Galactooligosaccharide Synthesis by Active β-Galactosidase Inclusion Bodies-Containing Escherichia coli Cells

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In this study, a galactooligosaccharide (GOS) was synthesized using active β-galactosidase (β-gal) inclusion bodies (IBs)-containing Escherichia coli (E. coli) cells. Analysis by MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) mass spectrometry revealed that a trisaccharide was the major constituent of the synthesized GOS mixture. Additionally, the optimal pH, lactose concentration, amounts of E. coli β-gal IBs, and temperature for GOS synthesis were 7.5, 500 g/l, 3.2 U/ml, and 37°C, respectively. The total GOS yield from 500 g/l of lactose under these optimal conditions was about 32%, which corresponded to 160.4 g/l of GOS. Western blot analyses revealed that β-gal IBs were gradually destroyed during the reaction. In addition, when both the reaction mixture and E. coli β-gal hydrolysatite were analyzed by high-performance thin-layer chromatography (HP-TLC), the trisaccharide was determined to be galactosyl lactose, indicating that a galactose moiety was most likely transferred to a lactose molecule during GOS synthesis. This GOS synthesis system might be useful for the synthesis of galactosylated drugs, which have recently received significant attention owing to the ability of the synthesis of galactosylated drugs, which have recently been developed [3, 7, 15, 25, 28, 40]. Typically, GOS is synthesized by a β-galactosidase (β-gal)-catalyzed enzymatic reaction with lactose [5, 11, 17, 24]. During this reaction (transgalactosylation), a galactose moiety is transferred to the hydroxyl group of lactose. There have been several studies of GOS production by β-galactosidase [4, 6, 18, 26] and immobilized β-galactosidase [15, 20-22, 28, 37, 40]. In addition, to increase production of GOS, various reactor systems and operational strategies have recently been developed [3, 7, 15, 25, 28, 40].

In our previous studies, active Escherichia coli (E. coli) β-gal inclusion bodies (IBs) were produced by the addition of a repressor (α-methyl D-glucopyranoside) or an inducer analog (α-fucose) after induction of the araBAD promoter system in E. coli [13, 14]. In addition, we successfully operated a packed-bed reactor for the hydrolysis of α-nitrophenyl-β-D-galactoside using immobilized β-gal IBs-containing E. coli cells [38] in the first reported operation of an enzyme reactor using our β-gal IBs. Recently, we demonstrated that immobilized β-gal IBs-containing E. coli cells were capable of hydrolyzing lactose for up to 240 h during repeated-batch operation [39].

It was previously reported that enzyme IBs in an E. coli expression system were expressed as biologically active IBs [1, 2, 9, 10, 16, 32, 36]. However, few studies have examined the potential for use of active IBs in an enzyme reactor. García-Fruitós et al. [8, 10] showed that only the β-gal-fused protein performed very efficiently as a catalyst in enzymatic reactions. In addition, they demonstrated that substrate hydrolysis mediated by the IBs was significantly faster than substrate hydrolysis mediated by the same amount of the soluble form, and that IBs catalyzed the formation of the product through conventional kinetics. Recently, Nahálka et al. [19] investigated a sialic acid synthesis process that used a cross-linked inclusion body.

Keywords: Galactooligosaccharide, β-galactosidase inclusion body, galactosyl lactose, araBAD promoter system, Escherichia coli

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which was mimicked by cross-linked enzyme aggregates technology.

This study was conducted to investigate whether β-gal IBs-containing E. coli cells can synthesize GOS through a transgalactosylation reaction, and to determine the optimal conditions for GOS synthesis by β-gal IBs-containing E. coli cells. Interestingly, this is the first study to report GOS synthesis by β-gal IBs. β-Gal IB utilization is potentially a more convenient and economic approach for enzymatic GOS synthesis, since no enzyme purification steps after the transgalactosylation reaction are required. That is, whole E. coli cells containing active β-gal IBs can be used directly for GOS synthesis.

