Biosci. Biotechnol. Biochem., **70** (9), 2296–2298, 2006

Note

Enzymatic Synthesis of L-Pipecolic Acid by Δ^1-Piperideine-2-carboxylate Reductase from *Pseudomonas putida*

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Received March 9, 2006; Accepted April 30, 2006; Online Publication, September 7, 2006
[doi:10.1271/bbb.60125]

L-Pipecolic acid is a chiral pharmaceutical intermediate. An enzymatic system for the synthesis of L-pipecolic acid from L-lysine by commercial L-lysine α-oxidase from *Trichoderma viride* and an extract of recombinant *Escherichia coli* cells coexpressing Δ^1-piperideine-2-carboxylate reductase from *Pseudomonas putida* and glucose dehydrogenase from *Bacillus subtilis* is described. A laboratory-scale process provided 27 g/l of L-pipecolic acid in 99.7% *e.e.*

Key words: Δ^1-piperideine-2-carboxylate reductase; *Pseudomonas putida*; L-pipecolic acid; Δ^1-piperideine-2-carboxylate

L-Pipecolic acid, a nonproteinogenic α-amino acid, is a key component of many bioactive molecules, such as the immunosuppressant FK506,[1)] the anticancer agent VX710,[2)] the antifungal antibiotic demethoxyrapamycin,[3)] the *N*-methyl-D-aspartate antagonist selfotel,[4)] the antitumor antibiotic sandramycin,[5)] the phytotoxic metabolite Cyl-2,[6)] the anesthetic bupivacaine,[7)] and the HIV protease inhibitor palinavir.[8)] There is an increasing demand for a convenient and efficient synthetic route to enantiomerically pure L-pipecolic acid because of its use in the synthesis of new medicaments.

Current methods to obtain a pure enantiomer of L-pipecolic acid involve chemical resolution,[9)] stereoselective transformation,[10)] the derivatization of natural amino acids,[11)] and enzymatic reactions,[12–16)] but most of these methods fail to provide a satisfactory solution for the synthesis of chiral pipecolic acid on an industrial scale due to certain limitations, such as tedious procedures, low yields, and unavailability of starting materials. Hence, new and convenient methods for the preparation of optically active pipecolic acid are required.

Recently, we identified and cloned the gene encoding Δ^1-piperideine-2-carboxylate (Pip2C) reductase from *Pseudomonas putida*.[17)] The enzyme, which belongs to a new NAD(P)H-dependent oxidoreductase family,[18,19)] catalyzes the reduction of Pip2C to L-pipecolic acid and is involved in D-lysine catabolism.[17)] In this paper, we describe the production of L-pipecolic acid by an enzyme-coupled system consisting of Pip2C reductase, glucose dehydrogenase (GDH) from *Bacillus subtilis*,[20)] and commercial L-lysine α-oxidase from *Trichoderma viride* (Yamasa, Hiroshima, Japan) (Scheme 1).

Recombinant *Escherichia coli* BL21(DE3) cells harboring both pDPKA,[17,21)] which carries a gene for Pip2C reductase, and pSTVbsGDH, which has a GDH[20)] gene between the EcoRI and PstI sites of pSTV28 (Takara Shuzo, Japan), were cultured in Luria-Bertani medium containing 100 μg/ml ampicillin and 25 μg/ml chloramphenicol at 37 °C for 16 h. Three h after induction of gene expression with 1 mM isopropyl-β-D-thiogalactopyranoside, the cells were harvested and washed twice with 20 mM Tris–HCl (pH 7.0). The washed cells were disrupted by sonication and centrifuged. The resulting crude extract was used directly for the production of L-pipecolic acid. The standard reaction mixture for the production of L-pipecolic acid contained L-lysine (55 mM), glucose (550 mM), $NADP^+$ (0.2 mM), FAD (1 mM), L-lysine α-oxidase (Yamasa, 3.0 U/ml), catalase from bovine liver (Sigma, St. Louis, MO, 50 U/ml), Tris–HCl (100 mM), and a cell-free extract (3.0 mg-protein/ml) at pH 7.5. The reaction was performed at 30 °C with reciprocal shaking. To prevent a decrease in pH during the reaction, the reaction mixture was adjusted to pH 7.5 with 10 M NaOH. Aliquots of the reaction mixture were removed for analysis of the product and the substrate. The concentrations of both L-pipecolic acid and L-lysine and the *e.e.* of L-pipecolic acid were determined by high-performance liquid chromatography (HPLC) (Wa-

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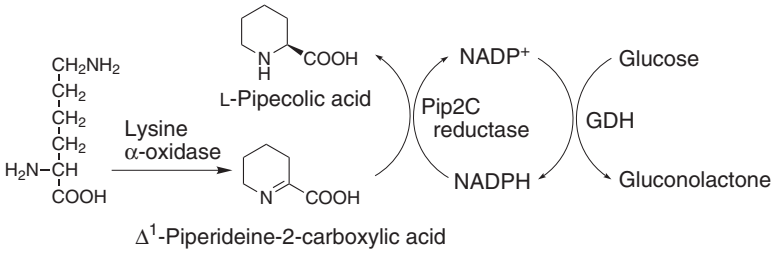
Abbreviations: Pip2C, Δ^1-piperideine-2-carboxylate; GDH, glucose dehydrogenase



Scheme 1. Bioconversion System from L-Lysine to L-Pipecolic Acid.

