BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Ectoine hydroxylase displays selective *trans*-3-hydroxylation activity towards L-proline

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Abstract

L-Hydroxyproline (Hyp) is a valuable intermediate for the synthesis of pharmaceuticals; consequently, a practical process for its production has been in high demand. To date, industrial processes have been developed by using L-Pro hydroxylases. However, a process for the synthesis of *trans*-3-Hyp has not yet been established, because of the lack of highly selective enzymes that can convert L-Pro to *trans*-3-Hyp. The present study was designed to develop a biocatalytic *trans*-3-Hyp production process. We speculated that ectoine hydroxylase (EctD), which is involved in the hydroxylation of the known compatible solute ectoine, may possess the ability to hydroxylate L-Pro, since the structures of ectoine and 5-hydroxyectoine resemble those of L-Pro and *trans*-3-Hyp, respectively. Consequently, we discovered that ectoine hydroxylases from *Halomonas elongata*, as well as some actinobacteria, catalyzed L-Pro hydroxylation to form *trans*-3-Hyp. Of these, ectoine hydroxylase from *Streptomyces cattleya* also utilized 3,4-dehydro-L-Pro, 2-methyl-L-Pro, and L-pipecolic acid as substrates. In the whole-cell bioconversion of L-Pro into *trans*-3-Hyp using *Escherichia coli* expressing the *ectD* gene from *S. cattleya*, only 12.4 mM *trans*-3-Hyp was produced from 30 mM L-Pro, suggesting a rapid depletion of 2-oxoglutarate, an essential component of enzyme activity as a cosubstrate, in the host. Therefore, the endogenous 2-oxoglutarate dehydrogenase gene was deleted. Using this deletion mutant as the host, *trans*-3-Hyp production was enhanced up to 26.8 mM from 30 mM L-Pro, with minimal loss of 2-oxoglutarate. This finding is not only beneficial for *trans*-3-Hyp production, but also for other *E. coli* bioconversion processes involving 2-oxoglutarate-utilizing enzymes.

Keywords *trans*-3-Hydroxy-L-proline · Hydroxyproline · Ectoine hydroxylase · 2-Oxoglutarate-dependent dioxygenase · Hydroxylation

Introduction

Owing to the growing importance of hydroxyproline (Hyp) as an ingredient in a wide range of pharmaceuticals, Pro hydroxylases capable of regio- and stereo-selective hydroxylation of the free-form L-Pro have been found in diverse microorganisms through extensive enzyme screening studies (Fig. 1). For example, *trans*-4-Hyp is a useful compound for the synthesis of carbapenem antibiotics and angiotensin-converting enzyme inhibitors (Remuzon 1996). *trans*-3-Hyp is also included as a constituent of microbial secondary metabolites (Hashizume et al. 2004; Schwartz et al. 1992; Sheehan et al. 1968; Suksamrarn et al. 2005). In naturally occurring Hyp isomers, the respective microbial L-Pro hydroxylases generate three Hyps (*trans*-4-Hyp, *cis*-4-Hyp, and *cis*-3-Hyp) from L-Pro in a regio- and stereo-selective manner (Hara and Kino 2009; Mori et al. 1997; Shibasaki et al. 1999a). However, finding an only *trans*-3-Hyp-forming Pro hydroxylase is considered to be the final research challenge, with both scientific significance and industrial application. *trans*-3-Hyp has attracted much attention in the field of synthetic chemistry as it serves as the scaffold for diverse pharmaceuticals, including peptide antibiotics (Davies et al. 2018; Kumar and Chandrasekhar 2012). Peterson et al. demonstrated slight L-Pro *trans*-3-


