

Cold-shock Protein Expression System Facilitates the Solubility of Human ST6Gal I in *Escherichia Coli*

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Abstract: The protein structures of most mammalian sialyltransferases have yet to be elucidated. Practical and convenient protein expression systems for soluble and active sialyltransferases will facilitate elucidation of the protein structures and catalytic mechanisms of these enzymes. The present study was performed to establish an efficient expression system for human ST6Gal I (hST6Gal I). cDNA encoding a soluble form of hST6Gal I was introduced into the bacterial expression vector pCold I carrying the cold shock promoter that is inducible by low-temperature conditions. The resultant DNA encodes the enzyme fused in frame with a maltose-binding protein (MBP) as a purification tag. This expression plasmid was introduced into the *E. coli* strain pGro7/BL21 harboring the molecular chaperones GroES and GroEL. Combined use of chaperone proteins and low-temperature cultivation during IPTG induction significantly improved the functional enzyme solubility in bacteria. The MBP-tagged hST6Gal I was efficiently purified by affinity chromatography using amylose-conjugated agarose.

Keywords: Sialyltransferase, cold shock vector, low-temperature induction, bacterial expression system.

INTRODUCTION

The sialyltransferase ST6Gal I catalyzes the transfer reaction of sialic acid to the non-reducing terminal Gal β 1-4GlcNAc residues of glycoconjugates with α 2,6 linkage [1]. Carbohydrate structures containing Neu5Ac α 2-6 residues play critical roles in many biological events such as cell-cell recognition, cancer metastasis, immunological responses, and cell-pathogen interactions [2-9]. In addition, medical applications of sialyltransferase, such as modification of therapeutic proteins, have attracted a great deal of attention in recent years [10-12]. To understand the catalytic mechanisms of action of sialyltransferase, it is indispensable to elucidate the protein structures of these enzymes. Very recently, the protein structures of some bacterial sialyltransferases [13-15] and a mammalian α 2,3 sialyltransferase [16] have been reported. However, the structures of other sialyltransferases, including human ST6Gal I (hST6Gal I), have not been determined. A convenient and efficient ST6Gal I expression system is indispensable to elucidate the protein structures and catalytic mechanisms of these enzymes.

In this study, we established an efficient expression and purification system for soluble and active recombinant hST6Gal I in *Escherichia coli* utilizing the bacterial expression vector pCold I. This vector significantly contributed to the production of soluble and active recombinant hST6Gal I.

MATERIALS AND METHODS

Materials

Pfu DNA polymerase was purchased from Promega (Madison, WI). Rat liver α 2,6 sialyltransferase was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Rabbit anti-MBP antibody was purchased from New England Biolabs, Inc. (Ipswich, MA). Biotin-conjugated lectin, *Sambucus sieboldiana* agglutinin (SSA), was purchased from Seikagaku Corporation (Tokyo, Japan). All other chemicals were of the highest purity available.

Cloning of the Gene Encoding MBP and Soluble form of ST6Gal I

The primers for polymerase chain reaction (PCR) were designed according to the nucleotide sequence of hST6Gal I. The sequences of primers used to amplify the soluble form of hST6Gal I cDNA were as follows: forward primer containing a *Bam*HI site (5'-TCATGGATCCGAATTCAGGT-GTTAAAGAG-3'), reverse primer containing an *Xba*I site (5'-TCATTCTAGATTAGCAGTGAATGGTCCGG-3'). PCR was performed using the cDNA in pSTMX [17] as a template for 25 cycles of denaturation (94°C, 1 min), annealing (51°C, 1 min), and extension (72°C, 1.5 min), followed by incubation at 4°C. The amplified DNA was ligated with linearized pGEM-T vector (Promega) according to the manufacturer's instructions. The nucleotide sequence of the isolated cDNA clone was confirmed by sequencing of both strands.

MBP was amplified by PCR using the following primers: forward primer containing an *Nde*I site (5'-TCATCATATG-

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AAAATAAAACAGGTGC-3'), reverse primer containing a *Bam*HI site and a cleavage site for HRV 3C protease (5'-TCATGGATCCGGAGGGCCCCTGGAACAGAACTTCCAGCGCCGCCGAGTCTGCGGTCTTTTCAG-3'). The amplified DNA fragment was ligated into the pGEM-T vector and confirmed by sequencing.