**MATERIALS AND METHODS**

**Materials**

Lactose, glucose, galactose, l-arabinose, α-fucose, glycerol, KH₂PO₄, Na₂HPO₄, phosphonomolybdic acid, ampicillin, and E. coli β-galactosidase were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Yeast extract was obtained from Becton Dickinson and Co. (Sparks, MD, USA). n-Butanol, ethanol, acetonitrile, and sulfuric acid were purchased from Daejung Chemical Co. (Shihueung, Gyeonggi-Do, Republic of Korea). B-PER II bacterial protein extraction reagent, SuperSignal West Pico chemiluminescent substrate, and Hyperfilm ECL were acquired from Thermo Fisher Scientific (Waltham, MA, USA). A thin-layer chromatography plate was obtained from Whatman (Maidstone, Kent, UK). Ce(SO₄)₂·4H₂O was obtained from Junsei Chemical Co. (Tokyo, Japan). A nitrocellulose membrane (Hybond-C Extra) was purchased from GE Healthcare (Piscataway, NJ, USA). Rabbit anti-β-galactosidase and goat anti-rabbit IgG-HRP (horseradish peroxidase) conjugate were obtained from Invitrogen (Carlsbad, CA, USA) and Abcam (Cambridge UK), respectively.

**Recombinant β-Gal-Expressing E. coli**

In this study, we used a recombinant E. coli in which the expression of β-gal was controlled by the araBAD promoter. The β-gal gene was cloned in an expression vector, pBAD/Myc-His/aZ (7.2 kb), which is one of the control vectors provided in the pBAD/Myc-His expression kit (Invitrogen) [13, 14, 38]. This expression vector contains the structural gene of β-gal (aZ), which is directed by the araBAD promoter and inserted into the SfsI site in a multicloning site. In addition, the positive regulator gene (araC), pBR322 origin, and ampicillin-resistant marker were contained in this vector. More details are described in the manual of the pBAD/Myc-His kit. E. coli MC1061 (F′araD139 Aekr-leu) 7696 galE15 galK16 Alac X74 rpsL (Str′) hsdR2 (r′ m−) mcrA mcrB1) was used as an expression host, in which the recombinant β-gal (120 kDa) was expressed by the addition of l-arabinose.

**Preparation of β-Gal IBs-Containing E. coli Cell**

Fermentor cultures were conducted in a 2.5 l jar fermentor (Applikon Biotechnology, The Netherlands) with a working volume of 1.0 l under conditions that have been described previously [13, 14, 38]. l-Arabinose induction was conducted at 0.05% after 3 h of culture, and 0.01% α-fucose was added after 3.5 h. After 5.5 h, the cells were collected by centrifugation, and the optical density at 600 nm (OD₆00) was adjusted to 2.0 using PBS (phosphate-buffered saline). For each sample, 1.5 ml of this standardized cell suspension was centrifuged, and the resulting cell pellets were stored at −20°C. The β-gal assay was conducted using a previously reported method [13, 14, 38]. One enzyme unit was defined as the amount of β-gal that resulted in the release of 1 nmol of α-nitrophenol per milliliter per minute at 37°C and pH 7.3.

For purification of the β-gal IB, the cell pellet was disrupted by a non-mechanical method using B-PER II bacterial protein extraction reagent. A detailed description of the IB purification method has been described previously [13, 14, 38].

**GOS Synthesis**

For MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) mass spectrometry, the substrate mixture was prepared in 15 ml conical tubes containing 10 ml of 50 mM phosphate buffer (pH 6.5) and 360 g/l lactose. This mixture was then sterilized by passing through a 0.22 µm filter. GOS synthesis was initiated by adding the cell suspension, which was prepared from 1.5 ml of the standardized cell suspension (5.3 U of β-gal). The temperature and shaking speed of the reaction mixture were maintained at 28°C and 70 rpm, respectively. After 96 h, the sample for MALDI-TOF mass spectrometry was collected.

