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A Simple Procedure for Selective Hydroxylation of L-Proline and L-Pipecolic Acid with Recombinantly Expressed Proline Hydroxylases

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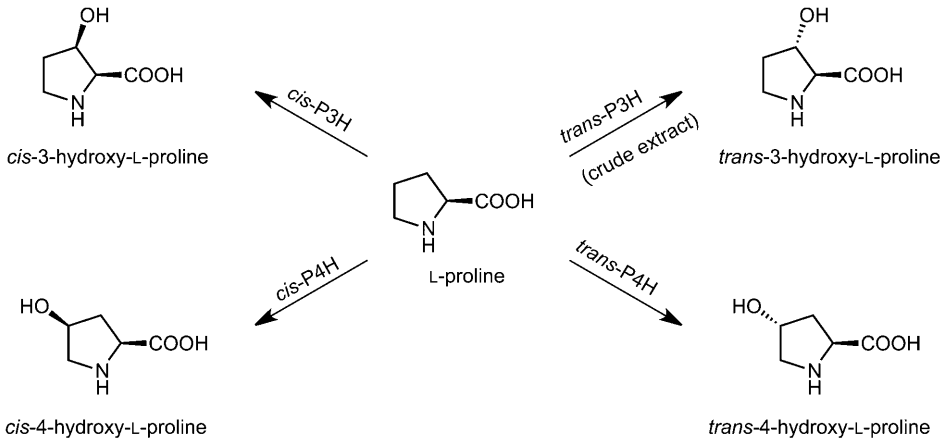
Abstract: Due to their diverse regio- and stereoselectivities, proline hydroxylases provide a straightforward access to hydroxprolines and other hydroxylated cylic amino acids, valuable chiral building blocks for chemical synthesis, which are often not available at reasonable expense by classical chemical synthesis. As yet, the application of proline hydroxylases is limited to a sophisticated industrial process for the production of two hydroxyproline isomers. This is mainly due to difficulties in their heterologues expression, their limited *in vitro* stability and complex product purification procedures. Here we describe a facile method for the production of *cis*-3-, *cis*-4- and *trans*-4-proline hydroxylase, and their application for the regio- and stereoselective hydroxylation of L-proline and its six-membered ring homologue L-pipecolic acid. Since *in vitro* catalysis with these enzymes is not very efficient and conversions are restricted to the milligram scale, an *in vivo* procedure was established, which allowed a quantitative conversion of 6 mM L-proline in shake flask cultures. After facile product purification *via* ion exchange chromatography, hydroxyprolines were isolated in yields of 35–61% (175–305 mg per flask). L-Pipecolic acid was converted with the isolated enzymes to prove the selectivities of the reactions. In transformations with optimized iron(II) concentration, conversions of 17–68% to hydroxylated products were achieved. The regio- and stereochemistry of the products was determined by NMR techniques. To demonstrate the applicability of the preparative *in vivo* approach for non-physiological substrates, L-pipecolic acid was converted with an *E. coli* strain producing *trans*-4-proline hydroxylase to *trans*-5-hydroxy-L-pipecolic acid in 61% yield. Thus, a synthetically valuable group of biocatalysts was made readily accessible for application in the laboratory without a need for special equipment or considerable development effort.

Keywords: asymmetric catalysis; enzyme catalysis; hydroxyprolines; α-ketoglutarate-dependent iron(II) oxygenases; regioselectivity; stereoselectivity

Introduction

Amino acids and their derivatives are valuable building blocks for organic synthesis. Especially proline with its rigid conformation has strong chirality inducing properties and is therefore widely used for stereoselective synthesis, both in stoichiometric amounts and as a chiral catalyst.[1] Thus, proline derivatives, especially those with substitutions at the carbon backbone, represent an interesting substance class for extension of the chiral pool. For instance, hydroxyprolines are used as building blocks for the synthesis of important pharmaceutical drugs such as carbapenem antibiotics, angiotensin-converting enzyme inhibitors and antispastic agents.[2] If not producible through collagen hydrolysis, functionalized proline derivatives are not readily available, either as natural products or by chemical synthesis. A promising approach for selective functionalization of amino acids is provided by the enzyme-catalyzed stereoselective hydroxylation. Especially α-ketoglutarate (α-KG) dependent Fe(II)-hydroxylases catalyze C–H activation reactions with high regio- and stereoselectivity,[3] and in contrast to the NAD(P)H-dependent P450 monooxygenases, their co-substrate α-KG is inexpensive so that a regeneration system is not required.

