Enzymatic Synthesis of 2-Phenoxyethanol Galactoside by Whole Cells of β-Galactosidase-Containing Escherichia coli

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We investigated whether β-galactosidase (β-gal)-containing Escherichia coli cells could transfer a galactose to 2-phenoxyethanol, resulting in 2-phenoxyethanol galactoside (PE-Gal). PE-Gal was confirmed by liquid chromatography-mass spectrometry. In addition, we also confirmed that a galactose molecule was covalently bonded with PE during thin-layer chromatography analysis of the β-gal hydrolysate of PE-Gal. The yield for PE-Gal synthesis was about 37.5% (weight basis), which was about 7–8 times greater than that of a previous report. In addition, the concentration of β-gal (0.96 U/ml) used in this PE-Gal synthesis was about 20 times less than that in a previous report.

Keywords: 2-Phenoxyethanol, β-galactosidase, transgalactosylation, 2-phenoxyethanol galactoside, Escherichia coli

Galactosylation reactions of biologically active molecules using the enzyme β-galactosidase (β-gal) are used to improve the biological activity and physicochemical properties of a molecule, such as active pharmaceutical ingredients [13, 18]. Some sources of β-gal have been introduced to be used for the galactosylation reaction [1, 12, 16, 19]. In particular, we used Escherichia coli (E. coli) β-gal in this study. This E. coli β-gal was produced in recombinant E. coli cells as inclusion bodies (IBs) [7, 8] and was used for studies on lactose hydrolysis [22], galactooligosaccharide (GOS) synthesis [11], and production of chlorphenesin galactoside (CPN-Gal) [9, 10]. The conversion yield of chlorphenesin (CPN) to CPN-Gal using E. coli β-gal was superior to those of other β-gals [3, 17], probably because there is more active E. coli β-gal in terms of specific activity [7, 8]. In addition, the β-gal-containing E. coli cell itself was directly used as a whole-cell enzyme for the galactosylation reaction without purifying β-gal.

In this study, we conducted the first investigation on galactosylation of 2-phenoxyethanol (PE, C\textsubscript{12}H\textsubscript{16}O\textsubscript{2}, CAS No. 122-99-6) (Fig. 1A) using β-gal-containing E. coli cells. PE has been widely used as a preservative for cosmetics [20]. However, some reports have indicated problems with PE, such as allergic reactions [4] and neurotoxicity [14]. In particular, many case reports on skin problems were found [2, 5, 6, 15]. Therefore, we conducted galactosylation of PE, in which a galactose molecule was transferred from lactose to the hydroxyl group of PE, because galactosylation of a drug molecule reduces its toxicity [13].

No reports are available on the galactosylation of PE. Only one Japanese patent (Donho M, Kimura T, Hayashi K, Nakajima H. 1996. Preparation of 2-phenoxyethyl β-D-galactopyranoside as antibacterial agent. JP 08225588), written in Japanese, showed that Aspergillus and Lipomyces β-gal can galactosylate PE, and the yield was 4.4–4.8%. In this study, we investigated 2-hydroxyethyl β-D-galactopyranoside (2-phenoxyethanol galactoside, PE galactoside, PE-Gal) production, purified the PE-Gal from reaction mixture, and confirmed whether a galactose molecule was covalently bonded to PE using β-gal-containing E. coli cells (as whole cells).

PE, silica gel (pore size 60Å, 200–400 mesh), phosphomolybdic acid, and E. coli β-gal were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ce(SO\textsubscript{4})\textsubscript{2}·4H\textsubscript{2}O was purchased from Junsei Chemical Co. (Tokyo, Japan). Thin-layer chromatography (TLC) plates (Partisil K5F) were purchased from Whatman (Maidstone, UK). All other reagents were reagent-grade.

We used recombinant E. coli in which β-gal expression was controlled by the araBAD promoter. The β-gal gene was cloned into the pBAD/Mye-His/lacZ vector (7.2 kb)
using the pBAD/Myc-His Expression Kit (Invitrogen, Carlsbad, CA, USA) [7, 8]. E. coli MC1061 was used as the expression host, and the induction was carried out by adding L-arabinose.

