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Novel Enzyme Family Found in Filamentous Fungi Catalyzing *trans*-4-Hydroxylation of L-Pipecolic Acid

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Hydroxypipecolic acids are bioactive compounds widely distributed in nature and are valuable building blocks for the organic synthesis of pharmaceuticals. We have found a novel hydroxylating enzyme with activity toward L-pipecolic acid (L-Pip) in a filamentous fungus, *Fusarium oxysporum* c8D. The enzyme L-Pip *trans*-4-hydroxylase (Pip4H) of *F. oxysporum* (*Fo*Pip4H) belongs to the Fe(II)/α-ketoglutarate-dependent dioxygenase superfamily, catalyzes the regio- and stereoselective hydroxylation of L-Pip, and produces optically pure *trans*-4-hydroxy-L-pipecolic acid (*trans*-4-L-HyPip). Amino acid sequence analysis revealed several fungal enzymes homologous with *Fo*Pip4H, and five of these also had L-Pip *trans*-4-hydroxylation activity. In particular, the homologous Pip4H enzyme derived from *Aspergillus nidulans* FGSC A4 (*An*Pip4H) had a broader substrate specificity spectrum than other homologues and reacted with the L and D forms of various cyclic and aliphatic amino acids. Using *Fo*Pip4H as a biocatalyst, a system for the preparative-scale production of chiral *trans*-4-L-HyPip was successfully developed. Thus, we report a fungal family of L-Pip hydroxylases and the enzymatic preparation of *trans*-4-L-HyPip, a bioactive compound and a constituent of secondary metabolites with useful physiological activities.

Hydroxypipecolic acids (HyPips) are naturally occurring six-membered heterocyclic hydroxy amino acids that are widely distributed in nature. For instance, 4-hydroxy-, 5-hydroxy-, and 4,5-dihydroxypipecolic acids have been found in various members of the plant family (1–4). Likewise, 5-hydroxypipecolic acid was found to be present in mammalian brain and blood (5). HyPips are also components of some peptide antibiotics, terpenoids, and alkaloids, for example, *trans*-3-hydroxy-L-pipecolic acid (*trans*-3-L-HyPip) in GE81112 (6), *cis*-3-hydroxy-L-pipecolic acid (*cis*-3-L-HyPip) in tetrazomine (7), *trans*-4-hydroxy-L-pipecolic acid (*trans*-4-L-HyPip) in ulleungamides (8) and damipipecolin (9), and *cis*-4-hydroxy-L-pipecolic acid (*cis*-4-L-HyPip) in halichonadins (10).

HyPips have also been used as valuable building blocks for organic synthesis in various industries, such as the pharmaceutical industry. For example, *cis*-4-L-HyPip is a component of palinavir, an HIV protease inhibitor (11, 12), and *cis*-5-hydroxy-L-pipecolic acid (*cis*-5-L-HyPip) is a precursor for the synthesis of the β-lactamase inhibitor MK-7655 (13). Thus, the development of processes for the industrial synthesis of HyPips may be of great significance.

The asymmetric biosynthesis of several L-HyPips was demonstrated by regio- and stereoselective hydroxylation of L-pipecolic acid (L-Pip) with enzymes belonging to the Fe(II)/α-ketoglutarate (αKG)-dependent dioxygenase (Fe/αKG-DO) superfamily. Three Fe/αKG-DOs, L-proline *cis*-3-hydroxylase (*cis*-Pro3H) of *Streptomyces* sp. strain TH1 (14), L-proline *trans*-4-hydroxylase (*trans*-Pro4H) of *Dactylosporangium* sp. strain RH1 (15), and L-proline *cis*-4-hydroxylase (*cis*-Pro4H) of *Sinorhizobium meliloti* (16), were found to possess hydroxylating activities toward L-Pip as well as L-proline and were utilized for the bioconversion of L-Pip into L-HyPip isomers. *cis*-Pro3H converted L-Pip into *cis*-3-L-HyPip with a yield of 17%, *cis*-Pro4H produced 33% *cis*-3-L-HyPip and 33% *cis*-5-L-HyPip from L-Pip in parallel, and *trans*-Pro4H converted L-Pip into *trans*-5-L-hydroxypipecolic acid (*trans*-5-L-HyPip) with a yield of 68% (17).

Thus, the enzymatic hydroxylation of L-Pip was achieved as a side reaction of L-proline hydroxylase. To date, specific L-Pip-hydroxylating enzymes have not been reported, despite the fact that HyPips are important metabolites found in plants and mammals. In the present study, we identified and characterized L-Pip hydroxylases broadly conserved in some filamentous fungi. We found that the newly discovered L-Pip hydroxylases were particularly suitable biocatalysts for the asymmetric hydroxylation of cyclic amino acids, such as pipecolic acids and prolines. Moreover, the widespread distribution of genes encoding L-Pip hydroxylases indicates some important physiological roles of fungal secondary metabolites related to *trans*-4-L-HyPip.

MATERIALS AND METHODS

Strains and culture. Microorganisms isolated from soil and plant samples were cultured in GP medium comprised of 0.15% (wt/vol) KH_2PO_4, 0.05% (wt/vol) K_2HPO_4, 0.1% (wt/vol) NH_4Cl, 0.03% (wt/vol) $MgSO_4 \cdot 7H_2O$, 0.01% (wt/vol) yeast extract, 0.025% (wt/vol) glucose, and 0.1% (wt/vol) L-Pip (pH 7.0) and grown at 28°C with shaking. *Escherichia coli* JM109 and Rosetta2(DE3) (Novagen, WI, USA) were used as host strains for the overexpression of the recombinant enzymes and cultured at 28°C in LB medium, comprised of 1% (wt/vol) tryptone, 0.5% (wt/vol) yeast extract, and 1% (wt/vol) NaCl, with the addition of appropriate antibiotics.

