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Expression of Mouse Galβ1,4GlcNAc α2,6-Sialyltransferase in an Insoluble Form in *Escherichia coli* and Partial Renaturation

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Abstract—Mouse Galβ1,4GlcNAc α2,6-sialyltransferase was produced in an insoluble form in *Escherichia coli* cells harboring expression plasmids. The insoluble protein was solubilized with 8 M urea and diluted for renaturation of the enzyme. The substrate specificity and kinetic parameters, except for the specific activity, of the renatured enzyme were similar to those of the enzyme obtained from rat liver. These results suggest that a bacterial expression system is a potentially powerful tool for the large scale production of sialyltransferases and for elucidating the molecular mechanisms of sialyltransferases.

Introduction

Galβ1,4GlcNAc α2,6-sialyltransferase (EC 2.4.99.1) catalyzes the incorporation of sialic acids at the terminal positions of glycoconjugates with NeuAc α2,6-Gal linkage. cDNA sequences from mouse,[1] rat,[2] human,[3] and chicken[4] along with the genomic DNA sequence,[5] and tissue specific alternative splicing in rat[6] have been described. Also, the domains responsible for localization to the Golgi apparatus and the acceptor substrate specificity, with synthetic acceptors, have been reported.[7,8] Cloned sialyltransferases have been expressed in cultured eukaryotic cells.[2,4] However, there are some advantages to producing the enzyme in bacteria, i.e. the low cost, easy handling and easy scale up for mass-production. To gain further insight into the structure and function relationship, we attempted to synthesize a recombinant sialyltransferase in *Escherichia coli.* Some other glycosyltransferases of eukaryotes have been expressed in bacteria as soluble forms in the cytosol[9] and periplasm,[10] and as insoluble forms.[11]

In this study, mouse Galβ1,4GlcNAc α2,6-sialyltransferase was produced in an insoluble form in *Escherichia coli,* and several renaturing conditions were examined as to formation of an active enzyme.

Materials and Methods

Rat liver Galβ1,4GlcNAc α2,6-sialyltransferase, fetuin, asialo-fetuin, bovine submaxillary mucin, α1-acid glycoprotein, galactose β1,3-*N*-acetylgalactosamine, lacto *N*-tetraose and *N*-acetyllactosamine were from Sigma (St Louis, U.S.A.). Urea was purchased from Wako Pure Chemicals (Osaka, Japan) and a solution was prepared just before use. CMP-[^{14}C]NeuAc (11 GBq/mmol) was from Amersham (U.K.). Bovine submaxillary asialo-mucin and asialo-α1-acid glycoprotein were obtained by mild acid treatment of the respective glycoproteins. *N*-Acetylgalactosamine β1,4-galactose was a kind gift from Dr Kajimoto (RIKEN). Pyridylamino oligosaccharides (PA-sugar 001, 021, 022 and 023) were from Takara (Kyoto, Japan). Protein concentrations were determined with a BCA protein assay kit (Pierce), with bovine serum albumin as the standard. Dialysis tubing (20/32) was from Viskase.

Plasmid construction

An initiation codon and cloning sites were attached by means of the PCR method to mouse brain Galβ1,4GlcNAc α2,6-sialyltransferase cDNA[1] with a sense primer (5'-TGGCATATGGGGAGCGACTATGAGGCTCT-3', containing an *Nde*I site) and an antisense primer (5'-ATGAGGATCCCTGGCTCAACAGCG-3' containing a *BamH*I site). The resulting PCR fragment (1152bp), that has the initiation codon and codes for a polypeptide from the 29th amino acid residue to the C-terminal end of the enzyme, which lacks the cytosolic and transmembrane domains, was incorporated into expression vector pET3b[12]

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Abbreviations: GalNAc, *N*-acetylgalactosamine; CMP-NeuAc, cytosine 5'-monophospho-*N*-acetylneuraminic acid; (all sugars are of the D-configuration); HPLC, high performance liquid chromatography; DTT, dithiothreitol; MOPS, 3-morpholinopropanesulfonic acid; MES, 3-morpholinoethanesulfonic acid.

at the *NdeI–BamHI* site that is located downstream of the T7 promoter to produce pET3-MBS.