Cultures were conducted in a 2.5 L jar fermentor (Applikon Biotechnology, Schiedam, The Netherlands) with a working volume of 1.0 L, under conditions described previously [7, 8]. L-Arabinose induction was performed at 0.05% after 3 h of culture, in which 0.01% D-fucose was added after 3.5 h. Cells were harvested after 5.5 h and collected by centrifugation, and the optical density at 600 nm was adjusted to 2.0 using phosphate-buffered saline. A 1.5 ml aliquot of this standardized cell suspension from each sample was centrifuged, and the resulting cell pellets were stored at -20°C. The stored E. coli cells were used for their enzymes in the PE-Gal synthesis reaction. The β-gal assay was conducted using a method reported previously [7, 8]. One enzyme unit was defined as the amount of β-gal that resulted in the release of 1 µmol of o-nitrophenol per milliliter per minute at 37°C and pH 7.3.

We synthesized PE-Gal using 2.5–40 g/l PE under conditions of 300 g/l lactose and 0.96 U/ml β-gal (as E. coli cells) in 10 ml of 50 mM phosphate buffer (pH 8.0) in a 15 ml conical tube to optimize the PE concentration. The synthesis was conducted on a shaking incubator (40°C, 100 rpm). After optimizing the PE concentration, PE-Gal was routinely synthesized in 40 ml of 50 mM phosphate buffer (pH 8.0) in a 50 ml conical tube. Because PE-Gal synthesis was monitored by TLC before purification, we presumptively designated PE-Gal as PE-Gal(p). PE-Gal(p) was quantified by TLC, the scanned images of the TLC bands were converted to peaks using AlphaEase FC software (Alpha Innotech, San Leonardo, CA, USA), and their quantities were determined.

A 20 × 10 cm TLC plate was used for the TLC analysis, with a 1.0 µl sample loading volume. Ethyl acetate: methanol: distilled water = 17:2:1 (v/v) was used as the mobile phase for PE and PE-Gal analysis, and the staining solution contained 2.5 g of phosphomolybdic acid, 1 g of Ce(SO₄)₂·4H₂O, and 6 ml of H₂SO₄ in 100 ml of distilled water. After developing the samples, the TLC plates were soaked in staining solution and dried in an oven at 80°C to

Fig. 1. Optimal PE concentration for PE-Gal(p) synthesis using β-gal-containing E. coli.

(A) Molecular structure of PE. (B) Relative PE-Gal(p) synthesis vs. PE concentration. Relative values were calculated based on a maximum synthesis of PE-Gal(p) of 1.0, in which PE-Gal(p) synthses were measured as scanned TLC peak areas. All measurements were performed three times using the same sample, and the average and standard deviation were calculated. (C) TLC for PE-Gal(p) synthesis using 2.5–40 g/l PE at 12 h. All samples were diluted 2-fold before TLC analyses.
visualize the bands.

Silica gel was packed into a 5-cm-high Econo-column (ID: 5 cm; L: 10 cm; maximum vol.: 193 ml; Bio-Rad, Hercules, CA, USA) with a 100 ml bed volume, and the column was washed once with mobile phase (ethyl acetate: methanol: distilled water = 17:2:1 (v/v)). Then, 8 ml of reaction mixture was loaded on the surface of the silica gel, and the mobile phase was flowed continuously by gravity. Each collected fraction was 80 ml. Then, we obtained the purified PE-Gal by evaporating the mobile phase using a rotary vacuum evaporator.

The purified PE-Gal and reaction mixture were applied to a high-speed liquid chromatography-mass spectrometer (LCMS-IT-TOF; Shimadzu Corp., Tokyo, Japan). The mobile phase was composed of a 30:70 (v/v) ratio of acetonitrile to distilled water. The mass spectrum was obtained in positive-ion mode.

Hydrolysis of PE-Gal was conducted using *E. coli* β-gal (Sigma), in which 100 µl of PE-Gal solution (0.03 mM) and 100 µl of *E. coli* β-gal solution (2 U/µl) were mixed in 50 mM phosphate buffer (pH 7.5), and the reaction proceeded at 37°C.