In the case of L-proline hydroxylation, different α-KG-dependent hydroxylases converting the free



Abbreviation	Enzyme	Source organism
cis-P3H	*cis*-3-proline hydroxylase (type I)	*Streptomyces* sp. TH1[4]
cis-P4H	*cis*-4-proline hydroxylase	*Sinorhizobium meliloti*[4]
trans-P3H	*trans*-3-proline hydroxylase	*Glarea lozoyensis* (fungus) crude extract[6]
trans-P4H	*trans*-4-proline-hydroxylase	*Dactylosporangium* sp. RH1[4]
		Glarea lozoyensis (fungus) crude extract[6]

Scheme 1. Proline hydroxylases cloned and expressed in *E. coli* and activities detected in the fungus *Glarea lozoyensis*.

amino acid have been identified in nature, generating all four possible regio- and stereoisomers of hydroxyproline (Scheme 1). To date, three types of bacterial enzymes, *cis*-3-, *cis*-4- and *trans*-4-proline hydroxylase (*cis*-P3H, *cis*-P4H and *trans*-P4H) have been cloned and overexpressed in *E. coli*.[4] *cis*-P3H (type II) from *Streptomyces* sp. strain TH1 was also characterized as the crystal structure of an iron complex.[5] An α-KG-dependent *trans*-P3H activity besides a *trans*-P4H activity was identified in protein crude extracts of the pneumocandin-producing fungus *Glarea lozoyensis*.[6]

In contrast to the prolyl hydroxylases involved in collagen biosynthesis, microbial proline hydroxylases exclusively hydroxylate the free amino acid. The proline hydroxylases characterized to date have been found in bacteria, where they are associated with the biosynthesis of peptide antibiotics. *cis*-P3H and *trans*-P4H were isolated from *Actinomycetales* including the producers of telomycin (*S. canus* ATCC 12647) and etamycin (*S. griseoviridus* P8648).[7] *cis*-P4Hs have been identified in two strains of the order Rhizobiales, *Mesorhizobium loti* and *Sinorhizobium meliloti*.[4]

The amino acids *trans*-4- and *trans*-3-hydroxyproline are available cost-efficiently by acidic hydrolysis of collagen. However, this process includes a complex purification procedure, much waste and the need for toxic solvents in the extraction process.[8] *cis*-4- and *cis*-3-hydroxyproline, which are less common or unknown in mammalian systems, can be obtained through epimerization reactions or chemical total synthesis.[9] They are being clinically evaluated as anticancer drugs and for the treatment of thickening of the basement membrane.[10] In an alternative synthetic approach, recombinant *E. coli* strains producing *trans*-P4H from *Dactylosporangium* sp. strain RH1 and *cis*-P3H from *Streptomyces* sp. strain TH1 are applied for the enzymatic production of *trans*-4- and *cis*-3-hydroxyproline, respectively.[11] The process was designed for the industrial scale using special expression vectors, optimized cultivation conditions and a complex product purification procedure.

It was also found that proline hydroxylases are not strictly substrate specific, but accept a set of proline congeners as long as the secondary amino acid moiety is untouched.[12] We were intrigued by the potential of these enzymes for functionalizing non-natural amino acids with diverse regio- and stereoselectivities and a comprehensive system for the production of these compounds on the laboratory scale was anticipated. However, several challenges had to be met: (i) The enzymes are virtually insoluble and not active when expressed in *E. coli* common expression vectors. (ii) They tend to denature rapidly when used for *in vitro* transformations. (iii) In the case of *in vivo* conversions, an elaborate work-up process to isolate the products from the medium is required.

Here we present a generally applicable method including cloning, protein expression, conversion, analytics and product purification, for the enzymatic production of three different hydroxyprolines on a preparative scale. The enzymes were also used *in vitro* to hydroxylate L-pipecolic acid, the six-membered ring congener of L-proline, and the products were characterized. Finally, an *in vivo* transformation of the non-

natural substrate on a preparative scale is demonstrated.

Results

Production of Recombinant Proline Hydroxylases

The proline hydroxylase genes were synthesized (in case of the GC-rich sequences *cis*-P3H and *trans*-P4H codon optimization for *E. coli* was applied), ligated into several commercially available expression vectors and tested for protein production. Strong expression was observed in most cases, however, the proteins were insoluble and no activity was detected. Variations of cultivation conditions or *E. coli* expression strains as well as denaturation techniques were not successful. An overnight expression with the cold shock vectors pCold I and pCold II at 16 °C gave a reasonable fraction of soluble and active protein (data not shown). Best results were obtained by coexpression of the genes in the expression vector pET28b with the chaperone system GroEL/GroES (Figure 1, **a**). *S. coelicolor* GroEL/GroES expressed in vector pETcoco-2 was used in this study,[13] even though comparable yields were also obtained with the commercially available chaperone vector pG-KJE8 encoding GroEL/GroES from *E. coli* among other proteins. Yields in a range of 5–10 mg protein/L medium were obtained from shake flask cultures after Ni-NTA purification and desalting for all three proteins (Figure 1, **b**).