Expression

pET3-MBS was transfected to JM109(DE3). The cells were cultured in 100 mL LB medium (with 100 μg/mL ampicillin) at 37 °C. When the optical density at 600 nm reached 0.2–0.4, production of the recombinant protein was initiated with induction of T7 RNA polymerase by the addition of 2 mM IPTG (isopropyl β-D-thiogalacto-pyranoside). After 2 h cultivation, the cells were harvested. The cells (*ca* 1 g wet weight) were then suspended in 10 mL of 20 mM Tris-HCl pH 8.0 and treated with lysozyme (0.1 mg/mL) and DNase I (0.01 mg/mL) for 30 min, and Triton X-100 was added to a final concentration of 1%. The insoluble fraction was collected by centrifugation at 12,000 *g* for 15 min at 4 °C. The precipitate was suspended in 3 mL of 10 mM Tris-HCl pH 7.4 and stored at -30 °C until use.

Solubilization and renaturation

To 0.5 mL of the above suspension, 0.48 g solid urea, 60 μL 5 M NaCl, 20 μL 1 M Tris-HCl, pH 7.4 and water were added to final volume of 1 mL (final concentration; 8 M urea, 0.3 M NaCl, 20 mM Tris-HCl, pH 7.4). The precipitate was extracted for 30 min at 10 °C, followed by centrifugation at 12,000 *g* for 15 min. The 0.1 mL aliquots of extract were diluted with the 1.9 mL of each renaturation buffer (standard composition; 0.5 M NaCl, 10 mM lactose, 0.5 mM EDTA, 20 mM MOPS–NaOH, pH 7.0) with 2 M urea (usually a final protein concentration was about 0.02 mg/mL). The solution was left at 4 °C for 12 h and then diluted again with an equal volume of the renaturation buffer to reduce the urea concentration to half (final concentration of urea was 1.2 M), and then left at 4 °C for a further 48 h. Then, sialyltransferase activity was measured to analyze the effects of the composition of the renaturation buffer at this point.

Finally, the enzymes were dialyzed against the renaturation buffer to gradually remove residual urea and reducing agents over 48 h at 4 °C. The samples were then concentrated approximately 20 times with Centricon-30 (Amicon).

Sialyltransferase assay

The activity of the sialyltransferase was measured with 50 μM CMP-[^{14}C]NeuAc (0.9 Bq/pmole) as a donor substrate, 5 mM Galβ1,4GlcNAc (*N*-acetyllactosamine) as an acceptor substrate, 1 mg/mL bovine serum albumin, 1 μL of the enzyme solution, and 50 mM sodium cacodylate, pH 6.0 in a total volume of 10 μL, with incubation at 37 °C for 1 h. Then the samples were subjected to HPTLC (silica gel 60, Merck), with development with ethanol/pyridine/*n*-butanol/acetic acid/water (100:10:10:3:30), and then the radioactivity transferred was determined with a radio image analyzer, BAS2000 (Fuji Film).[13] One unit of activity was defined as 1 μmol of sialic acid transferred per min.

The acceptor preference as to oligosaccharide branches was examined with an *N*-acetyllactosamine type biantennary pyridylamino-oligosaccharide as an acceptor substrate and analyzed fluorophotometrically by HPLC as described previously.[4]

Results

Expression and extraction of the sialyltransferase

The recombinant enzyme, which lacks the cytosolic and transmembrane domain was accumulated in the form of insoluble inclusion bodies in *E. coli* cells. The growth rate of the JM109(DE3) cells harboring pET3-MBS was indistinguishable from that of the original JM109(DE3) cells on both agar plates and liquid culture. The insoluble fraction of the cell lysate was washed with 1% Triton X-100 and then extracted by 8 M urea. Most of the extracted protein was a 42k dalton protein (Figure 1). Although only 8 M urea extract was used for renaturation experiment, 80% of the enzyme could be extracted with 5.7 M urea buffer.

Renaturation of the sialyltransferase

When the 8 M urea extract was dialyzed without dilution at 4 °C to slowly remove urea, the enzyme precipitated at a urea concentration of less than 0.5 M, and very little activity was recovered.

The activity was measured to determine the optimum dilution conditions at 48 h after second dilution (Table 1A). The maximum renaturation was observed at pH 7.0, with 0.5 M NaCl, and these compositions were chosen for further experiments. Three independent renaturation experiments, carried out with this condition, showed total recovered activities with 0.4–0.8 mU/0.1 mL extracts. At this stage of renaturation, the enzyme showed high K_m values for CMP-NeuAc and *N*-acetyllactosamine (0.14 mM and 20 mM, respectively). Also, under the conditions tested, reducing agents (DTT and β-mercaptoethanol) were inhibitory for the enzyme activity. This may have been due to the carry-over of urea, the concentration of which was 0.1 M in the assay mixture. Very little activity was observed at 12 h after the second dilution, indicating the refolding process is very slow at this temperature.