Construction of Expression Vector

The expression vector for recombinant hST6Gal I was prepared as follows. The DNA fragment encoding the soluble form of hST6Gal I was excised from hST6Gal I-pGEM-T with *Bam*HI/*Xba*I digestion, and purified by agarose gel electrophoresis. The DNA fragment encoding MBP was excised from MBP-pGEM-T with *Nde*I/*Bam*HI digestion, and purified by agarose gel electrophoresis. The hST6Gal I fragment and MBP fragment were then subcloned into the *Nde*I – *Xba*I sites of the expression vector pCold I.

Establishment of a Bacterial Clones Expressing hST6Gal I

The BL21-derived *E. coli* strain pGro7/BL21 (Takara Bio, Inc., Otsu, Japan) expressing GroEL and GroES was used as the host. We introduced a plasmid harboring the gene encoding the soluble form of hST6Gal I and MBP. The resultant clone was designated hST6Gal I/pGro7. We also introduced the same plasmid into similar *E. coli* strains, including pTf16/BL21, pG-Tf2/BL21, pKJE7/BL21, and pG-KJE8/BL21 and obtained hST6Gal I/pTf16, hST6Gal I/pG-Tf2, hST6Gal I/pKJE7, and hST6Gal I/pKJE8, respectively.

Expression of Soluble hST6Gal I Protein

In small-scale culture, a single colony of each clone was inoculated into Luria Broth (LB) medium supplemented with 100 µg/mL of ampicillin and 20 µg/mL chloramphenicol (LBampCm), and precultured at 37°C for overnight. Then, 0.5 mL of preculture was added to 9.5 mL of freshly prepared LBampCm supplemented with 0.5 mg/mL arabinose or 0.5 ng/mL tetracycline (according to the manufacturer's instructions), and cultured at 37°C until OD at 600 nm (OD₆₀₀) reached 0.4 – 0.5. The protein expression in bacteria was induced at 15°C for 24 h in the presence of IPTG at a final concentration of 1 mM. In large-scale culture for purification of the enzyme, several colonies on the clone plate were inoculated into 50 mL of LBampCm and cultured overnight at 37°C. Then, 25 mL of preculture was added to 475 mL of freshly prepared LBampCm supplemented with 0.5 mg/mL arabinose or 0.5 ng/mL tetracycline and cultured at 37°C until OD₆₀₀ = 0.4 – 0.5. The protein expression in bacteria was induced at 15°C for 24 h in the presence of IPTG at a final concentration of 1 mM.

ELISA-based Sialyltransferase Assay

Quantitative enzyme assay for immobilized glycoproteins as acceptors was carried out as described previously [18, 19]. Desialylated α1-acid glycoprotein as an acceptor at a concentration of 10 µg/mL was immobilized on 96-well plastic plates (Maxisorp; Nunc Inc., Roskilde, Denmark). The enzyme reaction was carried out in the wells according to the procedure of Weinstein *et al.* [20] with slight modifications. The reaction mixture had the following final concentrations in a total volume of 50 µL: 50 µM CMP-Neu5Ac, 0.5%

(w/v) Triton CF-54, 25 mM MES-NaOH, pH 6.5, and 0.6 µg of target protein.

Purification of Soluble Recombinant hST6Gal I

All operations described below were carried out at 4°C or on ice. For small-scale preparation of the soluble fraction, bacterial cell pellets were suspended in 10 mL of a sonication buffer containing 150 mM NaCl, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 20 mM Tris-HCl (pH 7.5) and pulse-disrupted using an ultrasonifier until the solution became clear. The solution was centrifuged and the supernatant was used for the sialyltransferase assay.