The water solubility of PE is 20–30 g/l at 20°C, but values differ in several reports. We first investigated the optimal concentration of PE during the galactosylation of PE using β-gal-containing *E. coli* cells. As shown in Figs. 1B and 1C, PE-Gal(p) was maximally synthesized at 10 g/l PE over 12 h under conditions of 300 g/l lactose and 0.96 U/ml β-gal (as *E. coli* cells) in 40 ml of 50 mM phosphate buffer (pH 8.0) in a 50 ml conical tube at 40°C; other variables except PE concentration were optimized previously [9, 11, 12]. It is probable that a decrease in water solubility of PE at >20 g/l led to the decrease in PE-Gal(p) synthesis.

We investigated the time-course profile of PE-Gal(p) synthesis by TLC (Fig. 2A). The spot of PE-Gal(p) reached a plateau at >24 h. This profile was very similar to that of

![Fig. 2. PE-Gal(p) synthesis and PE-Gal purification.](image)

(A) TLC analysis for PE-Gal(p) synthesis under optimal conditions over 36 h. (B) TLC analysis of the fractions from silica gel chromatography for purifying PE-Gal, where samples were at 12 h from panel A. (C) Mass spectrum data of purified PE-Gal from the LC-MS analysis. The mass spectrum was obtained in positive-ion mode. The preferred structure of PE-Gal is shown in the mass spectrum.
GOS [11] and CPN-Gal syntheses [9, 10]. Then, we conducted silica-gel column chromatography with an 8 ml reaction mixture collected at 12 h, and the fraction containing purified PE-Gal was observed between fractions 2 and 4 by TLC (Fig. 2B). In addition, we collected fractions 3 and 4 to obtain more purified PE-Gal, which did not contain residual PE. From this fraction, we obtained about 30 mg of purified PE-Gal after the mobile phase was evaporated. Finally, about 150 mg of purified PE-Gal was obtained after finishing the purification of the 40 ml reaction mixture collected at 12 h, and the synthesis yield of PE-Gal from 10 g/l PE in a 40 ml reaction mixture was about 37.5% (weight basis). This yield was remarkably higher than that of the previous Japanese patent (4.4–4.8%). That is, our yield was about 7–8 times greater than that of the Japanese patent report.

In this study, we used only 0.96 U/ml β-gal for PE-Gal synthesis, whereas 20 U/ml β-gal was used in the Japanese patent study. Thus, the β-gal concentration used in the PE-Gal synthesis reaction was about 20 times less than that of the Japanese patent study. When we synthesized CPN-Gal using our β-gal-containing E. coli cells in our previous reports [9, 10], about 4.2–4.6 times higher yield (64–67%) from CPN-Gal synthesis of CPN was observed, compared with that using β-gal from Kluyveromyces lactis (Lactozym 3000 L HP-G) [3], although we used about half the amount of β-gal.

We observed previously that enhanced specific β-gal activity occurs after adding D-fucose or methyl α-D-glucopyranoside (α-MG) after inducing l-arabinose when E. coli β-gal is expressed in the araBAD promoter system [7, 8]. In particular, this E. coli β-gal was expressed as insoluble inclusion bodies (IBs), and their enhancement was about 4–5 times higher than that when D-fucose or α-MG was not added. Additionally, we deduced that the looser structure of the β-gal IBs observed by slowing down the transcription rate after adding D-fucose or α-MG after induction resulted in enhanced β-gal IB enzymatic activity [21]. The reason for the higher yield from the PE-Gal synthesis was because β-gal-containing E. coli cells were prepared while adding D-fucose after induction, and specific β-gal activity was enhanced, as previously shown in the case of CPN-Gal synthesis from CPN.

