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Purification and characterization of a soluble recombinant human ST6Gal I functionally expressed in *Escherichia coli**

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A soluble and active form of recombinant human ST6Gal I was expressed in *Escherichia coli*. The gene encoding the soluble form of ST6Gal I lacking the membrane and cytosolic regions was introduced into a bacterial expression vector, pMAL-p2X, fused in frame with a maltose-binding protein (MBP) tag. Low-temperature cultivation at 13°C during IPTG-induction significantly improved both solubility and MBP-tagging of the recombinant enzyme expressed in bacteria. The supernatant prepared by disruption of the cells demonstrated sialic acid transfer activity to both an oligosaccharide and a glycoprotein, asialofetuin, indicating that the enzyme expressed in bacteria is soluble and active. The MBP-tagged enzyme was efficiently purified by a combination of cation-exchange column and amylase-conjugated agarose column chromatography. The purified recombinant enzyme exerted enzymatic activity even in the absence of detergents in the reaction mixture. Acceptor substrate specificity of the enzyme was marginally different from that of rat liver ST6Gal I. These observations suggest that membrane and cytosolic regions of ST6Gal I may affect the properties of the enzyme. The purified recombinant enzyme was applied to convert desialylated fetuin to resialylated fetuin. Lectin blotting demonstrated that resialylated fetuin possesses a single Neu5Ac α 2-6 residue. The resialylated fetuin efficiently blocked hemagglutination induced by influenza virus strain A/Memphis/1/71 (H3N2), indicating that resialylated carbohydrate chains on the protein are so active as to competitively inhibit virus-receptor interaction. In conclusion, soluble recombinant ST6Gal I obtained using our bacterial expression system is a valuable tool to investigate the molecular mechanisms of biological and pathological interactions mediated *via* carbohydrates.
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Keywords: **sialyltransferase, bacterial expression, influenza virus, sialic acid, resialylation**

Introduction

Sialic acid-containing glycoconjugates are involved in a number of biological and pathological events [1–5]. Many sialyltransferases involved in the synthesis of sialic acid-containing glycoconjugates have been identified [6–9]. The sialyltransferase ST6Gal I generates α2-6 linkage of sialic acid to non-reducing terminal Galβ1-4GlcNAc residues of oligosaccharides and glycoconjugates, such as glycoproteins and glycolipids [10–12]. Carbohydrate structures containing Neu5Acα2-6 residues play critical roles in cell-cell recognition and cell-pathogen interactions [3–5, 13–15]. The structures of these carbohydrate molecules are unique and their amounts in natural sources are small. Although there have been many attempts to synthesize such molecules chemically, it is still difficult to stereo-specifically generate functional sialylated carbohydrate molecules and to obtain the product in sufficient amounts to address the molecular mechanisms of the interaction. Attempts to synthesize sugar residues using specific glycosyltransferases have recently been reported [16–20]. However, as enzyme sources are limited, large-scale preparation of bio-active carbohydrates with these enzymes is still not effective with regard to

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either cost or quantity. To elucidate the biological and pathological significance of glycoconjugates, both quality and quantity of diverse sugar molecules are required.

Several systems for expression of recombinant proteins are available, based on *Escherichia coli*, vaccinia virus, *Pichia* yeast, baculovirus, and mammalian cells. Expression of proteins in *E. coli* is the most efficient, economical, and convenient approach to obtain large amounts of proteins of interest both for research and other applications. However, proteins expressed in *E. coli*, particularly those of mammalian origin, are readily transported into inclusion bodies [21,22]. Proteins in inclusion bodies can, to a very limited extent, be extracted with mild detergents or by ultra-sonication, which can be used to solubilize proteins in their native forms. Bacterial glycosyltransferases, but not those of mammalian origin, have been successfully expressed in *E. coli* as soluble and active forms [22–27]. Bacterial glycosyltransferases catalyze not only similar reactions to those in mammalian cells, but also unique reactions that generate characteristic carbohydrate structures seen in bacterial cells [23–26]. Due to the strong possibilities that these carbohydrates will show antigenic activity in human and animals, glycosyltransferases derived from non-animal sources should be avoided for the syntheses of (bio)active compounds. Although insoluble glycosyltransferases have been extracted from inclusion bodies with denaturants, such as urea and guanidinium salts and then re-folded under certain conditions [22], the yields of recombinant enzymes have not been enough to be applicable for mass production of (bio)active carbohydrates and investigation of catalytic mechanisms on sialylation by NMR.

In this study, we have expressed successfully a human glycosyltransferase, ST6Gal I, in *E. coli* as a soluble and active enzyme and established efficient procedures for large-scale purification of the recombinant enzyme. In addition, we have characterized the properties of the purified recombinant enzyme and utilized the enzyme for the generation of a glycoprotein with anti-influenza virus activity.

Materials and methods

Materials

Gene Pool Human Normal Liver cDNA and *Pfu* Turbo DNA Polymerase were obtained from Invitrogen Corporation and Stratagene (U.S.A.), respectively. Pyridylaminated (PA) sugar chains used this study were purchased from Takara Bio, Inc. (Japan). Fetuin was obtained from Sigma-Aldrich, Inc. (U.S.A.). Sialidase from *Arthrobacter ureafaciens* was kindly provided by Marukin Bio, Inc. (Japan). Rat liver α2-6sialyltransferase (ST6Gal I) was purchased from Wako Pure Chemical Industries, Ltd. (Japan). Rabbit anti-MBP antibody was purchased from New England Biolabs, Inc. (U.S.A). Biotin-conjugated lectins, *Maackia amurensis* agglutinin (MAA) and *Sambucus sieboldiana* agglutinin (SSA) were purchased from Seikagaku Corporation (Japan). All other chemicals were of the highest purity available.