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Fig. 1 Overview of diverse proline-related hydroxylases. **a** Simultaneous formation of *cis*-4-Hyp (major product) and *cis*-3-Hyp (minor product). **b** Simultaneous formation of *trans*-4-Hyp (major product) and *trans*-**3-Hyp (minor product)**
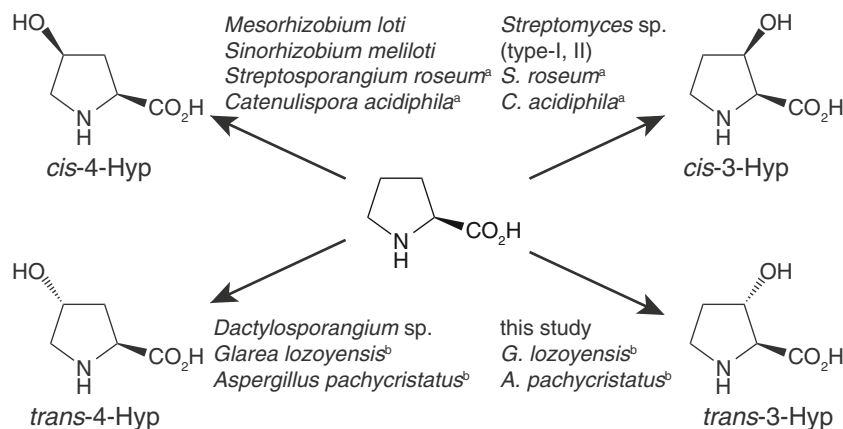
hydroxylation activity in a fungal cell extract of *Glarea lozoyensis*, which is a *trans*-3-Hyp-containing pneumocandin B$_0$ producer (Petersen et al. 2003), suggesting the involvement of a corresponding hydroxylase that catalyzed *trans*-3-Hyp formation. Recent genome sequence analysis of this fungus elucidated the pneumocandin biosynthetic gene cluster (Chen et al. 2013), and the *trans*-3-Hyp-generating hydroxylase (GloF) was found in this region. However, this enzyme enabled the simultaneous formation of both *trans*-4-Hyp and *trans*-3-Hyp, with the preferential formation of *trans*-4-Hyp with a molar yield of 8:1 (Houwaart et al. 2014). Recently, the orthologous enzyme, HtyE obtained from *Aspergillus pachycristatus*, has been reported; however, synthesis of *trans*-4-Hyp was still greater than *trans*-3-Hyp, with a molar yield of 2:1 (Mattay et al. 2018). This property seems unfavorable for yielding only *trans*-3-Hyp because chromatographic separations, at the very least, would likely be essential. Therefore, finding a bona fide enzyme for *trans*-3-Hyp synthesis remains a highly important target, as *trans*-3-hydroxylating enzymes would be useful to establish the enzymatic manufacturing of all known Hyp species.

We recently proposed a unique three-step reaction strategy for the selective synthesis of *trans*-3-Hyp (Hara et al. 2016). In brief, L-arginine was hydroxylated by L-arginine 3-hydroxylase (Yin and Zabriskie 2004) to form (2*S*,3*S*)-3-hydroxyarginine. The product was then converted to (2*S*,3*S*)-3-hydroxyorinithine by arginase and further converted to *trans*-3-Hyp by ornithine cyclodeaminase. This synthetic route is significantly shorter and more convenient than chemical synthesis processes that require (de)protection, harsh conditions, and complex purification. Indeed, the route may be useful for the selective synthesis of *trans*-3-Hyp; however, the final enzyme in the pathway, ornithine cyclodeaminase, showed relatively low activity toward (2*S*,3*S*)-3-hydroxyornithine compared with the other enzymes and resulted in a reduced yield. Thus, a more efficient and straightforward approach capable of *trans*-3-Hyp generation, such as

a one-step hydroxylation, is needed for practical *trans*-3-Hyp production.

Recent genomic analysis revealed that diverse halophilic or non-halophilic bacteria have the potential to synthesize 5-hydroxyectoine from ectoine (Czech et al. 2018); this biosynthetic route has been well investigated in *Salibacillus salexigens* (Bursy et al. 2007), *Pseudomonas stuzeri* (Seip et al. 2011), *Streptomyces* species (Bursy et al. 2008; Ikeda et al. 2014; Inbar and Lapidot 1988; Malin and Lapidot 1996; Prabhu et al. 2004), and *Sphingopyxis alaskensis* (Widderich et al. 2014). Of these, the X-ray crystallographic structures of EctD from *Virgibacillus salexigens* (Reuter et al. 2010) and *S. alaskensis* (Hoeppner et al. 2014) were solved. In general, ectoine and 5-hydroxyectoine are known as compatible solutes that serve as osmoprotectants and help organisms to survive under hyperosmotic environments, similar to glycine betaine, L-Pro, and trehalose (Pastor et al. 2010). Thus, these substances are so-called "chemical chaperones" that maintain proteins and cells against exogenous stresses, such as heat and osmotic stress. Owing to increased use of ectoine and hydroxyectoine in the healthcare and cosmetic industries (Lentzen and Schwarz 2006), there have been a number of reports on the functional analysis of hydroxyectoine biosynthesis, as well as applied studies of the use of hydroxyectoine in industrial processes (Kunte et al. 2014; Lentzen and Schwarz 2006). However, few studies have performed an in-depth investigation of the substrate range of EctD. The present study focused on the close relationship between the chemical structures of ectoine and L-Pro and their hydroxylated products 5-hydroxyectoine and *trans*-3-Hyp, especially in terms of the stereo configuration between the carboxy- and hydroxy-groups as *trans*-isomers. We thus hypothesized that EctD would accept L-Pro as a substrate, as well as ectoine, and that the hydroxylated product of L-Pro catalyzed by EctD would be *trans*-3-Hyp in this reaction scheme (Fig. 2).

