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Enzymatic Production of *trans*-4-Hydroxy-L-proline by Regio- and Stereospecific Hydroxylation of L-Proline

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A proline 4-hydroxylase gene, which was cloned from *Dactylosporangium* sp. RH1, was overexpressed in *Escherichia coli* W1485 on a plasmid under a tryptophan tandem promoter after the codon usage of the 5′ end of the gene was optimized. The proline 4-hydroxylase activity was1600-fold higher than that in *Dactylosporangium* sp. RH1. *trans*-4-Hydroxy-L-proline(Hyp) was produced and accumulated to 41 g/L (87% yield from L-proline) in 100 h when the recombinant *E. coli* was cultivated in a medium containing L-proline and glucose. 2-Oxoglutarate, which is necessary for the hydroxylation of L-proline by proline 4-hydroxylase, was apparently supplied from glucose through the cellular metabolic pathway. The *putA* mutant of W1485, which is not able to degrade L-proline, has allowed the quantitative conversion of L-proline to Hyp. The formation of other isomers of hydroxyproline was not observed. Productivity of Hyp was almost the same in a larger-scale culture. The method of manufacturing Hyp from L-proline was established.

Key words: hydroxyproline; proline; bioconversion; proline 4-hydroxylase; 2-oxoglutarate-dependent dioxygenase

trans-4-Hydroxy-L-proline (Hyp) is a useful chiral synthon for the chemical syntheses of pharmaceuticals such as antiphlogistics, carbapenems, and angiotensin-converting enzyme inhibitors.[1] It is also important as a starting material for synthesizing other hydroxyproline isomers.[1-3] Although it has been manufactured by acid hydrolysis of animal collagen, a better process has been desired because of the disadvantages of the method, a complex and long purification and much waste. Many processes of producing Hyp by microorganisms have been reported,[4-9] however, these processes can be hardly applied industrially because of the high cost and the low productivity of Hyp.

Since L-proline has been produced by fermentation at low cost, enzymatic conversion of L-proline to Hyp would be an economical process. The well-characterized prolyl hydroxylases, which hydroxylate peptidyl L-proline into peptidyl Hyp in the biosynthesis of collagen, cannot be used, because prolyl hydroxylases do not hydroxylate free L-proline.[10] Proline 4-hydroxylases, which hydroxylate free L-proline to free Hyp, have been found in some microorganisms.[11-14] Since the hydroxylation of L-proline requires 2-oxoglutarate, dioxygen, and ferrous ion, the enzymes were all categorized as 2-oxoglutarate-dependent dioxygenases (Fig. 1(a)). The gene for proline 4-hydroxylase had never been cloned until it was cloned and expressed in *Escherichia coli* from *Dactylosporangium* sp. RH1.[14]

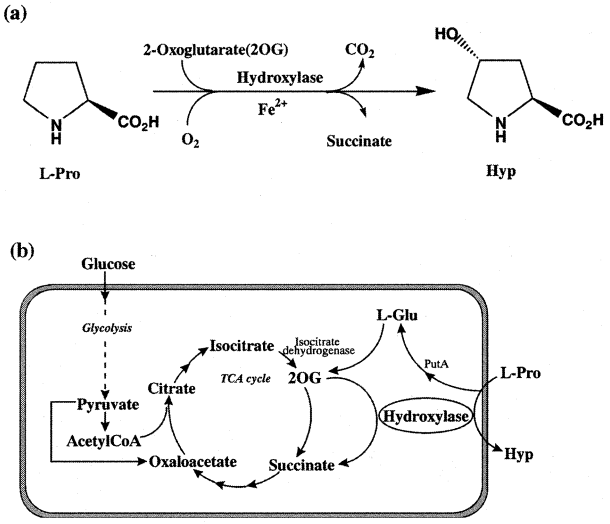

Fig. 1. Scheme of the Hydroxylation of L-Proline to Hyp by Proline 4-Hydroxylase.

(a) The reaction catalyzed by proline 4-hydroxylase, which is a 2-oxoglutarate-dependent dioxygenase. (b) *In vivo* Hyp production with the recycling of 2-oxoglutarate. AcetylCoA , TCA, and 2OG represent acetyl coenzymeA, tricarboxylic acid, and 2-oxoglutarate, respectively.

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Abbreviation: Hyp, *trans*-4-hydroxy-L-proline

Here we report a method for production of Hyp from L-proline and glucose. L-Proline was hydroxylated by the proline 4-hydroxylase expressed in the recombinant *E. coli* without the addition of 2-oxoglutarate. 2-Oxoglutarate was supplied from glucose through the metabolic pathway of *E. coli*.