Cloning of the gene encoding full-length ST6Gal I

The primers for polymerase chain reaction (PCR) were designed using the nucleotide sequence of human ST6Gal I [10]. The sequences of primers used to amplify the full-length human ST6Gal I cDNA were as follows: forward primer containing a *BamHI* site (5′-GGATCC ATGATTCACACCAACCTGAAGAAA-3′), reverse primer containing *SalI* site (5′-GTCGACTTAGCAGTGAATGGT CCGGAA-3′), corresponding to nucleotide positions 432–455 and 1631–1652 of human ST6Gal I, respectively. To readily express the wild-type enzyme in other systems, a *Bam HI* site was included in the forward primer. This site was not used in this study. PCR was performed for 30 cycles of denaturation (94°C, 1 min), annealing (60°C, 1 min), and extension (72°C, 4 min), followed by incubation at 72°C [28]. The 1241-bp DNA amplified using a combination of primers was ligated with linearized pGEM-T vector (Promega Corporation, U.S.A.) according to the manufacturer's instructions. The nucleotide sequence of the isolated cDNA clone was confirmed by sequencing of both strands [29].

Bacterial expression of soluble ST6Gal I protein

In small-scale culture, a single colony of pSTMX clone was inoculated into Luria Broth (LB) medium supplemented with 100 μg/ml of ampicillin (LBAmp) and precultured at 37°C for 14–16 h. One ml of preculture was added to 9 ml of freshly prepared LBAmp and cultured at the indicated temperature until mid-logarithmic phase (0.8–1.0 OD at 600 nm). In large-scale culture for purification of the enzyme, several colonies on the clone plate were inoculated into 150 ml of LBAmp and cultured at 37°C for 18 h. Ninety ml of preculture were added to 810 ml of freshly prepared LBAmp and cultured at 25°C until mid-logarithmic phase. The protein expression in bacteria was induced at the indicated temperature for 18 h in the presence of IPTG at a final concentration of 1 mM.

Extraction and purification of soluble recombinant ST6Gal I

All operations described below were carried out at 4°C or on ice. For preparation of soluble fraction, bacterial cell pellets were suspended in sonication buffer A containing 100 mM NaCl and 5 mM sodium phosphate, pH 7.0 and pulse-disrupted using an ultrasonifier until the solution became clear. The solution was centrifuged and the supernatant was used for sialyltransferase assays. For purification of the enzyme, the bacterial cell pellet corresponding to 9 liters of induced culture was suspended in 150 ml of sonication buffer B containing 150 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 1 mM NaN_3, and 20 mM Tris/HCl, pH 7.4 and pulse-disrupted with an ultrasonifier until the solution became clear. The solution was centrifuged for 90 min at 15,000 g. To remove contaminating nucleic acids, polyethylene imine was added to the supernatant at a final concentration of 0.7% and centrifuged for 30 min at 15,000 g.

Ammonium sulfate precipitation

Supernatant containing enzyme extracted by ultrasonication was precipitated by addition of solid ammonium sulfate up to 70% (w/v) saturation. The precipitates were collected by centrifugation, re-suspended in Buffer M (1 mM EDTA, 10 mM β-mercaptoethanol, 1 mM NaN_3, 20 mM MES, pH 6.0) containing 100 mM NaCl, and dialyzed against the same buffer.

CM-Sepharose column chromatography

The dialyzed fraction from the ammonium sulfate precipitation step was loaded on a column of CM-Sepharose equilibrated in Buffer M. The sialyltransferase activity was eluted with a linear NaCl gradient with 500 ml of Buffer M containing 100 mM NaCl as the initial buffer and 500 ml of Buffer M containing 500 mM NaCl as limit buffer, followed by an additional 200 ml of Buffer M containing 500 mM NaCl. The enzymatic activity was resolved by two pooled fractions (pool I and pool II). Pool I (80 ml) was further purified by affinity column chromatography.

Affinity column chromatography

Pool I was applied to a column of amylose-conjugated agarose equilibrated in buffer M containing 100 mM NaCl. The retained sialyltransferase was eluted with 150 ml of Buffer M containing 100 mM NaCl and 10 mM maltose. Fractions containing MBP-tagged ST6Gal I were pooled, mixed with glycerol and Triton CF-54 to give a final concentration of 50% and the indicated concentration, respectively, and stored at -20°C.

Sialyltransferase assay

The activity of sialyltransferase was measured with three assay methods (I-III). *(I) HPLC-based assay*: The assay for PA-oligosaccharide as an acceptor was carried out as described previously [30,31]. Sialylated products were resolved on an HPLC system equipped with an ion-exchange column, PALPAK Type N (6.4 mm $\times$ 25 cm) (Takara Bio, Inc.). Chromatography was performed at 40°C with a single solvent system of CH_3CN/500 mM Triethylamine-acetate buffer, pH 7.3 (55:45, by vol.) at a flow rate of 1 ml/min. *(II) Lectin blot assay*: The assay for desialylated fetuin as an acceptor was carried out according to the procedures of Weinstein *et al.*, Takeya *et al.*, and Malagolini *et al.* [11,12,32,33] with slight modifications. The reaction mixture had the following final concentration in a total volume of 20 μl: 0.5 mg/ml glycoproteins, 1 mM CMP-Neu5Ac, Triton CF-54 at the indicated concentrations, 1 mM $MnCl_2$, 25 mM sodium cacodylate-HCl buffer, pH 6.5, and enzyme fractions. The reaction mixture was subjected to SDS-PAGE and lectin blotting analysis. *(III) ELISA-based assay*: The quantitative enzyme assay for glycoproteins as acceptors was carried out according to the procedure of Mattox *et al.* [34]. The enzyme activity was determined in triplicate in each experiment.

