Bioscience, Biotechnology, and Biochemistry

ISSN: 0916-8451 (Print) 1347-6947 (Online) Journal homepage: http://www.tandfonline.com/loi/tbbb20

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To cite this article: Takeshi YAMAMOTO, Hideki NAGAE, Yasuhiro KAJIHARA & Ichiro TERADA (1998) Mass Production of Bacterial α2,6-Sialyltransferase and Enzymatic Syntheses of Sialyloligosaccharides, Bioscience, Biotechnology, and Biochemistry, 62:2, 210-214, DOI: 10.1271/bbb.62.210

To link to this article: http://dx.doi.org/10.1271/bbb.62.210

Biosci. Biotechnol. Biochem., **62** (2), 210–214, 1998

Mass Production of Bacterial α2,6-Sialyltransferase and Enzymatic Syntheses of Sialyloligosaccharides

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Received June 25, 1997

To supply α2,6-sialyltransferase for the large-scale synthesis of sialoside, we investigated culture conditions for the production of sialyltransferase 0160.

The addition of galactose and beef extract, and control of the pH of the culture medium were effective on the production of sialyltransferase 0160. The maximal enzyme productivity reached 550 units/L.

Using a crude extract of *Photobacterium damsela* JT0160 cells as an enzyme source, enzymatic syntheses were performed with mono- and di-saccharides as the sialyl acceptors. It was clarified that a crude extract of *P. damsela* JT0160 cells can be used as an synthetic catalyst for the enzymatic synthesis of sialyloligosaccharides. Furthermore, the enzyme assay showed that sialyltransferase 0160 could transfer NeuAc to not only N-linked but also O-linked carbohydrate chains.

These results indicated that an abundant supply of sialyltransferase 0160 and its broad specificity make possible the synthesis of sialoside on a large scale.

Key words: bacterial α2,6-sialyltransferase; mass production; enzymatic synthesis; *Photobacterium damsela*

Sialic acids exist in a variety of mammalian glycoproteins and glycolipids, and are usually attached at the terminals of oligosaccharide chains.[1] Recent studies concerned with glycobiology showed that sialic acids are important in a variety of biological and physiological events.[2–5] To clarify their role, many kinds of sialyloligosaccharide have been synthesized by chemical methods and enzymatic methods. A highly efficient sialylation method has been developed in recent studies.[6–10] However, the chemical formation of α-sialoside has been the most difficult glycosylation reaction to produce. For this reaction, enzymatic synthesis of sialyloligosaccharide using sialyltransferase has an advantage compared to chemical synthesis.

In general, bacterial enzymes are stable and are more productive compared to mammalian enzymes. Therefore, we screened for a bacterium which produces sialyltransferase and isolated a new β-galactoside α2,6-sialyltransferase (sialyltransferase 0160) from *Photobacterium damsela* JT0160.[11]

The sialyltransferase 0160 had a unique acceptor specificity compared with that of mammalian sialyltransferases. For example, mammalian enzymes did not transfer NeuAc to lactose, but, sialyltransferase 0160 recognizes lactose as an acceptor.[11] Further, sialyltransferase 0160 could transfer NeuAc to 2′-fucosyllactose and 3′-sialyllactose with high efficiency.[12]

Sialyl-Tn antigen is known to be a cancer associated antigen in epithelial cell cancers such as colon cancer[13] and is a glycoprotein that contains O-linked carbohydrate chains. In order to investigate the mechanism of malignant alteration and the antigenecity of the carbohydrate moiety of glycoprotein, a large amount of glycoprotein is needed. For this purpose, sialyltransferase has an advantage. We have reported that sialyltransferase 0160 could transfer NeuAc to N-acetylgalactosamine. So, it was suggested that an abundant supply of pure sialyltransferase 0160 would make it possible to synthesize target glycoproteins. Therefore, enzymatic syntheses were done using a crude enzyme to confirm the structure of products found when using mono- and di-saccharides as the sialyl acceptors. Furthermore, the enzymatic sialylation ability of this enzyme for glycoproteins was examined.