Characterization of Enzyme Reactions

Conversions of L-proline to hydroxyproline were measured by HPLC after FMOC-derivatization. The reverse-phase assay allows a baseline separation of all four hydroxyprolines and proline (Figure 2). Concentrations from 20–120 $\mu mol \cdot L^{-1}$ in the sample can be quantified by fluorescence detection.

For *in vitro* conversions we found that an appropiate concentration of $FeSO_4$ is crucial for proline hydroxylase activity, more than that of substrate and cosubstrate. Thus, the conversion was measured in dependence on the $FeSO_4$ concentrations without and in presence of L-ascorbate (Figure 3).

For all enzymes a distinct dependence of the total turnover to Fe(II) and ascorbate concentrations was found. Optimal Fe(II) concentrations were around 0.1 mM for the *cis*-selective enzymes whereas *trans*-P4H was slightly more iron tolerant and had an optimum at *ca.* 0.5 mM. In contrast to prolyl hydroxylases, ascorbate is not essential for catalysis with proline hydroxylases.[4,14] Nevertheless, the presence of L-ascorbate in an appropriate amount increases the production of hydroxyprolines significantly. This effect which is observed for many α-KG-dependent enzymes is generally explained by the ability of ascorbate to reduce iron(III) to the catalytically relevant iron(II) species.[15] At ascorbate concentrations >3 mM, however, an inhibitory effect is dominating. For instance, the conversion with *trans*-P4H at 6 mM ascorbate was lower than without (2 mM Fe^{2+} in both cases). The inhibitory effect was confirmed by measuring the total turnover of conversions with *trans*-P4H and 0.5 mM Fe^{2+} in dependence on the ascorbate concentration (see Supporting Information).

At adequate conditions a moderate, but virtually constant activity over two hours was observed for all

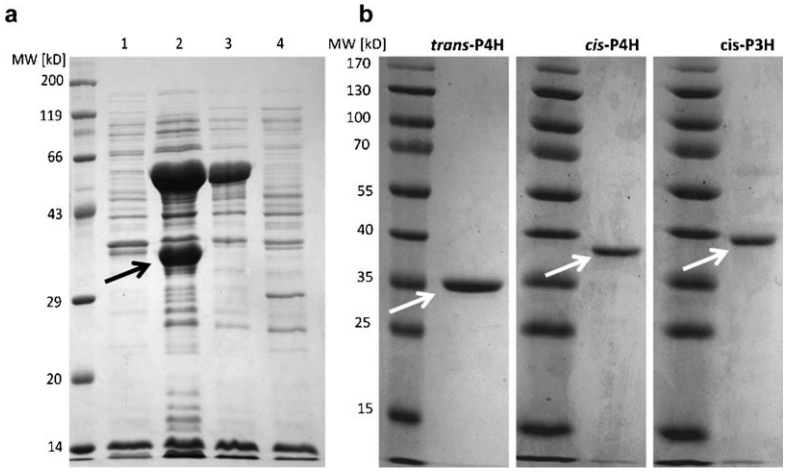


Figure 1. a) Expression of *trans*-P4H in *E. coli* BL21 Codon Plus RP, soluble fractions; lane 1: *trans*-P4H/pET28b(+); lane 2: *trans*-P4H/pET28b(+) + chaperones/pETcoco2; lane 3: chaperones only; lane 4: negative control. **b)** Purified *trans*-P4H, *cis*-4PH and *cis*-3PH. Proline hydroxylase bands are marked with arrows.

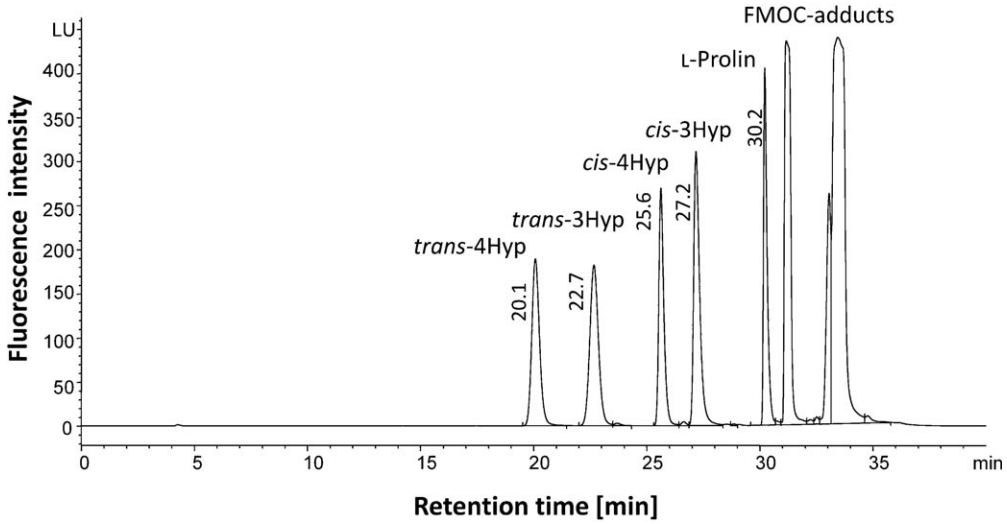


Figure 2. Separation of hydroxyprolines in HPLC-chromatogram (Hyp = hydroxyproline; wavelength: excitation = 254 nm, emission = 316 nm).

