Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–4, 2018

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Efficient monooxygenase-catalyzed piceatannol production: Application of cyclodextrins for reducing product inhibition

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Received 26 March 2018; accepted 24 April 2018
Available online xxx

Piceatannol is a rare, costly plant-based stilbene derivative and exhibits various health-enhancing properties. Recently, we demonstrated that piceatannol could be produced from resveratrol through site-selective hydroxylation using *Escherichia coli* cells expressing the monooxygenase HpaBC. However, piceatannol production ceased at approximately 25 mM, even when sufficient levels of the substrate resveratrol remained in the reaction mixture. In this study, we found that high concentrations (>20–25 mM) of piceatannol significantly inhibited the HpaBC-catalyzed reaction. Cyclodextrins (CDs) reportedly encapsulate various hydrophobic compounds. We found that the addition of β-CD or γ-CD to the reaction mixture reduced the inhibition caused by the product piceatannol. The effects of β-CD on piceatannol production were more pronounced than those of γ-CD at high concentrations of the substrate resveratrol and CDs. The production of piceatannol reached 49 mM (12 g L^{-1}) in the presence of β-CD, a level twice that achieved in the absence of β-CD. The technique described here might be applicable to the bioproduction of other stilbenes and structurally related compounds.

[**Key words:** Cyclodextrin; Monooxygenase; Piceatannol; Product inhibition; Resveratrol; Whole-cell catalyst]

Piceatannol (3,4,3′,5′-tetrahydroxy-*trans*-stilbene) is a naturally occurring hydroxylated analogue of resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) that has attracted increased attention due to reports that it has various health-enhancing properties (1–3). For example, piceatannol inhibits melanogenesis, promotes collagen synthesis, and exhibits vasorelaxant effects (4,5). More recent reports indicate that piceatannol not only induces expression of sirtuin but also inhibits adipogenesis and reduces blood glucose levels (6–8). Furthermore, in rats, piceatannol is absorbed with higher metabolic stability than resveratrol (9). These beneficial properties encourage the use of piceatannol in health and functional foods. Although piceatannol is produced in a variety of plants, including grapes, Japanese knotweed, and passion fruit, the accumulation levels are generally low (1,10,11).

Methods employing biotechnological processes have shown promise for the production of piceatannol. In contrast to piceatannol, resveratrol accumulates at high levels in grapes and Japanese knotweed, making this compound readily available and relatively inexpensive (10–12). Thus, microorganisms and enzymes that covert resveratrol to piceatannol represent a potentially efficient vehicle for the production of piceatannol (Fig. 1). Several studies have reported that *Streptomyces* strains convert resveratrol to piceatannol (13,14). In addition, several monooxygenases, including cytochrome P450s, tyrosinases, and flavin-dependent enzymes, catalyze the reaction (15–17). We recently found that the two-component flavin-dependent monooxygenase HpaBC from *Pseudomonas aeruginosa* exhibits high activity in catalyzing the regioselective hydroxylation of resveratrol to piceatannol (Fig. 1) (18,19). HpaBC consists of a flavin-dependent monooxygenase (HpaB) and an NAD(P)H:flavin oxidoreductase (HpaC) (20,21). Using *Escherichia coli* cells expressing HpaBC as a biocatalyst, piceatannol production reached 23 mM (5.2 g L^{-1}) (18). However, we also noted that piceatannol production ceased at approximately 25 mM, even when sufficient levels of the substrate resveratrol remained in the reaction mixture (18).

In this study, we investigated a method to increase the yield of piceatannol. We found that high concentrations (>20–25 mM) of piceatannol significantly inhibit the HpaBC-catalyzed reaction. Piceatannol interacted non-covalently with cyclodextrins (CDs) to form piceatannol/CD complexes in the previous reports (22,23). We hypothesized that the addition of CDs to the reaction mixture would reduce the product inhibition due to the formation of piceatannol/CD complexes. Herein, we demonstrate that the use of CDs significantly enhances piceatannol production. This technique might be widely applied in the bioproduction of other stilbenes and structurally related compounds.

MATERIALS AND METHODS

Chemicals Resveratrol and piceatannol were purchased from Tokyo Kasei (Tokyo, Japan). Tween 80 was purchased from MP Biomedicals (Illkirch, France).