For large-scale production, the bacterial cell pellet was suspended in 100 mL of sonication buffer and pulse-disrupted with an ultrasonifier until the solution become clear. The solution was centrifuged and the supernatant was subjected to affinity column chromatography as follows. The supernatant of induced *E. coli* was applied to a column of amylase-conjugated agarose equilibrated in a buffer (TBS) containing 150 mM NaCl, 25 mM Tris-HCl (pH 7.5). The retained sialyltransferase was eluted with TBS containing 10 mM maltose. Fractions containing hST6Gal I were collected and dialyzed against TBS. Then, the fraction was reloaded onto the amylase conjugated agarose in a similar manner. Finally, hST6Gal I was eluted, mixed to give a final concentration of 50% glycerol and 0.08% Triton CF-54, and stored at –80°C, until use.

Digestion with HRV 3C Protease

One unit of HRV 3C protease (Novagen, Darmstadt, Germany) was used for digestion of purified hST6Gal I. HRV 3C was added to purified hST6Gal I in HRV 3C protease cleavage buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5). The reaction mixture was incubated overnight at 4°C. The reaction products were then analyzed by SDS-PAGE and Coomassie brilliant blue staining.

RESULTS

Strategy for Cloning of the Gene Encoding Soluble hST6Gal I Into the Bacterial Vector pCold I

The soluble form of the enzyme was fused to maltose-binding protein (MBP) as a purification tag. We introduced the genes encoding MBP and the soluble form of hST6Gal I into the plasmid expression vector pCold I (Fig. 1). The plasmid DNA was introduced into the BL21-derived *E. coli* strain pGro7/BL21, which expresses GroEL and GroES. The resultant host bacteria carry the plasmid harboring the gene encoding the soluble form of hST6Gal I and MBP, and the obtained clone was designated hST6Gal I/pGro7. We also introduced the same plasmid DNA into other *E. coli* strains, *i.e.*, pTf16/BL21, pG-Tf2/BL21, pKJE7/BL21, pG-KJE8/BL21, resulting in hST6Gal I/pTf16, hST6Gal I/pG-Tf2, hST6Gal I/pKJE7, and hST6Gal I/pKJE8.

Expression of the Soluble form of MBP-tagged hST6Gal I

When BL21 was used as the bacterial host, MBP-tagged hST6Gal I was not expressed in the soluble fraction (data not shown). Therefore, we attempted to express MBP-tagged hST6Gal I in the soluble fraction using the chaperone coex-

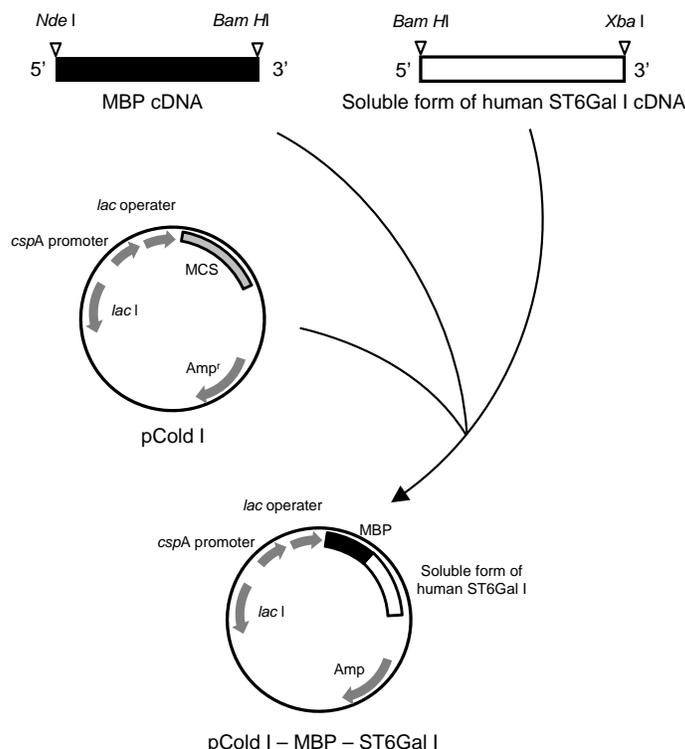


Fig. (1). Plasmid map of the expression vector. The genes coding MBP and hST6Gal I were inserted into the cloning site of the bacterial expression vector pCold I.