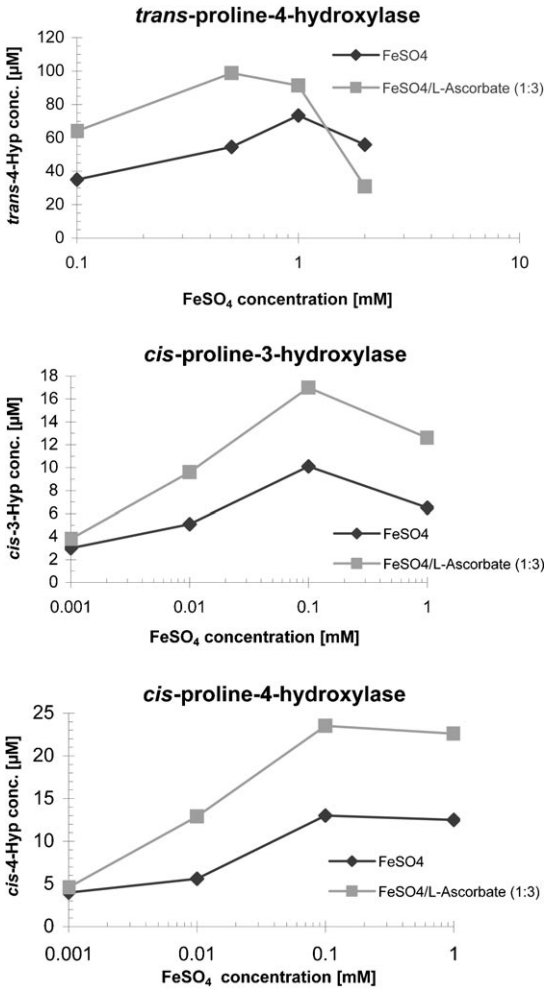


Figure 3. Hydroxyproline production at different concentrations of $FeSO_4$ alone and in the presence of L-ascorbate (molar ratio $FeSO_4$/L-ascorbate = 1:3). Since concentrations range over three orders of magnitude, a logarithmic scale was used.

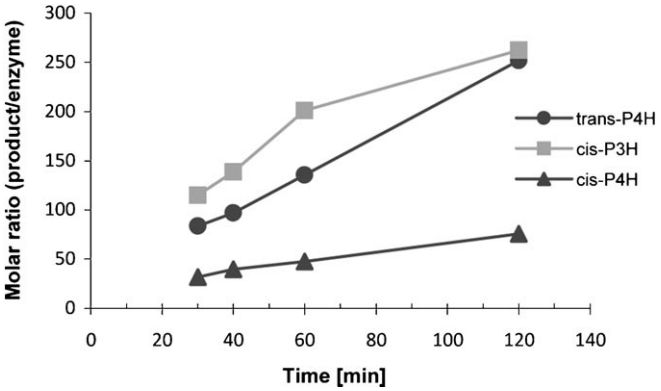


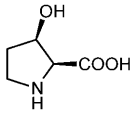
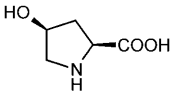
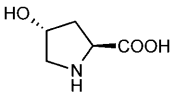
Figure 4. Hydroxyproline production with *trans*-P4H, *cis*-P4H and *cis*-P3H. Optimal temperatures according to the literature were applied for each enzyme.[4,11]

three proline hydroxylases, although some protein precipitated already minutes after start of the reaction (Figure 4). After 24 h no further activity could be detected. Attempts to stabilize the enzymes with additives were not successful (data not shown). Nevertheless, a product concentration of 8 mM hydroxyproline was achieved in the presence of approx. 0.5 mg·mL^{-1} *trans*-P4H. With the *cis*-selective enzymes activities and total turnover were slightly lower under comparable conditions.

In vivo Production of Hydroxyprolines

Hydroxyprolines were generated by feeding L-proline (6.25 mM) to an exponentionally growing culture of the corresponding *E. coli* strain and incubation at 28 °C for three days. HPLC analysis of the product mixture revealed quantitative substrate conversion

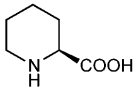
Table 1. Hydroxylation of L-proline in *E. coli* cultures expressing *trans*-4-, *cis*-3- and *cis*-4-proline hydroxylases.