Western blotting (Lectin blotting)

The proteins were resolved by SDS-PAGE under reducing conditions [35], electro-transferred and incubated with primary probes (anti-MBP antibody or biotin-conjugated lectins) after blocking of the membrane with 5% non-fat milk for anti-MBP antibody or with 1% BSA for biotin-conjugated lectins. The immune complexes were visualized using an AP-detection system (Promega, U.S.A.).

Preparation of resialylated fetuin

Preparation of desialylated fetuin

In a total volume of 7.5 ml, fetuin at a concentration of 10 mg/ml was incubated at 37°C for 3 h with sialidase (5 U/ml) from *Arthrobacter ureafaciens*.

Resialylation of desialylated fetuin with purified recombinant ST6Gal I

The reaction mixture for resialylation had the following final concentration in a total volume of 3 ml: 6.6 mg/ml desialylated fetuin, 0.5 mM CMP-Neu5Ac, 0.25% Triton CF-54, 1 mM $MnCl_2$, 10 μM 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (Neu2en5Ac), 25 mM sodium cacodylate-HCl buffer, pH 6.5, and 0.27 mg/ml purified recombinant enzyme. The reaction was carried out at 37°C for 3 h [36]. Removal of Triton CF-54 from the reaction mixture was performed by Bio-beads SM-2 (5 g dried weight) according to the manufacturer's instruction. Finally, Neu2en5Ac was added to resialylated fetuin at a final concentration of 10 μM and stored at 4°C until use.

Quantitative analysis of sialic acid bound to glycoproteins

Determination of sialic acid bound to glycoproteins was carried out by the method of Hayakawa *et al.* [37] with slight modifications. Glycoproteins (10–50 μg) were hydrolyzed at 80°C for 18 h in 500 μl of a solution containing 25 mM H_2SO_4. A fluorometric derivatizing reagent was added directly to the hydrolyzed samples and further incubated at 60°C for 2.5 h. Fluorescent derivatives were resolved by HPLC in a system equipped with a reverse phase column, TSK-Gel ODS-80TS (4.6 mm $\times$ 15 cm) (Tosoh Corporation, Japan) at 25°C with a single solvent system of H_2O/MeOH/CH_3CN (10:3:1, by vol.) at a flow rate of 0.5 ml/min. *N*-acetyl-neuraminic acid was used to produce the standard curve.

Hemagglutination inhibition (HAI) assay

The human influenza virus used in this study was A/Memphis/1/71 (H3N2). The virus strain was propagated as described previously [38,39].

Hemagglutination inhibition (HAI) assay was carried out as described previously [40]. The maximum dilution of the agents showing complete inhibition of hemagglutination was defined as the titer of hemagglutinin inhibition.

Results

Strategy of cloning of the gene encoding soluble ST6Gal I into a bacterial expression vector, pMAL-p2X

The present study was designed to express the soluble form of the enzyme fused with a purification tag, maltose-binding protein (MBP), which facilitated not only purification of the protein from bacterial cells without any detergents but also efficient removal of the enzyme from the sialylation mixtures (Figure 1). Complementary DNA encoding the full length of ST6Gal I was amplified by PCR using human liver total cDNAs as the template. A unique *Eco*RI site at position of 130 from the initiation codon was located downstream of the 3′-terminus of the coding sequences of the cytoplasmic region and transmembrane domain. A DNA fragment encoding the entire catalytic domain of the enzyme double-digested with *Eco*RI and *Sal*I was cloned into a bacterial expression vector, pMAL-p2X, resulting in a clone designated pSTMX (Figure 1).

Low temperature induction improved solubility and MBP-tagging of the enzyme

Under induction at 37°C, neither sialyltransferase activity nor MBP-tagged proteins were detected in the soluble fraction (data not shown). The induced protein was detected in the insoluble fractions in which the enzyme was not tagged with MBP or not solubilized as active form with the non-ionic detergent Triton X-100. When proteins derived from eukaryotes are expressed in bacterial cells, they are often recovered in insoluble inclusion bodies, which are resistant to solubilization with mild detergents [21,22]. Low temperature induction of proteins in bacterial cells has resolved this problem to some extent

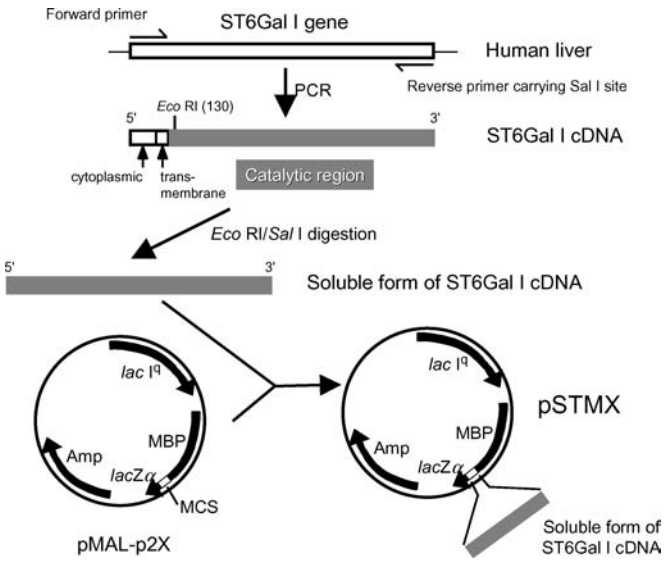

Figure 1. Scheme of cloning of cDNA encoding a soluble form of human ST6Gal I into the bacterial expression vector, pMAL-p2X.