In this paper, we report on (i) the culture conditions for production of sialyltransferase 0160 from *P. damsela* JT0160; (ii) the synthesis of sialyloligosaccharides using a crude extract of *P. damsela* JT0160 cells as the enzyme source.; and (iii) the specificity of sialyltransferase 0160 for glycoproteins.

Materials and Methods

Materials. CMP-[4,5,6,7,8,9-^{14}C]-NeuAc was purchased from Du Pont; CMP-NeuAc, asialo-fetuin, asialo-bovine submaxillary mucin, α1 acid-glycoprotein, and methyl-β-D-galactopyranoside were obtained from Sigma; asialo-α1 acid glycoprotein was prepared by mild acid hydrolysis (0.05 M sulfuric acid, 1 h, 80°C) of the α1-acid glycoprotein; lactose was from Kanto Chemicals; colominic acid and N-acetylgalactosamine were from Nakarai Tesque; 6′-sialyllactose was from Oxford Glycosystems; marine broth 2216, proteose peptone, soytone, casamino acids, tryptone, yeast extract, and beef extract were from Difco Laboratories;

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nutrient broth was from Oxoid; Polypeptone Y was from Nippon Seiyaku; Clear-sol 1 (scintillation cocktail) was from Nakarai Tesque and all other reagents were of analytical grade and commercially available. Neuraminidase was from Seikagaku Kogyo; Sephadex G-50 was from Pharmacia; Dowex 1 × 8 was from Dow Chemical Co.; radioactivity was measured using a Packard model TR 1900 liquid scintillation counter. Proton NMR spectra were obtained using a Bruker AM500 spectrometer.

Bacterial strain and cultivation. A strain of *P. damsela* JT0160 was isolated from seawater from Sagami Bay, Kanagawa, Japan.[11] Artificial seawater was used for preparation of the media, except for the marine broth 2216 and the nutrient broth. The pre-culture was prepared as follows: 0.5 ml of glycerol solution (40%, v/v) in which the *P. damsela* JT0160 was stored, was inoculated into 100 ml of marine broth 2216 medium in a 500-ml flask, and cultivated at 30°C for 8 h on a rotary shaker at 150 rpm. This seed culture was transferred to 1 L of medium in a 3-L flask or 3 L of medium in a 5-L jar fermentor, at the ratio of 1%. Unless otherwise stated, the culture temperature was kept constant at 30°C and the cultivation continued for 8 h. For the jar culture, the fermentor was agitated at 600 rpm and aerated at a rate of 1.5 L/min.

Preparation of the crude extract and purification of sialyltransferase 0160. P. damsela JT0160 cells were harvested by centrifugation (6,000 × *g*, 20 min) from the culture medium. The 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 until absorbance at 660 nm became 30% of that of the cell suspensions. The sonicated solution was centrifuged (100, 500 × *g*, 60 min) and the obtained supernatant was dialyzed in a cellulose tube against 20 mM sodium cacodylate buffer (pH 6.0) containing 0.2% Triton X-100. After dialysis, precipitation was removed by centrifugation (100, 500 × *g*, 60 min) to obtain a clarified extract. This solution was used as the crude extract of *P. damsela* JT0160.

The purification of sialyltransferase 0160 was done as described previously.[11]

Standard sialyltransferase assay. Sialyltransferase activity was measured as described previously.[11] One unit (U) is defined as the amount that transferred 1 μmole of NeuAc per min to the lactose.