pression system. After protein induction in the above bacterial strains at 15°C, bacteria were sedimented and the pellets were disrupted by ultrasonication. Expression of MBP-tagged hST6Gal I in each bacterial host was analyzed by western blotting using anti-MBP antibody (Fig. 2). MBP-tagged hST6Gal I was expressed at high levels in hST6Gal I/pGro7 (lane 1) and hST6Gal I/pG-KJE8 (lane 4), and low-level expression was observed in hST6Gal I/pKJE7 (lane 3). On the other hand, no expression was detected in either hST6Gal I/pG-Tf2 (lane 2) or hST6Gal I/pTf16 (lane 5). Sialyltransferase activities in the supernatants were determined by ELISA-based assay as described previously [18, 19]. As shown in Fig. (3), the supernatant of hST6Gal I/pGro7 showed the highest levels of activity (55.7 μ U/mg protein/min). hST6Gal I/pG-KJE8 and hST6Gal I/pKJE7 showed lower levels of enzyme activity. These data were in good agreement with those obtained by western blotting analysis (Fig. 2). These observations indicated that hST6Gal I/pGro7 was the most effective host for expression of the MBP-tagged hST6Gal I. Although hST6Gal I/pG-Tf2 or hST6Gal I/pTf16 had slightly enzyme activity, the signals corresponded to the enzyme were not detected on a membrane with anti-MBP antibody. It was because the enzyme protein expression in those host bacteria may be less than the detection level by immunoblotting analysis.

Purification of a Soluble form of MBP-tagged hST6Gal I from Bacteria

hST6Gal I/pGro7 pellets prepared from 500 mL of culture were disrupted with ultrasonication. The supernatant thus obtained was subjected to amylose-conjugated agarose column chromatography. MBP-tagged hST6Gal I was par-

tially purified by affinity chromatography (Fig. 4, lane 1). Western blotting analysis of the purified fraction with anti-MBP antibody showed that the maltose-eluted fraction contained MBP-tagged ST6Gal I (Fig. 4 lane 2). In this step, the tagged enzyme was obtained in a yield of 41.1% and with

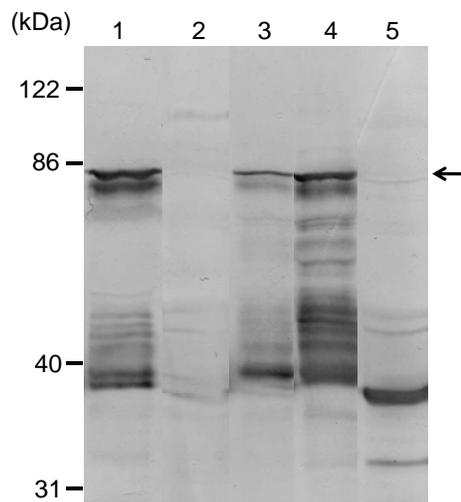


Fig. (2). ST6Gal I protein expression in chaperone-coexpressing *E. coli*. The protein expression in bacteria was induced at 15°C for 24 h in the presence of IPTG at a final concentration of 1 mM. Induced bacterial cells were disrupted by ultrasonication. Crude lysates cleared by sedimentation were collected and subjected to SDS-PAGE, followed by western blotting analysis using anti-MBP antibody. Lane 1, pGro7/BL21; lane 2, pG-Tf2/BL21; lane 3, pKJE7/BL21; lane 4, pG-KJE8/BL21; lane 5, pTf16/BL21. The arrow indicates MBP-tagged ST6Gal I.

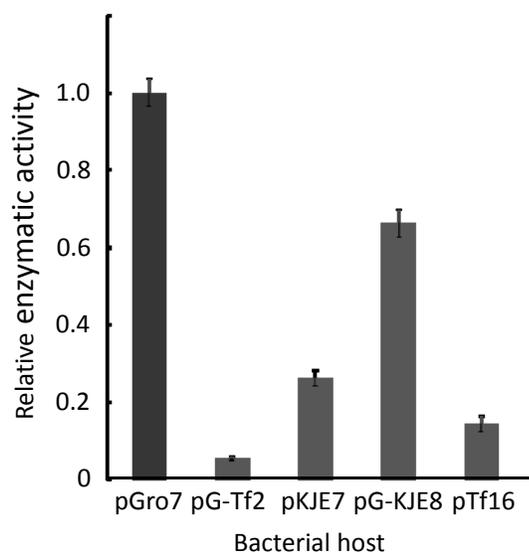


Fig. (3). Relative enzymatic activity of crude lysates prepared from host bacterial cells. The quantitative enzyme assay was carried out as described in Materials and methods. Activity of the host is shown relative to pGro7/BL21 as 1.0 (55.7 μ U/mg protein/min).

