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Immobilized sialyltransferase fused to a fungal biotin-binding protein: Production, properties, and applications

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A β-galactoside α2,6-sialyltransferase (ST) from the marine bacterium *Photobacterium* sp. JT-ISH-224 with a broad acceptor substrate specificity was fused to a fungal biotin-binding protein tamavidin 2 (TM2) to produce immobilized enzyme. Specifically, a gene for the fusion protein, in which ST from *Photobacterium* sp. JT-ISH-224 and TM2 were connected via a peptide linker (ST-L-TM2) was constructed and expressed in *Escherichia coli*. The ST-L-TM2 was produced in the soluble form with a yield of approximately 15,000 unit/300 ml of the *E. coli* culture. The ST-L-TM2 was partially purified and part of it was immobilized onto biotin-bearing magnetic microbeads. The immobilized ST-L-TM2 onto microbeads could be used at least seven consecutive reaction cycles with no observed decrease in enzymatic activity. In addition, the optimum pH and temperature of the immobilized enzyme were changed compared to those of a free form of the ST. Considering these results, it was strongly expected that the immobilized ST-L-TM2 was a promising tool for the production of various kind of sialoligosaccharides.

[**Key words:** Sialyltransferase; Tamavidin; Immobilized enzyme; Photobacterium; Sialyllactose]

Sialic acid is a family of over 50 naturally occurring monosaccharides that exist mainly in mammals and bacteria (1,2). One of the major sialic acid existing in mammals and bacteria is *N*-acetylneuraminic acid (Neu5Ac). It has been clearly demonstrated that oligosaccharide chains in glycoproteins and glycolipids containing Neu5Ac play an important role in many biological processes including inflammatory and immunological responses, cell–cell recognition, cancer metastasis, and viral infection (3,4). In addition, it has been also reported that sialyllactose (mixture of 3′- and 6′-sialyllactose), Neu5Ac and so on could be candidates for prophylactic drugs to GNE myopathy, which is a moderately progressive autosomal recessive myopathy (5). Specifically, a prophylactic treatment was tested to confirm effectiveness in a mouse model of GNE myopathy and it was confirmed that oral treatment with the sialyllactose, Neu5Ac and *N*-acetylmannosamine, precursor of Neu5Ac, completely precluded the development of the myopathic phenotype in the model mice (6). Furthermore, it was demonstrated that treatment with 6′-sialyllactose ameliorated muscle atrophy and degeneration in symptomatic GNE myopathy mice (7). Thus, it is strongly expected that GNE myopathy can be treatable even at a progressive stage by oral administration of 6′-sialyllactose. For these reasons, Neu5Ac and sialyloligosaccharides are thought to be important biological components.

Producing a large variety of sialyloligosaccharides is needed for clarifying the relationship between the structure and function of the oligosaccharide chains. Also, an abundant supply of the oligosaccharide is indispensable for their industrial application. Chemical and enzymatic sialylation are the two major routes for preparation of sialyloligosaccharides (8). Although several highly efficient chemical sialylation methods have been developed, chemical preparation of sialyloligosaccharides is still complicated because these methods require multiple protection and deprotection steps (9,10). In contrast, enzymatic sialylation using sialyltransferase is a single-step process that affords high positional and anomer selectivity as well as a high reaction yield (8,11). Therefore, enzymatic sialylation is advantageous compared with chemical sialylation. To date, we have isolated over 20 marine bacteria that produce sialyltransferases. Many of these bacteria are classified in the genus *Photobacterium* or the closely related genus *Vibrio* (12,13). Since α2,6- and α2,3-sialyltransferases from these marine bacteria show broad donor and acceptor substrate specificity, they would be applied for producing a diverse of Neu5Ac-containing molecules. We have established *Escherichia coli* protein production systems that highly produce these recombinant enzymes (14,15).

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Recently, we have reported the novel avidin-like biotin-binding proteins, tamavidin 1 (TM1) and tamavidin 2 (TM2) from a basidiomycete fungus, *Pleurotus cornucopiae*, known as the Tamogitake mushroom in Japan (16). Like other biotin-binding proteins (e.g., avidin and streptavidin), TM1 and TM2 are tetramers and bind biotin with very high affinity. Moreover, these proteins possess two attractive features. First, unlike any other biotin-binding proteins, TM1 and TM2 are produced as soluble proteins at a high level in *E. coli* protein production system. Second, these proteins, especially TM2, are thermally stable than biotin-binding proteins found in a bird and bacteria (16).