The addition of 1 μM and 1 mM reducing reagent, and subsequent removal of both urea and the reducing agent gave about the same specific activity as without a reducing agent (Table 1B).

Characteristics of the renatured sialyltransferase

The substrate specificity and kinetic parameters were similar to those of the enzyme obtained from rat liver (Tables 2 and 3).

Galβ1,4GlcNAc α2,6-sialyltransferase was reported to distinguish between the different branches of biantennary glycopeptides of the *N*-acetyllactosamine type.[4,14,15] The renatured enzyme also prefers galactose residues on Manα1,3 branches over ones on Manα1,6 branches (Figure 2).

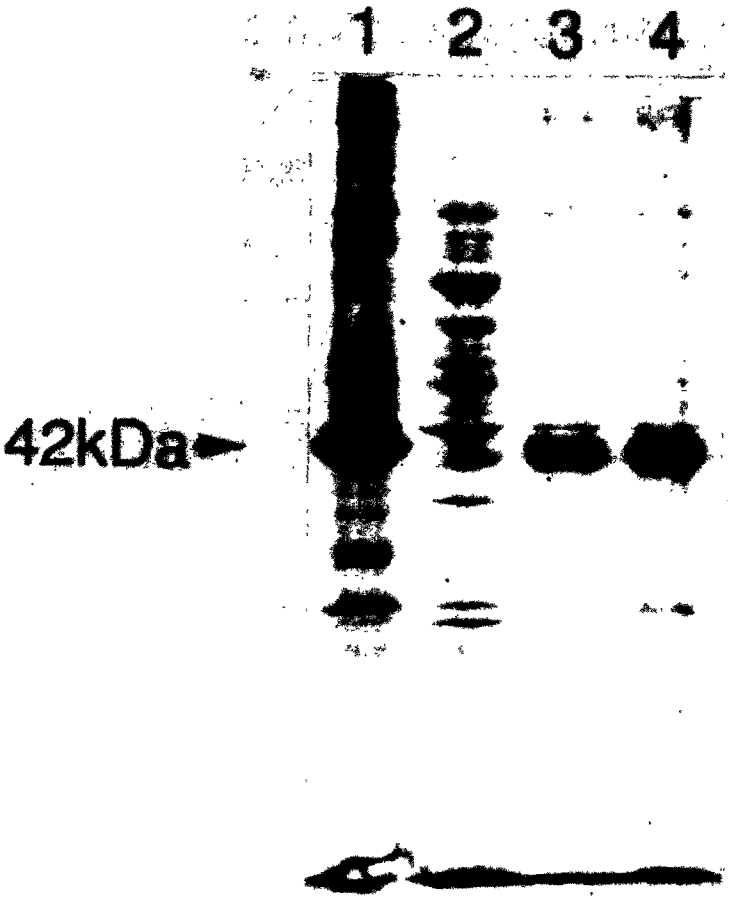

Figure 1. Expression of a mouse Galβ1,4GlcNAc α2,6-sialyltransferase in *Escherichia coli*, and its extraction. SDS-polyacrylamide gel (10%) electrophoresis of various fractions of *E. coli* lysate. Lane 1, insoluble fraction of the cell lysate; lane 2, soluble fraction plus Triton X-100 extract; lane 3, 5.7 M urea extract; lane 4, 8 M urea extract

Table 1. The effects of various conditions on renaturation of Galβ1,4GlcNAc α2,6-sialyltransferase

(A) The 8M urea extract was diluted with 20 vol. of the various renaturation buffer. The samples were left at 4 °C for 12 h then diluted again with respective renaturation buffer to reduce the urea concentration to half, left again for further 48 h, and then were analyzed. The standard renaturation buffer contained; 2 M urea, 20 mM Tris-HCl pH 7.4, 0.3 M NaCl, 20 mM lactose and 0.5 mM EDTA. The deviation from the standard composition is indicated.

