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Short communication

Identification and characterization of 2-oxoglutarate-dependent dioxygenases catalyzing selective *cis*-hydroxylation of proline and pipecolinic acid from actinomycetes

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ABSTRACT

Microbial hydroxylases were screened for the capacity to effect direct hydroxylation of proline and pipecolinic acid, based on genomic information. Of the eight candidates screened, 2-oxoglutarate-dependent hydroxylase from *Streptosporangium roseum* NBRC 3776[T] and aspartyl/asparaginyl β-hydroxylase from *Catenulispora acidiphila* NBRC 102108[T] showed both proline and pipecolinic acid hydroxylation activities. In the case of L-proline hydroxylation, both enzymes catalyzed the simultaneous formation of *cis*-3-hydroxy-L-proline and *cis*-4-hydroxy-L-proline, and *cis*-4-hydroxy-L-proline was preferentially produced. In the case of L-pipecolinic acid hydroxylation, both enzymes catalyzed the simultaneous formation of *cis*-3-hydroxy-L-pipecolinic acid and *cis*-5-hydroxy-L-pipecolinic acid. While the former enzyme preferentially produced *cis*-3-hydroxy-L-pipecolinic acid, the latter enzyme preferentially produced *cis*-5-hydroxy-L-pipecolinic acid.

The direct hydroxylation of a compound by using molecular oxygen has attracted much attention in the chemical industry, because it is generally difficult to achieve by a conventional chemical synthesis, as it requires an oxidizing agent or a complex catalyst. In microorganisms, hydroxylation is a common reaction and is usually mediated by a hydroxylase. Generally, hydroxylases play an important role in the primary or secondary metabolism of amino acids, fatty acids, nucleic acids, antibiotics, and aromatic compounds. To date, several classes of hydroxylases, including mono- and dioxygenases, have been known. Among these enzymes, microbial 2-oxoglutarate (2-OG)-dependent dioxygenase is considered an attractive candidate for industrial use, because it does not require heme or a redox partner protein that is essential for other enzymes such as cytochrome P450 monooxygenase, and does not use NAD(P)H, which is rather expensive for commercial use, as a cofactor. Instead, 2-OG-dependent dioxygenase uses non-heme iron(II) as a cofactor and 2-OG as a cosubstrate for the activation of molecular oxygen.

Recent studies on amino acid hydroxylation have shown that diverse microbial hydroxylases, including phenylalanine hydroxylase (Nakata et al., 1979), proline hydroxylase (Hara and Kino, 2009; Lawrence et al., 1996; Mori et al., 1997; Shibasaki et al., 1999a), arginine hydroxylase (Yin and Zabriskie, 2004), asparagine hydroxylase (Strieker et al., 2007), and isoleucine hydroxylase (Kodera et al., 2009), are present in various microorganisms. Interestingly, all these enzymes, except phenylalanine hydroxylase, are 2-OG-dependent dioxygenases. However, the classes of enzymes that catalyze amino acid hydroxylation are relatively restricted, when compared with other classes of hydroxylases or oxygenases that act on other aliphatic and aromatic compounds. Recently, we identified and characterized novel L-proline *cis*-4-hydroxylases from the root nodule bacteria *Mesorhizobium loti* and *Sinorhizobium meliloti*. Based on our results, a method for the production of hydroxyprolines (Hyps) such as *cis*-3-Hyp, *cis*-4-Hyp, and *trans*-4-Hyp; and hydroxypipecolinic acids (HPAs) such as *cis*-3-HPA, *cis*-5-HPA, and *trans*-5-HPA, in a growing culture of *Escherichia coli* was developed (Huttel and Klein, 2011). In the bioconversion of L-pipecolinic acid by using *E. coli* expressing the L-proline *cis*-4-hydroxylase gene, *cis*-3-HPA and *cis*-5-HPA were produced in the same molar ratio (Huttel and Klein, 2011). *cis*-5-HPA is used as a pharmaceutical intermediate (Bailey and Bryans, 1988) and antifungal agent (Brenner and

Abbreviations: 2-OG, 2-oxoglutarate; Hyp, hydroxproline; HPA, hydroxypipecolinic acid; SrPH, EEP13595; CaPH, ACU72347; SmP4H, L-proline *cis*-4-hydroxylase from *Sinorhizobium meliloti*; FDAA, N^{α}-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide.

