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Modification of Artificial Oliogosaccharides in Recombinant *Escherichia coli* Cells

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Abstract: Artificial oligosaccharides were modified using recombinant *Escherichia coli* cells that overexpress sialidase. Based on the principle of the saccharide primer method by using bacterial cells overexpressing enzymes related to oligosaccharide modification. **Problem statement:** It is very hard to obtain oligosaccharides, because they have complex and diverse structures with different linkage patterns and monosaccharide components. **Approach:** It had been known that various oligosaccharides can be synthesized in mammalian cells from saccharide primers. We attempted to modify oligosaccharides by using bacterial cells overexpressing enzymes related to oligosaccharide modification instead of mammalian cells. **Results:** The glycosphingolipid-like derivative GM3 was absorbed by the cell and desialylated by the expressed sialidase and the desialylated product was then secreted into the medium. The GM3-type oligosaccharides were not detected from the cell fraction of recombinant *E. coli* cells that overexpress sialidase differently from recombinant *E. coli* carrying only vector DNA (pET-19b). **Conclusion/Recommendations:** *E. coli* as well as mammalian cells may be used as a biocatalyst for oligosaccharide modification and production of artificial functional oligosaccharides.

Key words: Oligosaccharide, *escherichia coli*, sialidase, saccharide primer

INTRODUCTION

Oligosaccharides are expressed on the cell surface in all living organisms. Oligosaccharides have complex and diverse structures with different linkage patterns and monosaccharide components. In recent years, their physiological roles have been studied extensively and they have been successfully characterized. Cell surface oligosaccharides play various essential roles in proliferation, differentiation, cell-cell and cell-substrate interactions, carcinogenesis, bacterial or viral infections, immune response and inflammation[1].

Structurally complex oligosaccharides present in higher organisms are difficult to extract using common methods such as extraction from natural organic resources[2,3], chemical synthesis methods[4,6] and extraction by a sequence of enzymatic reactions[7,10]. In the early 1990's, the saccharide primer method was developed as a novel method for oligosaccharide synthesis. With this method, various oligosaccharides could be synthesized in mammalian cells from saccharide primers such as dodecyl β-lactoside (Lac-C12) supplemented in the culture medium[11,13]. This method is very convenient because oligosaccharide products are secreted into the medium and can be separated from the other cellular molecules[14]. In this paper, we attempted to modify oligosaccharides based on the principle of the saccharide primer method by using bacterial cells overexpressing enzymes related to oligosaccharide modification.

MATERIALS AND METHODS

Arthrobacter ureafaciens was obtained from the Riken Bioresource Center (Saitama, Japan). GM3-type oligosaccharides were synthesized using the saccharide primer method described in literature[15]. n-Dodecyl β-lactoside was glycosylated in mouse melanoma B16 cells and the GM3-type oligosaccharide was collected from the culture medium. The GM3-type oligosaccharide was concentrated by using a polystyrene adsorption resin, Diaion HP20 (Mitsubishi Chemical, Tokyo, Japan) and purified using centrifugal partition chromatography. High Performance Thin-Layer Chromatography (HPTLC) plates were purchased from Merck (Darmstadt, Germany).

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Expression of sialidase genes in *E. coli*: DNAs coding isoenzymes L and M1 were amplified using the chromosomal DNA of *A. ureafaciens* as a template. The nucleotide sequences of deoxyoligonucleotide primers used for PCR are shown in Table 1. The amplified fragments were digested with *Nde*I and *Xho*I and inserted into the pET-19b vector, for which *E. coli* BL21(DE3)pLysS (Novagen, Madison, WI, U.S.A.) was used as a host. Luria-Bertani (LB) broth (Difco, Detroit, MI, U.S.A.) was used for the culture of *E. coli*. Ampicillin and chloramphenicol at final concentrations of 100 and 34 μg mL^{-1}, respectively, were used. The transformants were cultivated in 2 mL LB medium and stimulation with isopropyl-β-D-thiogalactopyranoside (IPTG) was carried out for protein expression according to the manufacturer's instructions.

Modification of oligosaccharide in *E. coli cells:* The transformants were cultured in 2 mL LB medium at 37 ºC with shaking at 120 rpm. When the optical density at 660 nm reached 0.6, IPTG and GM3-type oligosaccharide were added at final concentrations of 0.4 and 0.25 mM (or 1 mM), respectively. The modified oligosaccharides were obtained after incubation at 37 ºC for 3 h.