We conducted LC-MS to verify the mass of the purified PE-Gal and observed a peak in positive-ion MS mode (Fig. 2C). In addition, a single LC peak was also observed on the chromatogram (data not shown). The peak showed a mass of 323.0197 (m/z), and it was deduced that the mass peak was the sodium ion adduct of PE-Gal, because the calculated mass of the sodium ion adduct of PE-Gal was 323.275 [138.16 (PE) + 180.15 (galactose) − 18.015 (water) + 22.98 (sodium) = 323.275]. This kind of sodium ion adduct was often observed in our previous reports [9–11].

Additionally, we hydrolyzed PE-Gal using commercially available E. coli β-gal to confirm that a galactose molecule was transferred from lactose to PE during the β-gal transgalactosylation reaction. As shown in Fig. 3, a galactose molecule was released at 24 (lane 5) and 48 h

![Fig. 3](image-url)
(lane 6), respectively, during the hydrolysis reaction, and we confirmed that the Rf value of the sugar moiety (lanes 5 and 6) released from purified PE-Gal was exactly the same as that of a standard galactose molecule (lane 3). We also observed a PE molecule (lanes 5 and 6) and a PE-Gal molecule at time zero (lane 4).

The β-gal transgalactosylation reaction occurs as a galactose molecule is transferred from lactose to the hydroxyl group of an acceptor molecule [3, 9, 17]. Because the PE molecule has one hydroxyl group, it is likely that a galactose molecule was transferred to the hydroxyl group of PE, as shown in Fig. 1A. Finally, based on the results of Figs. 2C and 3, we conclude that the purified PE-Gal was synthesized by a galactose molecule bonded covalently at the hydroxyl group of PE. We inserted the preferred structure of the sodium ion adduct of PE-Gal in Fig. 2C.

However, we found a shoulder peak on the LC chromatogram at the left side of the PE-Gal peak as the reaction proceeded. Although we do not show the TLC data after 36 h, we extended the reaction to >36 h. This shoulder peak was distinctively larger at 72 h. As shown in Fig. 4A, we observed a shoulder peak (peak 3) on the LC chromatogram in the 72 h sample together with PE (peak 1) and PE-Gal (peak 2). Then, we found a mass peak of 485.0396 (m/z) from the MS analysis of peak 3 (Fig. 4B). It was deduced that the mass peak was the sodium ion adduct of PE-Gal-Gal (PE galactoside, which accepted two galactose molecules), because the calculated mass of the PE-Gal-Gal sodium ion adduct was 485.41 [138.16 (PE) + 2 × 180.15 (galactose) – 2 × 18.015 (water) + 22.98 (sodium) = 485.41]. We have previously investigated GOS synthesis using our β-gal-containing E. coli cells [11], where we observed the galactose transfer reaction to lactose. That is, a galactose molecule of lactose was transferred to a new lactose molecule, which is a trisaccharide (Lactose-Gal). In addition, a tetrasaccharide (Lactose-Gal-Gal) was found during GOS synthesis, as a tetrasaccharide was synthesized by the two galactose molecules being transferred. Therefore, based on our previous results and MS data, it was deduced that the shoulder peak (peak 3 in Fig. 4A) was PE-Gal-Gal, which is PE galactoside bonded to two galactose molecules and PE. We inserted the preferred structure of the PE-Gal-Gal sodium ion adduct in Fig. 4B.

In conclusion, we demonstrated that β-gal-containing E. coli cells could successfully transfer galactose from lactose to PE, resulting in PE-Gal. We verified the PE-Gal synthesis by LC-MS analysis of the purified PE-Gal and TLC analysis of the PE-Gal β-gal hydrolysate, respectively. Our PE-Gal synthesis yield was about 37.5% (weight basis), which was 7–8 times greater than that of a previous Japanese patent report. In addition, our concentration of β-gal (0.96 U/ml) used in PE-Gal synthesis was about 20 times less than that of the Japanese patent (20 U/ml) result. For further study, we will investigate the minimum inhibitory concentrations (MICs) of PE-Gal against bacteria. The decrease in cytotoxicity to human skin cells [9] will also be examined. We expect that the usefulness of these β-gal-containing E. coli cells will be extended to synthesize some galactoside derivatives of the ingredients contained in cosmetics, foods, and drugs, as shown in this and a previous study [9].

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References