Materials and Methods

Materials. Chemicals were purchased from Nacalai Tesque (Kyoto, Japan) unless otherwise stated. L-Proline was from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan).

Enzyme assay. Cellular activities of proline 4-hydroxylase were measured as reported before.[14] The reaction mixtures containing 240 mM of 2-(*N*-morphorino)ethane-sulfonic acid (MES), pH 6.5, 20 mM of L-proline, 40 mM of 2-oxoglutarate, 4 mM of ferrous sulfate, and cells were incubated at 35°C for 10 min with shaking. L-Proline, Hyp, and other isomers of hydroxyproline were measured with HPLC.[15] The amount of the enzyme which forms 1 nmol of Hyp in one minute was defined as 1 unit(U).

Plasmid construction. Plasmids pTrS31 (*trp* promoter) and pTrS32 (*trp* tandem promoter) were constructed from pKYP10 and pKYP200.[16] A plasmid, pBTac1 (*tac* promoter), was from Boehringer-Mannheim. DNA oligomers were synthesized as follows; S1[5′-GTGAGGAAAGCTTATGCTGACCCCGACGGAGCTCAAG-3′], S2[5′-GTGAGGAGAATTCATGCTGACCCCGACGGAGCTCAAG-3′], A1[5′-GCCTGCGGGATCCTAGACGGGCTGGGCCAGCGCGAA-3′], and A2[5′-CCGCCTGAAGCTTCCTAGACGGGCTGGGCCAGCGCGAA-3′]. The gene for proline 4-hydroxylase (GenBank Accession D78338) was amplified by PCR with pRH71[14] as a template. The amplified DNA fragment and a plasmid were digested with the two restriction enzymes followed by ligation. For pTrS31 and 32, primers S1 and A1 and restriction enzymes *Hin*dIII and *Bam*HI were used. For pBTac1, primers A2 and S2 and restriction enzymes *Eco*RI and *Hin*dIII were used.

To construct the plasmid pWFH1 (Fig. 2), the plasmid pTr2-4OH was digested with *Bam*HI and *Pvu*II and blunted with a DNA blunting kit (Takara, Kyoto, Japan) followed by self-ligation to construct pTr2-4OH*Δ*. The two DNA oligomers synthesized were M1 [5′-GTGAGGAAAGCTTATGCTGACCCCGACCGAACTGAAACAGTATCGTGAAGCGGGCTATCTGCTGA-3′] and M2 [5′-CCGGAATTCGTCGACTTCACGCGGGCCCAGGCCATCTTCAATCAGCAGATAGCCCGCTTCACGATA-3′]. M1 corresponds to 1-17 N-terminal amino acids of proline 4-hydroxylase with the optimized codons and 7 bases plus *Hin*dIII site at the 5′ end. M2 corresponds to 28-10 amino acids of proline 4-hydroxylase with the optimized codons and 9 bases at the 5′ end. M1 and M2 have 25 complementary bases at each 3′ end so that the double stranded DNA corresponding to 1-28 amino acids is amplified when PCR is done with M1 and M2. The amplified DNA replaced the corresponding part of pTr2-4OH*Δ* to construct pWFH1.

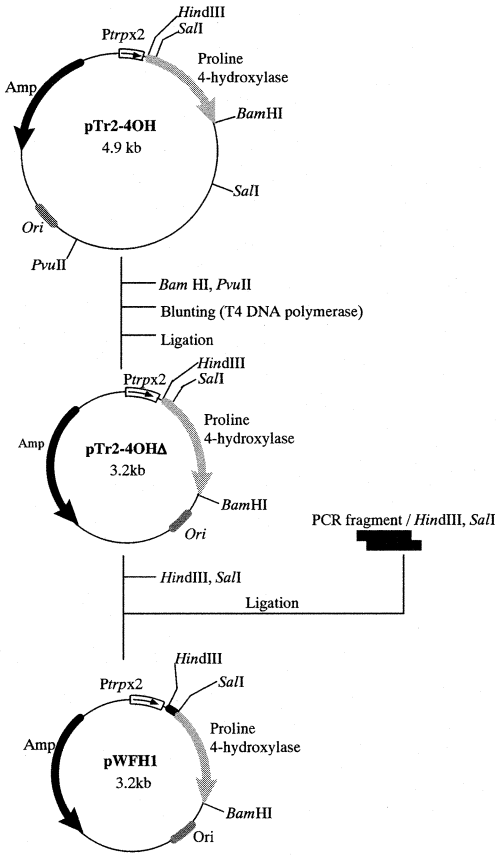

Fig. 2. Construction of the Proline 4-Hydroxylase Expression Plasmid pWFH1.