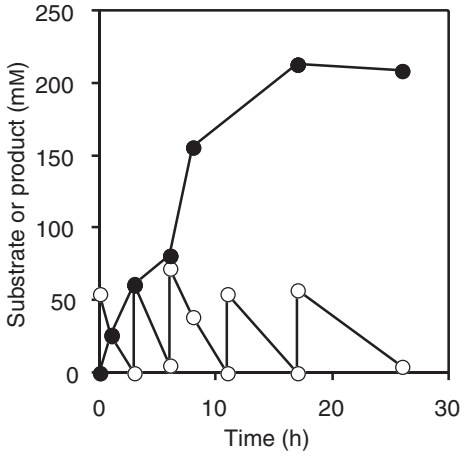


Fig. 1. Time Course of the Production of L-Pipecolic Acid.

Production of L-pipecolic acid was performed at 30 °C at pH 7.5 in a reaction mixture containing L-lysine (55 mM), glucose (550 mM), $NADP^+$ (0.11 mM), FAD (1 mM), L-lysine α-oxidase (3.0 U/ml), catalase (50 U/ml), Tris–HCl (100 mM), and a cell-free extract (3.0 mg-protein/ml). L-Pipecolic acid (●) and L-lysine (○) were determined by HPLC.

ters Alliance 2695; Waters, Milford, MA) with a Chiralpak WE column (4.6 × 250 mm, Daicel Chemical Industries, Tokyo) and 2 mM $CuSO_4$ as the eluent at a flow rate of 0.75 ml/min at 50 °C, monitored at 254 nm. Standard curves for L-lysine and L-pipecolic acid were linear over ranges of 0–10 mM ($R^2 = 0.998$) and 0–16 mM ($R^2 = 0.999$) respectively. We synthesized L-pipecolic acid in a 10-ml standard reaction mixture. L-Lysine was added to the reaction mixture stepwise. After a 17-h reaction, 210 mM (27 g/l) of L-pipecolic acid was produced (Fig. 1). The molar yield of L-pipecolic acid as to L-lysine was 90%. The *e.e.* of the product obtained by the 17-h reaction was determined by HPLC. The retention time of L-pipecolic acid was 14.7 min, and that of D-pipecolic acid was 18.0 min. D-Pipecolic acid was not detected in the reaction mixture by HPLC analysis. L-Pipecolic acid was obtained with satisfactorily high optical purity (> 99.7% *e.e.*). L-Pipecolic acid can be isolated from the resultant reaction solution by commonly used methods, such as ion exchange chromatography and crystallization, as described previously.[22)]

Fujii *et al.* reported the synthesis of L-pipecolic acid from L-lysine using recombinant *E. coli* cells expressing L-lysine 6-aminotransferase and pyrroline-5-carboxylate reductase.[12,13)] In their system, at most 16 g/l of L-pipecolic acid is synthesized in a 110-h reaction.[13)] Thus our system can produce a higher amount of the product in a shorter reaction time than the previously reported system, indicating that it is applicable in industrial production of L-pipecolic acid. The L-lysine α-oxidase reaction employed as the first step in our system can be more efficient than the aminotransferase reaction in the system by Fujii *et al.*, because of the low activity of the recombinant aminotransferase in *E. coli*.[23)] An additional potential advantage of our system is that the product generated from L-lysine by L-lysine α-oxidase is almost completely cyclized to Pip2C: no intermediates, α-imino acid or α-keto-ε-aminocaproate, were detected in the reaction mixture. This suggests that cyclization occurs in the active site of the enzyme before release of the product into solution, which is consistent with an observation by Danson *et al.*[24)] Previously we found that Pip2C reductase from *P. putida* also has *N*-methyl-L-amino acid dehydrogenase activity[21)] and can be used for the synthesis of *N*-methyl-L-phenylalanine.[25)] Thus the enzyme is unique in that it is useful for the production of different types of chiral compounds.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (B) 15370043 and 17370037 (to N.E.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by a Grant-in-Aid for the Encouragement of Young Scientists 15780070 (to H.M.) from the Japan Society for the Promotion of Science, by the National Project on Protein Structural and Functional Analyses, and by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (21st Century COE on the Kyoto University Alliance for Chemistry).

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