When determining the optimal conditions for GOS synthesis, the experimental procedure was the same as described above. To determine the optimal pH, GOS syntheses were conducted at various pHs in 50 mM phosphate buffer containing 360 g/l lactose, and a single cell pellet prepared from the standardized cell suspension (5.3 U of β-gal) was used as a source of β-gal. To determine the optimal lactose concentration, GOS syntheses were conducted at 400–700 g/l in 50 mM phosphate buffer (pH 8.0), and a single cell pellet prepared from the standardized cell suspension (5.3 U of β-gal) was used as a source of β-gal. To optimize the amount of β-gal, GOS syntheses were conducted in the presence of various amounts of the cell pellet prepared from standardized cell suspension in 50 mM phosphate buffer (pH 7.5) containing 500 g/l of lactose. To determine the optimal temperature, GOS syntheses were conducted at the optimized pH, lactose concentration, and amount of β-gal at the following temperatures: 28, 37, and 40°C. All reactions were stopped after 120 h by heating and the samples were then stored at 4°C.

**MALDI-TOF Mass Spectrometry**

To prepare the sample for MALDI-TOF mass spectrometry analysis, 7.0 ml of GOS-containing solution that was prepared by the above described protocol was filtered through a 0.22 µm filter. Next, 200 µl of this filtered solution was diluted by adding 800 µl of 50 mM phosphate buffer (pH 6.5), after which 1.0 ml of this diluted solution was lyophilized in a 15 ml conical tube. The MALDI-TOF mass spectra were then measured on a Bruker Ultraflex III (Germany) in reflector mode with positive polarity, using 2,5-dihydroxybenzoic acid (DHB) as a matrix.

**Hydrolysis of GOS by E. coli β-Gal**

The synthesized GOS mixture, which was obtained from the 120 h reaction described above under optimal conditions, was hydrolyzed by E. coli β-gal. Next, 500 µl of the GOS sample and 500 µl of the
E. coli β-gal solution (1,000 U), which were prepared in 50 mM phosphate buffer (pH 7.5), were mixed and incubated at 37°C. The hydrolysis reaction was stopped after 96 h.

**Thin-Layer Chromatography**

Lactose, galactose, glucose, and GOS were analyzed via thin-layer chromatography (TLC) using a 20 × 10 cm Partisil K5F (Whatman) TLC plate, butanol–ethanol solution [butanol:ethanol:distilled water = 5:3:2 (v/v)] for the mobile phase, and a sample loading volume of 1.0 µl. To improve separation, development of the mobile phase was conducted three times. To visualize the bands, TLC plates were soaked in staining solution (2.5 g of phosphomolybdic acid, 1.0 g of Ce(SO₄)₂·4H₂O, and 6 ml of H₂SO₄ in 100 ml of distilled water), and then baked at 75°C for 15 min.

**High-Performance Thin-Layer Chromatography**

The conditions used for high-performance thin-layer chromatography (HP-TLC) were the same as those used for TLC. HP-TLC was conducted on a CAMAG system (Switzerland) equipped with an automatic developing chamber, automatic TLC sampler, TLC scanner, and winCATS software. For the quantitative analyses of lactose, galactose, glucose, and GOS, the images on the TLC plate were converted to peaks using a scanner, and the peak areas were measured with the winCATS software, after which the lactose, galactose, glucose, and GOS concentrations were quantitatively determined using the approach described by Robyt and Mukerjea [27].

**Western Blotting**

After the cell pellet from the GOS-containing reaction solution was disrupted by a non-mechanical method [13, 14, 38], soluble and insoluble fractions were separated by 12.5% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). For Western blotting, proteins were transferred onto nitrocellulose membranes, followed by sequential immunoprobing with 1:5,000 rabbit anti-β-gal and 1:10,000 goat anti-rabbit IgG-HRP (horseradish peroxidase) conjugate. Immunoreactive bands were visualized using the SuperSignal West Pico chemiluminescent substrate, followed by exposure on Hyperfilm ECL.