This paper demonstrates that EctD hydroxylates L-Pro and the well-known EctD from *Halomonas elongata* was reevaluated as the standard enzyme for the formation *trans*-
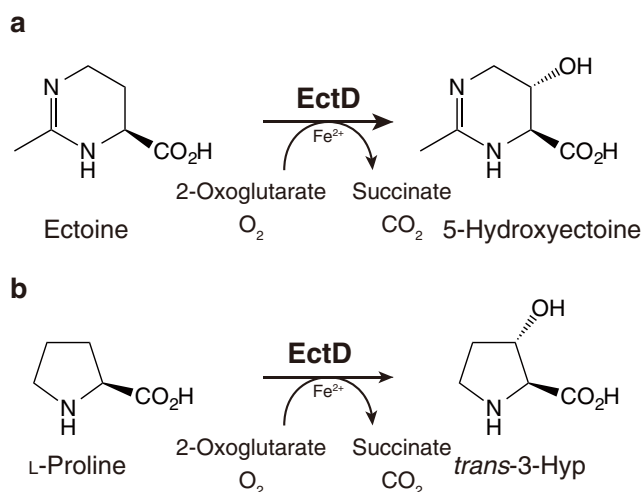
Fig. 2 The 2-OG-dependent hydroxylation reaction catalyzed by EctD. **a** Physiological reaction, **b** alternative reaction

3-Hyp. Moreover, we gained a deeper insight into several EctD enzymes from non-halophilic actinobacteria, as only limited information on the substrate specificity of EctD is available in these organisms, except for the ectoine hydrolases of *S. chrysomallus* (Prabhu et al. 2004) and *S. coelicolor* A3(2) (Bursy et al. 2008). These two enzymes were previously reported to have no substrate specificity for L-Pro. We confirmed that *trans*-3-hydroxylating activity in EctD could promote the bioproduction of *trans*-3-Hyp.

Materials and methods

Chemicals

trans-3-Hyp, ectoine, 5-hydroxyectoine, and 2-methyl-L-Pro were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were analytical grade and purchased from Wako Pure Chemical Industries (Osaka, Japan) or Tokyo Chemical Industry (Tokyo, Japan), unless otherwise mentioned.

Bacterial strains

We selected EctD from *H. elongata* as a model enzyme and other EctDs were chosen from four publicly available actinomycetes with a known genome sequence. *H. elongata* NBRC 15536[T], *Streptomyces coelicolor* A3(2) (synonym *S. violaceoruber* NBRC 15146), *S. avermitilis* NBRC 14893[T], *S. cattleya* NBRC 14057[T], and *Saccharopolyspora erythraea* NBRC 13426[T] were obtained from the Biological Resource Center, NITE (Chiba, Japan). *Escherichia coli* JM109 was purchased from Nippon Gene (Tokyo, Japan), and *E. coli* Rosetta 2(DE3) was obtained from Merck (Darmstadt, Germany).