Table 1: Primers used for cloning of sialidase DNA

Primer	Oligonucleotide sequence (5' to 3')	Used for
Neu-F	TTTTTCATATGAG ATCCAACAGCACCTCCG	Cloning isoenzymes L and M1
NeuL-R	TTTTTCTCGAGTTAATCG CGGACCAACAGGTCCAC	Cloning isoenzyme L
NeuM1-R	TTTTTCTCGAGTTACGAA ATCAGGCCATCCCGCAG	Cloning isoenzyme M1

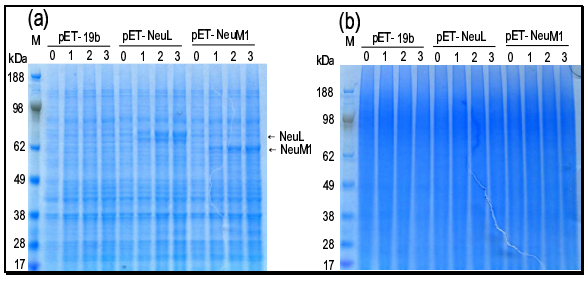

Fig. 1: SDS-PAGE of *A. ureafaciens* sialidase isoenzymes L and M1. (a) Cell fraction; (b) Medium fraction. M: Marker; 0, 1, 2 and 3: Incubation times after adding IPTG (h). The proteins were electrophoresed on NuPAGE 4-12% Bis-Tris Gels (Invitrogen, Tokyo, Japan) and then stained with Coomassie Brilliant Blue

Analysis of modified oligosaccharides: After incubation, the culture media were collected and the cells were washed with PBS (-). The oligosaccharides collected from the culture media were suspended in PBS (-) and purified using a Sep-Pak C18 column (Waters, Milford, MA, U.S.A). After elution from the column, the solution was evaporated and dissolved in 250 or 1000 μl of chloroform/methanol (2:1, v/v) for HPTLC analysis. The cells were suspended in 1 mL of chloroform/methanol (2:1, v/v). After sonication and centrifugation at 15,000 rpm for 30 min each, the extracts were transferred to a new tube. The remaining lipids from the cells were extracted again with 1 mL of chloroform/2-propanol/water (7:11:2, v/v/v). The first and the second extracts were pooled. After evaporation, the resultant pellet was dissolved in 250 or 1000 μl of chloroform/methanol (2:1, v/v) and analyzed using HPTLC. Glycolipids from the cell homogenate and culture medium fraction were analyzed by HPTLC with chloroform/methanol/0.25% aqueous KCl (5:4:1, v/v/v) as the developing solvent. The HPTLC plates were sprayed with resorcinol regent and heated at 120 ºC to detect the separated glycolipids[15]. A densitogram was obtained from HPTLC by using Scion Image Software (Scion Corporation, http://www.scioncorp.com) to quantify the glycolipids.

RESULTS

Expression of sialidase genes in *E. coli*: The genes coding the sialidase isoenzymes L, M1, M2 and S in *A. ureafaciens* were cloned and their activities toward gangliosides were compared with those of the recombinant enzymes. Isoenzymes L, M1 and M2 exhibit higher activity toward GM1 than isoenzyme S[16]. DNAs coding the isoenzymes L and M1 (GeneBank/NCBI accession number AB193297) were amplified with PCR by using *A. ureafaciens* genomic DNA as the template. The amplified DNA fragments were ligated into the expression pET-19b vector (Novagen, Madison, WI, U.S.A.) using the *Nde*I and *Xho*I sites (pET-NeuL, pET-NeuM1) and transformed into *E. coli* BL21(DE3)pLysS. The transformants were cultivated in 2 mL of LB medium and proteins were expressed upon stimulation with IPTG. The cells were then separated from the culture medium by centrifugation and the cells and medium fractions were analyzed by SDS-PAGE. As shown in Fig. 1, the recombinant proteins were localized in the *E. coli* cells.