Enzyme immobilization is attractive for industrial applications (17–19), because immobilized enzymes facilitate the purification of reaction products as well as the enzyme re-utilization. Previously, we showed a method for immobilization and purification of recombinant proteins including a sialyltransferase (ST) using TM2 as an affinity tag. Also, we demonstrated that the importance of some parameters, including linker length between TM2 and a fusion partner, and that between biotin and solid phase, for tight biotin-binding (20). However, the enzymatic characterization of the ST fused to TM2 (ST-L-TM2) and its application for producing sialosides have not been reported. In this study, we show the enzymatic characteristics of the immobilized ST-L-TM2, and its application in the production of sialyloligosaccharides.

MATERIALS AND METHODS

Expression cassette for the ST-L-TM2 The construction of the expression cassette for ST-L-TM2 was described previously (20). The gene encoding ST-L-TM2 was integrated in an expression vector pTrc99A to yield pSL5T2 (plasmid harboring the sequence of ST-L-TM2). Nucleotide sequences of the genes for ST from *Photobacterium* sp. JT-ISH-224, TM2 from a basidiomycete fungus *P. cornucopiae* and ST-L-TM2 are available in the DDBJ/EMBL/GenBank databases as AB293985, AB102785 and AB531016, respectively. *Photobacterium* sp. JT-ISH-224 was deposited as NITE BP-87 in National Institute of Technology and Evaluation of Japan (NITE).

Expression and purification of ST-L-TM2 A single colony of *E. coli* (strain TB1) harboring pSL5T2 was inoculated in 6 ml of liquid lysogeny broth (Becton–Dickinson, Sparks, MD, USA) containing 100 μg/ml ampicillin and was cultivated at 30°C until the absorbance at 600 nm reached to 0.5. After the cultivation, this seed culture was added to 300 ml of the same culture medium containing 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG, Wako Pure Chemicals, Osaka, Japan). The culture medium was incubated for 15 h at 30°C. After the cultivation, bacteria were harvested by centrifugation at 8000 ×*g* at 4°C and obtained pellet was suspended in 6.5 ml of 20 mM Bis-Tris buffer (pH 6.0) containing 0.3% Triton X-100 (buffer A). The pellet was lysed by sonication on ice and cellular debris was removed by centrifugation at 100,000 ×*g* for 60 min at 4°C. The supernatant was filtered through a 0.45-μm cellulose acetate membrane. The filtrate containing ST-L-TM2 was purified on a HiLoad 26/10 Q Sepharose HP column (2.6 cm × 10 cm; GE Healthcare Science, Tokyo, Japan) equilibrated with buffer A. The enzyme was eluted with a linear gradient of 0–1 M NaCl in buffer A and the fractions containing fusion sialyltransferase ST-L-TM2 were collected.

Immobilization of ST-L-TM2 on magnetic beads Immobilization of the ST-L-TM2 was carried out according to the manufacture's instruction. To state briefly, BioMag Biotin beads (200 μl; Polysciences, Inc., Warrington, PA) were pre-washed twice with 20 mM Bis-Tris buffer (pH 6.0). The partially purified ST-L-TM2 described above (200 U, 1.35 mg protein) was added to the BioMag Biotin beads solution and the mixture was gently rotated for 2 h at 4°C. After the reaction, the beads were collected with a magnet (Adem-Mag SV, Ademtech SA, France) and were washed twice with 20 mM Bis-Tris (pH 6.0) containing 1 M NaCl before being suspended in 200 μl of 20 mM Bis-Tris (pH 6.0).

Sialyltransferase assay Sialyltransferase activity was assayed using a standard method (21) by measuring [4,5,6,7,8,9-^{14}C]-Neu5Ac transferred from cytidine monophosphate (CMP)-[4,5,6,7,8,9-^{14}C]-Neu5Ac (DuPont, Boston, MA, USA) as a donor substrate to lactose (Wako Pure Chemicals, Osaka, Japan). One unit (U) was defined as the amount of enzyme that transferred 1 μmol of Neu5Ac from CMP-Neu5Ac to lactose per minute. Radioactivity was measured using a Packard model TR 1900 liquid scintillation counter.

pH and temperature profiles of ST-L-TM2 and protein determination Assays for determining the pH profile of the immobilized ST-L-TM2 were performed using the following buffers: 100 mM sodium acetate buffer (pH 4.0–5.0), 100 mM sodium cacodylate buffer (pH 5.0–6.0), 100 mM Bis-Tris buffer (pH 6.0–7.0), and 100 mM phosphate buffer (pH 7.0–8.0). Assays were performed with gently rotating in triplicate. To investigate the optimum temperature of the enzyme activity, the enzyme reaction was carried out at 20°C, 25°C, 30°C, 35°C, 40°C, and 45°C according to the method described in sialyltransferase assay. Assays were performed in triplicate.