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Table 1
Hydroxylase gene candidates screened and characterized in this study.

Accession number[a]	Source microorganism	Strain	Annotation	Motif[b]	Identity with SmP4H[c] (%)	Hydroxylase activity
EFV12517	*Segniliparus rugosus*	JCM 13579^T	Aspartyl/asparaginyl β-hydroxylase	IIP**H**R**D**YLE. . . V**H**S	33	–
EEP13595	*Streptosporangium roseum*	NBRC 3776^T	2-Oxoglutarate-dependent hydroxylase	IVP**H**R**D**FLE. . . I**H**A	30	+
ACU72347	*Catenulispora acidiphila*	NBRC 102108^T	Aspartyl/asparaginyl β-hydroxylase	IVP**H**R**D**FLE. . . I**H**S	29	+
AEA44416	*Fluviicola taffensis*	NBRC 106335^T	Aspartyl/asparaginyl β-hydroxylase	IKP**H**T**D**HEL. . . V**H**S	26	–
ABG60121	*Cytophaga hutchinsonii*	NBRC 15051^T	Conserved hypothetical protein	IKE**H**T**D**NDT. . . P**H**F	23	–
ABQ08010	*Flavobacterium johnsoniae*	NBRC 14942^T	Aspartyl/asparaginyl β-hydroxylase	IKE**H**V**D**NDT. . . P**H**S	23	–
EFK57969	*Sphingobacterium spiritivorum*	NBRC 14948^T	β-Hydroxylase, aspartyl/asparaginyl family	IKP**H**R**D**PGC. . . V**H**W	22	–
ACU63324	*Chitinophaga pinensis*	NBRC 15968^T	Aspartyl/asparaginyl β-hydroxylase	IKP**H**K**D**PGC. . . T**H**A	20	–

[a] Accession number in the NCBI protein database.
[b] The bold-typed amino acids indicate essential residues that are conserved in non-heme iron(II) enzymes (Koehntop et al., 2005).
[c] Calculated by the fast homology search function in GENETYX software ver. 10 using the FASTP program (GENETYX corporation, Tokyo, Japan).

Romeo, 1986). However, the selective synthesis of *cis*-5-HPA is still difficult owing to the simultaneous production of *cis*-3-HPA. This study was undertaken to screen novel microbial hydroxylases for regioselectivity of L-pipecolinic acid hydroxylation for selective synthesis of *cis*-5-HPA.

While identifying potential microbial hydroxylases for screening, we focused on candidates having some degree of amino acid sequence identity with L-proline *cis*-4-hydroxylase from *S. meliloti* (SmP4H) and the conserved motif that is present within 2-OG-dependent dioxygenases, the 2-His-1-carboxylate facial triad (Koehntop et al., 2005). Based on these criteria, we selected 8 candidate proteins (Table 1). JCM and NBRC strains were obtained from RIKEN Bioresource Center (Ibaraki, Japan), and Biological Resource Center, NITE (Chiba, Japan), respectively. The genomic DNA from the microorganisms was extracted according to a standard protocol (Sambrook and Russell, 2001). Putative hydroxylase genes were amplified by PCR using KOD FX Neo (Toyobo, Osaka, Japan) DNA polymerase. The primer sequences used are listed in Table S1. In a previous study, we cloned, overexpressed, and purified L-proline *cis*-4-hydroxylase from *M. loti* and *S. meliloti* (Hara and Kino, 2009). In brief, *E. coli* Rosetta 2 (DE3) (Merck, Darmstadt, Germany) carrying pET-21d(+) vector (Merck) that contained a hydroxylase gene was cultivated on Luria-Bertani medium (per liter: 10 g Bacto Tryptone, 5 g Bacto Yeast extract, 10 g NaCl, pH 7.0) supplemented with 50 μg/mL ampicillin and 34 μg/mL chloramphenicol. Recombinant His_6-tagged enzymes were purified with a HisTrap HP column (GE Healthcare, Fairfield, CT) followed by a PD-10 column (GE Healthcare). In the present study, enzyme overexpression and purification was carried out by the same procedure.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2013.12.003.

