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Microbial screening in hydroxylation of L-proline

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Abstract—Microbial screening of 250 wild type strains resulted in identification of five strains with the activity of prolyl hydroxylase. All five strains hydroxylated regioselectively and enantioselectively L-proline into 4(*R*)-*trans*-hydroxy-L-proline **1**. The best conversions were obtained with a wild type of *Aeromonas caviae*. 3-Hydroxylase activity was not detected.

The importance of enzymatic hydroxylations is in their ability to introduce one or more OH groups at inactivated centers in hydrocarbons. First microbial hydroxylations described in the literature were with steroids as substrates.[1–4] Hydroxylations of hydrocarbons other than steroids have been extensively studied with $P450_{cam}$ from *Pseudomonas putida*, which transforms D-camphor into 5-*exo*-hydroxycamphor. Small and medium-size cycloalkenes have been hydroxylated and mixtures of allyl hydroxylated products accompanied with epoxydes and/or cis-diols have been obtained.[5,6] In the series of aromatic compounds, the hydroxylation is often the initial step in the degradation and detoxification in both procaryotes and eucaryotes yielding a mixture of phenols and catechols. Some microorganisms hydroxylate benzylic positions and other heteroaromatic side chains. Thus hydroxylation of ethyl benzene, for example, leads to enantiomerically enriched phenylethanols.[7] Hydroxyprolines, 4(*R*)-*trans*-hydroxy-L-proline (**1**) and 3(*R*)-*cis*-hydroxy-L-proline (**2**), Figure 1, are useful chiral synthons in the synthesis of pharmaceuticals. They play an important role in the preparation of anti-inflammatory pharmaceuticals like oxaceprol, inhibitors of angiotensin converting enzyme like fosinopril, or even food additives.

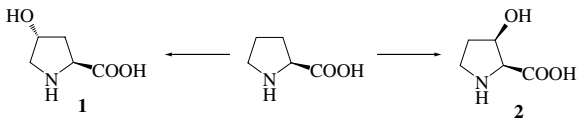

Figure 1.

The enzymes responsible for hydroxylation of L-proline are prolyl 3-hydroxylase and prolyl 4-hydroxylase, respectively. Both enzymes were described in *Streptomyces*,[8–11,13,14b] *Bacillus*,[8,12,15] *Dactylosporangium*[14a,b] and *Amycolatopsis*[14a] species and are not very abundant. The screening of more than 3000 microorganisms resulted in only eight strains able to produce prolyl hydroxylase activities. The activity of prolyl hydroxylase was very weak in some cases and thus detectable only by using C-14 labeled substrate.[10,11] *Dactylosporangium* sp. proline 4-hydroxylase gene has been cloned and expressed in *Escherichia coli*[14a] as a fused protein that had 13-fold higher activity then the enzyme in the original strain. Recombinant 4-hydroxylase from *Dactylosporangium* sp. and 3-hydroxylase from *Streptomyces* sp. have been assayed for their selectivity with different substrates.[16] Prolyl hydroxylases were partially purified and characterized. Both prolyl hydroxylases require 2-oxoglutarate as a cosubstrate and Fe^{2+} as a cofactor. EDTA, Zn^{2+}, Cu^{2+}, Co^{2+} and Ba^{2+} are reported to be the enzyme inhibitors.[10] The crystal structure of the first proline 3-hydroxylase with and without complexed iron from *Streptomyces* sp. has been elucidated.[17] There is no data on three dimensional structures of other 2-oxoglutarate oxygenases. Thus the authors suggest the possibility of the convergent evolution to a mechanism/active site chemistry with different overall folds. Among fungi, 3-hydroxylase and 4-hydroxylase have been detected in the pneumocanding-producing strain of *Glarea lozoyensis*.[18] The enzymes converted L-proline to both *trans*-3- and *trans*-4-hydroxy-L-prolines in the reaction dependent on 2-oxoglutarate, ascorbate, dithiotreitol and Fe^{2+}.