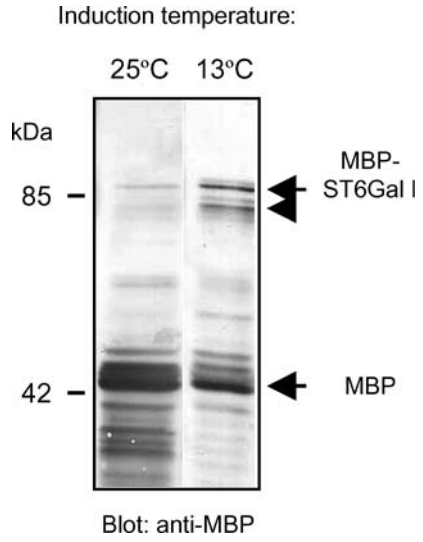

Figure 2. Improvement of solubility and MBP-tagging of ST6Gal I by low temperature cultivation of cloned bacteria transformed with pSTMX during IPTG induction. The protein expression in bacteria was induced at 13 or 25°C for 18 h in the presence of IPTG at a final concentration of 1 mM. Extraction of proteins was carried out as described in Materials and methods. The proteins were resolved by SDS-PAGE and transferred electrophoretically onto PVDF membranes. Detection of MBP-tagged ST6Gal I was performed by incubation with anti-MBP monoclonal antibody, according to the manufacturer's instructions.

[41,42]. Thus, we examined induction of MBP-tagged ST6Gal I at both 25°C and 13°C. Although the protein of interest were weakly induced at 13°C and partially degraded (Figure 2, arrow head), western blotting using anti-MBP antibody demonstrated that MBP-tagged ST6Gal I with a predicted molecular mass of 87 kDa was only detected in the fraction prepared from bacteria induced at 13°C, but not in that from those induced at 25°C (Figure 2). On the other hand, HPLC-based sialyltransferase analysis commonly revealed activity in the soluble fraction prepared under either temperature condition (representative data shown in Figure 4). Extraction of protein from bacterial cells induced at 25°C under different buffer systems including protease inhibitors or lysozyme did not improve tagging of ST6Gal I with MBP (data not shown). These results also suggest that expressed soluble recombinant MBP-tagged ST6Gal I is cleaved in bacterial cells. Taken together with the results of the low temperature experiments, induction at temperatures lower than 25°C (13°C in this study) improved both solubility of active enzyme and tagging of the enzyme with MBP.

Detection of enzyme activity of soluble sialyltransferase expressed in *E. coli*

Sialyltransferase activity was determined the conventional assay using pyridylaminated (PA) sugars as acceptor substrates

PA-sugar	Structure
PA-001	Galβ1-4GlcNAcβ1-2Manα1 $\searrow$ 6 / 3 $\swarrow$ Manβ1-4GlcNAcβ1-4GlcNAc-PA Galβ1-4GlcNAcβ1-2Manα1
PA-002	Galβ1-4GlcNAcβ1-2Manα1 $\searrow$ 6 Galβ1-4GlcNAcβ1 $\searrow$ 4 Manα1 $\swarrow$ 3 Manβ1-4GlcNAcβ1-4GlcNAc-PA Galβ1-4GlcNAcβ1 $\swarrow$ 2
PA-026	Galβ1-4Glc-PA
PA-028	Galβ1-3GalNAcβ1-4Galβ1-4Glc-PA
PA-029	NeuAcα2-3Galβ1-4Glc-PA
PA-041	Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA
PA-042	Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA

Figure 3. Pyridylaminated oligosaccharides (PA-sugars) used in this study. PA-sugar chain 001 was used for determination of sialyltransferase activity unless otherwise indicated.

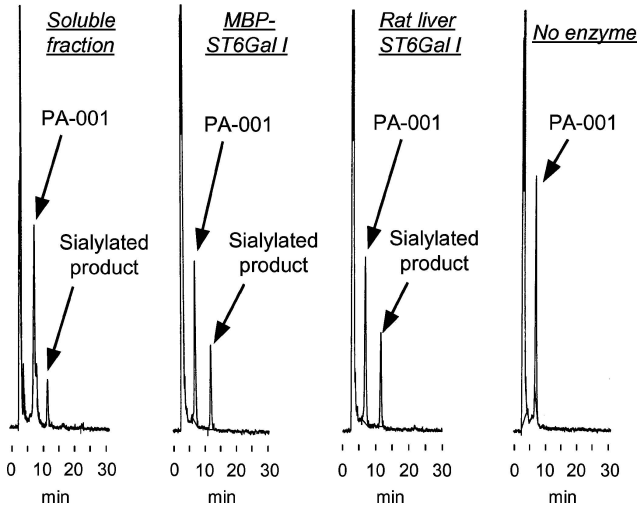

Figure 4. Sialylation of PA-sugar by soluble fraction prepared by disruption of cloned bacteria. Sialylation of pyridylaminated sugar chain (PA-001) as the substrate. Detection of sialylated products was performed by HPLC as described in Materials and methods. Elution profiles of PA-sugars shown are representative data of independent HPLC analyses. Sialylation of PA-001 in the mixture containing soluble fraction prepared from bacteria induced at 13°C, the purified MBP-tagged ST6Gal I or rat liver ST6Gal I as enzyme sources. No enzyme indicates that reaction mixtures do not contain any enzymes.