*A value of 0 indicates less than 5% of the control

Renaturation conditions	Relative activity (compared to standard)
standard composition	1
pH9.5, Tris-HCl 20mM	0*
pH8.0, Tris-HCl 20mM	0.6
pH7.0, MOPS-NaOH 20mM	2.5
pH6.0, MES-NaOH 20mM	1.5
0.5M NaCl	2
0.1M NaCl	0.2
0.01M NaCl	0
0mM lactose	0.5
1M urea	1.5
0M urea	0.6

(B) The 8M urea extract was diluted with 20 vol. of the renaturation buffer, 2 M urea, 20 mM MOPS–NaOH, pH 7.0, 0.5 M NaCl, 20 mM lactose and 0.5 mM EDTA, with or without reducing reagents. The samples were left at 4 °C for 12 h and then diluted again to reduce the urea concentration to half, and then dialyzed against the renaturation buffer to remove residual urea and reducing reagents, and finally analyzed.

Reducing reagent	Specific activity (mU/mg)
None	7
1μM DTT	6
1mM DTT	12

Table 2. The substrate specificity of renatured mouse Galβ1,4GlcNAc α2,6-sialyltransferase

The assays were carried out with 2 mg/mL of each substrates as described in Materials and Methods. HPTLC was performed with ethanol/pyridine/*n*-butanol/acetic acid/water (100:10:10:3:30) when oligosaccharides and glycoproteins were used as acceptors, and with chloroform/methanol/0.5% $CaCl_2$ (55:45:8) when glycolipids were the acceptors.

*The activity is expressed relative to that of Galβ1,4GlcNAc.
**A value of 0 indicates less than 2% of the control.

Substrate	Relative Activity*	
	Renatured mouse Galβ1,4GlcNAc α2,6-sialyltransferase	Rat liver Galβ1,4GlcNAc α2,6-sialyltransferase
fetuin	0.25	0**
asialofetuin	1.5	0.97
α1 acid glycoprotein	0.1	0.1
asialo-α1 acid glycoprotein	2.1	1
bovine submaxillary mucin	0	0
bovine submaxillary asialo-mucin	0	0
lacto *N*-tetraose	0	0
Galβ1,4GlcNAc	1	1
Galβ1,3GalNAc	0	0
GalNAcβ1,4Gal	0	0
Galβ1,4Glc	0	0
Gal	0	0

Table 3. The kinetic parameters of renatured Galβ1,4GlcNAc α2,6-sialyltransferase

*Measured with *N*-acetyllactosamine as the acceptor.
**Concentration expressed as terminal galactose residues.

Substrate	Km (mM)	
	Renatured mouse Galβ1,4GlcNAc α2,6-sialyltransferase	Rat liver Galβ1,4GlcNAc α2,6-sialyltransferase
CMP-NeuAc*	0.08	0.04
N-acetyllactosamine	6.5	5
asialo-α1 acid glycoprotein**	0.4	0.2

Table 4. The effects of various reagents on Galβ1,4GlcNAc α2,6-sialyltransferase activity

The activity is expressed relative to that of a no reagent control.

Reagent	Renatured mouse Galβ1,4GlcNAc α2,6-sialyltransferase	Rat liver Galβ1,4GlcNAc α2,6-sialyltransferase
Reducing agent		
DTT (1mM)	1.0	0.9
(1μM)	1.1	1.2
Mercaptoethanol (1mM)	1.1	1.1
(1μM)	1.0	1.1
Detergent		
Triton X-100 (1%)	1.5	0.8
(0.5%)	1.4	1.4
(0.1%)	1.3	1.3
Divalent cations		
$MgCl_2$ (5mM)	11	1.0
$MnCl_2$ (5mM)	13	1.1
EDTA (5mM)	1.7	0.9