Keywords: L-Proline; Hydroxylation; Microbial screening.
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We describe in this letter a new, rapid and original screening approach. Although the described methodology was applied in the microbial screening of prolyl hydroxylase activity, it can be applied to any kind of bioconversion. Two hundred and fifty wild type strains have been screened for their ability to selectively hydroxylate L-proline. A rapid screening was set up on a small scale (96-well format, Fig. 2) with the cultures grown on the solid medium as described in the experimental part. The first and the most important reason for using solid agar plugs rather than liquid cultures in the screening is the presence of, extracellular and intracellular enzymes. Moreover, this approach allows the long term storage of biocatalysts with a minimal loss of activity. As the plugs are kept frozen in the sterile buffer of the desired pH they are ready to be used when needed.

When microorganisms grew and covered completely the surface of the medium on Petri plate they were cut and placed in the sterile buffer of defined pH depending on the reaction searched. Among 250 strains screened for prolyl hydroxylase activity, 31 strains transformed more than 30% of L-proline. However, only five microorganisms (Table 1) gave the product whose retention time, UV and mass spectra correspond to 4(*R*)-*trans*-4-hydroxy-L-proline **1**, while the remaining 26 strains metabolized L-proline without the appearance of detectable product(s). L-Proline was stable under the same reaction conditions and no decomposition or degradation was detected when the biocatalyst was omitted. Similarly, strains alone did not produce any detectable hydroxyl proline when the substrate was omitted.

The screening was realized in 1 ml reaction volume and the positive results were reproduced several times in a 24-well format with 10 ml reaction volume. The best reproducibility was obtained with *Aeromonas caviae*, and this strain was used for the flask experiments. Quantitative HPLC analysis obtained with *A. caviae* shows 68% of conversion of starting substrate. However, the 4(*R*)-*trans*-hydroxy-L-proline **1** was obtained in only 10% of yield. The product was quantified from HPLC/UV spectra after its transformation into 2,4-dinitrofluorobenzene derivative. The identification of the biotransformation product was based on comparison of its MS spectra with those of the commercial samples after the transformation into 2,4-dinitrofluorobenzene derivative. The HPLC spectra of one example of *A. caviae* biotransformation products and of the standard commercial samples are given in Figures 3 and 4. The crude lyophilized product from a culture broth of *A. caviae* was analyzed by NMR and compared to commercial hydroxyl prolines. The NMR analysis confirmed the 4(*R*)-*trans*-hydroxy-L-proline **1**.

Other microorganisms which hydroxylated L-proline into *trans*-4-hydroxyproline (*Bacillus subtilis*, *Fusarium solani* and *Gordonia rubripertincta*) did not grow better and moreover showed lower activity then *A. caviae*. In order to better exploit the enzymatic potential of *A. caviae* prolyl hydroxylase on a large scale we put

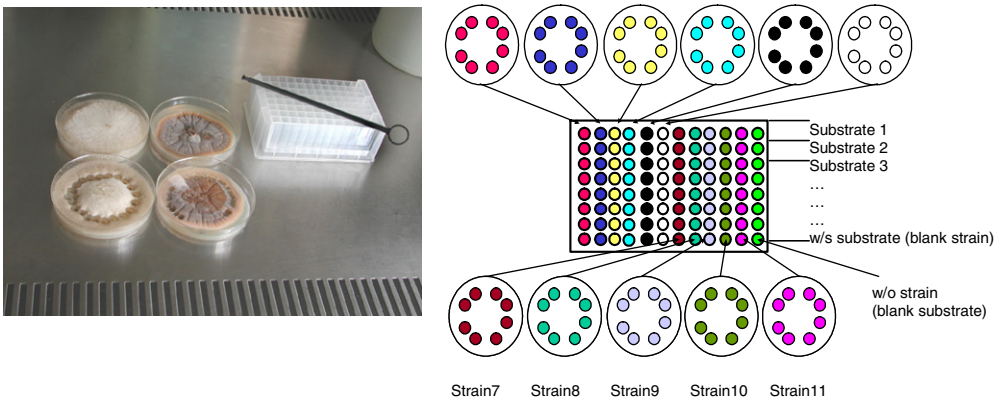

Figure 2.