**RESULTS AND DISCUSSION**

**Determination of Molecular Weight of GOS**

The recombinant E. coli cells expressed β-gal as IBs, which was consistent with our previous reports [13, 14, 38]. To verify whether these E. coli β-gal IBs and β-gal IBs-containing E. coli cells could synthesize GOS like many other β-gals [4, 6, 15, 18, 20, 21, 25, 26, 28, 37, 40], the enzymatic GOS synthesis reaction was catalyzed using E. coli β-gal IBs or β-gal IBs-containing E. coli cells. As shown in Fig. 1, a newly synthesized molecule was detected in the TLC analysis after 360 g/l of lactose and E. coli β-gal IBs or β-gal IBs-containing E. coli cells were reacted for 96 h. MALDI-TOF mass spectrometry was then conducted on those reaction mixtures to determine which GOS molecules were formed during the reaction. The sodium cation adducts of lactose (m/z =365.14 in Fig. 2) and GOS (m/z =527.188 and 689.227 in Fig. 2, and m/z =527.185 and 689.228 in Fig. 3) were observed. This sodium adduct was frequently detected when the biological molecules were analyzed [12, 23, 34].

![Fig. 1. GOS syntheses by E. coli β-gal IBs and β-gal IBs-containing E. coli cells.](image1)

Reactions were conducted in 50 mM phosphate buffer (pH 6.5) containing 360 g/l of lactose. The GOS-containing reaction solution was collected after 96 h. The sample was then diluted five-fold before analysis. Lane 1 (Glc), 2 (Gal), 3 (Lac), 4 (IB), and 5 (Cell) indicate 1% glucose, 1% galactose, 1% lactose, E. coli β-gal IBs, and β-gal IBs-containing E. coli cells, respectively. The arrow indicates a newly synthesized molecule that was determined to be the GOS.

![Fig. 2. MALDI-TOF mass spectrometry of the GOS-containing reaction solution in which GOS was synthesized by E. coli β-gal IBs.](image2)
The MALDI-TOF mass spectrometry data revealed that at least two GOS molecules were present. This conclusion was based on the difference in the molecular weights of the GOS and sodium. That is, the sodium cation adducts that had a molecular weight of 527.188 and 527.185, respectively, were trisaccharides, whereas those that had a molecular weight of 689.227 and 689.228, respectively, were tetrasaccharides (Fig. 2 and 3). Because the tetrasaccharides were detected as a somewhat smaller peak in Fig. 2 and 3, it was deduced that the spot underneath the lactose spot on the TLC in Fig. 1 (i.e., the spot arrowed in Fig. 1) was a trisaccharide. Specifically, for GOS synthesis by *E. coli* β-gal IBs, or β-gal IBs-containing *E. coli* cells, a trisaccharide was determined to be the major constituent of the produced GOSs. Meanwhile, when commercially available GOSs were analyzed by TLC, they were found to be a mixture of various GOSs with different molecular weights (Fig. 4), which is quite different from the results obtained upon analysis of the GOS produced in this study. In previous studies, tri-, tertra-, and pentaoligosaccharides and much higher molecular-weighted GOSs were reported to be synthesized by microbial β-gal [4, 6, 15, 18, 22, 26, 28, 37, 40]. However, it was deduced that the GOS synthesis method used in this study primarily resulted in the production of trisaccharides (i.e., monosaccharide-added lactose). Therefore, our GOS synthesis system might be a useful tool for synthesis of glycosylated drugs, which have recently received significant attention owing to the ability of galactose molecules to improve the solubility of drugs while decreasing their toxicity.

