio-Science and Technology for valuable advice. We also thank Mr. Masashi Mizutani, R&D Group, Product Quality Research Division, Japan Tobacco, Inc., for conducting NMR spectroscopy.

References

22) Yamamoto T, Hamada Y, Ichikawa M, Kajiwara H, Mine T,


