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Enantioselective synthesis of (*S*)-phenylephrine by whole cells of recombinant *Escherichia coli* expressing the amino alcohol dehydrogenase gene from *Rhodococcus erythropolis* BCRC 10909

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ABSTRACT

(*R*)-phenylephrine [(*R*)-PE] is an α_1-adrenergic receptor agonist that is widely used in over-the-counter drugs to treat the common cold. We found that *Rhodococcus erythropolis* BCRC 10909 can convert detectable level of 1-(3-hydroxyphenyl)-2-(methylamino) ethanone (HPMAE) to (*S*)-PE by high performance liquid chromatography tandem mass spectrometry analysis. An amino alcohol dehydrogenase gene (*RE_AADH*) which possesses the ability to convert HPMAE to (*S*)-PE was then isolated from *R. erythropolis* BCRC 10909 and expressed in *Escherichia coli* NovaBlue. The purified RE_AADH, tagged with 6×His, had a molecular mass of approximately 30 kDa and exhibited a specific activity of 0.19 μU/mg to HPMAE in the presence of NADPH, indicating this enzyme could be categorized as $NADP^+$-dependent short-chain dehydrogenase reductase. *E. coli* NovaBlue cell expressing the *RE_AADH* gene was able to convert HPMAE to (*S*)-PE with more than 99% enantiomeric excess (ee), 78% yield and a productivity of 3.9 mmol (*S*)-PE/L h in 12 h at 30 °C and pH 7. The (*S*)-PE, recovered from reaction mixture by precipitation at pH 11.3, could be converted to (*R*)-PE (ee > 99%) by Walden inversion reaction. This is the first reported biocatalytic process for the production of (*S*)-PE from HPMAE.

1. Introduction

Phenylephrine (PE), a sympathomimetic agonist, has a wide variety of surgical applications, such as counteracting hypotension. It also is an active ingredient used in eye drops to dilate the pupil and in topical nasal decongestants [1,2]. Although the systemic bioavailability of PE in nasal decongestants is only about 38% that of orally administered *d*-pseudoephedrine (*d*-PDE) [3,4], PE does not cause the release of endogenous noradrenaline [5] and is less likely to cause side effects like central nervous system stimulation, insomnia, anxiety, irritability and restlessness [2]. Moreover, the Combat Methamphetamine Epidemic Act of 2005 permits the banning of over-the-count sales of medications containing *d*-PDE, in order to control the clandestine manufacture of methamphetamine from *d*-PDE in the USA. This has resulted in the substitution of (*R*)-PE for *d*-PDE in many nasal decongestants [6,7].

PE contains a chiral center in the C_α of the side chain, and (*R*)-PE exhibits more potent than the *S* enantiomer in activating of α_1-adrenergic receptors [2]. (*R*)-PE can be synthesized by symmetric hydrogenation and chemical resolution [8–10]. However, a racemic mixture typically results from symmetric hydrogenation, and repeat kinetic resolution to obtain the desired optical product often is both expensive and tedious. In recent years, several chemical asymmetric hydrogenation methods have been developed to allow for more direct (*R*)-PE synthesis [11–14]; but these processes usually require high pressure, high temperature, and several organic solvents that are not environmentally friendly.

Chiral aryl alcohols are important chiral building blocks for the production of optically active drugs in the pharmaceutical and agrochemical industries [15–17]. They can be obtained by asymmetric reduction of prochiral aryl ketones using oxidoreductases. Chiral 2-chloro-1-phenylethanol is a key intermediate during the preparation of anti-depressants and potential therapeutic agents of cocaine-abuse [18]. It can be converted from 2-chloro-1-phenylethanone by asymmetric reduction, using *Candida magnoliae* $NADP^+$-dependent carbonyl reductase [19] or *Ralstonia* sp. $NADP^+$-dependent alcohol dehydrogenase [20]. *Corynebacterium* sp. ST-10 with NAD^+-dependent phenylacetaldehyde reductase activity can be used in the conversion of 2-chloro-1-(3-chlorophenyl)ethanone to 2-chloro-1-(3-chlorophenyl)ethanol, a precursor for the synthesis of β_3 adrenergic receptor agonists [21,22]. Very recently, *Escherichia coli* cells that express the L-1-

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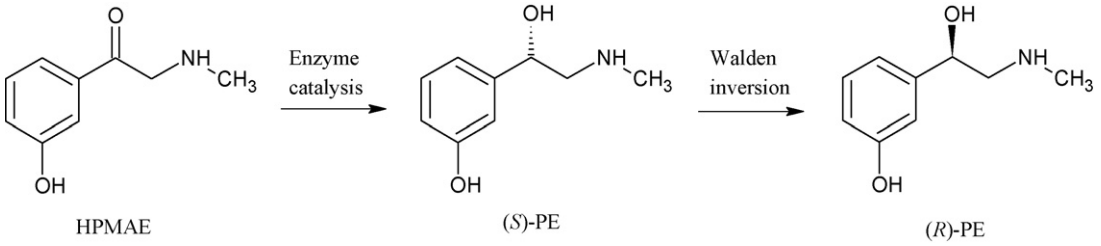