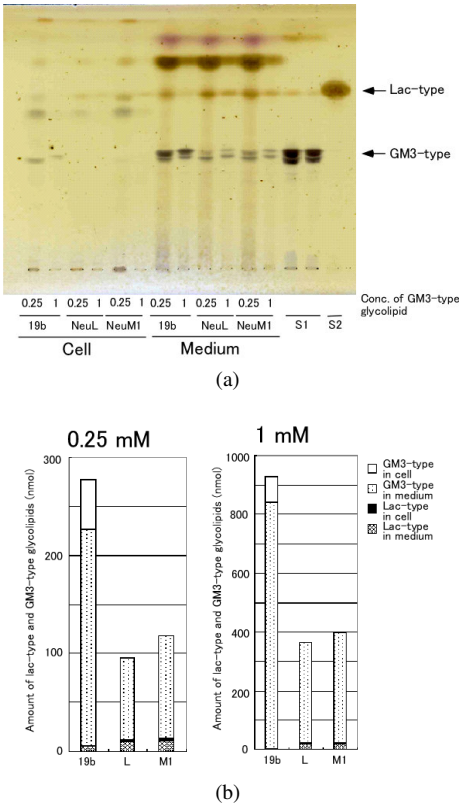

Fig. 2: Desialylation with recombinant *E. coli* cells. (a) HPTLC results of glycolipids extracted from the cell and culture medium fractions. Samples were analyzed using HPTLC with chloroform/methanol/0.25% aqueous KCl (5:4:1, v/v) as the developing solvent. The HPTLC plates were sprayed with resorcinol and heated at 120ºC to detect the separated glycolipids. S1: GM3-type glycolipid standard and S2: Lac-type glycolipid standard. (b) The amount of GM3-type and Lac-type glycolipids

Modification of GM3-type oligosaccharide in *E. coli* cells: *E. coli* BL21(DE3)pLysS carrying the pET-19b (control), pET-NeuL and pET-NeuM1 vectors were cultured in 2 mL of LB medium at 37ºC with shaking at 120 rpm. At an optical density of 0.6 at 660 nm, the IPTG and GM3-type oligosaccharides were added to a final concentration of 0.4 mM and 0.25 (or 1 mM), respectively. After 3 h, the cells were separated from the culture medium by centrifugation. After washing the cells with PBS (-), lipids were extracted from the cell and medium fractions and analyzed by HPTLC. As shown in Fig. 2, desialylated GM3-type oligosaccharide was found to be produced in the culture medium of recombinant *E. coli* carrying either the pET-NeuL or

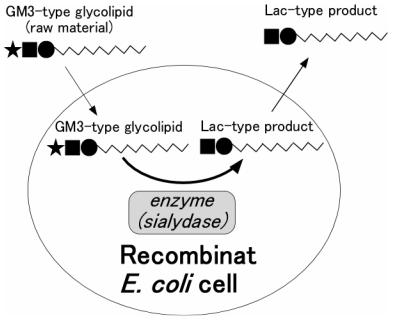

Fig. 3: Models of modification of artificial oligosaccharides in recombinant *E. coli* cells. ★: Sialic acid, ■: Galactose, ●: Glucose

pET-NeuM1 vectors more efficiently than in the pET-19b vector (control). Interestingly, GM3-type oligosaccharides were detected from the cell fraction of recombinant *E. coli* carrying pET-19b (control). This result suggested that GM3-type oligosaccharides could be internalized into *E. coli* cells. GM3-type oligosaccharides were not detected from the cell fraction of recombinant *E. coli* carrying either pET-NeuL or pET-NeuM1 vectors. This was considered to be due to desialylation by the sialidase expressed in *E. coli* cells. The reaction yields by recombinant *E. coli* carrying pET-NeuL and pET-NeuM1 were 2.1 and 2.5%, respectively, when 0.25 mM GM3-type oligosaccharide was added into the culture medium.

DISCUSSION

The saccharide primer method has been developed as a method for oligosaccharide synthesis. With this method, various oligosaccharides could be synthesized in mammalian cells from saccharide primers such as dodecyl β-lactoside (Lac-C12). It has been reported that the reaction yield of GM3-type oligosaccharide from Lac-C12 by mouse B16 melanoma cells was 5.6 %[17] . In this paper, the maximam reaction yield by recombinant *E. coli* was 2.5%. The reaction yield by recombinant *E. coli* cells was lower than it by B16 cells. It is thought that the reason may be because *E. coli* degraded the GM3-type saccharide primer. If the GM3-type oligosaccharide primer degradation activity of *E. coli* could be decreased, it might be improved at reaction yield more.

CONCLUSION

In this study, we demonstrated that GM3-type oligosaccharides could be modified using recombinant *E. coli* cells as well as mammalian cells. *E. coli* cells could internalize the saccharide primer and the products

were secreted into the culture medium similar to that in mammalian cells (Fig. 3). Modification of saccharides by using recombinant *E. coli* cells will facilitate the low-cost mass production of glycolipid analogues because the cost for *E. coli* cultivation is considerably lower than that for mammalian cell cultivation. Moreover, the equipment for cultivation can be easily scaled up for mass production.

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