(Figure 3). Previous studies demonstrated that PA-sugar chains are good substrates for glycosyltransferases [22,30,31]. In Figure 4, under our HPLC conditions, PA-001 was commonly eluted around at 7 min post injection. The positive control enzyme, rat liver ST6Gal I consistently demonstrated a major sialylated product that was eluted at 11–12 min post injection. In some cases, a very weak signal was detected around at 23 min post injection, suggesting that the slower eluted signal is corresponded to di-sialylated PA-001 as described previously [22]. The reaction without enzyme did not showed any signals at either 11–12 min or 23 min, meaning that the detected signals are specifically derived from sialylation of PA-001 with enzyme added in the reaction mixtures. The soluble fraction from bacterial cells clearly showed a sialylated product around at 11 min post injection, similarly to rat liver ST6Gal I, indicating that the soluble fraction contains sialyltransferase activity. The MBP-tagged ST6Gal I purified by affinity column chromatography also demonstrated sialyltransferase activity. The slower signal eluted around at 23 min was similarly observed by the reaction with the purified MBP-tagged ST6Gal I when higher concentration of the enzyme was used for sialylation of PA-001 (data not shown). Taken together, these results indicate that the cloned bacteria induced at 13°C produce soluble and active form of the enzyme.

Purification of a soluble form of MBP-Tagged ST6Gal I from bacterial cells

The purification of MBP-tagged ST6Gal I from 9 liters of bacterial culture is summarized in Table 1. Two sialyltransferase activities were resolved by CM-Sepharose chromatography. Based upon the deduced amino acid sequences, the isoelectric points of MBP-tagged ST6Gal I and soluble ST6Gal I without MBP were 6.72 and 8.79, respectively. Under these elution conditions, MBP-tagged ST6Gal I was expected to be eluted faster

Table 1. Purification of a recombinant MBP-ST6Gal I

Step	*Volume (ml)*	*Protein (mg)*	*Activity (units[a])*	*Yield (%)*	*Specific activity (units/mg)*	*Purification*
Ultrasonic disruption	145.0	536.5	1.38	100	0.0026	1
Ammonium sulfate precipitation	27.0	299.7	N.T.[b]	–	–	–
CM-Sepharose (Fr. I)	80.0	8.7	0.78	56.3	0.095	36.5
Amylose resin	10.0	2.4	0.67	48.2	0.27	103.8

[a] One unit of activity is defined as 1 μmol/min of product formed with asialo-fetuin as substrate in 37°C, pH 6.0. Enzyme activities were determined by ELISA-based assay described in Materials and methods.
[b]Not tested.

than the soluble ST6Gal I. Western blotting of pooled fractions with anti-MBP antibody clearly showed that the faster peak (pool I) contained MBP-tagged ST6Gal I (lane 3 in Figure 5). On the other hand, there were no signals detected in the slower peak (pool II, lane 4 in Figure 5), indicating that the cleaved enzyme was still active. In this step, the tagged enzyme was obtained with a yield of about 60% with good purification (36.5-fold). The resolution of the enzyme by amylose-agarose chromatography was very sharp. Western blotting with anti-MBP antibody demonstrated that the peak obtained by maltose elution contained the MBP-tagged ST6Gal I (lane 5 in Figure 5). In this final purification step, the MBP-tagged ST6Gal I was obtained in a yield of 48% with good purification (over 100-fold). Although this preparation contained MBP, which was cleaved from the tagged enzyme, the active enzyme was purified (lane 5 in Figure 5). Typical data by HPLC-based sialyltransferase assay using the purified MBP-tagged ST6Gal I are shown in Figure 4. Overall, the purification of the tagged enzyme with a combination of CM-Sepharose and amylose-agarose was effective with regard to both time and effort. The specific activity

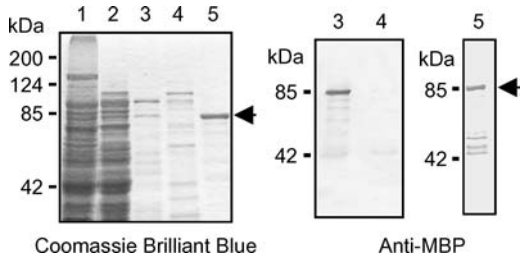

Figure 5. Purification of MBP-tagged ST6Gal I by CM-Sepharose and amylose-conjugated agarose. CM-Sepharose and Affinity column chromatography was performed as described in Materials and methods. In fractions from CM-Sepharose, the two sialyltransferases (I and II) were pooled as indicated and subjected to western blotting using anti-MBP antibody. The sialyltransferase resolved by amylose-conjugated agarose was pooled and subjected to western blotting using anti-MBP antibody. Lane 1, soluble fraction; lane 2, ammonium sulfate precipitates; lane 3, pool I in CM-Sepharose; lane 4, pool II in CM-Sepharose; lane 5, elution from amylose-conjugated agarose.