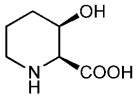
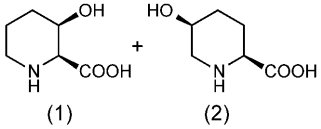
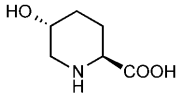
Enzyme	Product	Conversion to product[a]	Isolated[b]
cis-P3H		69%	61% (305 mg)
cis-P4H		41%	35% (175 mg)
trans-P4H		63%	51% (255 mg)

[a] 500 mg L-proline were converted in 800 mL culture for 3 days at 28 °C. The conversion was determined by HPLC.
[b] Since the product still contained inorganic salts after ion exchange chromatography, yields were determined by HPLC.

and product concentrations >3.75 mM (60%) in the case of *trans*-P4H and *cis*-P3H and 2.56 mM (41%) for *cis*-P4H (Table 1). The loss of substrate is due to metabolization by *E. coli.* It can be substantially reduced by deletion of the proline dehydrogenase gene *putA* in *E. coli*.[11a] To circumvent a laborious product purification including a separation from other amino acids, an amino acid-free minimal medium based on glycerol, acetate and ammonium sulfate was used. Glucose was also avoided, since it suppresses the amplification of the pETcoco-based chaperone vector. The concentration of L-proline was set low enough to ensure a quantitative conversion within 72 h incubation time. After that the hydroxyprolines were isolated from the culture broth by two steps of ion-exchange chromatography. In this way 80–90% of the corresponding product was obtained. In the medium and also in the isolated product only the expected regio- and stereoisomer for the corresponding enzyme was found. Some inorganic salts, especially sodium chloride, were co-eluted with the product, but did not affect follow up chemistry. If the salt-free compound is required, this can be readily separated by further chromatography or recrystallization. Occasionally proteinogenic amino acids, especially valine, were enriched in the medium and coeluted with hydroxyproline. So far the reason for this is not fully understood, but it is evident that the amount of other amino acids can be reduced to traces at optimal fermentation conditions (*cf.* ^{1}H NMR spectra in Supporting Information).

Table 2. Conversion of L-pipecolic acid with *trans*-4-, *cis*-3- and *cis*-4-proline hydroxylases.

Substrate: 

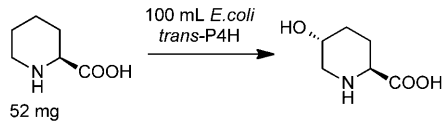
Hydroxylase	Product	Conversion to product[a]
cis-P3H		17%
cis-P4H	 (1) (2)	33% (1) + 33% (2)
trans-P4H		68%

[a] 8 mg (1 mM) pipecolic acid were incubated with fresh enzyme preparation for 14 h at 37 °C (*cis*-P3H, *trans*-P4H) and 25 °C (*cis*-P4H), respectively. No other products were detected in this assay (HPLC, NMR).

In vitro Hydroxylation of L-Pipecolic Acid

In the scope of this project it should be demonstrated that our system also applies to non-natural substrates. In the case of *trans*-P4H and *cis*-P3H a relatively relaxed substrate specificity has been found for proline derivatives.[12] Especially the six-membered homologue of L-proline, L-pipecolic acid, appeared to be a good substrate for these enzymes, and the hydroxylated products are of synthetic interest too. Thus L-pipecolic acid was chosen as model substrate. The selectivity of the reactions was confirmed by NMR spectroscopy of the isolated products. In coincidence with the literature, *trans*-P4H gave *trans*-5-hydroxypipecolic acid in a good yield (Table 1) whereas the conversion with *cis*-P3H was rather poor.[12] Yet the product could be unambiguously identified as the *cis*-3-hydroxylated pipecolic acid by ^{1}H-^{1}H-COSY-NMR spectroscopy. The coupling constant between H-2 and H-3 was 1.6 Hz, which is in good agreement with the *cis*-product (7.1 Hz for the corresponding *trans*-product; see also Supporting Information).[16] With *cis*-P4H, the conversion of L-pipecolic acid was about 55% compared to that observed for L-proline under the identical conditions. Two products were obtained in an approximate 1:1 ratio (Table 2 and HPLC chromatogram in Supporting Information). To the best of our knowledge, all conversions with proline hydroxylases reported so far gave a single product exclusively. Through ^{1}H-^{1}H-COSY-NMR spectroscopy the compounds were identified as the 3- and 5-hydroxylated

Table 3. Hydroxylation of L-pipecolic acid with recombinant *E. coli* producing *trans*-P4H.

Reaction	Conversion to product	Isolated yield[a]
 52 mg	78%	61% (=35 mg)

[a] Eluted in a fraction together with 13 mg *trans*-4-hydroxyproline. Product concentrations were determined by HPLC.

congeners. In the HPLC assay one of the products had the same retention time as *cis*-3-hydroxypipecolic acid and the ^{1}H NMR coupling constants were identical (see Supporting Information). The second compound was eluted approx. 2 min later than *trans*-5-hydroxy-L-pipecolic acid indicating that the product is *cis*-5-hydroxylated. In the ^{1}H NMR spectrum the coupling constants were in good agreement with those of *cis*-5-hydroxy-L-pipecolic acid reported in the literature.[17] So for both products a *cis*-configuration can be assigned, coinciding with the *cis*-selectivity of the enzyme.