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Conditions for amino acid analysis by HPLC. Amino acids were derivatized using an AccQ-Tag derivatization kit (Waters, MA, USA) according to the manufacturer's instructions and analyzed with an Alliance 2695 high-performance liquid chromatography (HPLC) system (Waters). An XBridge C_{18} column (particle size, 5 μm; 2.1 by 150 mm; Waters) was used for separation at 40°C. The mobile phases were 10 mM ammonium acetate at pH 5.0 (eluent A) and methanol (eluent B), and the flow rate of the eluent was 0.3 ml/min. The eluent gradients were 0 to 1% (vol/vol) eluent B for 0 to 0.5 min, 1 to 5% eluent B for 0.5 to 18 min, 5 to 9% eluent B for 18 to 19 min, 9 to 17% eluent B for 19 to 29.5 min, 17 to 60% eluent B for 29.5 to 40 min, and 60% eluent B for 40 to 43 min. The AccQ-Tag derivatives were detected with a fluorescence detector (excitation, 250 nm; emission, 395 nm). Electrospray ionization (ESI) mass spectrometry (MS) was carried out using an LCMS-2010A liquid chromatography (LC)-MS system (Shimadzu, Kyoto, Japan). MS conditions were as follows: block temperature, 200°C; curved desolvation line (CDL) temperature, 250°C; detector voltage, 1.5 kV; and nebulizing gas flow, 1.5 liters/min.

Conditions for succinate analysis by ion chromatography (IC). Succinate was quantitatively analyzed by use of a Prominence HPLC system (Shimadzu) equipped with a Shim-pack IC-SA2 column (150 by 4.6 mm; Shimadzu) at 50°C. The sample was isocratically eluted in the mobile phase, consisting of 1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$, at a flow rate of 1.0 ml/min. The succinate concentration was determined with a suppressed conductivity detector.

Assay of L-Pip hydroxylase activity. The L-Pip hydroxylase activity in the samples, such as wet cell pellets of the microorganisms, their cell lysates, and protein solutions, was assayed. The standard reaction conditions were as follows: the reaction mixture consisted of 10 mM L-Pip, 15 mM αKG, 0.5 mM $FeSO_4 \cdot 7H_2O$, 5 mM ascorbate, and 1 mM dithiothreitol (DTT) in 50 mM MES (morpholineethanesulfonic acid) buffer (pH 6.5), and the reaction was carried out at 20°C for 10 min with shaking at 300 rpm. After the reaction, hydroxylation activity was determined by measurement of the amount of succinate produced by succinate analysis and/or the amino acid content of the samples by amino acid analysis. In order to determine the catalytic properties of L-Pip hydroxylases, 1 mg/ml of purified enzyme was used for the reaction. The effects of pH on hydroxylation activity were examined by varying the reaction pH between pH 3.0 and 10.0. pH stability was examined by measuring the residual activity after incubation in each buffer for 1 h. The effects of temperature on hydroxylation activity were examined by varying the reaction temperature between 10°C and 70°C. The temperature stability was examined by measuring the residual activity after incubation at each temperature for 1 h.

For substrate specificity analysis, various kinds of substrates were used instead of L-Pip. To determine the kinetic parameters, the reaction was carried out at the optimum pH and temperature for each enzyme and with substrate concentrations that varied from 0.25 to 10 mM. Apparent K_m and V_{max} values were determined by use of Lineweaver-Burk plots of the data. Enzyme activity is expressed in micromoles per minute per milligram (units per milligram).

Microbial screening for hydroxylation activity of L-Pip. About 100 kinds of soil and plant samples were used as the source of microorganisms. A small amount of untreated sample was added into a test tube containing 5 ml isolation medium, comprised of 0.15% (wt/vol) KH_2PO_4, 0.05% (wt/vol) K_2HPO_4, 0.1% (wt/vol) NH_4Cl, 0.03% (wt/vol) $MgSO_4 \cdot 7H_2O$, 0.01% (wt/vol) yeast extract, and 0.1% (wt/vol) L-Pip (pH 7.0), and incubated at 28°C with shaking for a few days. Fifty microliters of the culture broth was transferred into 5 ml of fresh isolation medium and further cultivated for a few days. Then, the culture was streaked onto an agar plate with isolation medium and cultivated at 28°C for a few days. After cultivation, more than 2,500 colonies appeared, and these colonies were isolated as L-Pip-assimilating microorganisms. These microorganisms were cultured in GP medium and grown at 28°C with shaking for a few days. Microbial cells were harvested by centrifugation at 3,000 rpm for 10 min. The wet cell pellet was applied to the L-Pip hydroxylase activity assay under the standard reaction conditions, except that a reaction time of 16 h was used.

Microbial conversion of L-Pip. *Fusarium oxysporum* c8D was cultured in GP medium at 28°C with shaking for a few days. The wet cell pellet was reacted with L-Pip under the standard reaction conditions, except that a reaction time of 16 h was used. In order to identify the reaction products, the solution remaining after the reaction was centrifuged, and the supernatant was adjusted to pH 11 using 1 M KOH and applied to negative ion-exchange resin (Dowex 2 × 8; Dow Chemical, Midland, USA) that had previously been equilibrated with distilled water. The reaction product of L-Pip was eluted using 3% (wt/vol) KCl and freeze-dried. The dried powder was dissolved in distilled water and separated using a Prominence HPLC system (Shimadzu) equipped with a TSKgel Amide-80 column (7.8 mm by 300 mm; Tosoh, Tokyo, Japan) at 40°C. The mobile phase was 1.5 mM ammonium acetate (pH 5.0) in 85% acetonitrile, and the flow rate was 0.6 ml/min. Amino acids were detected by determination of the UV absorbance at 210 nm. The eluate containing the isolated amino acids was freeze-dried and dissolved in D_2O. Proton and carbon nuclear magnetic resonances (^1H and ^{13}C NMRs, respectively) were recorded on an Avance 500 spectrometer (Bruker, Billerica, MA, USA).