Table 1. HPLC results of biotransformation of L-proline

Strain	Origin of strains	[Dry biomass] (mg/ml)	Conv.[a] (%)	4-OH-Pro (%)
Bacillus subtilis	ATCC 21967	7.00	88	8
Aeromonas caviae	CRTL	5.75	68	10
Fusarium solani	CRTL	5.80	93	6
Gordonia rubripertincta	CRTL	7.90	74	7
Staphylococcus capitis	CRTL	5.15	96	6
Blank (substrate w/o strain)[b]	—	—	0	0

[a] HPLC conversion based on L-Pro being consumed.
[b] L-Proline was stable under the same reaction conditions. No decomposition or degradation was detected when the biocatalyst was omitted.

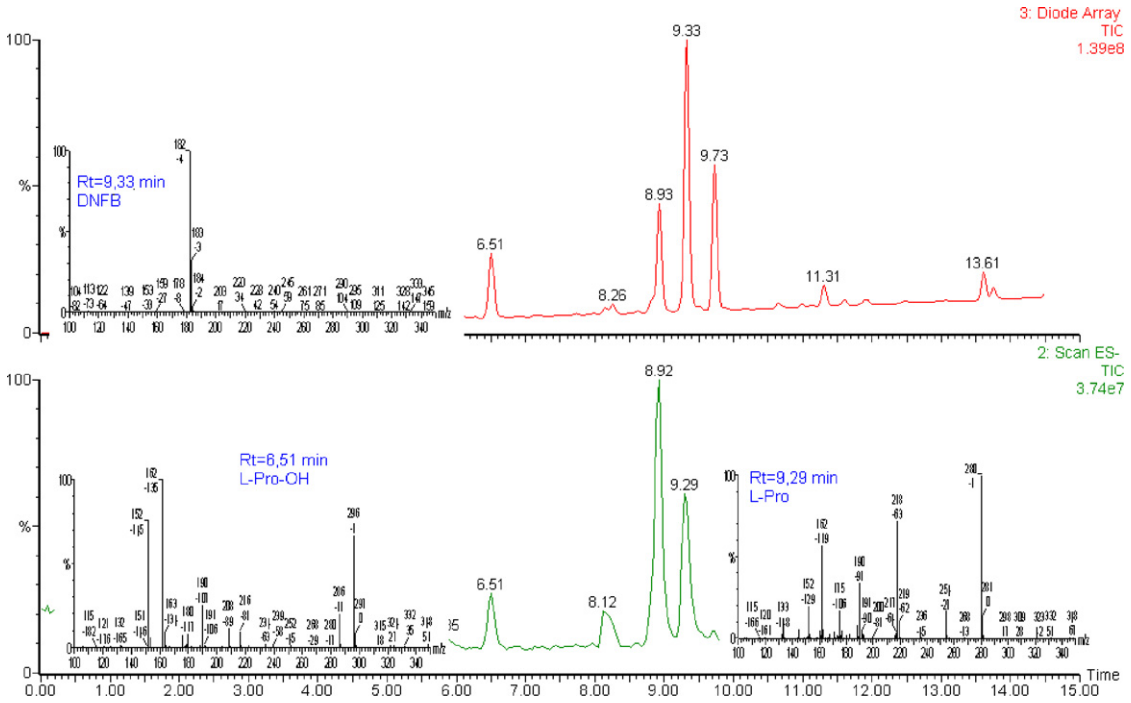

Figure 3. UV chromatograms and MS spectra of DNP-derivatives obtained by hydroxylation of L-proline with *Aeromonas caviae*: DNP-L-Pro = 9.29 min ($M^- = 280$); DNP-**1** = 6.51 min ($M^- = 296$).