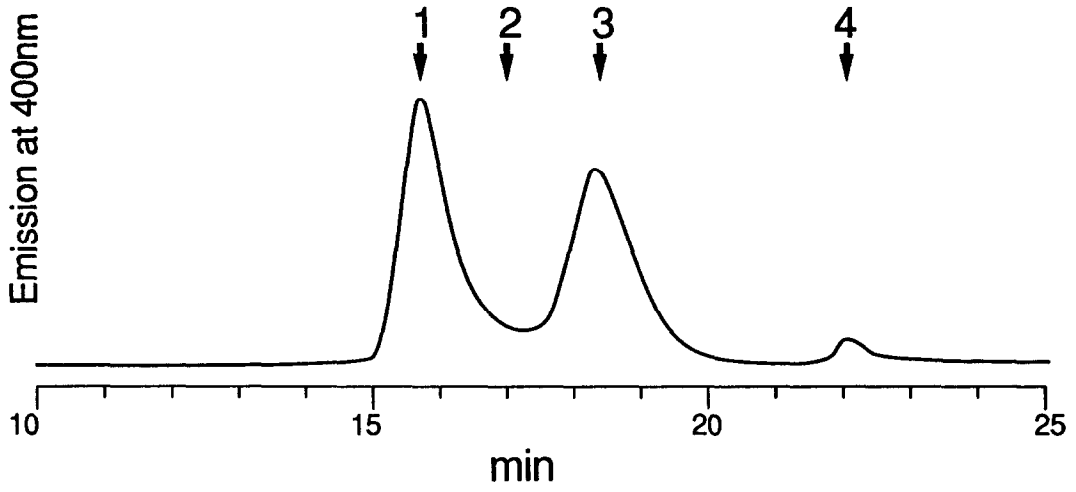

Figure 2. HPLC analysis of the sialylated PA-oligosaccharides. A desialylated biantennary PA-oligosaccharide (001) was sialylated and then analyzed with HPLC. The assays were performed with 10 pmoles of acceptor substrates and 0.1 mM CMP-NeuAc in a final volume of 5μL. The assay mixtures were incubated at 37 °C for 1 h, and the reaction was stopped with 90 μL of cold water. Each assay mixture was then analyzed to identify sialylated pyridylamino oligosaccharides with an HPLC (Tosoh, Japan) equipped with a reversed-phase column (Shimpack CLC-ODS, 0.6 cm x 15 cm Shimazu, Japan). The column was equilibrated with a mixture of 70% solvent A (10 mM sodium phosphate, pH 3.8) and 30% solvent B (0.5% *n*-butanol, 10 mM sodium phosphate, pH 3.8), and eluted at the flow rate of 1 mL/min with a linear gradient of solvent B to 60% over 30 min at 55 °C. Pyridylamino oligosaccharides were detected fluorophotometrically with excitation at 320 nm and emission at 400 nm. The arrows, 1, 2, 3 and 4 indicate the elution position of the standard PA-sugars, 022 (monosialylated at the Manα1,3 branch), 023 (disialylated), 001 (desialylated) and 021 (monosialylated at the Manα1,6 branch), respectively

On the complete removal of urea, the enzyme regained its resistance to reducing agents (Table 4). Remarkably, over 10 times activation was observed with divalent cations. It is assumed that prolonged dialysis against buffer containing 0.5 mM EDTA in the presence of urea may cause detachment of tightly bound divalent cations, which are required to maintain the proper conformation of the enzyme. The enzyme in the renaturation buffer with 1.2 M urea was also activated by these divalent cations (data not shown).

The specific activity of the renatured enzyme was 0.15 U/mg protein (measured with 5 mM $MnCl_2$), which is about 2% of that of the enzyme obtained from rat liver.[16] The overall recovery of the enzyme was 0.1 U/100 mL culture medium.

Discussion

In this study, mouse Galβ1,4GlcNAc α2,6-sialyltransferase was produced in an insoluble form in *Escherichia coli* cells. The product was purified as an insoluble protein, solubilized with 8 M urea, and renatured to give the active enzyme.

The specific activity of the renatured enzyme was much lower than that of the rat liver enzyme, indicating the renaturation is only partial and further optimization of the renaturing conditions is required. Sialyltransferases so far known have at least one highly conserved pair of cysteines, which are suggested to form a disulfide linkage.[17] Therefore, the reduction followed by the oxidation of disulfide linkages during the denaturation–renaturation process was expected to be essential and effective for recovery of the enzyme. Although our results in Table 1B did not support the idea, it is still possible that careful titration of the reduced/oxidized ratio of thiol reagents during the renaturation process could improve the recovery, as in the case of some proteins.[11,18]

It was reported that the attached carbohydrate chain is important in controlling the activity of the sialyltransferase and that it is part of the mechanism regulating the compartment specific expression of the activity in the Golgi apparatus.[19] Our results indicate the possibility that the enzyme can be activated without the carbohydrate chain under certain conditions. In addition, the susceptibility to reducing agents in partially denatured state and the requirement of divalent cations for the proper conformation provided some clue for understanding the structure–function relationship of this enzyme.

It should be noted that unlike other glycosyltransferases, sialyltransferases share very conserved regions (sialyl-motif)[20] within their active domains, and are expected to form similar higher-order structures.[17] It is conceivable that the renaturing procedure for one sialyltransferase is applicable to other sialyltransferases.

Although the recovery rate so far obtained is far from satisfactory, the results suggested the potential of a bacterial expression system in the production of large amounts of functional sialyltransferases.

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