Measurement of L-Pip hydroxylation activity using *F. oxysporum* c8D cell lysate. *F. oxysporum* c8D was cultivated in a medium comprised of 0.15% (wt/vol) KH_2PO_4, 0.05% (wt/vol) K_2HPO_4, 0.1% (wt/vol) NH_4Cl, 0.03% (wt/vol) $MgSO_4 \cdot 7H_2O$, 0.01% (wt/vol) yeast extract, and 0.025% (wt/vol) glucose (pH 7.0) and grown at 28°C with shaking. In order to induce L-Pip hydroxylation activity, 0.1% (wt/vol) L-Pip was added to the medium. At several time points during cultivation, *F. oxysporum* c8D cells were collected by centrifugation and disrupted with glass beads at 4°C using a multibead shocker (Yasui Kikai, Osaka, Japan). The cell lysate was centrifuged at 12,000 × *g* for 30 min, and the resulting supernatant was applied to the L-Pip hydroxylase activity assay. To test the effects of different cofactors on activity, $FeSO_4 \cdot 7H_2O$, αKG, or ascorbate was eliminated from the reaction mixture.

Purification of L-Pip *trans*-4-hydroxylase (Pip4H) from *F. oxysporum* c8D cells (*Fo*Pip4H). *F. oxysporum* c8D was cultivated for 40 h in 500 ml of GP medium. The cell suspension was centrifuged; the cell pellet (2.5 g [wet weight] of cells) was resuspended in 25 ml of MD buffer, consisting of 40 mM MES buffer (pH 6.5), 1 mM DTT, and Complete protease inhibitor cocktail (Roche Diagnostic, Basel, Switzerland) at the manufacturer's recommended concentration; and the cell pellet was disrupted with glass beads at 4°C using a multibead shocker (Yasui Kikai). The cell lysate was centrifuged at 12,000 × *g* for 30 min, and the resulting supernatant was dialyzed against MD buffer. The protein solution was applied into a Toyopearl CM-650M column (3.5 by 150 mm; Tosoh, Tokyo, Japan) that had been equilibrated with MD buffer and eluted with MD buffer containing 1 M NaCl. The fractions showing L-Pip-hydroxylating activities were collected and dialyzed against MD buffer. The proteins were sequentially separated with a Toyopearl DEAE-650M column (3.5 by 150 mm; Tosoh) and a Mono Q column (5 by 50 mm; GE Healthcare Biosciences, Uppsala, Sweden) as described above. The active fractions were collected and applied onto a Phenyl Superose column (GE Healthcare Biosciences) that had been equilibrated with MD buffer containing 1.8 M $(NH_4)_2SO_4$ and eluted using a linear gradient of 1.8 to 0 M $(NH_4)_2SO_4$ in MD buffer. The active fractions were then concentrated using an Ultracal-10K membrane (Millipore, MA, USA) and separated using a Superdex 200 column (GE Healthcare) in MD buffer containing 150 mM NaCl.

Amino acid sequencing of *Fo*Pip4H. The purified *Fo*Pip4H enzyme was freeze-dried and dissolved in 25 μl of 50 mM Tris buffer (pH 9.0) containing 8 M urea, and the solution was incubated at 37°C for 1 h. Then, 25 μl of 50 mM Tris buffer (pH 9.0) and 6 nmol of lysyl endopeptidase were added to the solution and the mixture was incubated at 30°C for 6 h. The resulting peptides were separated with a Prominence HPLC system (Shimadzu) equipped with a Jupiter C_{18} column (250 by 4.6 mm; Phe-

nomenex, CA, USA) at 35°C, using 10 mM trifluoroacetic acid in H_2O (eluent A) and 10 mM trifluoroacetic acid in acetonitrile (eluent B) as the mobile phases. A linear gradient (10 to 45% eluent B over 20 min) was carried out at a flow rate of 1.0 ml/min. The eluate was monitored by determination of the UV absorbance at 210 nm, and the fractions containing the peptides were collected. The N-terminal amino acid sequences of the peptides were determined by automated Edman degradation with a PSQQ-30 protein sequencer (Shimadzu).

Construction of strains for expression of L-Pip hydroxylases. The *Fo*Pip4H gene was amplified by PCR using the following primers: AAGG ATCCATGGCCGCCCTCAACGCAGA and CCAAGCTTTTAAGCCTT AGCTTCAGCAG. PCR was carried out with Tks Gflex DNA polymerase (TaKaRa Bio, Shiga, Japan) under the following conditions: 30 cycles of 10 s at 98°C, 15 s at 56°C, and 40 s at 68°C. For the expression of N-terminally 6×His-tagged proteins, the PCR product was digested with BamHI and HindIII endonucleases and cloned into the expression vector pQE80L (Qiagen, CA, USA), which had previously been digested with the same endonucleases. The resultant plasmid, pQE-*Fopip4H*, was transformed into *E. coli* JM109.

Codon-optimized genes encoding the proteins of the following filamentous fungi homologous to *Fo*Pip4H were synthesized and ligated into the pJexpress411 vector by DNA2.0 Bioengineering Solutions (CA, USA): *Colletotrichum gloeosporioides* Nara gc5 (*Cg*Pip4H; GenBank accession no. XP_007276452), *Aspergillus oryzae* RIB40 (*Ao*Pip4H; GenBank accession no. XP_001827566), *Talaromyces marneffei* ATCC 18224 (PMAA_075210; GenBank accession no. XP_002146999), *Aspergillus flavus* NRRL3357 (AFLA_066710; GenBank accession no. XP_002380230), *Penicillium rubens* Wisconsin 54-1255 (*Pr*Pip4H; GenBank accession no. XP_002558179), *Fusarium graminearum* PH-1 (*Fg*Pip4H; GenBank accession no. XP_011322545), and *Aspergillus nidulans* FGSC A4 (*An*Pip4H; GenBank accession no. XP_659994). The resultant plasmids were designated pJ-*Cgpip4H*, pJ-*Aopip4H*, pJ-*PMAA_075210*, pJ-*AFLA_066710*, pJ-*Prpip4H*, pJ-*Fgpip4H*, and pJ-*Anpip4H*, respectively, and transformed into *E. coli* Rosetta2 (DE3).