**Optimal Conditions of GOS Synthesis by β-Gal IBs-Containing *E. coli* Cells**

To assess the practical application of our *E. coli* β-gal, we decided to use whole cells containing *E. coli* β-gal IBs for the synthesis of GOS. We optimized the GOS synthesis conditions including the pH, lactose concentration, amounts of β-gal IBs-containing *E. coli* cells, and temperature. As shown in Fig. 5, the optimal pH, lactose concentration, amounts of *E. coli* β-gal, and temperature for GOS synthesis were determined to be 7.5, 500 g/l, 3.2 U/ml, and 37°C, respectively. GOS synthesis was quantified using the approach described by Robyt and Mukerjea [27], in which glucose, isomaltodextrin, and maltodextrin gave the same level of intensity or density on the TLC plate, where the density was linearly proportional to the weight. Previous studies have also quantitatively analyzed and monitored GOS synthesis by microbial β-gal using TLC [26, 37]. Using this analytical approach, the total GOS yield was determined to be about 32% under the optimal conditions described above when the reaction time was 120 h (Fig. 6). A TLC plate containing the GOS synthesized under the optimal conditions is shown in Fig. 7. In addition, the amount of GOS at 72 h, when the plateau of the profile began, was estimated quantitatively using 1% lactose as a standard. Based on this estimation, 32% GOS at 72 h corresponded to 160.4 g/l, indicating that the GOS production under the optimal conditions was 160.4 g/l.

Thermostable β-gal has typically been used for the synthesis of GOS at reaction temperatures of 40–60°C [4, 6, 15, 18, 22, 26, 28, 37].
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Fig. 5. Optimal conditions for GOS synthesis by β-gal IBs-containing E. coli cells.
Values of relative GOS synthesis were calculated on the basis that the maximum synthesis of GOS was 1.0 under each condition, in which GOS syntheses were measured as scanned peak areas by HP-TLC. Before HP-TLC was conducted, samples were diluted 5-fold. (A) pH optimization. (B) Optimization of lactose concentration. (C) Optimization of the amount of β-gal IBs-containing E. coli cells. (D) Temperature optimization. All measurements were conducted three times (n=3) using the same sample, and the average and standard deviation were calculated.

Fig. 6. Profiles of lactose, galactose, glucose, and GOS during GOS synthesis under the optimal conditions using β-gal IBs-containing E. coli cells. Percent of lactose (▼), galactose (○), glucose (●), and GOS (▽) indicate their percent peak areas determined by HP-TLC. Before HP-TLC was conducted, samples were diluted 10-fold. The sample indicated by the arrow (at 72 h) was used to estimate the amount of GOS. All measurements were taken three times (n=3) using the same sample, and the average and standard deviation were calculated.

Fig. 7. TLC of GOS synthesis under optimal conditions. Samples were collected at 72 h in Fig. 6. Glc, Gal, and Lac indicate 1% glucose, 1% galactose, and 1% lactose standards, respectively. Lanes 1, 2, 3, and 4 were diluted 5-, 10-, 20-, and 40-fold, respectively, before samples were analyzed.
6, 15, 18, 20–22, 26, 28, 37, 40]. In addition, the optimal lactose concentration and pH have been reported to vary from 250–600 (g/l) and 4.0–7.5, respectively, and the optimal pH was shown to depend on the microbial source of β-gal. The amounts of β-gal used in these reactions ranged from 8–32 U/ml [18] and 3–9 U/ml [4]. The GOS yield was previously reported to be 17% when the reaction time was 120 min [18], and 55% when the reaction time was 400 h [40]. A GOS yield of 17–41% has commonly been reported in previous studies [4, 6, 15, 21, 28, 37]. Therefore, in this study, the GOSs were synthesized relatively slowly, but the amount of GOS was comparable to previous results (Fig. 6). However, E. coli β-gal started to deactivate at 40°C because the β-gal-containing E. coli cells were not thermophiles (Fig. 5D).