Syntheses of sialyloligosaccharides using a crude extract of P. damsela JT0160. The enzymatic syntheses were done using N-acetylgalactosamine, methyl-β-D-galactopyranoside, and lactose as acceptor substrates. The reaction mixture was composed of each acceptor substrate (30 μmol), CMP-NeuAc (16 μmol, 9.6 mg), and 100 mU of crude extracts of *P. damsela* JT0160 in 0.5 ml of 20 mM sodium cacodylate buffer (pH 5.0) containing 0.02% Triton X-100. Each reaction mixture was incubated at 30°C for 4 h. The products were purified as follows: after the reaction, each reaction mixture was diluted with 10 ml of distilled water and put on separate columns (1.5 × 9 cm) of a Dowex 1 × 8 (phosphate form, 200–400 mesh). The columns were washed with distilled water, and then the product was eluted with 5 mM of sodium phosphate buffer (pH 6.8; total volume 60 ml). The fractions containing glycosidic NeuAc were pooled and evaporated to dry residues. Each dried residue was dissolved in 5 ml of solvent A (AcOEt:MeOH:H_2O = 3:2:1), and then put on columns (1.5 × 6.8 cm) of silica gel. The product was eluted with solvent A. The fractions found to contain glycosidic NeuAc were pooled and evaporated to dry residues. The purified products were analyzed by ^{1}H-NMR spectroscopy.

Sialyltransferase assay for glycoprotein. Sialyltransferase activity with glycoprotein was assayed by measuring transferred [4,5,6,7,8,9-^{14}C]-NeuAc from CMP-[4,5,6,7,8,9-^{14}C]-NeuAc to glycoprotein as an acceptor substrate. The reaction mixture contained the following components: 70 nmol of CMP-[4,5,6,7,8,9-^{14}C]-NeuAc (642 cpm/nmol), 2 mg of asialo-glycoprotein and enzyme solution in 0.1 ml of 20 mM sodium cacodylate buffer (pH 5.0) containing 0.02% Triton X-100. The enzyme reaction was done at 30°C. All assays were done in duplicate. After the reaction, the reaction mixture was put on a column of a Sephadex G-50 (super fine, 0.8 × 14 cm) equilibrated with 0.1 M NaCl, and eluted with 0.1 M NaCl. All the sialylated product was eluted in a 2–4 ml fraction of this column. The fraction (2 ml) was collected directly into a scintillation vial for counting. The amount of NeuAc transferred to the acceptor was calculated.

Neuraminidase digestion of the sialylated-glycoprotein by sialyltransferase 0160 was done. The fraction that contained the sialylated glycoprotein (containing ^{14}C-labeled NeuAc) obtained by the method described above was collected and dialyzed, using a cellulose tube, against 20 mM sodium cacodylate buffer (pH 5.5). After dialysis, the fraction was concentrated to 100 μl by ultrafiltration using Molecut L (Millipore, exclusion molecular mass 10 kDa). Then, neuraminidase (50 mU) was added to the concentrated fraction and the solution was incubated at 37°C for 5 h. After the reaction, the reaction mixture was put on a column of a Sephadex G-50 and eluted with 0.1 M NaCl. The fraction which contained glycoprotein was collected directly into a scintillation vial for counting.

Other assays. Neuraminidase activity of the crude extract was assayed using colominic acid and 6′-sialyllactose as the substrates.[14] The sialic acid released was measured using the thiobarbituric acid assay (TBA assay) method described by Aminoff.[15] The growth of cells was measured by measuring the optical density at 660 nm.

Results

Culture conditions for the production of sialyltransferase 0160

First, the optimum temperature and pH of the culture medium for cultivation of *P. damsela* JT0160 were ex-

amined using marine broth 2216 as the culture medium. The optimum temperature and pH of the culture medium were 30°C and pH 8, respectively. Second, the relationship between cell concentration in a culture medium and the productivity of sialyltransferase 0160 was examined using marine broth 2216 and nutrient broth (containing 3.0% NaCl) as the culture media. As a result, it was found that the productivity of sialyltransferase 0160 depended on the cell concentration in the culture medium. Then, the effects of various organic compounds on the production of sialyltransferase 0160 were examined. As shown in Table I and Fig. 1, the addition of galactose and beef extract were effective. The addition of yeast extract (0.1%, W/V) was indispensable for effective growth of *P. damsela* JT0160.