Expression and purification of recombinant Pip4H enzymes. Each of the *E. coli* transformants expressing the Pip4H enzymes was cultured at 28°C in LB medium, comprised of 1% (wt/vol) tryptone, 0.5% (wt/vol) yeast extract, and 1% (wt/vol) NaCl, with the addition of appropriate antibiotics. At an optical density at 600 nm (OD_{600}) of 1.0, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and the cultures were incubated for 16 h at 28°C with shaking at 300 rpm. The cell suspension (250 ml) was centrifuged at 3,000 rpm for 10 min, and the cell pellet was suspended in 10 ml of binding buffer containing 20 mM Tris-HCl buffer (pH 7.4), 0.5 M NaCl, 20 mM imidazole, and 1 mM DTT and disrupted for 1 h by sonication with an Insonator 201R sonicator (Kubota, Osaka, Japan). The lysate was centrifuged at 12,000 × *g* for 30 min, and the supernatant was filtered through a 0.45-μm-pore-size Millex syringe-driven filter unit (Millipore). The protein solution was applied to an Ni-Sepharose column (His Trap HP 5 ml; GE Healthcare Bioscience) that had previously been equilibrated with binding buffer. The column was washed with binding buffer, and the proteins were eluted with a linear gradient of 0.02 to 0.50 M imidazole in binding buffer. The fractions containing recombinant Pip4H enzyme were pooled, concentrated by ultrafiltration, and used as purified enzyme.

Preparative-scale production of *trans*-4-L-HyPip by bioconversion with *Fo*Pip4H-expressing *E. coli* cells. For the production of *trans*-4-L-HyPip from L-Pip, the reaction was performed in an 80-ml mixture composed of 100 mM L-Pip, 150 mM αKG, 1 mM $FeSO_4 \cdot 7H_2O$, 10 mM tris(2-carboxyethyl)phosphine (TCEP), 10 mM trisodium citrate, 6 g (wet weight) of cells of *E. coli* JM109/pQE-*Fopip4H*, and 50 mM bis-Tris buffer (pH 6.5) at 15°C for 6 h with vigorous shaking. The reaction mixture was centrifuged, and the supernatant was adjusted to pH 2.0 with 1 M HCl, applied to positive ion-exchange resin (Dowex 50W × 8; Dow Chemical) which had been equilibrated with distilled water, and eluted with 1 M NH_3 solution. The fractions containing the oxidized amino acid products were passed through negative ion-exchange resin (Dowex 2 × 8; Dow Chemical) which had been equilibrated with distilled water and eluted with 1 M HCl. The fractions containing the products were combined and freeze-dried.

Nucleotide sequence accession number. The *Fo*Pip4H gene has been submitted to GenBank and may be found under accession no. LC076551.

RESULTS

L-Pip hydroxylation activities of filamentous fungi isolated from soil. We isolated more than 2,500 microorganisms assimilating L-Pip from soil and plant samples. These microorganisms, including bacteria, actinomycetes, and filamentous fungi, were screened for hydroxylation activity toward L-Pip. In reaction mixtures containing wet cells, two filamentous fungal isolates in particular, c7D and c8D, produced the same L-Pip-derived reaction product, as observed by amino acid analysis. The molecular mass of this product was greater than that of L-Pip by 16 Da, which indicated that the hydroxylation reaction was catalyzed in the reaction mixtures containing these fungal isolates. According to the morphological characteristics and taxonomic identification obtained using the internal transcribed sequence-5.8S ribosomal DNA sequence by the identification services of TechnoSuruga Laboratory Co., Ltd. (Shizuoka, Japan), strain c7D was identified to be *Fusarium merismoides* and strain c8D was identified to be *Fusarium oxysporum*. Because of its higher activity on amino acid analysis, *F. oxysporum* c8D was chosen for further investigation.

Structural analysis of the product of L-Pip hydroxylation by *F. oxysporum* c8D. The hydroxylated product of L-Pip in the reaction mixture with *F. oxysporum* c8D cells was purified and subjected to structural analysis (see Fig. S1 in the supplemental material). The chemical shifts in NMR analysis were as follows: ^1H NMR (500 MHz, D_2O) δ 1.82 to 1.95 (3H, m), 2.19 (1H, d, J = 14.6 Hz), 3.27 (2H, m), 3.88 (1H, dd, J = 11.5, 3.1 Hz), 4.20 (1H, s). ^{13}C NMR (500 MHz, D_2O) δ 28.18, 32.96, 38.72, 54.14, 62.03, 174.52. On the basis of the information from these NMR spectra and published data from a previous study (18), the reaction product of L-Pip was determined to be *trans*-4-hydroxy-L-pipecolic acid (*trans*-4-L-HyPip).

L-Pip-hydroxylating activity of *F. oxysporum* c8D. The hydroxylating activity for the production of *trans*-4-L-HyPip from L-Pip was measured using the lysate of *F. oxysporum* c8D cells collected at various points during cell cultivation. *F. oxysporum* c8D cells cultured without L-Pip showed no activity throughout the growth period. When L-Pip was added to the culture medium, strong hydroxylating activity was observed during late log phase and stationary phase; the activity then gradually diminished after L-Pip depletion (Fig. 1). These results indicate that the L-Pip-hydroxylating activity of *F. oxysporum* c8D is inducible and strictly regulated by the presence of L-Pip during cultivation. In contrast to the effect of L-Pip, no inductive effect of L-Pro on the activity of *F. oxysporum* c8D was observed. Moreover, the addition of ferrous iron, αKG, and ascorbate to the reaction mixture was required for the maximum activity of the *F. oxysporum* c8D cell lysate. Since similar observations have been made for other amino acid hydroxylases belonging to the Fe/αKG-DO superfamily (19–21), these results suggest that the enzyme responsible for L-Pip-hydroxylating activity belongs to the Fe/αKG-DO superfamily.