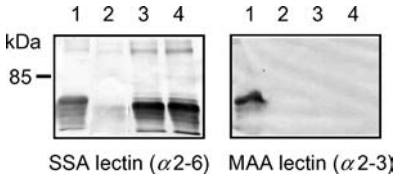

Figure 6. Resialylation of desialylated fetuin as a substrate. Detection of sialylated proteins was carried out using lectins, which react specifically with Neu5Acα2-6 (SSA lectin) and Neu5Acα2-3 (MAA lectin) residues as described in Materials and methods. Aliquots of reaction mixtures were subjected to SDS-PAGE on 8% separation gels. Proteins resolved on gels were then electrophoretically transferred onto PVDF membranes, followed by incubation with biotinylated lectins. Sialylated proteins were detected by incubation of the membranes with alkaline phosphatase-conjugated streptavidin. Lane 1, fetuin; lane 2, desialylated fetuin; lane 3, resialylation products from desialylated fetuin by rat liver ST6Gal I; lane 4, resialylation products from desialylated fetuin by recombinant MBP-tagged ST6Gal I.

of final preparation of MBP-tagged ST6Gal I is 0.27 U/mg protein. This value is apparently lower than purified rat liver ST6Gal I. However, lectin blotting and quantitative analyses revealed that the levels of incorporation of sialic acid into resialylated fetuin with the same concentration of both enzymes are similar (Figure 6). There is one possible explanation that sialylation assay conditions and donor substrate which define enzyme activity unit are different.

Characterization of the purified MBP-tagged ST6Gal I

Substrate specificity of the enzyme was determined by HPLC-based assay with the PA-sugar chains listed in Figure 3. The kinetic property was determined as the K_m value of purified MBP-tagged ST6Gal I against the substrate, PA-001. In the presence of Triton CF-54, the calculated K_m value against PA-001 was 5.3 μM. As shown in Table 2, similarly to rat liver ST6Gal I, longer and bi- or triantennary carbohydrate structures that commonly have Galβ1-4GlcNAc terminal residues were equally utilized by the purified MBP-tagged ST6Gal I. The reactivity against a shorter and linear carbohydrate with the same terminal residue as PA-001 or PA-002 showed much less activity for

Table 2. Substrate specificity and detergent requirement of MBP-ST6Gal I

	Rat liver ST6Gal I[a]		*MBP-ST6Gal I*[b]	
PA-sugar	*(+)*	*(−)*	*(+)*	*(−)*
PA-001	100	28.9	100	32.4
PA-002	108	28.9	76.5	17.6
PA-041	18.4	N.D.	14.7	N.D.
PA-042	N.D.	N.D.	N.D.	N.D.
PA-026	N.D.	N.T.	N.D.[c]	N.T.
PA-028	N.D.	N.T.	N.D.[c]	N.T.
PA-029	N.D.	N.T.	N.D.[c]	N.T.

[a,b]Values are indicated as relative activities of rat liver ST6Gal I and purifiedMBP-ST6Gal I to those with PA-001 as substrate in the presence of Triton CF-54 (0.25%), respectively.
[c]Enzymatic activities are measured using bacterial crude lysates containingMBP-ST6Gal I.
All enzymatic activities were measured by HPLC analysis described in Materials and methods.
Parentheses indicates the existence of Triton CF-54 (0.25%) in the reaction mixture.
N.D., Not detected; N.T., Not tested.

the enzyme. Other types of carbohydrate structures examined were not recognized or sialylated by the enzyme. These results indicated that the MBP-tagged ST6Gal I specifically transfers Neu5Ac to the non-reducing terminal of type II sugar chains, such as Galβ1-4GlcNAc residues. Table 2 also shows that Triton CF-54 is not an essential requirement for enzyme activity of MBP-tagged ST6Gal I although the activity was reduced in the absence of the detergent. To further investigate the requirement for Triton CF-54 for optimized enzyme activity, an ELISA-based assay was performed with immobilized asialofetuin as the substrate. MBP-tagged ST6Gal I demonstrated the transfer of Neu5Ac to immobilized asialofetuin even in buffer without detergent to the same extent as that in the presence of detergent at a concentration of 0.25% (data not shown). In contrast, the activity of ST6Gal I from rat liver was not detected in buffer without Triton CF-54, suggesting that the rat enzyme is susceptible to the presence of Triton CF-54. With the exception of the Triton CF-54 requirement for asialofetuin, the specific activity of soluble MBP-tagged ST6Gal I was comparable to that of ST6Gal I purified from the rat liver. The enzyme exerted 96% of original activity over 1 month later when MBP-tagged ST6Gal I was stored at -20°C in 100 mM NaCl, 1mM EDTA, 10 mM β-mercaptoethanol, 1 mM NaN_3, 50%(v/v) glycerol, 20 mM MES/NaOH buffer (pH 6.0), and 0.08% Triton CF-54.