Composition of the optimum medium

To examine the effects of the composition of the medium on sialyltransferase 0160 production, several types of media were prepared by varying the amounts of polypeptone Y, yeast extract, beef extract, and galactose. The pH of the culture media were all adjusted to 8. These examinations were done in a 3-L flask. All of the prepared media contained 100 mM tricine to maintain the pH of the media. These results are also shown in Table II. The optimum medium for sialyltransferase 0160 production was judged to be the 3PYBG medium. In a 3-L flask culture with 3PYBG medium, the sialyltransferase activity of the culture medium was found to be 550 units/L. The same result was obtained in the jar culture using 3PYBG medium without the addition of tricine to the medium.

Table I. Effects of Various Peptones on Growth of *P. damsela* JT0160

Nitrogen sources	Ratio of maximum cell concentration (relative)
Marine broth 2216 (control)	1.00
Polypeptone Y	2.16
Proteose peptone	1.15
Soytone	1.23
Casamino acids	2.55
Tryptone	1.60
Polypeptone Y+Beef extract	3.55
Casamino acids+Beef extract	3.51

Basal medium consisted of 0.1% yeast extract in artificial seawater. The peptones (0.5%, w/v) and beef extract (0.3%, w/v) were added.

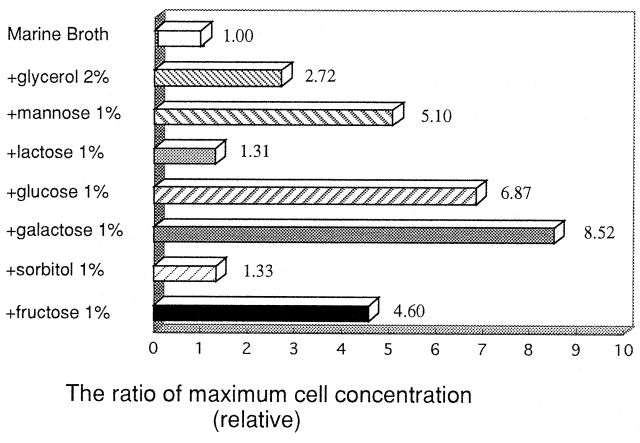

Fig. 1. Effects of Various Saccharides on Growth of *P. damsela* JT0160.

Marine broth 2216 was used as basal medium. The amount of saccharides added are indicated.

Table II. Culture Media for the Production of Sialyltransferase 0160

Medium Name	Amount (g/L)				Activity (U/L)
	Polypeptone Y	Yeast ext.	Beef ext.	galactose	
PYB1/2G	5	1	3	5	110
PYBG	5	1	3	10	200
PYB2G	5	1	3	20	280
2PYBG	10	2	6	20	400
3PYBG	15	3	9	30	550

Purification of sialyltransferase 0160

Purification of sialyltransferase 0160 was done using the cells obtained from 2 L of 3PYBG medium. The total enzyme amount of the crude extract was about 1,100 units. The enzyme was purified 750-fold, with a yield of 18%. The purified enzyme migrated as a single polypeptide with a molecular mass of 61 kDa under denaturing conditions (data not shown). The specific activity of the purified enzyme was 6.8 U/mg.