Identification of an L-Pip *trans*-4-hydroxylase from *F. oxysporum* c8D. An L-Pip *trans*-4-hydroxylase, *Fo*Pip4H, was purified from *F. oxysporum* c8D cells by five steps of column chromatog-

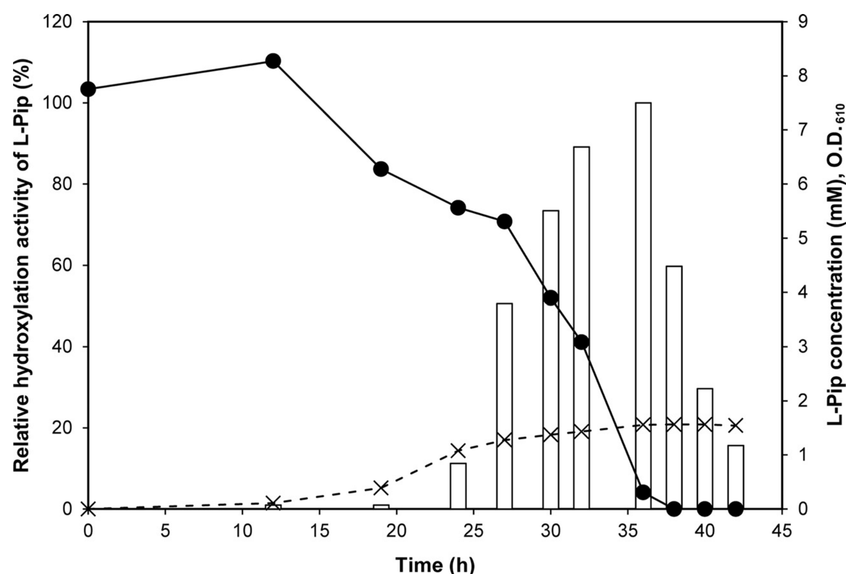

FIG 1 L-Pip hydroxylation activity in *F. oxysporum* c8D cells cultivated with L-Pip. Lysates of *F. oxysporum* c8D cells, collected at several time points during cultivation in medium containing L-Pip, were used for the L-Pip hydroxylation reaction. The relative activity for L-Pip hydroxylation in the cell lysates (white bars), the concentration of L-Pip in the medium (closed circles), and cell densities at an OD_{610} (multiplication signs) are shown.

raphy (Table 1) and had an approximate molecular mass of 40 kDa, as determined by SDS-PAGE analysis (data not shown). Although the N terminus of *Fo*Pip4H was blocked from Edman degradation, its internal amino acid sequences were successfully determined to be MLAK, TRDIK, and GYEKGLGPLKTRDIKE TVD. A BLAST search of these sequences revealed that they correspond to the amino acid sequence for a protein of unknown function, FOXB_08233 (GenBank accession no. EGU81245.1), of *F. oxysporum* Fo5176. The genome region encoding *Fo*Pip4H was sequenced in its entirety and found to include one exon without introns. The full-length amino acid sequences of *Fo*Pip4H and FOXB_08233 were identical. The amino acid sequence of *Fo*Pip4H also contained the motifs required for ferrous iron binding (His^1-XAA-Asp/Glu-XAA_n-His^2) and αKG binding (Arg) that are conserved in the Fe/αKG-DO superfamily (22), providing further evidence that *Fo*Pip4H is a member of the Fe/αKG-DO superfamily. *Fo*Pip4H was heterologously expressed in *E. coli* and purified for use in the enzyme assay, where it was confirmed to have hydroxylating activity toward L-Pip and produce *trans*-4-L-HyPip (Fig. 2A).

Phylogenetic analysis of proteins homologous to *Fo*Pip4H. Proteins homologous to *Fo*Pip4H were obtained by use of a search

TABLE 1 Purification of L-Pip-hydroxylating enzyme from *F. oxysporum* c8D

Step	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Yield (%)
Cell lysate	1.26	45	0.028	100
Toyopearl CM-650M	0.50	9.1	0.055	40
Toyopearl DEAE-650M	0.33	5.1	0.065	26
Mono Q	0.30	1.9	0.16	24
Phenyl Superose	0.067	0.32	0.21	5.3
Superdex 200	0.0022	0.0030	0.74	0.18

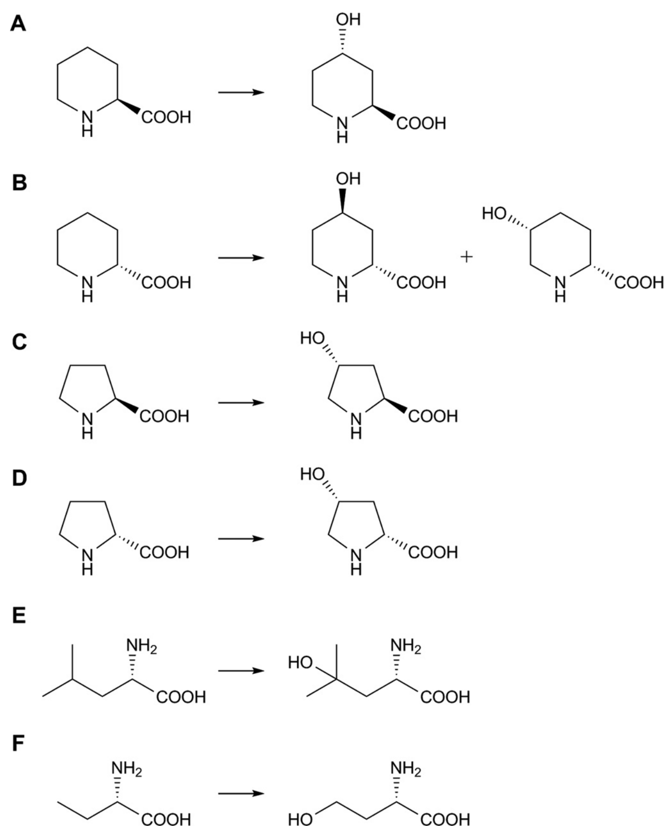

FIG 2 Hydroxylating reactions catalyzed by Pip4Hs. (A) Hydroxylation of L-Pip into *trans*-4-L-HyPip by *Fo*Pip4H and *An*Pip4H; (B) hydroxylation of D-Pip into *trans*-4-D-HyPip and *cis*-5-D-HyPip by *An*Pip4H; (C) hydroxylation of L-Pro into *trans*-4-L-HyPro by *Fo*Pip4H and *An*Pip4H; (D) hydroxylation of D-Pro into *cis*-4-D-HyPro by *An*Pip4H; (E) hydroxylation of L-Leu into 4-hydroxy-L-Leu by *Fo*Pip4H; (F) hydroxylation of L-AABA into L-homoserine by *Fo*Pip4H.