Initially, whole-cell reaction was performed to evaluate the hydroxylation activity toward L-proline and L-pipecolinic acid under the following conditions: 5 mM L-proline or L-pipecolinic acid, 10 mM 2-OG, 1 mM L-ascorbic acid, 0.5 mM $FeSO_4$, and whole

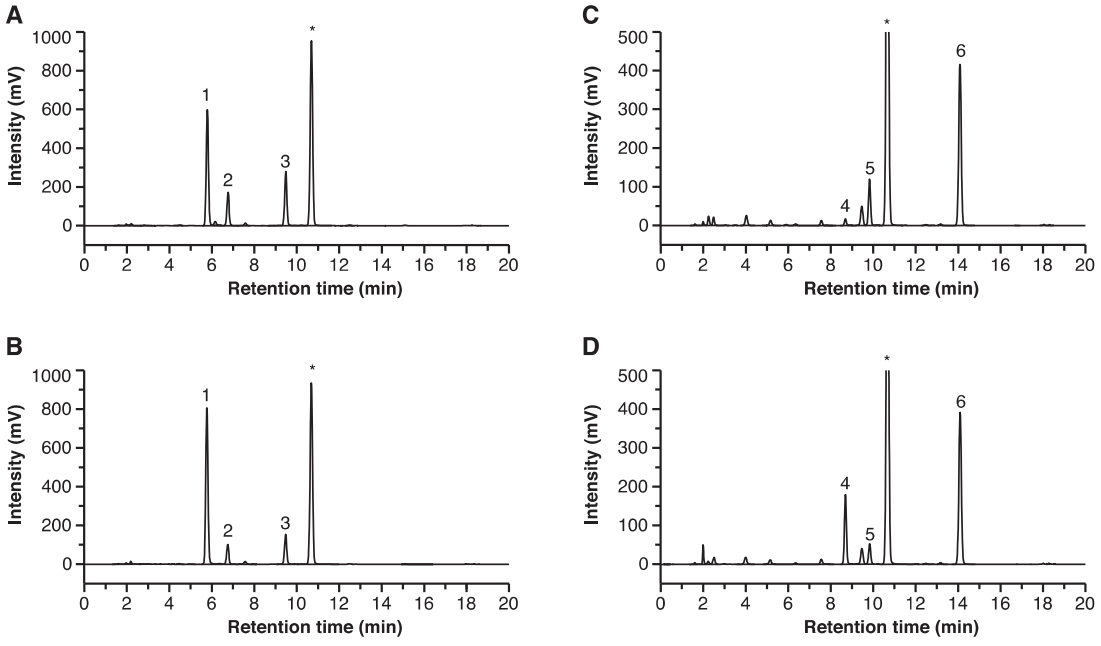

Fig. 1. Analysis of regio- and stereoisomers of hydroxyproline and hydroxypipecolinic acid. (A) L-Proline hydroxylation by SrPH, (B) L-proline hydroxylation by CaPH, (C) L-pipecolinic acid hydroxylation by SrPH, (D) L-pipecolinic acid hydroxylation by CaPH. The peak numbers indicate the following compounds: (1) *cis*-4-Hyp; (2) *cis*-3-Hyp; (3) L-proline; (4) *cis*-5-HPA; (5) *cis*-3-HPA; (6) L-pipecolinic acid. Asterisks denote the derivatization reagent, FDAA.