P*trp*×2 represents the tryptophan tandem promoter from *E. coli*. Nucleotide sequences of *Hin*dIII-*Sal*I fragment of pTr2-4OH and pWFH1 and Shine-Dalgano sequence were shown in Fig. 3.

Production of Hydroxyproline. The recombinant *E. coli* was cultivated on LB plates containing 50 μg/ml ampicillin at 37°C. Cells were inoculated into 50 ml of Med 4G (polypepton 10 g/l, Bactoyeast extract 5 g/l, NaCl 10 g/l, glucose 20 g/l, $CaCO_3$ 10 g/l) in 300-ml Erlenmeyer flasks and cultivated at 30°C for 16 hours. One hundred ml of the culture broth was inoculated into 2 liters of Med7 (Glucose 20 g/l, $(NH_4)_2SO_4$ 10 g/l, K_2HPO_4 1 g/l, NaCl 2 g/l, $MgSO_4$ 0.5 g/l, $FeSO_4$ 0.278 g/l, $CaCl_2$ 0.015 g/l, peptone 8 g/l, pH8.0) containing 200 mM L-proline in 5-liter jar fermentors, and cultured at 33°C, with agitation at 400 rpm, and aeration of 2 l/min. The pH of the culture was kept at 6.5 with 14% NH_4OH.

Construction of putA and icd mutant. Mutation of *putA* and *icd* were introduced into *E. coli* W1485 by P1 phage transduction.[17] A disrupted *putA* was from *E. coli* SBS821 [*pyrD*34 *trp*-45 *his*-68 *thyA*25 *thi deoR*33 *galK*35 *xyl*-7 *mtl*-2 *malA*1 *rpsL*118$\lambda^R(\lambda)^-$ *appA*1 *putA*::Tn*5*(*kan*^{r}) Mu$^+$].[18] The *putA* mutants grew as white colonies on 2,3,5,-triphenyl tetrazolium chloride plates containing L-proline.[19] P1 phage lysate of RS3263 [F+:*fadR*::Tn*5* *bef*-108::Tn*10* (*tet*^{r})]-derived *icd*-mutated strain was used as a source of the *icd* mutation. The *icd* mutants grew on the M9 plate containing 0.5 g/l of 2-oxoglutarate, but not on the M9 plate.

Results and Discussion

Construction of proline 4-hydroxylase expression plasmids

In our previous paper, we reported the gene expression as a fused protein with a part of β-galactosidase (0.4 U/mg wet cells).[14] Although the cellular activity was 13.6-fold higher than that of *Dactylosporangium* sp. RH1, it was not enough to produce Hyp efficiently. To overexpress the proline 4-hydroxylase gene, the selection of a host strain and promoter were done first.

Several *E. coli* strains were compared with respect to the expression of proline 4-hydroxylase by pES1-23a.[14] *E. coli* W1485 was chosen because its recombinant showed the highest activity of the hydroxylase (data not shown).

As to promoters, a *trp* tandem promoter was selected among three potent promoters, *trp*, *trp* tandem,[16] and *tac* promoters[20] since the expression plasmid with the *trp* tandem promoter, pTr2-4OH, had higher activity than that of other promoters.

Since the gene was from an actinomycete, it had a high G+C content (74%) and contained codons rare for *E. coli*, which are considered to be unfavorable to gene expression in *E. coli*.[21] The codon usage of 5′ end of proline 4-hydroxylase gene on pTr2-4OH was optimized for the expression in *E. coli* by cassette mutagenesis as in Fig. 2. Optimal codons were chosen according to the codon usage of *E. coli* genes in the GenBank.[22] The substituted part of the gene was sequenced as previously reported[14] to be identical to the designed one (Fig. 3). The optimized plasmid, pWFH1, had 7.7-fold higher activity than the pre-optimized plasmid, pTr2-4OH (Table 1). This result would be in accordance with the study by E. Goldman *et al.*, in which the expression level is more affected by the codons near the 5′ than the 3′ end.[23]

Production of hydroxyproline

To hydroxylate L-proline to Hyp by proline 4-hydroxylase, 2-oxoglutarate is necessary (Fig. 1). It is, however, much more expensive than glucose as an industrial raw material. 2-Oxoglutarate is a metabolic intermediate of TCA cycle in *E. coli*, and its