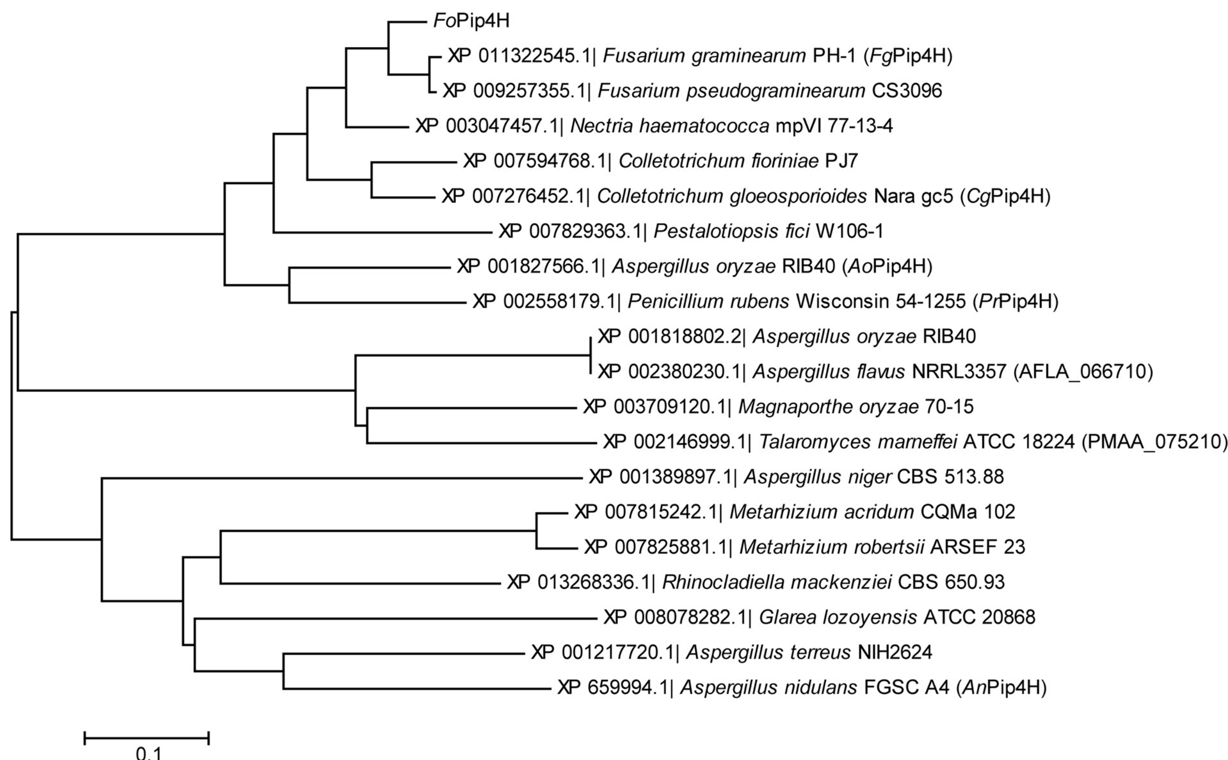

FIG 3 Phylogenetic tree of amino acid sequences of proteins homologous to *Fo*Pip4H. The proteins sharing over 40% identity with *Fo*Pip4H from filamentous fungi were subjected to phylogenetic analysis using the neighbor-joining method.

of the amino acid sequence of *Fo*Pip4H against the sequences in protein reference sequence databases with the BLASTP program. All proteins sharing over 40% identity with *Fo*Pip4H were from filamentous fungi and were subjected to phylogenetic analysis using the neighbor-joining method (Fig. 3). The proteins homologous to *Fo*Pip4H were widely distributed among several classes of filamentous ascomycetes, such as Sordariomycetes (*Fusarium*, *Nectria*, *Colletotrichum*, *Pestalotiopsis*, *Magnaporthe*, *Metarhizium*), Eurotiomycetes (*Aspergillus*, *Penicillium*, *Talaromyces*, *Rhinocladiella*), and Leotiomycetes (*Glarea* genus). Furthermore, all had the conserved amino acid motifs of the Fe/αKG-DO superfamily. This would suggest that these homologous proteins may have hydroxylation activities similar to the hydroxylation activity of *Fo*Pip4H and play an important common role in filamentous ascomycetes.

L-Pip hydroxylation activity of proteins homologous to *Fo*Pip4H. Among the 20 proteins shown in Fig. 3, 7 diverse homologues of *Fo*Pip4H were selected to be assayed for hydroxylation activity toward L-Pip: *Fg*Pip4H, *Cg*Pip4H, *Ao*Pip4H, *Pr*Pip4H, AFLA_066710, *An*Pip4H, and PMAA_075210. Four of the homologues, *Fg*Pip4H, *Cg*Pip4H, *Ao*Pip4H, and *Pr*Pip4H, which had a high degree of similarity to *Fo*Pip4H (with over 67% identity), catalyzed the hydroxylation of L-Pip and produced *trans*-4-L-HyPip like *Fo*Pip4H (Table 2). On the other hand, the two proteins with lower degrees of homology, AFLA_066710 and PMAA_075210 (with 44% and 41% identities to *Fo*Pip4H, respectively), showed no activity toward L-Pip, even though the successful expression of these proteins was confirmed. Interestingly, *An*Pip4H, which also had a low level of homology with *Fo*Pip4H (42% identity), was found to possess hydroxylation activity and produce *trans*-4-L-HyPip. Thus, *Fo*Pip4H and *An*Pip4H were used for further characterization of the enzyme activity. The optimal pH and temperature for L-Pip hydroxylation activity were pH 7.0 and 15°C, respectively, for *Fo*Pip4H and pH 6.5 and 37°C, respectively, for *An*Pip4H. *Fo*Pip4H maintained over 80% of its maximum activity after 1 h of incubation at pH 5 to 7 and a

TABLE 2 Proteins homologous to *Fo*Pip4H and their L-Pip-hydroxylating activities

Enzyme	Origin	% identity to *Fo*Pip4H	L-Pip-hydroxylating activity[a]
*Fo*Pip4H	*Fusarium oxysporum* c8D	100	+
*Fg*Pip4H	*Fusarium graminearum* PH-1	92	+
*Cg*Pip4H	*Colletotrichum gloeosporioides* Nara gc5	81	+
*Ao*Pip4H	*Aspergillus oryzae* RIB40	71	+
*Pr*Pip4H	*Penicillium rubens* Wisconsin 54-1255	67	+
AFLA_066710	*Aspergillus flavus* NRRL3357	44	−
*An*Pip4H	*Aspergillus nidulans* FGSC A4	42	+
PMAA_075210	*Talaromyces marneffei* ATCC 18224	41	−

[a] *trans*-4-L-HyPip production was detected (+) or not detected (−) by amino acid analysis.