Construction of expression plasmids

To construct the expression plasmids for *ectD* genes fused with C-terminally His6-tag in *E. coli*, the five candidate genes from *H. elongata* (HeEctD, accession number HELO_4008), *S. cattleya* (ScaEctD, accession number SCATT_10620), *S. erythraea* (SerEctD, accession number SACE_0486), *S. avermitilis* (SavEctD, accession number SAV_6395), and *S. coelicolor* A3(2) (ScoEctD, accession number SCO1867) were amplified by polymerase chain reaction (PCR) from chromosomal DNA with the KOD-plus DNA polymerase (Toyobo, Osaka, Japan) for *H. elongata* or GC-rich PCR system (Roche, Basel, Switzerland), for other actinomycete strains, using the oligonucleotide primers listed in Table 1. PCR was conducted on an iCycler (Bio-Rad Laboratories, Hercules, CA, USA) using the following program: 94 °C for 2 min, and 25 cycles each of 94 °C for 15 s, 50 °C for 30 s, and 68 °C for 1 min. Subsequently, the amplified DNA was digested with *Nde*I and *Xho*I for ScaEctD, SerEctD, SavEctD, and ScoEctD. *Nde*I with *Hin*dIII for HeEctD were inserted into the same restriction sites of pET-21a(+) plasmid (Merck). The amplified *ScaEctD* gene, using the primer set listed in Table 2, was introduced directionally into the pQE-30 plasmid (Qiagen, Hilden, Germany) between the *Bam*HI and *Hin*dIII sites. The resulting plasmids were introduced into *E. coli* JM109 and amplified; the structure was confirmed by restriction digestion and DNA sequencing.

Gene expression in *E. coli* and enzyme purification

To overexpress the *ectD* gene, *E. coli* Rosetta 2(DE3) harboring an expression vector was grown on 5-mL Luria-Bertani medium (Green and Sambrook 2012) containing 50 μg mL^{-1} ampicillin and 30 μg mL^{-1} chloramphenicol at 37 °C for 8 h at 280 rpm. Subsequently, 1 mL of the cell culture was transferred into 100 mL of the same medium, and cells were cultivated until the OD$_{600}$ value reached approximately 0.5. Subsequently, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM. The incubation was continued for another 8 h at 25 °C; after this, the cells were collected by centrifugation at 5000×*g* for 10 min at 4 °C. The cell pellet was stored at −80 °C until use for enzyme purification. The enzyme purification procedure was carried out as described previously (Hara et al. 2015).

Enzyme assay

The hydroxylation activities of ectoine and L-Pro with purified EctD were assayed by determining the conversions of ectoine to 5-hydroxyectoine and L-Pro to *trans*-3-Hyp by using high-performance liquid chromatography (HPLC) in comparison with authentic standards. To test whether the enzymes accepted ectoine and L-Pro as substrates, the hydroxylation reaction

Table 1 Oligonucleotide primers used in this study

Description	Sequence (5′ to 3′)	Restriction site
Cloning into pET-21a(+)[a]		
HeEctD_forward	ATCTT*CATATG*TCAGTGCAGACATCGTCCAACCG	*Nde*I
HeEctD_reverse	ATTCG*AAGCTT*GCCATCCGGCGACCACGCCTC	*Hin*dIII
ScoEctD_forward	ATCTT*CATATG*GCCACCCCTCGGCAGGAC	*Nde*I
ScoEctD_reverse	ATTCG*CTCGAG*CTTCACCGGCGTGAAGTCCCG	*Xho*I
SavEctD_forward	ATCTT*CATATG*ACCACGATCACCGACCTGTACCC	*Nde*I
SavEctD_reverse	ATTCG*CTCGAG*CCGCACCGGCGTGAAGTCCCGC	*Xho*I
SerEctD_forward	ATCTT*CATATG*ACCGTCGCCGATCTGAGTACTC	*Nde*I
SerEctD_reverse	ATTCG*CTCGAG*GCGGCGGACCGGAGTGAAGTCG	*Xho*I
ScaEctD_forward 1	ATCTT*CATATG*ACCACCGCCCCCGAGCGC	*Nde*I
ScaEctD_reverse 1	ATTCG*CTCGAG*GCGCACCGGGGTGAAGTCCCGC	*Xho*I
Cloning into pQE-30[a]		
ScaEctD_forward 2	AGATA*GGATCC*ATGACCACCGCCCCCGAGCGC	*Bam*HI
ScaEctD_reverse 2	ATTAG*AAGCTT*ATTAGCGCACCGGGGTGAAGTCCCGC	*Hin*dIII
Gene disruption[b]		
sucAB deletion_forward	<u>GGTAGTATCCACGGCGAAGTAAGCATAAAAAAGATGCTTAAGGGATCACG</u>ATTAACCCTCACTAAAGGGCG	
sucAB deletion_reverse	<u>GAAGGCGATAATGCCTTATCCGGTCTACAGTGCAGGTGAAACTTAAACTA</u>TAATACGACTCACTATAGGGCTC	

[a] Italics indicate digestion sites by restriction enzymes

[b] Underlines indicate the flanking region of chromosomal *sucAB* gene

was conducted as follows: a 200-μL reaction mixture containing 100 mM potassium phosphate buffer (pH 7.5), 5 mM ectoine or L-Pro, 10 mM 2-OG, 1 mM L-ascorbate, 0.5 mM FeSO$_4$, and 20 μg of each purified enzyme was incubated at 30 °C for various intervals of up to 1 h. The reaction was terminated by heat denaturation (80 °C, 10 min), immediately followed by centrifugation to assay the supernatant.