Inhibition of influenza virus-induced hemagglutination with fetuin resialylated by the action of MBP-tagged ST6Gal I

Fetuin, a glycoprotein that contains three $N-$glycosylated sugar chains with both Neu5Acα2-6 and α2-3 residues, has been used to investigate the interaction between influenza viruses and host cell surface receptors [16]. Although this molecule can bind to both human and animal types of influenza virus, it contains two different Neu5Ac linkages, Neu5Acα2-6 and Neu5Acα2-3 [43–47]. Fetuin artificially generated to possess a single Neu5Ac linkage could be an ideal tool to study the molecular interactions between receptors and viruses. We applied the purified MBP-tagged ST6Gal I for generation of resialylated fetuin carrying a single Neu5Acα2-6Gal linkage. First, we prepared asialofetuin, designated desialylated fetuin, from fetuin with sialidase (*Arthrobacter ureafaciens*) and confirmed desialylation with lectins, MAA and SSA [48–51]. In Figure 6, desialylated fetuin was not significantly detected with MAA lectins (lane 2 on right panel). On the other hand, SSA lectin was observed to weakly react with desialylated fetuin (lane 2 on left panel). Since this lectin can also bind to Galβ1-4Glc or Galβ1-4GlcNAc terminal residue with much lower affinity [48,49], faint signal detected on the membrane was thought to be derived from desialylated terminal carbohydrate residues of fetuin, but not Neu5Acα2-6Gal residue. In fact, a highly sensitive fluorometric analysis did not show the existence of any residual sialic acid associated with desialylated fetuin (data not shown). These findings strongly suggested that sialidase treatment completed desialylation of fetuin under our experimental conditions. The desialylated fetuin did not affect influenza virus-mediated hemagglutination (Figure 7).

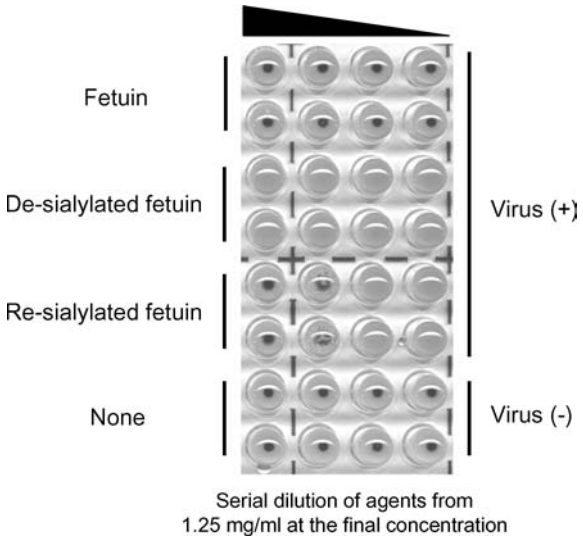

Figure 7. Inhibition of influenza virus-induced hemagglutination by fetuin resialylated with purified MBP-tagged ST6Gal I. Desialylated fetuin (2 mg) was incubated at 37°C for 3 h in a mixture (total volume 3 ml) containing purified MBP-tagged ST6Gal I (0.8 mg), 0.25 (w/v) % Triton CF-54, 1 mM $MnCl_2$, 500 μM CMP-Neu5Ac, 10 μM Neu2en5Ac, and 25 mM cacodylate buffer (pH 6.5). Resialylated fetuin was purified using Bio-beads SM-2 and amylose-conjugated agarose. Details of purification are described in Materials and Methods. Hemagglutination inhibition assay was carried out as described in Materials and methods. Test agents were serially diluted from protein at a final concentration of 1.25 mg/ml. The agents were pre-incubated at 4°C for 1 h with influenza virus, A/Memphis/1/71 (H3N2) and then subjected to hemagglutination assay.

Using MBP-tagged ST6Gal I, desialylated fetuin was resialylated to generate a homogeneous Neu5Acα2-6Gal linkage pattern (Figure 6, lane 4 on left panel), similarly to that using rat liver ST6Gal I (Figure 6, lane 3 on left panel). After resialylation of desialylated fetuin, the enzyme was readily removed by amylose-agarose chromatography. By a quantitative analysis of sialic acid, the level of incorporation of sialic acid into resialylated fetuin was 3% of that of native fetuin. Although the incorporation rate was low, the resialylated fetuin was then evaluated in hemagglutination inhibition assay. Figure 7 shows that resialylated fetuin was restored to inhibit virus-mediated hemagglutination at a protein concentration of 0.625 mg/ml. In conclusion, the purified MBP-tagged ST6Gal I generated an artificial fetuin carrying a homogeneous Neu5Acα2-6 linkage pattern that can be a valuable tool for the study of human influenza virus-receptor interactions.

Discussion

Functional soluble glycosyltransferases, including sialyltransferases, derived from mammalian species have not been successfully expressed in *E. coli.* Previous studies demonstrated that the recombinant enzymes are expressed but transported into inclusion bodies from which the proteins cannot be solubilized with mild detergents [22,27]. Here, we examined the expression of both active and soluble forms of recombinant ST6Gal I, a human-derived sialyltransferase, in bacterial cells. For our purposes, both the cytosolic region and transmembrane domain were removed, resulting in a soluble form of the enzyme containing the catalytic site. The bacterial clone, pSTMX harboring the gene encoding a truncated enzyme fused with MBP as a purification tag, was established and used for expression of the enzyme. The low temperature induction method improved solubility and tagging of the recombinant enzyme expressed in *E. coli*.

The expressed protein was effectively purified to nearly homogeneity by a combination of CM-Sepharose and amylose-agarose column chromatography. In the protein fraction purified by amylose-agarose column chromatography, a few minor bands with faster mobility on the gel were stained with anti-MBP antibody, indicating that they were originally derived from MBP-tagged ST6Gal I and then proteolytically degraded during purification. To enhance the yields of the protein, further improvements of induction and purification conditions are required. For further purification, factor Xa protease could be useful. Human ST6Gal I does not contain recognition sequences by this protease. On the other hand, a single cleavage site is located in the junction of MBP and ST6Gal I. The combination of factor Xa digestion and gel exclusion chromatography could provide us higher purified enzyme suitable for studies such as NMR or X-ray diffraction.