In vivo Hydroxylation of L-Pipecolic Acid with *trans*-P4H

Although some milligrams of hydroxypipecolic acids were obtained through catalysis with isolated enzymes, this method cannot be considered as an efficient synthetic approach and there is little potential for up-scale. In the longer term, an *in vivo* method as used for hydroxyprolines is desirable. However, L-pipecolic acid is not a proteinogenic amino acid and the uptake of the compound by living cells might be a limiting factor. To test the general feasibility of this approach, a 4 mM solution of L-pipecolic acid was converted with an *E. coli* strain expressing *trans*-P4H under the same conditions as applied for proline hydroxylation.

A surprisingly high product concentration was found in the fermentation broth (3.12 mM) and after ion exchange chromatography the product was obtained in 61% yield (Table 3). Neither substrate nor other regio- or stereoisomers of the product were found in the amino acid isolate. Since endogenously produced L-proline was also hydroxylated by *E. coli*, *trans*-4-hydroxyproline was obtained in the same fraction as the product after chromatographic work-up (see HPLC chromatogram and ^{1}H NMR spectrum in Supporting Information).

Discussion

Whereas heterologous expression of proline hydroxylases in *E. coli* with standard vectors yielded almost exclusively insoluble protein, coexpression with the chaperone system GroES/GroEL gave a soluble protein fraction of up to 10 mg·L^{-1} medium. In analytical conversions the best total turnover was observed at iron(II) concentrations of 0.1–0.5 mM, lower than applied for these enzymes in the literature.[4,11] It was found that iron(II) concentrations already in a low millimolar range have a strong inhibitory effect. Besides L-proline, the non-physiological substrate L-pipecolic acid was tested. From all conversions enough product for a comprehensive chemical analysis including 2D-NMR analysis was obtained, even though the enzymatic activity was rather poor with *cis*-P3H. However, isolated proline hydroxylases have a relatively low specific activity and tend to denaturation under reaction conditions, so that *in vitro* conversions in production scale are not profitable. In contrast, hydroxylation of L-proline with recombinant *E. coli* strains proved to be very efficient in an optimized industrial process.[11] Thus, we established an analogue method for our strains applicable in a laboratory scale. L-Proline in a concentration of 6.25 mM was quantitatively converted in shake flask cultures. Hydroxyprolines were obtained as single regio- and stereoisomers in 41–69% yield in the medium depending on the enzyme. Since an amino acid-free minimal medium was used, the complex down-stream process for product purification applied in industry could be replaced by two straightforward ion exchange chromatography steps, from which 80–90% of the product was recovered. The product fractions still contained inorganic salts, especially sodium chloride, which did not interfere in follow-up chemistry. With regard to organic compounds hydroxyprolines could be obtained in purities >95%. In a few cultures valine was found in considerable amounts as a by-product, however, this can be avoided by careful control of the fermentation conditions. The transformations described here were not optimized for maximal yields and we expect that product concentrations far beyond 0.6 g·L^{-1} are feasible after some optimization.

Surprisingly, conversions of L-pipecolic acid with *cis*-P4H yielded *cis*-3-hydroxy-L-pipecolic acid besides the expected *cis*-5-isomer. This demonstrates that the catalytic properties of this enzyme are very similar to that of *cis*-P3H, although the sequence identity is rather moderate (34%).[18] In contrast, *trans*-P4H is not related to any of the *cis*-selective enzymes (11–14% sequence identity), so that a different evolutionary origin can be postulated for this enzyme.

In the longer term, we are interested in the selective functionalization of non-physiological substrates on a preparative scale. Therefore, we tested the feasibility of our *in vivo* approach by converting a 4 mM solution of L-pipecolic acid with *trans*-P4H producing *E. coli*. 78% of the substrate was hydroxylated selectively to *trans*-5-hydroxy pipecolic acid. This shows that in principle amino acids other than L-proline can be oxidized on a preparative scale too. Hydroxyproline produced by *E. coli* was found as a side product in this culture which was isolated with the product after ion exchange chromatography. Currently we are investigating whether an advanced separation procedure is required for product purification or if the formation of hydroxyproline can be suppressed by optimization of the fermentation conditions.