Protein determination was performed using a Coomassie Protein Assay Reagent (Pierce, Rockford, IL) according to the manufacturer's instructions. Bovine serum albumin was used as a standard.

Sialyllactose production by immobilized ST-L-TM2 A reaction solution (125 μl) consisting of 55.2 mM lactose, 91.2 mM CMP-Neu5Ac, 100 mM Bis-Tris buffer (pH 6.0), 0.5 M NaCl, and 50 μl (0.5 U) of immobilized ST-L-TM2 was incubated for 2 h at 30°C. Then the immobilized ST-L-TM2 was collected with a magnet, and the supernatant containing sialyllactose was recovered. The produced sialyllactose in the supernatant was measured as follows. The supernatant was diluted with 15 mM KH_2PO_4 (pH 5.2) containing 75% CH_3CN and then was applied to a TSK-Gel Amide-80 column (4.6 × 250 mm, Tosoh, Tokyo, Japan) at room temperature with a flow late of 1 ml/min. According to the methods reported previously (22), the sialyllactose was eluted and monitored with ultraviolet (195 nm) and fluorescence (Ex: 300 nm, Em: 360 nm) detectors. For repeated production of sialyllactose, the re-collected immobilized enzyme was added to a fresh reaction solution for each subsequent run of the reaction. The entire process of 7 reaction cycles was carried out in triplicate.

RESULTS AND DISCUSSION

Production and immobilization of ST-L-TM2 A β-galactoside α2,6-sialyltransferase (ST) from the marine bacterium *Photobacterium* sp. JT-ISH-224 was genetically fused to the fungal biotin-binding protein tamavidin 2 (TM2) via a peptide linker [L, (Gly-Gly-Gly-Gly-Ser)×5] (20). Specifically, a gene for the ST-L-TM2 was constructed and expressed in *E. coli*. The ST-L-TM2 was produced in the soluble form with a yield of 14,985 U in 300 ml of *E. coli* culture.

The ST-L-TM2 was partially purified and incubated with biotin-bearing magnetic microbeads to promote the binding between TM2 moiety and biotin on the beads. Then, the fusion protein that binds the beads was washed thoroughly. Specific activity of the immobilized ST-L-TM2 was 11.3 U/ml beads, enough for further investigation. However, the efficiency of immobilization was low compared to the number of biotin on the magnetic beads. So, optimization of immobilization method is indispensable.

pH and temperature profiles of the immobilized ST-L-TM2 Generally, solid support systems stabilize the structure of the enzymes and immobilized enzymes by solid support usually maintain their activities (19). In addition, as compared to free form of enzymes in solution, it is well-known that immobilized enzymes are more robust and more resistant to environmental changes in many cases. Previously, we have demonstrated that the fusion sialyltransferase used in this study was produced in the soluble form in *E. coli* and could be immobilized on biotinylated beads in a single-step process (20). However, pH and temperature profiles of the immobilized ST-L-TM2 have not been clarified. Thus, the pH and temperature profiles of immobilized ST-L-TM2 were investigated.

As shown in Fig. 1a and b, the maximum activities of the immobilized ST-L-TM2 were observed at pH 6.0 and at 35°C, respectively. The enzymatic activity of the immobilized ST-L-TM2 did not decrease between pH 6.0 and 8.0 (Fig. 1a). On the other hand, in case of the free form of the recombinant β-galactoside α2,6-sialyltransferase from marine bacterium *Photobacterium* sp. JT-ISH-224, an obvious decrease in the enzymatic activity was observed at pH 8 (23). CMP-Neu5Ac, common donor substrate of sialyltransferases, is more stable at pH 8 than acidic condition and sialosides are also more stable under basic conditions. Therefore, sialyltransferases having its optimum pH under basic condition like ST-L-TM2 is thought to be a useful tool.

With regard to temperature profiles of the free form of the recombinant β-galactoside α2,6-sialyltransferase from marine bacterium *Photobacterium* sp. JT-ISH-224, the enzyme showed a 90% decrease in activity at 40°C compared to that observed at 30°C as