Since recombinant proteins expressed in *E.coli* are unglycosylated, there is a possibility that defect in glycosylation alters solubility and stability of MBP-tagged ST6Gal I. Under our conditions, low temperature induction significantly improved the solubility of the enzyme. A simple solubilization method, ultra-sonication was effective to solubilize the enzyme from bacterial cells without detergents. In terms of stability, no significant loss of enzyme activity was observed over 1 month from enzyme preparation. These results suggest that defect in glycosylation does not seriously affect properties of MBP-tagged ST6Gal I.

A previous study has demonstrated that removal of up to 80 amino acid residues from N-terminus of human ST6Gal I does not alter the recognition parameters such as substrate specificity and K_m value [52]. MBP-tagged ST6Gal I lacks of 43 amino acid residues from N-terminus of wild type protein. As expected, the enzymatic properties of the soluble MBP-tagged ST6Gal I were comparable to those of rat liver ST6Gal I, with regard to substrate specificity and stability. K_m value of the recombinant enzyme (5.3 μM) against PA-sugar 001 was similar level to those of other species of ST6Gal I and truncated ST6Gal I expressed in mammalian or yeast cells [22,52,53]. The detergent requirement of the protein was different from those of ST6Gal I purified previously [11,22]. The most significant characteristic was that MBP-tagged ST6Gal I did not have an absolute for detergent, such as Triton CF-54, for its enzyme activity, implying that the soluble enzyme obtained in this study could also be used for cellular experiments, e.g., sialylation of the cell surface directly.

Yield of recombinant soluble ST6Gal I expressed in mammalian cells is much higher than that in our expression system [52]. There seems several reasons. First, in mammalian expression, the genes introduced into cells were transiently expressed. Second, under our expression conditions, low temperature induction improved solubility and tagging of activity of the enzyme, but considerably reduced yield of the enzyme protein. However, bacterial expression system we established in this study is possibly valuable to produce large amount of the enzyme according to the following points. (i) It is not necessary to transfect the gene in each experiment for production of the enzyme. (ii) The effort and cost of large culture of bacterial cells is much lower than those of mammalian cells. (iii) Specific equipments and facilities for large culture such as clean room, clean bench and CO_2 incubator are not required to produce the enzyme expressed in *E. coli*.

In terms of large production of soluble ST6Gal I, Malissard et al have expressed the enzyme in yeast cells [54]. Although yield (0.3 units/liter) of the enzyme expressed in yeast is twice higher than that expressed in *E. coli*, the enzyme produced in bacteria has some merits to apply for generation of artificial carbohydrate molecules. First, the effort and cost of culture of bacterial cells are lower than those of yeast cells. Second, in the cases of *in vivo* and *in vitro* applications of artificial carbohydrates sialylated with recombinant enzymes, N-glycan on ST6Gal I expressed in yeast cells would be immunogens against animals and would affect carbohydrate-mediated phenomena if the enzyme were not completely removed from sialylated products. Carbohydrate products sialylated with the enzyme expressed

in bacteria might be easily handled for purification and application.

To evaluate the utility of the expressed enzyme, we thought it of value to explore its application in an examination of the influenza virus-receptor interaction with a sialylated structure. Several lines of evidence have demonstrated that ST6Gal I catalyzes sialylation of *N*-linked carbohydrate chains of glycoproteins, particularly Galβ1-4GlcNAc terminal residues, but not *O*-linked residues [11,12,53,55,56]. The results of lectin blotting suggest that the transfer of sialic acid residues with soluble recombinant ST6Gal I results in the expression of Neu5Acα2-6Galβ1-4GlcNAc terminal residues on *N*-linked carbohydrates of fetuin. The incorporation rate of sialic acid into desialylated fetuin was only 3% of that of native fetuin. This degree of incorporation corresponded to about 0.3 mol of sialic acid/mol fetuin. It was demonstrated previously that ST6Gal I purified from the bovine colostrums transferred sialic acid into asialofetuin at 7.5 mol/mol fetuin [56]. There is one possible explanation for the low incorporation rate observed in this study. As our sialylation mixture contained residual sialidase, which was used to generate desialylated fetuin, it might still effectively hydrolyze sialic acid residues transferred into desialylated fetuin to some extent during purification and storage even in the presence of Neu2en5Ac. In this case, removal of sialidase from the mixtures containing desialylated fetuin by α1-acid glycoprotein-conjugated agarose column chromatography as described previously [56] may promote the incorporation rate of sialic acid into fetuin. In the present study, although the degree of sialylation was not high, resialylated fetuin significantly blocked the interaction of human-type influenza virus with erythrocytes. These results indicated that Neu5Acα2-6Galβ1-4GlcNAc terminal residues on N-linked carbohydrates of fetuin are sufficiently functional for analysis of virus-receptor interactions.

In conclusion, the bacterial expression systems established in this study provide soluble and active glycosyltransferases as well as MBP-tagged ST6Gal I. The recombinant enzymes could be used for wide range of studies on the mechanisms of not only virus-cell interactions but also cell-cell interactions and the molecular mechanisms on catalytic reaction.

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