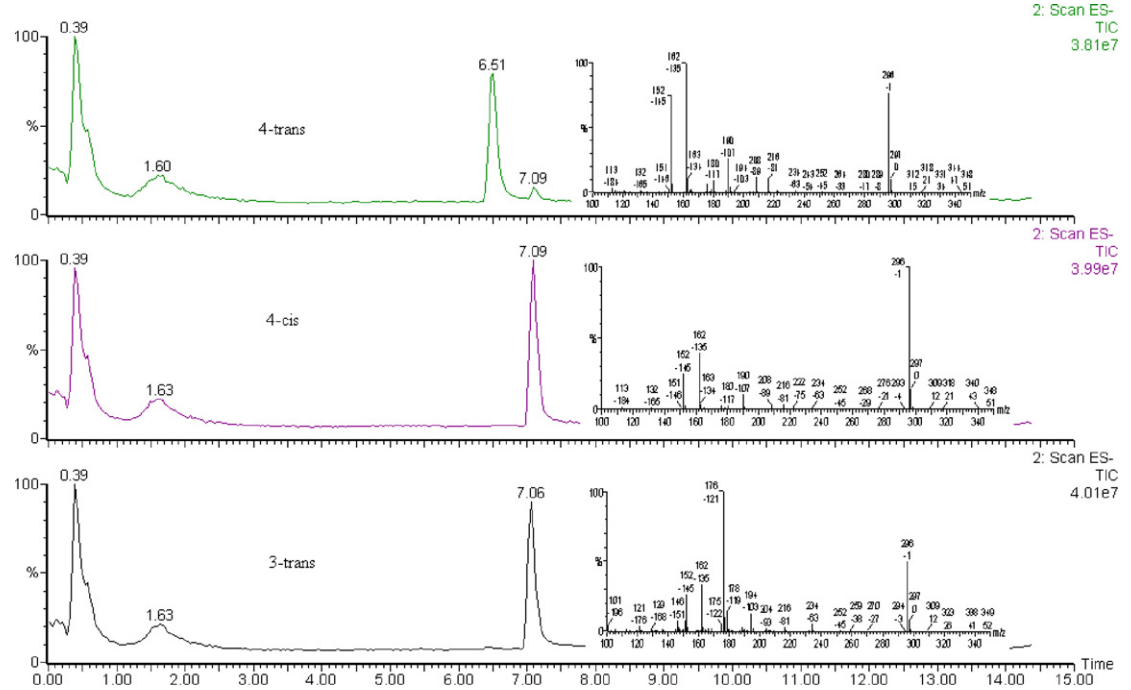

Figure 4. UV chromatograms and MS spectra of DNP-derivatives of standard samples (4-*trans*-hydroxy-L-proline **1**, 4-*cis*-hydroxy-L-proline and 3-*trans*-hydroxy-L-proline **2**) (the separation of 4-*cis* and 3-*trans* isomers is difficult under the HPLC conditions. However, it is possible to distinguish the two isomers based on different fragmentation in mass spectra).

our efforts to gene cloning and its heterologous expression.

Five strains (2%) out of 250 showed the activity of prolyl hydroxylase. All five strains gave the product of hydroxylation at the C-4 position or 4(*R*)-*trans*-hydroxy-L-proline **1** in 5–10% yield as determined by quantitative HPLC analysis. No 3-hydroxylase activity was detected. In conclusion, the reaction product in all five positive hits corresponds to the dinitrophenyl (DNP) derivative of 4(*R*)-*trans*-hydroxy-L-proline. The best conversions were obtained with *A. caviae*. To this date no prolyl

hydroxylase nor its gene was described for *Aeromonas* sp. However, it was not possible to optimize the productivity or to develop large scale fermentation due to the poor growth of *A. caviae*, the lack of data concerning the expression and the regulation of proline hydroxylase gene. To optimize the enzyme production, our efforts are oriented towards genetic engineering and recombinant strain construction of a high density biomass and a tight control of gene expression.

Typical experimental procedure: PDA (potato-dextros-agar) was supplied by Difco. TES (*N-tris*[hydroxymethyl]methyl-2-aminoethane sulfonic acid) was purchased from Sigma. All chemicals and solvents were used without any further purification if not stated otherwise. NMR spectra were recorded in DMSO-d_6 solutions on a Bruecker instrument operating at 27 °C and 300 MHz for a proton NMR and 75.5 MHz for the carbon NMR. Chemical shifts (δ) are expressed in ppm relative to HMDS (proton NMR) and TMS (carbon NMR). Reactions were followed by HPLC/UV/MS on a Watres Aliance 2790 separation module equipped with 996 PDA detector and micromasse ZQ using RP18 column (100 mm, 5 μm particle size) under the following conditions: flow rate 1 ml/min; solvents: A: H_2O/0.1% HCOOH and B: acetonitrile/0.1% HCOOH. Gradient was programmed from 100% A to 80% B over 20 min. Retention times (t_r in minutes) of DNP-derivatives were: 6.51 (4-*trans*-hydroxy-L-proline), 9.29 (L-proline) and 9.33 (2,4-dinitrofluorobenzene). 2,4-Dinitrophenyl derivative of L-proline (DNP-L-Pro) was synthesized, purified and a calibration curve was obtained for a concentration range between 1 and 50 mg/l. The conversion was measured based on the transformation of the starting substrate.