Conclusions

In summary, a comprehensive approach to three different proline hydroxylases including protein expression, biotransfomation, product purification, analytics and their application in biocatalysis is presented. It allows the synthesis of a diversity of hydroxyprolines and hydroxylated proline derivatives with standard laboratory equipment. It is highly flexible and new isoenzymes or substrates are easily integrated. Whereas the scale of *in vitro* production is restricted by the intrinsically limited stability of the enzymes, hydroxyprolines can be obtained efficiently on a preparative scale through *in vivo* transformation of proline in simple shake flask cultures. First experiments with L-pipecolic acid suggest that non-natural substrates can also be hydroxylated on a preparative scale.

Experimental Section

Chemicals and Culture Media

Chemicals, antibiotics and culture media were purchased from Sigma Aldrich Chemie GmbH (Munich, Germany), A. Hartenstein GmbH (Würzburg, Germany) and Carl Roth GmbH & Co. KG (Karlsruhe, Germany).

Bacterial Strains, Plasmids and Proline Hydroxylase Genes

Escherichia coli BL21 Codon Plus RP with chaperone expression vector pL1SL2 was kindly provided by Peter F. Leadly (University of Cambrige, U.K.).[13] The chaperone encoding plasmid pG-KJE8 (Takara Bio Inc., Japan) was also used for coexpression. Expression plasmid pET-28b (+) (Novagen, Darmstadt, Germany) was applied for gene cloning and expression. The genes of *trans*-P4H (GenBank No. BAA20094) and *cis*-P3H type I (Protein Data base: 1E5RA) were synthesized by ATG:biosynthetics GmbH (Merzhausen, Germany) under optimized codon usage for *E. coli*. The gene of *cis*-P4H (GenBank No. CAC47686) was synthesized by the same company without C-terminal stop codon for expression of the C-terminal HisTag in pET-28b (+). All three genes were initially inserted into the SmaI site of pBSK II. For cold-shock expression the vectors pCold I and pCold II (Takara Bio Inc., Japan) were used and gene expression was performed according to the manufacturer's instructions.

Construction of the Expression Plasmids

The proline hydroxylase genes were amplified from the corresponding pBSK II-based plasmid by PCR with *Phusion*® High-Fidelity DNA Polymerase (New England Biolabs, NEB, Ipswich, Massachusetts) using T7 and T3 standard primers. The PCR products were digested with SalI and NotI (NEB) in case of *trans*-P4H and *cis*-P3H and with NotI and HindIII in case of *cis*-P4H. The DNA fragments were ligated into the corresponding sites of pET-28b (+) and verified by sequencing (Eurofins MWG Operon, Ebersberg, Germany). The plasmids were transformed into *E. coli* BL21 Codon Plus RP containing pL1SL2, an expression vector for the *Streptomyces coelicolor* chaperones GroEL 1, GroEL 2, and GroES.[13]

Preparation and Purification of Recombinant Enzymes

Recombinant *E. coli* cells were grown aerobically at 37 °C and 140 rpm in 800 mL Terrific Broth (TB) with ampicillin (100 $\mu g\,mL^{-1}$), chloramphenicol (34 $\mu g\,mL^{-1}$), kanamycin (50 $\mu g\,mL^{-1}$) and L-arabinose (0.01% w/v). At an OD_{600} = 1.2, isopropyl-ß-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM and the cultures were incubated for 16 h at 28 °C and 140 rpm. The cells were harvested by centrifugation (3300 × *g*, 20 min, 4 °C) and resuspended in 15 mL equilibration buffer [imidazole (20 mM), glycerol (20% v/v), Mes (50 mM, pH 6.5), Tween 20 (0.1% v/v)] and sonicated on ice for 7 × 10 s in 30 s intervals (Branson Sonifier 250, Danbury, USA). After centrifugation (9290 × *g*, 60 min, 4 °C), the enzymes were isolated from the cell-free extract using Ni-NTA superflow chromatography according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany). Imidazole was removed *via* PD-10 desalting columns (GE Healthcare, Piscataway, USA) so that the enzymes were eluted in Mes buffer (50 mM, pH 6.5). The concentrations of purified protein were measured with a Qubit™fluorometer (Invitrogen, Paisley, UK). For conversions described here fresh enzyme preparations were used. However, enzyme solutions or lyophyllisates can

be stored for months at −80 °C without significant loss off activity.