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1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2018.04.016

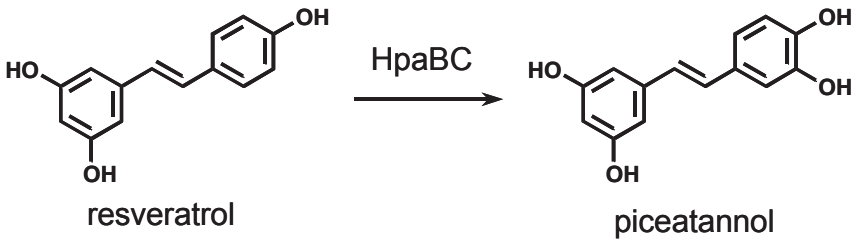

FIG. 1. HpaBC-catalyzed conversion of resveratrol to piceatannol.

α-CD, β-CD, and γ-CD were purchased from Wako Pure Chemicals (Osaka, Japan). All other chemicals were of analytical grade.

Preparation of whole cells The previously constructed pETDhpaBC plasmid encoding the *hpaB* and *hpaC* genes was introduced into *E. coli* BL21 Star(DE3) cells (Invitrogen, Carlsbad, CA, USA) (24). The transformed *E. coli* cells were cultivated at 25°C in LB medium containing (per liter) Bacto tryptone (10 g), Bacto yeast extract (5 g), and NaCl (10 g) (pH 7.0), and ampicillin (100 μg mL^{-1}). After cultivation for 12 h (OD_{600} = 0.8–1.0), isopropyl-β-D-thiogalactopyranoside (1 mM) was added to the medium, and cultivation was continued for an additional 12 h at 25°C. Cells were harvested by centrifugation and washed with potassium phosphate buffer (50 mM, pH 7.5) containing glycerol (10% v/v). These cells were used for whole-cell reactions.

Reactions using whole cells The reaction mixture (100 μL) contained transformed *E. coli* cells (32 g of dry cell weight per liter), the substrate resveratrol (10–50 mM), dimethylsulfoxide (4% v/v), Tween 80 (1% v/v), and potassium phosphate buffer (200 mM, pH 7.5) containing glycerol (10% v/v). We previously reported that glycerol was effective as an energy source in supporting NADH-dependent HpaBC-catalyzed oxidation (24). Piceatannol (10–30 mM) was added to the reaction mixture when assessing the inhibitory effect of the product. When assessing the effect of CDs on the bioconversion reaction, α-CD, β-CD, or γ-CD (10–50 mM) was added to the reaction mixture. Solubilities (%, w/v) of α-CD, β-CD, and γ-CD in water at 25°C are 14.5, 1.85, and 23.2, respectively (25). High concentrations of CDs resulted in a colloidal emulsion. The reactions were carried out at 30°C with vigorous shaking using a microtube shaker.

Flask-scale production of piceatannol The reaction was carried out in a 500-mL flask containing transformed *E. coli* cells (32 g of dry cell weight per liter), resveratrol (50 mM), dimethyl sulfoxide (4% v/v), Tween 80 (1% v/v), β-CD (50 mM), and potassium phosphate buffer (200 mM, pH 7.5) containing glycerol (10% v/v) in a volume of 20 mL. *E. coli* cells used (0.64 g) was collected from ca. 400 mL of culture broth. The reactions were carried out at 30°C with reciprocal shaking at 240 rpm.

Product analysis High-performance liquid chromatography (HPLC) analysis was performed using an 1100 series HPLC system (Agilent, Palo Alto, CA, USA) equipped with an XTerra MS C18 IS column (4.6 × 20 mm; particle size, 3.5 μm; Waters, Milford, MA, USA), as described previously (18,26). The reaction mixture (100 μL) was acidified by the addition of HCl (pH 2–3), and methanol (500 μL) and water (400 μL) were then added. The solution was vigorously shaken and centrifuged. The resulting supernatant was appropriately diluted and injected into the HPLC system. Mobile phase A consisted of a mixture of acetonitrile/methanol/potassium phosphate buffer (10 mM, pH 2.7) at a ratio of 2.5:2.5:95, and mobile phase B consisted of acetonitrile. The samples were eluted with 0% B for 3 min, followed by a linear gradient of 0–70% B for 9 min at a flow rate of 1 mL min^{-1}. Compounds were detected spectrophotometrically at a wavelength of 254 nm. Resveratrol and piceatannol were detected at 7.1 min and 6.6 min, respectively, regardless of the addition of CDs to the reaction mixture.