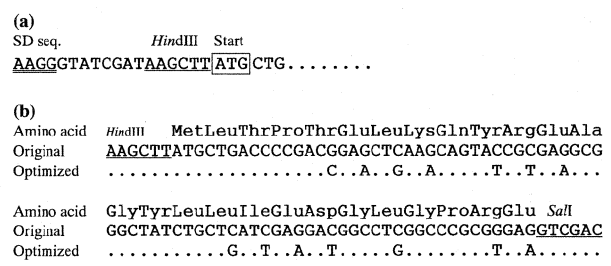

Fig. 3. Nucleotide Sequence of the 5′ End and Its Upstream Region of the Gene for Proline 4-Hydroxylase on pWFH1.

(a) Nucleotide sequence between Shine-Dalgano sequence and start codon of proline 4-hydroxylase gene on pWFH1. (b) Codon optimization of the 5′ end region of the gene for proline 4-hydroxylase. In the row of "Optimized", only the substituted bases were shown, dots showing no substitution. The fourteen nucleotides' substitutions were limited to the third letter without changing the coding amino acids. The sequence was confirmed after the construction of pWFH1 in both directions.

Table 1. Summary of Hydroxyproline Production by *E. coli* Recombinants

Plasmid	Host	Hyp (g/l)	Hyp (mM)	Consumed L-Pro (mM)	Yield[a] (%)	A600[b]	Plasmid stability[c] (%)	Act.[d] (U/mg wet cell)
none	W1485	0.0	0.0	180	0.0	36	—	0.0
pTrS-32	W1485	0.0	0.0	150	0.0	20	88	0.0
pTr2-4OH	W1485	15	110	220	51	48	88	6.0
pTr2-4OHΔ	W1485	6.9	53	210	25	25	100	2.0
pWFH1	W1485	41	310	360	87	53	100	46
pWFH1	W1485 *putA*	41	310	310	100	41	100	43
Dactylosporangium sp. RH1[e]								0.028

[a] Yield of Hyp against the consumed L-proline.
[b] Absorbance of culture broth of 100 h at 600 nm.
[c] Samples of culture broth at 100 h were diluted and placed on the plates of LB medium. After incubation at 30°C overnight, colonies on the LB-plate were transferred to ampicillin (50 μg/ml)-containing LB plates. The ratio of the number of colonies grown on Amp plates against that of the transferred colonies is shown by percentage as plasmid stability.
[d] Cellular proline 4-hydroxylase activities of the 72-hours' culture were measured as in Materials and Methods.
[e] See ref. 14.

production from glucose was reported.[24] Therefore, the 2-oxoglutarate required for proline 4-hydroxylase reaction in recombinant *E. coli* cells was expected to be supplied from glucose through the metabolic pathway (Fig. 1(b)). In fact, L-proline was hydroxylated to Hyp in the resting cell reaction without the addition of 2-oxoglutarate (Fig. 4).

Then, production of Hyp was examined by usig the growing cells. *E. coli* W1485/pWFH1 was cultivated for 100 h in a medium containing L-proline, glucose, and ferrous ion. Hyp was produced in a medium at an amount of 41 g/l (87% yield from L-proline) (Table 1). Formation of other hydroxyproline isomers and accumulation of succinate were not observed. Recombinant *E. coli* cells harboring pWFH1 or pTr2-4OH grew better than host *E. coli* cells. Although the reason why pTr2-4OHΔ showed the lower activity of proline 4-hydroxylase than pTr2-4OH is unclear, the cell growth and productivity of Hyp seems to be related to the activity. Proline 4-hydroxylase may increase the cell growth by increasing the material flow from 2-oxoglutarate to succinate in the presence of L-proline by bypassing 2-oxoglutarate dehydrogenase.

Culture conditions for the production of Hyp were examined. The initial concentration of L-proline, the agitation of jar fermentor, temperature, and pH were varied in the range of 0–200 mM, 200–700 cycles/min, 30–37°C, and 6.0–7.0, respectively. The optimized conditions were 200 mM, 400 cycles/min, 33°C, and 6.5, respectively (data not shown).