Derivatization and HPLC Assay

Hydroxyprolines and L-proline were analyzed by HPLC after precolumn derivatization (FMOC/ADAM) as follows.[19] To 40 μL of sample solution, 40 μL of sodium borate buffer (0.5 M, pH 7.7) were added. Then 80 μL of 9-fluorenyl-methoxycarbonyl chloride (FMOC-Cl, 1.5 mM in acetone) were added and the mixture was vortexed for 60 s. After that 100 μL of 1-adamantanamine (ADAM, 40 mM in acetone and sodium borate buffer (pH 7.7), 1:1) were added and the mixture was vortexed for 45 s. For analysis, the mixture was diluted with 140 μL HPLC buffer A (20% acetonitril/80% 50 mM sodium acetate pH 5). HPLC analysis was performed with an Agilent 1100 series HPLC system (Agilent Technologies, Santa Clara, USA) equipped with an Agilent fluorescence detector (1100 series) and a LiChrosorb RP 18–5 μ column (250 × 4.0 mm, CS GmbH, Langerwehe, Germany) maintained at 45 °C. A gradient of buffer A and buffer B (80% acetonitrile/20% 50 mM sodium acetate pH 5) was applied (gradient program 1, buffer A: 1. 0–2 min 100%; 2–17 min: 100% → 91%; 17–25 min: 91% → 70%; 25–27 min: 70% → 0%; 27–32 min: 0%; 32–34 min: 0% → 100%; 34–40 min: 100%). The injection volume was 10 μL and the flow rate 0.75 mL min^{-1}. Derivatized hydroxylprolines were detected fluorometrically at 254 nm excitation wavelength and at 316 nm emission wavelength.

Assay for L-Proline

The *in vitro* activity assay of proline hydroxylases were carried out in a reaction mixture containing Mes buffer (50 mM, pH 6.5), L-proline (8 mM), α-KG (14 mM), ferrous sulfate (0.5 mM), L-ascorbate (1.5 mM) and enzyme preparation in a final volume of 1 mL. Reactions with *trans*-P4H and *cis*-P3H were carried out at 37 °C and reactions with *cis*-P4H at 25 °C. The hydroxyproline concentration was measured by HPLC as described above.

Assay for L-Pipecolic Acid

Conversions of L-pipecolic acid were performed in Mes buffer (50 mM, pH 6.5), L-pipecolic acid (1 mM), α-KG (10 mM), ferrous sulfate (0.5 mM), L-ascorbate (1.5 mM) and enzyme preparation in a final volume of 60 mL (4 × 15 mL). Reactions with *trans*-P4H and *cis*-P3H were carried out at 37 °C for 14 h and reactions with *cis*-P4H at 25 °C for 14 h. Reaction products were isolated by ion-exchange chromatography as described below. The hydroxylated L-pipecolic acids were analyzed by HPLC as Fmoc derivatives (gradient program 2, buffer A: 0–2 min: 100%; 2–19 min: 100% → 75%; 19–25 min: 75% → 45%; 25–27 min: 45% → 0%; 27–32 min: 0%; 32–34 min: 0% → 100%; 34–40 min: 100%), by mass spectroscopy (photospray ionization, Agilent Technologies, Santa Clara, USA) and NMR spectroscopy (Bruker Avance DRX 400, Buker Biospin GmbH, Rheinstetten, Germany).

In vivo Production of Hydroxyprolines

E. coli production strains were grown aerobically in a 2-L flask with 800 mL medium 5: glycerol (1% v/v), $(NH_4)_2SO_4$ (10 g/L), NaCl (2 g/L), K_2HPO_4 (1 g/L), $MgSO_4$ (1 g/L), NH_4Cl (5 g/L), sodium acetate (5 g/L), supplemented with ampicillin (100 μg/mL), chloramphenicol (34 μg/mL), kanamycin (50 μg/mL) and L-arabinose (0.01% w/v) at 37 °C and 140 rpm. At $OD_{600} = 1.1–1.4$ IPTG was added to a final concentration of 0.2 mM. Subsequently, 500 mg L-proline (6.25 mM), 111 mg $FeSO_4 \cdot 7H_2O$ (0.5 mM) and 1.45 g α-KG disodium salt·$2H_2O$ (8 mM) were added. The cells were incubated for further 72 h at 28 °C and 140 rpm. After 24 h and 48 h 4 mL glycerol (6.6 mL/L) were supplemented.

In vivo Production of *trans*-5-Hydroxypipecolic Acid

The transformation was performed as described for the production of hydroxyprolines, but on a 100 mL scale. L-Pipecolic acid (52 mg, 4 mM) was added to the culture instead of L-proline.

Isolation of Hydroxylated Products

The cultures were centrifuged (3300 × *g*, 45 min, 4 °C) and the supernatant was adjusted with HCl to pH 1.3–1.6 and passed through a column with 110 mL of cation-exchange resin [Dowex® 50 W × 8, 50–100 mesh (H^+ form)]. The fractions containing hydroxylation products were passed through a second column with 140 mL of anion-exchange resin [Dowex® 1 × 8, 20–50 mesh (OH^- form)]. The product fractions were combined and concentrated under reduced pressure and air-dried to obtain the products as a brownish solid containing inorganic salts. Isolated yields determined by HPLC: *cis*-3-Hyp: 305 mg (61%), $[\alpha]_D^{20}$: −61.3 (*c* 0.3, H_2O); *cis*-4-Hyp: 175 mg (35%), $[\alpha]_D^{20}$: −43.9 (c 0.3, H_2O); *trans*-4-Hyp: 255 mg, (51%).

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