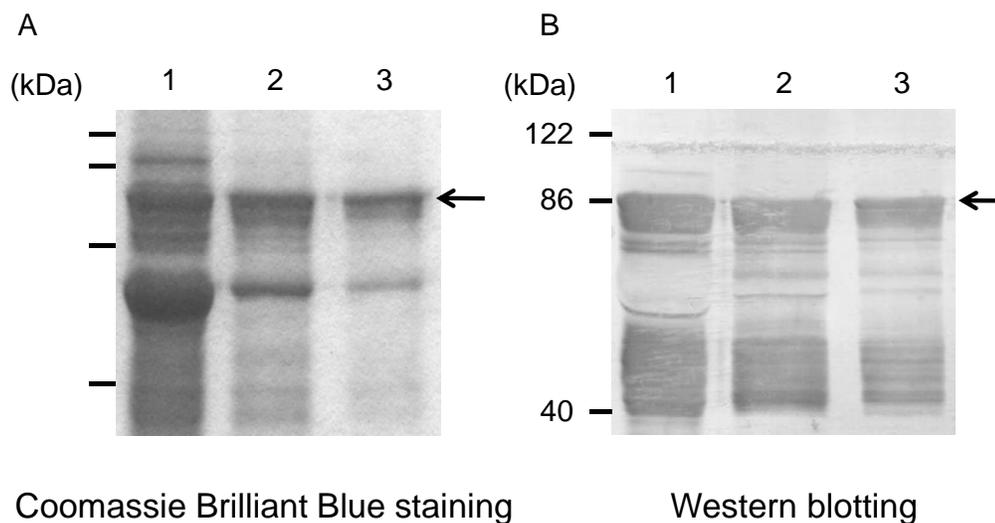


Fig. (4). Purification of MBP-tagged hST6Gal I by affinity chromatography using amylose-conjugated agarose. Crude lysate (lane 1) was loaded onto an amylose-conjugated agarose column. MBP-tagged ST6Gal I was eluted with TBS containing 10 mM maltose. Elution (lane 2) was dialyzed against TBS to remove maltose. The obtained fraction was reloaded onto the amylose-conjugated agarose column. MBP-tagged ST6Gal I was eluted with TBS containing 10 mM maltose (lane 3). Fractions were analyzed by SDS-PAGE (A) and Western blotting using anti-MBP antibody (B). Arrows indicate MBP-tagged ST6Gal I.

3.88-fold purification. The eluted fraction was dialyzed against MBS (150 mM NaCl, 20 mM MES-NaOH, pH 6.0). The dialyzed fraction was again subjected to amylose-conjugated agarose column chromatography. Western blotting analysis with anti-MBP antibody demonstrated that the major fraction obtained by maltose elution contained the MBP-tagged ST6Gal I (Fig. 4 lane 3). In this final purification step, the MBP-tagged ST6Gal I was obtained in a yield of 20.3% and with 6.12-fold purification. The purification of MBP-tagged ST6Gal I is summarized in Table 1. The specific activity of the recombinant enzyme was 0.3 mU/mg protein/min. The overall yield of the enzyme was 1.13 mU from 500 mL of culture medium. The specific activity was lower than that described in our previous study [17]. This is

the reason why purification level of the enzyme was relatively lower.