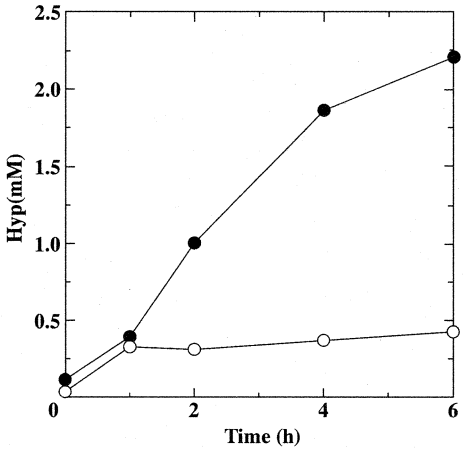

Fig. 4. Resting Cell Reaction for Hydroxyproline Production by *icd*$^+$ or *icd*$^-$ Host Strain.

E. coli W1485 [*putA*]/pWFH1 (●) and *E. coli* W1485 [*putA icd*]/pWFH1 (○) were cultivated at 30°C on LB plates which contained 50 μg/ml of ampicillin and 50 μg/ml of ampicillin plus 12.5 μg/ml of tetracycline, respectively. The grown cells were transferred to the liquid LB medium which contains 100 μg/m of ampicillin and 100 μg/ml of ampicillin plus 25 μg/ml of tetracycline, respectively and cultivated at 30°C for 18 hours. The cells harvested were suspended with 0.85% of NaCl aqueous solution, and kept in ice water before use. The reaction mixtures contains 250 mM of TES buffer, pH=7.0, 100 mM of L-proline, 2% of glucose, 5 mM of $FeSO_4$, and cells that had an OD_{600}=10. The pH of the mixture was controlled to be above 6.5 with 5 N NaOH solution. The glucose concentration was monitored each 1 hour with glucose oxidase-based analyzer and was kept manually around 1–2% with 50% glucose solution.

As described, hydroxylation of L-proline to Hyp did not proceed quantitatively (Table 1). L-Proline was reported to be degraded to L-glutamate through the action of a bifunctional enzyme encoded by *putA*.[25] Introduction of a *putA* mutation into host cells *E. coli* W1485 improved the yield of hydroxylation of L-proline, suggesting that the degradation of L-proline contributed to lower the hydroxylation yield. L-Proline was hydroxylated to Hyp quantitatively (100% yield from L-proline) in the culture of *E. coli* W1485 *putA*/pWFH1 (Table 1). Productivity of Hyp was almost the same in a larger-scale culture.

Analysis of the metabolic pathways supplying 2-oxoglutarate

2-Oxoglutarate was expected to be supplied from glucose through the action of the tricarboxylic acid cycle, where isocitrate dehydrogenase encoded by *icd* is directly responsible for 2-oxoglutarate formation (Fig. 1(b)). In the culture of the *icd* mutant of W1485/pWFH1, Hyp was still produced, but the yield of Hyp from L-proline was 52%. The result suggested that there would be another route effective to supply 2-oxoglutarate. Then, we examined Hyp production from L-proline in an *icd*, *putA* double mutant in the resting cell reaction. Hyp was not produced except for a small amount in the beginning of the reaction, probably because the residual cellular 2-oxoglutarate was used for Hyp production, while Hyp was produced quantitatively up to approximately 2.2 mM for 6 h in a *putA* mutant (Fig. 4). These results suggest that 2-oxoglutarate can be supplied by two pathways, through the action of PutA from L-proline and through the action of Icd from glucose. A part of 2-oxoglutarate was apparently reproduced from succinate through the tricarboxylic acid cycle because the produced succinate was not accumulated (Fig. 1(b)). This recycling use of succinate may be the reason why the hydroxylation proceeds efficiently.

Production of other hydroxylated imino carboxylic acids

cis-3-Hydroxy-L-proline was also efficiently produced from L-proline by using the proline 3-hydroxylase gene[26,27] after optimizing its expression in the same manner as described above. After 100 h of culture of the *putA* mutant harboring the proline 3-hydroxylase expression plasmid, *cis*-3-hydroxy-L-proline was accumulated to 68 g/l from 59 g/l of L-proline (yield 100%). As reported previously, proline 3-hydroxylase and 4-hydroxylase can catalyze regio- and stereospecific hydroxylation or epoxidation of some cyclic imino carboxylic acids other than L-proline.[27] We have already examined the properties of

hydroxylation of various substrates, isolated the hydroxylated products, and confirmed the chemical structures of the products in small scale preparations.[27] The results described in this report would provide the manufacturing method to produce hydroxylated cyclic imino carboxylic acids, such as hydroxyazetidinecarboxylic acid, epoxyprolines, and hydroxypipecolic acids. These hydroxylated imino carboxylic acids would be also useful as chiral building blocks.

Acknowledgments

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