Fig. 1. Schematic diagram showing the conversion of HPMAE to (*S*)-PE by asymmetrical biocatalysis and the conversion of (*S*)-PE to (*R*)-PE by Walden inversion reaction.

amino-2-propanol dehydrogenase (*AADH*) gene from *Rhodococcus erythropolis* MAK154 have been demonstrated to possess the capacity to reduce (*S*)-1-phenyl-2-methylamino-propan-1-one (MAK) to *d*-PDE [23].

PE and *d*-PDE are almost identical in their structure, except that PE contains one hydroxyl group in the phenyl moiety, and *d*-PDE contains one methyl group at C_β in the side chain. To the best of our knowledge, no bioconversion method for the production of chiral PE from 1-(3-hydroxyphenyl)-2-(methylamino) ethanone (HPMAE) has been reported. In this study, we describe that *E. coli* cells expressing amino alcohol dehydrogenase gene (*RE_AADH*) from *R. erythropolis* BCRC 10909 are able to produce (*S*)-PE from HPMAE, and the resulting (*S*)-PE could subsequently be converted to (*R*)-PE, a clinically useful sympathomimetic agonist, by Walden inversion reaction (Fig. 1).

2. Materials and methods

2.1. Chemicals, bacterial strains and media

(*R*)-PE and other general chemicals were purchased from Sigma–Aldrich (St. Louis, MO). All solvents used in HPLC analysis were of LC grade and purchased from Merck (Darmstadt, Germany). HPMAE and (*S*)-PE were obtained from Industrial Technology Research Institute (Hsinchu, Taiwan). The chemicals required for protein assay and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad (Hercules, CA). Culture media were obtained from Becton, Dickinson and Company (Sparks, MD). Bacterial strains were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). The expression vector pQE-30 was purchased from QIAGEN (Hilden, Germany).

2.2. Analysis of HPMAE and PE

A high performance liquid chromatography tandem mass spectrometry (HPLC–MS-MS) system consisting of two Perkin-Elmer Series 200 Micro LC pumps, a Series 200 Autosampler (Perkin-Elmer Co., Waltham, MA), and an AB-Sciex API-2000 triple quadrupole mass spectrometer with TurboIonSpray probe (Applied-Biosystems, Foster City, CA), was used to rapidly identify HPMAE and PE in the reaction mixture. Data processing was performed using Analyst version 1.3.1 software (Applied-Biosystems). HPLC analysis was performed on a reversed-phase Polaris C-18A column (2 mm i.d. × 50 mm; particle size, 3 μm) (Varian Inc. Palo Alto, CA). The mobile phase was 0.1% formic acid and methanol at a ratio of 15:85, and the flow rate was 100 μl/min. For MS-MS detection, the TurboIonSpray, orifice voltages, temperature, collision energy, and entrance potential were set at 5500 V, 80 V, 200 °C, 28 eV, and −9 V, respectively. The collision gas (nitrogen) was maintained at a pressure of 2.3×10^{-5} torr. The positive ion mode and multiple-reaction monitoring (MRM) mode were used to detect the HPMAE (*m*/*z* 166.1–148.1) and PE (*m*/*z* 168.1–150.1), respectively. Total analysis time was 5 min for each sample.

The chirality of PE was analyzed by HPLC with a chiral column (chiral-HPLC). Analytical HPLC was performed utilizing a Waters 2695 LC/Waters 996 Photodiode Array detector system with a CYCLOBOND I 2000 AC column (3.2 × 250 mm, Astec Inc., Whippany, NJ), the mobile phase consisted of methanol and 0.5% sodium acetate (pH 5.5) at a ratio of 5:95 under the following conditions at room temperature: flow rate of 0.7 ml/min, detection at 215 nm, scanning from 200 to 400 nm for compound identification, and retention times of 13.5 min for HPMAE, 14.8 min for (*R*)-PE, and 18.6 min for (*S*)-PE. Concentrations of HPMAE, (*S*)-PE and (*R*)-PE in the reaction mixture were also measured by chiral-HPLC, which was calibrated using several standard solutions of HPMAE, (*S*)-PE and (*R*)-PE, with their concentrations varied from 1 to 100 mM. The standard solutions were prepared freshly prior to each experiment. The values of enantiomeric excess of (*S*)-PE were calculated using the equation: $ee^{(S)\text{-PE}} = [(S\text{-enantiomer}) - (R\text{-enantiomer})]/[(S\text{-enantiomer}) + (R\text{-enantiomer})] \times 100\%$.