Syntheses of sialyloligosaccharides using a crude extract of P. damsela *JT0160*

Neuraminidase activity was not observed in the crude extract. Using crude extract as the enzyme source, the enzymatic syntheses were done. The products were isolated and analyzed by ^{1}H-NMR spectroscopy (in D_2O, 298 K). The product formed with N-acetylgalactosamine as the acceptor turned out to be NeuAcα2-6GalNAc and 4.6 mg of product (1, 9.0 μmol, yield 56.2%) was obtained. The structural reporter group signals were as follows: δ 5.22 (d, 0.5 H, J=3.7 Hz, GalNAc α H-1), δ 4.63 (d, 0.5 H, J=8.4 Hz, GalNAc β H-1), δ 2.74 (dd, 0.5 H, J=4.6 Hz, 12.5 Hz, NeuAc H-3 eq), δ 2.73 (dd, 0.5 H, J=4.6 Hz, 12.4 Hz, NeuAc H-3 eq), δ 2.05, 2.04 (each 3 H, Ac), δ 1.71 (dd, 0.5 H, J=12.2 Hz, 12.2 Hz, NeuAc H-3 ax), δ 1.70 (dd, 0.5 H, J=12.1 Hz, 12.1 Hz, NeuAc H-3 ax). The product formed with methyl-β-D-galactopyranoside as the acceptor turned out to be NeuAcα2,6Gal-OMe, and 5.0 mg of product (2, 10.4 μmol, yield 65.0%) was obtained. The structual reporter group signals were as follows: δ 4.31 (d, 1 H, J=7.94 Hz, Gal H-1), δ 3.57 (s, 3 H, OMe), δ 3.49 (dd, 1 H, J=8.0 Hz, 9.9 Hz, Gal H-2), δ 2.73 (dd, 1 H, J=4.68 Hz, 12.5 Hz, NeuAc H-3 eq), δ 2.04 (s, 3 H, Ac), δ 1.70 (dd, 1 H, J=12.2 Hz, 12.2 Hz, NeuAc H-3 ax). The product formed with lactose as the acceptor turned out to be NeuAcα2-6Galβ1-4Glc, and 6.4 mg of product (3, 10.2 μmol, yield 63.6%) was obtained. The structual reporter group signals were as follows: δ 5.21 (d, 0.4 H, J=3.5 Hz, Glc α H-1), δ 4.66 (d, 0.6 H, J=8.0 Hz, Glc β H-1), δ 4.42 (d, 1 H, J=7.8 Hz, Gal H-1), δ 3.30 (dd, 0.6 H, J=8.4 Hz, 8.4 Hz, Glc β H-2), δ 2.70 (dd, 1 H,

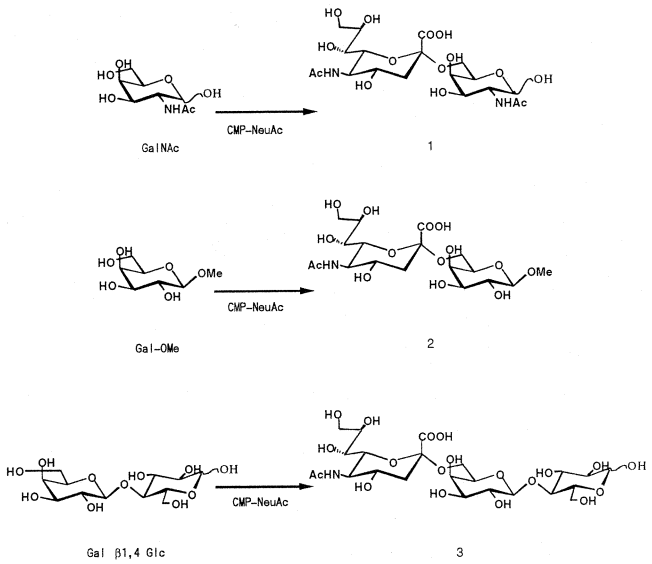

Table III. Acceptor Specificity of Sialyltransferase 0160 for Glycoproteins

Acceptor	Transferred NeuAc (nmol)	
	Reaction time 5 min	60 min
Asialo-fetuin	3.3	11.2
Asialo-α1 acid-glycoprotein	3.1	10.9
Asialo-bovine submaxillary mucin	0.9	3.5

Transfened NeuAc (nmol) represents the amount of transferred NeuAc to 2 mg of acceptor.

J=4.5 Hz, 12.35 Hz, NeuAc H-3 eq), δ 2.02 (s, 3 H, Ac), δ 1.73 (dd, 1 H, J=12.3 Hz, 12.3 Hz, NeuAc H-3 ax). The ^{1}H-NMR spectrum of each product showed good agreement with those of reported NeuAcα2-6GalNAc,[16] NeuAcα2-6Gal-OMe,[17] and NeuAcα2-6Galβ1-4Glc,[18] respectively.