DISCUSSION

There have been no previous reports of functional and soluble expression of mammalian glycosyltransferases, including sialyltransferases, in *E. coli*. When expressed in *E. coli*, the recombinant enzyme was transported into inclusion bodies, making it resistant to solubilization with mild detergents [21]. We established a soluble expression system for human sialyltransferase ST6Gal I in *E. coli* [17]. However, the yield of the recombinant protein was still low in this system. As a result, a large culture volume was required to obtain the enzyme in sufficient amounts for *in vitro* applica-

Table 1. Summary of Purification of a MBP-tagged hST6Gal I

Step	Volume (ml)	Total Protein (mg)	Specific Activity ^a (μ units/mg protein/min)	Total Activity ^a (μ units/min)	Yield (%)	Fold
Crude lysate	100	100.6	55.7	5.57	100	1
Partially purified enzyme (first elution)	55	10.58	216	2.29	41.1	3.88
Partially purified enzyme (second elution)	35	3.31	341	1.13	20.3	6.12

^aOne unit of activity is defined as 1 μ mol of sialic acid transfer to acceptor substrate per 1 min at 37°C.

tions. The present study was performed to improve the expression of the soluble form of recombinant human ST6Gal I in bacteria. For this purpose, we used the cold shock plasmid vector pCold I, which has high efficiency of protein expression at temperatures below 15°C. Our previous study demonstrated that induction below 25°C contributes to expression of soluble hST6Gal I in *E. coli* and prevents its digestion by proteases [17]. Therefore, protein expression at low temperatures with cold shock vector is advantageous for expression of soluble hST6Gal I. Some mammalian proteins such as human β -actin have been expressed using the cold shock vector [22]. Our results showed that combined use of cold shock vector and chaperone-coexpressing *E. coli* strains significantly improved solubilization of the recombinant protein. The chaperone-coexpressing *E. coli* strain pGro7/BL21 was the best host for expression of hST6Gal I using pCold I vector (Figs. 2 and 3).

The expressed protein was effectively purified by amylose-conjugated agarose column chromatography. The protein purified by amylose-conjugated column chromatography contained a few minor components with faster mobility on the gel detected with anti-MBP antibody, indicating that they

were originally derived from MBP-tagged ST6Gal I and proteolytically degraded during purification. For further purification, HRV 3C protease could be useful as hST6Gal I does not contain sequences for recognition by this protease. A single cleavage site was introduced into the junction of MBP and hST6Gal I. We found that MBP-tagged hST6Gal I was digested to MBP and hST6Gal I by HRV 3C protease (Fig. 5). The combination of HRV 3C digestion and other types of affinity chromatography could provide highly purified enzyme in sufficient amounts for crystallization experiments.

In conclusion, an efficient bacterial expression system for soluble hST6Gal I was established. The recombinant enzymes could be used to study the molecular mechanisms of the catalytic reaction.

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CONFLICT OF INTEREST

None.

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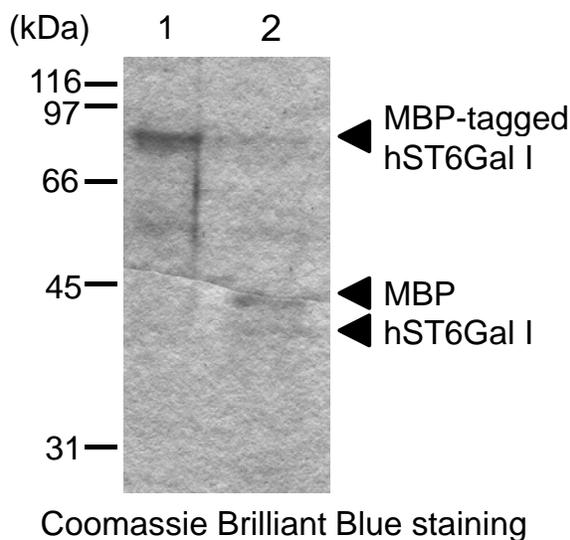


Fig. (5). Purified MBP-tagged hST6Gal I were treated with HRV 3C protease. Purified MBP-ST6Gal I were mixed with HRV3C protease (1 unit/20 mg of the protein of interest) and incubated overnight at 4°C. Lane 1, purified MBP-tagged hST6Gal I; lane 2, purified MBP-tagged hST6Gal I treated with HRV 3C protease. Samples were analyzed by SDS-PAGE, followed by staining of the gel with coomassie brilliant blue reagent.

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