2.3. Screening of bacterial strains capable of converting HPMAE to PE

Bacterial colonies were inoculated into 50 ml Luria-Bertani (LB) medium and incubated at a given temperature for each microorganism with vigorous shaking until an OD_{600} of approximately 2.0 was achieved. Cells were harvested by centrifugation at 12,000 × *g* for 10 min. Cell pellets were resuspended in 10 ml of 100 mM sodium phosphate buffer (pH 7) or sodium citrate buffer (pH 6) containing 2% glucose and 10 mM HPMAE, and then incubated at 28 or 37 °C for 2 h. Once the reaction was completed, the cells were removed by centrifugation. The reaction solution was filtered through a 0.22-μm membrane and subjected to HPLC–MS-MS analysis.

2.4. Expression of the RE_AADH gene in E. coli

Genomic DNA of *R. erythropolis* BCRC 10909 was isolated using a MasterPure Gram Positive DNA Purification Kit (Epicentre Biotechnologies, Madison, WI). The open reading frame (ORF) of *RE_AADH* was obtained by PCR using *R. erythropolis* genomic DNA as the template, and primers designed on the basis of the *R. erythropolis* MAK154 *AADH* gene [23]: forward primer (5′-CGGGATCCATGTTCAACTCCATTGAAG), which introduces the sequence for a unique *Bam* HI site (underlined); and reverse primer (5′-CCCAAGCTTTTACAGTTCGCCGAGCGCCAT), which incorporates sequences from a unique *Hin*d III site (underlined). The PCR product was cloned as a *Bam* HI-*Hin*d III fragment into the corresponding site of pQE-30 to generate the plasmid pQE-*aadh*, thereby allowing high-level expression of the N-terminal 6×His-tagged RE_AADH in *E. coli*. The recombinant plasmid was introduced into *E. coli* NovaBlue. For high-level expression of the *RE_AADH* gene, *E. coli* NovaBlue (pQE-*aadh*) was grown in LB broth containing ampicillin (100 μg/ml) at 37 °C for 16 h with shaking. The culture was diluted (1:100) with 100 ml of LB and grown at 37 °C to an OD_{600} of approximately 0.6–0.8. To induce gene expression, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the cultivation continued at 30 °C for an additional 6 h. Cells were collected by centrifugation at 12,000 × *g* and 4 °C for 10 min. Bacterial pellets were washed twice with 25 ml of ice-cold 100 mM sodium phosphate buffer (pH 7.0) and resuspended in the same buffer. The IPTG-induced cells were disrupted by sonication and the cell debris pelleted by centrifugation at 4 °C for 15 min. The resulting supernatant was subjected to a Ni-NTA column (QIAGEN, Hilden, Germany), and the purification procedure carried out in accordance with the manufacturer's instructions. The expression level of the *RE_AADH* gene was analyzed by SDS-PAGE. The concentration of purified protein was measured with a protein assay kit (Bio-Rad, Hercules, CA), using bovine serum albumin as the standard. Reduction activity of RE_AADH was measured by determining the production of PE from HPMAE by chiral-HPLC. The assay mixture (500 μl), containing the appropriate amount of enzyme, 5 mM HPMAE, and 1 mM NADPH in 100 mM sodium phosphate buffer (pH 6.0), was incubated at 30 °C. The enzyme activity of one unit (U) was defined as the amount of enzymes that result in the formation of 1 μmol PE from HPMAE per minute at 30 °C.

2.5. Production of (S)-PE using recombinant E. coli cells

A glass vessel (250 ml) equipped with a propeller-type impeller and an automatic pH controller (pH/ORP controller PC310, Suntex CO., Taipei, Taiwan) was used for the conversion of HPMAE to (*S*)-PE. A reaction mixture (100 ml) containing 1 g (wet weight) of IPTG-induced *E. coli* NovaBlue (pQE-*aadh*), 2% glucose, 20 mM KCl, 18 mM NH_4Cl, and various concentrations of HPMAE in 100 mM sodium phosphate buffer (pH 6.5 or 7.0) was incubated at 30 °C. The consumption of HPMAE and the production of (*S*)-PE were measured using chiral-HPLC.

2.6. Recovery of (S)-PE and Walden inversion reaction

Recovery of (*S*)-PE from the reaction mixture and conversion of (*S*)-PE to (*R*)-PE were performed mainly according to the method described by Dorokhova et al. [24]. Briefly, 200 ml solution containing 48 mM (*S*)-PE, which were prepared from the reaction mixture containing 60 mM HPMAE, was centrifuged at 12,000 × *g* and 4 °C for 20 min. The supernatants were filtered with a 0.22 μm membrane. The pH of the filtrate was adjusted to 10.5–11.5 using 10 N NaOH. As a result, the (*S*)-PE precipitated out, which was collected by centrifugation and then washed with cold water. Washed precipitates were dried by vacuum evaporator for Walden inversion reaction. To perform the Walden inversion reaction, (*S*)-PE, acetic anhydride, and