TABLE 3 Substrate specificity analysis and kinetic parameters of *Fo*Pip4H and *An*Pip4H[b]

Substrate	*Fo*Pip4H		*An*Pip4H	
	Sp act (U/mg)	K_m (mM)	Sp act (U/mg)	K_m (mM)
L-Pip	1.6 ± 0.1	1.5 ± 0.1	0.76 ± 0.01	1.4 ± 0.0
D-Pip	ND		0.18 ± 0.01[a]	
L-Pro	0.55 ± 0.03	3.1 ± 0.2	0.37 ± 0.02	1.4 ± 0.1
D-Pro	ND		0.044 ± 0.008	3.8 ± 0.7
L-Leu	tr		ND	
L-AABA	tr		ND	
cis-5-L-HyPip	0.50 ± 0.05[a]		0.28 ± 0.05[a]	

[a] Enzyme activities were analyzed by succinate analysis.
[b] All measurements were performed at least three times. ND, not detected; tr, trace activity detected only in the amino acid analysis.

temperature of 4 to 30°C, and *An*Pip4H maintained over 80% of its maximum activity after 1 h of incubation at pH 6 to 8 and a temperature of 4 to 40°C.

Asymmetric hydroxylation of amino acids with *Fo*Pip4H. Purified recombinant *Fo*Pip4H was used to analyze substrate specificity (Table 3). Proteinogenic L-amino acids, nonproteinogenic L-amino acids, D-amino acids, amino acid derivatives, and piperidine derivatives were used as the substrates. Enzymatic activities were confirmed by both amino acid analysis and succinate analysis. In the reaction mixture containing *Fo*Pip4H, succinate production and amino acid conversion were detected when L-Pro or *cis*-5-L-HyPip was used as a substrate, as well as when L-Pip was used as a substrate. Although succinate formation was not detected in reaction mixtures containing L-leucine (L-Leu) or L-2-aminobutyrate (L-AABA) due to the detection limit of the succinate analysis used in this study, a single peak of a newly produced amino acid was observed by amino acid analysis, as was the case with L-Pip, L-Pro, or *cis*-5-L-HyPip. The molecular masses of all these amino acid products were greater than those of their original substrates by 16 Da, indicating that *Fo*Pip4H catalyzed the hydroxylation of these substrates (see Fig. S2 in the supplemental material). In the amino acid analysis, the retention times and molecular masses of the products from L-Pro, L-Leu, and L-AABA corresponded to those of authentic samples of *trans*-4-hydroxy-L-Pro (*trans*-4-L-HyPro), 4-hydroxy-L-Leu, and L-homoserine, respectively (see Fig. S2D, F, and G in the supplemental material); however, we were unable to identify the hydroxylated product of *cis*-5-L-HyPip without some authentic samples, such as dihydroxylated compounds of L-Pip (see Fig. S2C in the supplemental material). We determined that *Fo*Pip4H introduced a hydroxyl group onto the carbon at the 4 position of L-amino acids, such as L-Pip, L-Pro, L-Leu, and L-AABA (Fig. 2A, C, E, and F). Specific activities and K_m values were determined to be 1.6 U/mg and 1.5 mM, respectively, for L-Pip and 0.55 U/mg and 3.1 mM, respectively, for L-Pro. These results indicate that, compared to L-Pro, L-Pip was the preferred substrate for *Fo*Pip4H.

Asymmetric hydroxylation of amino acids with *An*Pip4H. Substrate specificity analysis of *An*Pip4H was performed in a manner similar to that described above (Table 3). *An*Pip4H reacted with L-Pip, D-pipecolic acid (D-Pip), L-Pro, D-proline (D-Pro), and *cis*-5-L-HyPip. On the basis of the results of amino acid analysis (see Fig. S2 in the supplemental material), the reaction products were all hydroxylated compounds corresponding to the original substrates, and *trans*-4-L-HyPip, *trans*-4-L-HyPro, and *cis*-4-D-HyPro were identified to be products from L-Pip, L-Pro, and D-Pro, respectively (see Fig. S2A, D, and E in the supplemental material). Interestingly, two kinds of hydroxylated compounds were formed when D-Pip was used as a substrate, and they were identified to be *trans*-4-hydroxy-D-pipecolic acid and *cis*-5-hydroxy-D-pipecolic acid by the correspondence of the retention times with those of their enantiomeric counterparts, *trans*-4-L-HyPip, and *cis*-5-L-HyPip, respectively (see Fig. S2B in the supplemental material). Likewise, although a single peak of an hydroxylated compound was detected in the reaction mixture containing *cis*-5-L-HyPip, the structure has not yet been identified (see Fig. S2C in the supplemental material). We determined that *An*Pip4H introduced a hydroxyl group onto the carbon at the 4 or 5 position of amino acids such as L-Pip, D-Pip, L-Pro, D-Pro, and L-Leu (Fig. 2A to D). Specific activities and K_m values were determined to be 0.76 U/mg and 1.4 mM, respectively, for L-Pip; 0.37 U/mg and 1.4 mM, respectively, for L-Pro; and 0.04 U/mg and 3.8 mM, respectively, for D-Pro. These results demonstrated that, compared to all substrates used here, L-Pip was the principal substrate for *An*Pip4H, similar to the finding for *Fo*Pip4H.

