CHARACTERIZATION OF MICROBIAL HYDROXYLASES FOR EFFICIENT SYNTYESIS OF VALUABLE HYDROXY IMINO ACIDS

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Key words: hydroxyproline, hydroxypipecolinic acid, hydroxylase

Introduction. Hydroxy imino acids are industrially important compounds, especially in pharmaceutical field. *trans*-4-Hydroxy-proline (*trans*-4-Hyp) serves as a material for the synthesis of carbapenem antibiotic and antiphlogistic, whereas *cis*-4-Hyp itself is useful for an antitumor drug. To expand the kind of hydroxy imino acids, we screened proline hydroxylases based on microbial genome information. Here, we demonstrate microbial proline hydroxylases applicable to the synthesis of a variety of hydroxy imino acids, including hydroxypipecolinic acid.

Methods. DNA manipulation and gene expression procedure were described elsewhere (1). All microbes were obtained from National Institute of Technology and Evaluation (Chiba, Japan) and Japan Collection of Microorganisms (Ibaraki, Japan). Hydroxylase genes were amplified by PCR and cloned into appropriate site of pET-21a(+) or -21d(+) plasmids. The resulting plasmids were introduced into *Escherichia coli* Rosetta 2 (DE3). For gene expression, inducible T7 expression system was used. Overexpressed His_6-tagged enzymes were purified by Ni^{2+} affinity and gel filtration chromatography, followed by imino acid hydroxylation reaction. Substrates and products were determined using HPLC, MS, and NMR analysis.

Results. To evaluate hydroxylase activity, 8 candidate proteins were examined in the reaction mixtures supplemented with 5 mM proline, respectively. Among candidates, 2 proteins, which were from *Streptosporangium roseum* NBRC 3776 (StP4H) and *Catenulispora acidiphila* NBRC 102108 (CaP4H), showed proline hydroxylation activity with a 2-oxoglutarate-dependent manner. According to the HPLC analysis, the reaction products were identified as *cis*-4-Hyp and *cis*-3-Hyp with a molar ratio of 3:1 (StP4H) and 4:1 (CaP4H). Next, substrate specificity was investigated in detail. These enzymes hydroxylated various proline analogs, including pipecolinic acid, 3,4-dehydroxproline and *cis*-3-Hyp, as well as proline. Only CaP4H hydroxylated azetidine-2-carboxylic acid (table 1). The reaction products from corresponding substrates were identified as follows: *cis*-5- and *cis*-3-hydroxypipecolinic acid from pipecolinic acid, *cis*-3,4-epoxyproline from 3,4-dehydroproline, *cis*-2,3-*cis*-3,4-diHyp from *cis*-3-Hyp, and *cis*-3-hydroxyazetidine-2-carboxylic acid from azetidine-2-carboxylic acid. Interestingly, when StP4H was used, *cis*-5- and *cis*-3-hydroxypipecolinic acid were co-produced with a molar ratio of 18:82, however, when CaP4H was used, the products ratio was 73:27. CaP4H was able to hydroxylate pipecolinic acid with the highest molar yield for the synthesis of *cis*-5-hydroxypipecolinic acid among P4Hs including rhizobia P4Hs.

Table 1. Substrate specificity of proline hydroxylases.

	StP4H	CaP4H
Proline	+	+
Pipecolinic acid	+	+
3,4-Dehydroproline	+	+
cis-3-Hyp	+	+
Azetidine-2-carboxylic acid	–	+

Conclusions. We found two proline hydroxylases, SrP4H and CaP4H, from *S. roseum* and *C. acidiphila*, respectively. These enzymes produced *cis*-4-Hyp and *cis*-3-Hyp simultaneously in a different yield from proline. Especially, CaP4H gave higher a yield of *cis*-5-hydroxypipecolinic acid than other P4Hs. Hence, it should be possible to produce *cis*-5-hydroxypipecolinic acid efficiently by CaP4H .

Acknowledgements. This work was financially supported by Adaptable and Seamless Technology Transfer Program through target-driven R&D, JST. We thank KYOWA HAKKO BIO Co., Ltd for providing us with reagents and for helpful discussions.

References.

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