Unfortunately, the lactose gradually precipitated during the reaction, although the amount of precipitated lactose depended on the concentration of lactose. In addition, GOS production increased very much slowly after 24 h, as a function of reaction time (Fig. 6). As shown in Fig. 8, this occurred because β-gal IBs were gradually destroyed during the reaction and the β-gal was functioning in an IB during the entire reaction (Fig. 8).

Hydrolysis of GOS by E. coli β-Gal
To determine which monosaccharide moiety was transferred to the lactose molecule when GOS was synthesized, the GOS-containing reaction mixture prepared under optimal conditions was subjected to HP-TLC analysis (Fig. 9A). Because the galactose peak (peak number 2) was smaller than the glucose peak (peak number 1), the galactose was most likely transferred to the lactose molecule during GOS synthesis (peak number 4). In addition, as shown in Fig. 6, the relative amount of galactose during GOS synthesis was always less than that of glucose. These findings provide further evidence that a galactose moiety was transferred to lactose.

To further confirm these results, HP-TLC was conducted after the reaction mixture in Fig. 9A was hydrolyzed using E. coli β-gal (Fig. 9B). When the peaks in Fig. 9A were compared with those in Fig. 9B, the galactose peak (peak number 2) in Fig. 9B was found to be higher after the hydrolysis reaction, and the galactose peak (peak number 2) was higher than the glucose peak (peak number 1). Because the lactose molecule was hydrolyzed to glucose and galactose at a 1:1 ratio by E. coli β-gal, it was deduced that the greater increase in the galactose peak (peak number 2) in Fig. 9B occurred because the GOS molecules contained more galactose molecules. Thus, the greater increase in the galactose peak (peak number 2) provides direct evidence that a galactose moiety was transferred to lactose during GOS synthesis. Therefore, the trisaccharide GOS synthesized in this study was determined to be galactosyl lactose. In other words, this GOS contained two molecules of galactose and one molecule of glucose; however, the position of the chemical linkage between galactose and lactose was not determined in this study.

Fig. 8. Western blotting analysis of β-gal IBs in E. coli cells during 120 h. M, standard E. coli β-gal (Sigma); S, soluble fraction of E. coli cells; I, insoluble fraction of E. coli cells.

Fig. 9. Comparison of (A) the scanned TLC profile of the reaction mixture prepared by β-gal IBs-containing E. coli cells under the optimized conditions and (B) the scanned TLC profile of a GOS-hydrolyzed mixture prepared by E. coli β-gal (Sigma). The GOS-containing reaction mixture in (A) was collected after 120 h. hydrolyzed mixture of GOS by E. coli β-gal (Sigma) was prepared after 96 h. Scans were acquired using the HP-TLC system. Samples were diluted 20-fold. Peaks 1, 2, 3, and 4 indicate glucose, galactose, lactose, and GOS, respectively.
Some studies have shown that 6′ galactosyl lactoses (n-Galp-(1→6)-O-Lac) [18] and 3′ and 2′ galactosyl lactoses [4] were produced during GOS synthesis. Therefore, it is likely that one of those linkages is involved in our GOS. When a galactosylated drug is synthesized by E. coli β-gal IBs in a future study, the position of the chemical linkage between the galactose and drug will be investigated thoroughly.

Using MALDI-TOF mass spectrometry, a trisaccharide was determined to be the major constituent synthesized by β-gal IBs-containing E. coli cells. The total GOS yield was determined to be about 32% under the optimal reaction conditions. When both the reaction mixture and its E. coli β-gal hydrolytase were analyzed by HP-TLC, the trisaccharide was shown to be synthesized by the transfer of a galactose molecule to a lactose molecule during GOS synthesis. This GOS synthesis system might be a useful tool for synthesis of galactosylated drugs, which have recently received a great deal of attention owing to the ability of the galactose molecules to improve the drugs solubility while decreasing their toxicity. We recently showed that lactose hydrolysis was possible by the immobilized β-gal IBs-containing E. coli cells [39]. It is expected that the results of this study will be applicable to the enzyme reactor system.

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References