Preparative-scale production of *trans*-4-L-HyPip. The biotransformation of L-Pip to obtain *trans*-4-L-HyPip was carried out using *E. coli* cells expressing *Fo*Pip4H as the biocatalyst. As shown in Fig. 4, 100 mM L-Pip was linearly converted into *trans*-4-L-HyPip and was completely consumed within 5 h. As a result, *trans*-4-L-HyPip was successfully produced in the reaction mixture with a conversion ratio of 91%. Therefore, a total of 23 g of L-Pip was used as a substrate for the biotransformation, and the resultant *trans*-4-L-HyPip was purified by ion-exchange chromatography. Consequently, 18 g of *trans*-4-L-HyPip was obtained, and its final production yield was 81%. According to the ^{1}H NMR spectrum, the compound was confirmed to consist of *trans*-4-L-HyPip with a >99% diastereomeric excess. Thus, the asymmetric hydroxylation of L-Pip was achieved in the biotransformation by taking advantage of the high regio- and stereoselectivity of *Fo*Pip4H.

DISCUSSION

Along with L-proline hydroxylases, many Fe/αKG-DOs are known to hydroxylate free amino acids, such as L-asparagine, L-

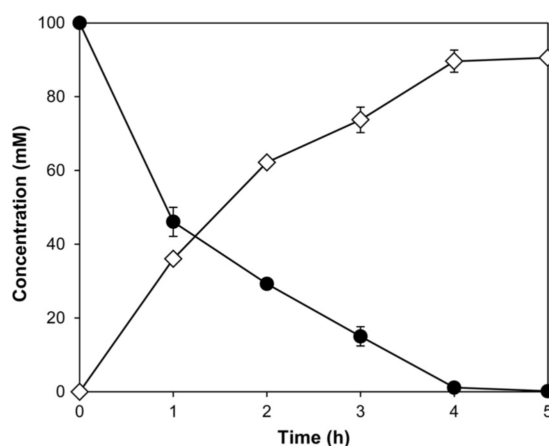

FIG 4 Preparative-scale production of *trans*-4-L-HyPip using *Fo*Pip4H. The hydroxylation of L-Pip (closed circles) into *trans*-4-L-HyPip (open diamonds) with *Fo*Pip4H-expressing *E. coli* cells is shown. All measurements were performed at least three times.

lysine, L-arginine, L-Leu, and L-isoleucine (23–26). Until recently, all Fe/αKG-DOs had been found in bacteria; however, an L-proline hydroxylase was identified to be the first Fe/αKG-DO from a fungus, *Glarea lozoyensis* (27). Here, we have reported six novel fungal Fe/αKG-DOs, Pip4Hs, which had hydroxylation activities toward L-Pip and were widely distributed among several classes of filamentous ascomycetes. Although both *Fo*Pip4H and *An*Pip4H had catalytic properties similar to those of bacterial proline hydroxylases with respect to kinetic parameters and optimal reaction conditions (14, 16), their amino acid sequences showed no homology to those of known bacterial proline hydroxylases. More importantly, *Fo*Pip4H and *An*Pip4H preferentially reacted with the substrate L-Pip rather than L-Pro, in contrast to bacterial proline hydroxylases, which favor L-Pro over L-Pip (17, 28). Furthermore, we found that *Fo*Pip4H is an inducible enzyme strictly regulated by L-Pip but not by L-Pro (Fig. 1). Additionally, we obtained preliminary results by substrate specificity analysis of all Pip4Hs (see Table S1 in the supplemental material). According to those results, *Cg*Pip4H showed hydroxylation activity toward both L-Pip and L-Pro, as was the case with *Fo*Pip4H and *An*Pip4H, but *Fg*Pip4H, *Ao*Pip4H, and *Pr*Pip4H did not react with L-Pro. Taken together, these results suggest that Pip4Hs primarily catalyze the hydroxylation of L-Pip.

Under natural circumstances, it is presumed that *trans*-4-L-HyPip is formed as a secondary metabolite of filamentous ascomycetes possessing Pip4Hs. Here we showed that *F. oxysporum* c8D produced *trans*-4-L-HyPip in the culture medium using the substrate of Pip4Hs, L-Pip. Now then, L-Pip is produced by microorganisms, plants, and animals and is therefore readily available for use by fungi (29). Alternatively, L-Pip might be synthesized by these fungi themselves. Indeed, the gene set encoding key enzymes for L-Pip biosynthesis, L-α-aminoadipate aminotransferase, L-aminoadipate-semialdehyde dehydrogenase, and pyrroline-5-carboxylate reductase (30), is found in full in the genome sequences of the ascomycetes listed in Table 2. Thus, it is not unexpected that *F. oxysporum* c8D and other fungi might produce *trans*-4-L-HyPip as a metabolite, though its role in fungi is still unknown.

As shown in Fig. 4, the preparative-scale production of optically pure *trans*-4-L-HyPip was possible using *Fo*Pip4H as the biocatalyst. Some reports have indicated that *trans*-4-L-HyPip has physiological functions. In *Acacia maidenii*, significant osmotic adjustment was caused by increases in *trans*-4-L-HyPip levels (31). It was also reported to be a relatively selective agonist of an *N*-methyl-D-aspartate receptor subtype prevalently found in the mouse cortex (32). Additionally, *trans*-4-L-HyPip has antidiabetic and antioxidative effects in mice (33) and stimulated glucose uptake and glucose transporter-4 translocation from an intracellular location to the cell surface in skeletal muscle cells (34). Moreover, *trans*-4-L-HyPip has been found to be present as a component of various bioactive compounds. For example damipipecolin, a bromopyrrole alkaloid isolated from the sponge *Axinella damicornis*, displayed a modulating effect on the serotonin receptor (9); halichonadin K, a dimeric sesquiterpenoid isolated from a marine sponge (*Halichondria* sp.), had cytotoxicity against human epidermoid carcinoma KB cells (10); and ulleungamide A, a cyclic depsipeptide isolated from a *Streptomyces* sp., displayed growth-inhibitory activity against some bacteria (8). Thus, there may be an increasing demand for *trans*-4-L-HyPip, and our production method is promising for its high efficiency and regio- and stereoselectivity.

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