General screening conditions: Microorganisms were grown on sterile PDA medium for 48–72 h. Two plugs of 5 mm diameter (pieces of medium covered with microbial culture) were cut and transferred to 96-deep well screening plates. A sterile buffer (TES, 1 ml, pH 7) was added, and thus the prepared biocatalysts were stored frozen until used. For the activity assay, biocatalysts were de-frozen by incubation at 37 °C step, followed by the addition of L-proline (10 mM), 2-ketoglutaric acid (10 mM), L-ascorbic acid (5 mM) and ferrous sulfate (1 mM). Reaction mixtures were incubated at 25 °C and 130 rpm for 24 h. Samples (50 μl) were withdrawn and following reactants were added: 0.1 ml of 2% $NaHCO_3$, 1 ml of dry ethanol and 0.3 ml of a 0.25% solution of 2,4-dinitrofluorobenzene in ethanol. The mixtures were shaken at 43 °C and 1000 rpm for 2 h. Reactions were stopped by adding 50 μl of 1 M HCl and the samples were analyzed by HPLC without further treatment.

References and notes

1. Kramli, A.; Horvath, J. *Nature London* **1949**, *163*, 219.
2. Hechter, O.; Jacobsen, R. P.; Jeanloz, R.; Levy, H.; Marshall, C. W.; Pincus, G.; Schenker, V. *J. Am. Chem. Soc.* **1949**, *71*, 3261–3262.
3. Peterson, D. H.; Murray, H. C. *J. Am. Chem. Soc.* **1952**, *74*, 1871–1872.
4. Schull, G. M.; Kita, D. A. *J. Am. Chem. Soc.* **1955**, *77*, 763–764.
5. Groves, J. T.; Subramanian, D. V. *J. Am. Chem. Soc.* **1984**, *106*, 2177–2181.
6. Ziffer, H.; Gibson, D. T. *Tetrahedron Lett.* **1975**, *16*, 2137–2138.
7. Holland, H. L.; Bergen, E. J.; Chenchaiah, P. C.; Khan, S. H.; Munoz, B.; Ninniss, R. W.; Richards, D. *Can. J. Chem.* **1987**, *65*, 502–507.
8. Mori, H.; Shibasaki, T.; Uozaki, Y.; Ochiai, K.; Ozaki, A. *Appl. Environ. Microbiol.* **1996**, *62*, 1903–1907.
9. Mori, H.; Shibasaki, T.; Yano, K.; Ozaki, A. *J. Bacteriol.* **1997**, *179*, 5677–5683.
10. Onishi, M.; Okamura, Y.; Okamoto, R.; Ishikura, T. *Biochem. Biophys. Res. Commun.* **1984**, *120*, 45–51.
11. Katz, E.; Kamal, F.; Mason, K. *J. Biol. Chem.* **1979**, *254*, 6684–6690.
12. US 6413748 B1, 2002, Kyowa Hakko Kogyo Co. JP.
13. EP 0641862 A2, 2001, Kyowa Hakko Kogyo Co. JP.
14. (a) Shibasaki, T.; Mori, H.; Chiba, S.; Ozaki, A. *Appl. Environ. Microbiol.* **1999**, *65*, 4028–4031; (b) EP 0826773A2, 1998, Kyowa Hakko Kogyo Co. JP.
15. CA 2131776, 1995, Kyowa Hakko Kogyo Co. JP.
16. Shibasaki, T.; Sakurai, W.; Hasegawa, A.; Uosaki, Y.; Mori, H.; Yoshida, M.; Ozaki, A. *Tetrahedron Lett.* **1999**, *40*, 5227–5230.
17. Clifton, I. J.; Hsueh, L.-C.; Baldwin, J. E.; Harlos, K.; Schofield, C. J. *Eur. J. Biochem.* **2001**, *268*, 6625–6636.
18. Petersen, L.; Olewinski, R.; Salmon, P.; Connors, N. *Appl. Microbiol. Biotechnol.* **2003**, *62*, 263–267.