RESULTS AND DISCUSSION

Inhibition of bioconversion by the product piceatannol *E. coli* cells expressing HpaBC completely converted 10 mM resveratrol to piceatannol within 2 h (19). However, when whole cells were incubated with 30 mM resveratrol, the production of piceatannol ceased at approximately 25 mM, even though sufficient substrate remained in the reaction mixture (18). We also confirmed that resveratrol concentrations >30 mM led to the same results (see Fig. 5 for 50 mM resveratrol). These results suggest that the product piceatannol inhibits the HpaBC-catalyzed reaction. We previously reported that HpaBC also catalyzes the hydroxylation of piceatannol to 3,4,5,3′,5′-pentahydroxy-*trans*-stilbene (PHS) (19). However, HpaBC cannot catalyze the piceatannol hydroxylation when sufficient resveratrol exists in the reaction mixture (19).

We first examined the effect of piceatannol on the HpaBC-catalyzed reaction. HpaBC whole-cell catalyst was incubated with 10 mM resveratrol in the presence of 10–30 mM piceatannol. As the concentration of piceatannol increased, less resveratrol was converted by the catalyst (Fig. 2). Although 10 mM piceatannol inhibited only 25% of the activity compared with that in the absence of piceatannol, piceatannol at 20 mM inhibited 85% of the activity. Furthermore, at 30 mM, piceatannol completely inhibited the reaction. These results indicate that at high concentrations, piceatannol completely inhibits the HpaBC-catalyzed reaction.

Reduction of piceatannol-mediated inhibition by CDs Piceatannol reportedly interacts non-covalently with CDs to form piceatannol/CD complexes (22,23). CDs are a family of cyclic oligosaccharides consisting of glucopyranose monomers. Typical CDs are α-CD, β-CD, and γ-CD, which consist of six, seven, and eight glucopyranose monomers, respectively (25,27). The hydroxy groups in CDs are oriented to the outside of the cyclic molecules. Thus, the outer surface of the molecules is hydrophilic, whereas the inner cavity is hydrophobic. This molecular structure enables CDs to encapsulate various hydrophobic compounds. α-CD, β-CD, and γ-CD each forms a 1:1 complex with piceatannol (22,23). We hypothesized that the addition of CDs to the reaction mixture would reduce the inhibition caused by piceatannol through the formation of the piceatannol/CD complexes. After the reaction, piceatannol can be readily collected from the complexes because the interaction is non-covalent.

To examine the effect of CDs on the piceatannol-mediated inhibition of resveratrol conversion, HpaBC whole-cell catalyst

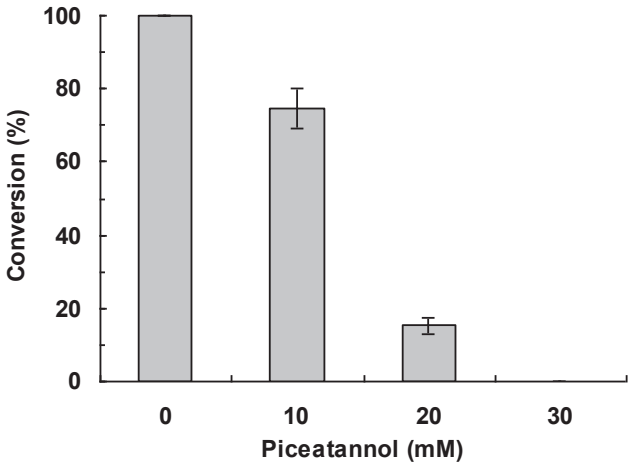

FIG. 2. Effect of piceatannol concentration on the HpaBC-catalyzed reaction. After a 6-h reaction of *E. coli* cells expressing HpaBC with 10 mM resveratrol in the presence of various concentrations of piceatannol, consumption of the substrate resveratrol was determined. Data are the average from three independent experiments; error bars indicate standard deviations from the means.

Please cite this article in press as: Furuya, T., et al., Efficient monooxygenase-catalyzed piceatannol production: Application of cyclodextrins for reducing product inhibition, J. Biosci. Bioeng., (2018), https://doi.org/10.1016/j.jbiosc.2018.04.016