Acceptor specificity for glycoproteins

Using the purified sialyltransferase 0160, enzyme assays were done with glycoproteins as acceptors for 5 min and 1 h. The results showed that sialyltransferase 0160 transferred NeuAc from CMP-NeuAc to asialo-fetuin, asialo-α1 acid-glycoprotein, and asialo-bovine submaxillary mucin (Table III). It may be possible to increase the amount of transferred NeuAc by extension of the reaction time.

To confirm the formation of glycosidic NeuAc on glycoproteins, neuraminidase digestion of the sialylated-glycoproteins by sialyltransferase 0160 was done. No radioactivity was observed in the gel-filtration fraction that contained neuraminidase-treated glycoprotein. These results indicated that the glycosidic NeuAc on glycoproteins was formed by sialyltransferase 0160.

Discussion

As reported previously,[11] production of sialyltransferase 0160 was limited to 20 units per 1 L of culture broth before this study. To improve this low enzyme productivity, we investigated the culture conditions for production of sialyltransferase 0160. The maximum enzyme productivity reached 550 U/L in the culture medium, which corresponds to 27.5 times higher yield than that before the optimization. Mass production of sialyltransferase 0160 by flask culture has a great advantage in the synthesis of sialyloligosaccharides, because the culture of *P. damsela* JT0160 under optimum conditions can be done by a simple procedure and a large amount of enzyme can be obtained. From an optimized culture medium, purified sialyltransferase 0160 was obtained with a yield of 18% by the simple method reported previously.[11]

It is clearly demonstrated in this study that a crude extract of *P. damsela* JT0160 cells can be used as a synthetic catalyst for the enzymatic synthesis of sialyloligosaccharides. Using 0.1 U of the crude enzyme, 4.6 mg of NeuAcα2-6GalNAc, 5.0 mg of NeuAcα2-6Gal-OMe, and 6.4 mg of NeuAcα2-6Galβ1-4Glc were obtained under the conditions described above. The conditions for the synthesis of sialyloligosaccharide was not optimized. So, it may be possible to increase the yield by extension of the reaction time. Gram scale synthesis of sialyloligosaccharide may be possible with crude enzyme prepared from 1 L of culture broth by this simple and convenient method. Moreover, it was found that sialyltransferase 0160 can act as GalNAcα2,6-sialyltransferase. Up to the present, three types of GalNAc α2,6-sialyltransferases have been reported.[19-21] All of these GalNAc α2,6-sialyltransferases transfer NeuAc through the α2,6-linkage onto a GalNAc residue, which is O-glycosidically linked to Ser/Thr of glycoproteins. It was suggested that these enzymes recognize the protein moiety of glycoproteins as acceptors. So, these enzymes cannot transfer NeuAc to free GalNAc. However, sialyltransferase 0160 can transfer NeuAc through the α2,6-linkage onto free GalNAc. This result indicated that sialyltransferase 0160 is the only sialyltransferase that can make oligosaccharide which contains the NeuAcα2,6-GalNAc structure.

The acceptor specificity of sialyltransferase 0160 for glycoprotein was unique compared with that of mammalian sialyltransferases. It was find that sialyltransferase 0160 could transfer NeuAc to not only N-linked but also O-linked carbohydrate chains. These results indicate that sialyltransferase 0160 may be useful in the preparation of sialyl Tn antigen and its analogues enzymatically for investigating the mechanism of malignant alteration and the antigenecity of the carbohydrate moiety of glycoprotein. Futhermore, these results showed the possibility that sialyltransferase 0160 can modify both N-linked and O-linked type carbohydrate chains of glycoproteins to confer additional functions.

Mass production of sialyltransferase 0160 and the broad specificity of sialyltransferase 0160 for acceptor substrates make possible the enzymatic and/or chemoenzymatic synthesis of sialoside that previously could not be synthesized on a large scale using mammalian α2,6-sialyltransferase.

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