The effects of pH and temperature on enzyme activity were determined by ectoine hydroxylation reaction under the above conditions with the exception of various pH values (5–10) and temperatures (10–60 °C).

To determine the effect of chemicals on enzyme activity, ScaEctD was used as a representative example, and 2 mM chelating reagents (EDTA and 1,10-phenanthroline), 2 mM metal ions (MgSO$_4$, MnCl$_2$, FeCl$_3$, CoCl$_2$, ZnSO$_4$, CuSO$_4$, and NiSO$_4$), or 100 mM salts (NaCl and KCl) were added individually to the ectoine hydroxylation reaction mixture, as described above.

The steady-state kinetic analysis of EctD for ectoine and L-Pro was determined by varying concentrations of ectoine and L-Pro between 0 and 50 mM. Other conditions were the same as described above.

Preparation of *E. coli* cells for *trans*-3-Hyp production

E. coli endogenous *sucAB* gene encoding enzymes that generate succinyl-CoA from 2-oxoglutate were disrupted by the Quick and Easy *E. coli* Gene Deletion Kit (Gene Bridges, Heidelberg, Germany) as per the manufacturer's instructions. The deletion cassette was constructed by PCR amplification using FRT-PGK-gb2-neo-FRT as the template DNA with the primer pair listed in Table 1.

E. coli harboring ScaEctD gene on pQE-30 plasmid was inoculated into 5 mL of LB medium, including 50 μg mL^{-1} ampicillin, and incubated aerobically at 37 °C for 16 h at 280 rpm. This culture was transferred into 1 L of fresh terrific

Table 2 Kinetic parameters of hydroxylases for ectoine and l-Pro hydroxylation

Enzyme	Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
ScaEctD	Ectoine	1.7 ± 0.1	$5.5 \times 10^{-1} \pm 0.1 \times 10^{-1}$	3.3×10^{-1}
	L-Pro	17 ± 2.2	$1.1 \times 10^{-1} \pm 0.1 \times 10^{-1}$	6.8×10^{-3}
HeEctD	Ectoine	$5.6 \times 10^{-1} \pm 0.3 \times 10^{-1}$	$2.2 \times 10^{-1} \pm 0.03 \times 10^{-1}$	3.9×10^{-1}
	L-Pro	5.5 ± 0.6	$2.9 \times 10^{-2} \pm 0.1 \times 10^{-2}$	5.3×10^{-3}

broth (Green and Sambrook 2012) containing 50 μg mL^{-1} ampicillin and cultivated using a model MDL-200 jar fermentor (B.E. MARUBISHI, Tokyo, Japan). The following parameters were used: temperature, 37 °C; agitation, 600 rpm; and air flow rate, 1 L min^{-1}. When the OD_{600} reached approximately 4, 1 mM IPTG was added to the medium, and the temperature was lowered to 25 °C. After cultivation for 12 h, the cells were harvested by centrifugation at 12,000×g for 5 min at 4 °C. The collected cells were stored frozen at −80 °C until use.

For bioconversion of L-Pro, the thawed cells, with an OD_{600}: 150 were suspended in the reaction mixture containing 30 mM L-Pro, 30 or 60 mM 2-OG, 10 mM L-ascorbate, and 5 mM $FeSO_4$ in a total volume of 50 mL. The reaction was conducted in the enzyme catalysis device model BME (ABLE Corporation, Tokyo, Japan) at the following conditions: temperature, 20 °C; agitation, 1500 rpm; air flow rate, 200 mL min^{-1}; pH, 7.0 adjusted by 1 M HCl. A small portion of the mixture was withdrawn at each time interval, and concentrations of amino acids and organic acids in the supernatant after centrifugation were determined by HPLC.

HPLC and MS analyses

L-Pro and Hyp were determined as described previously (Hara et al. 2016). Ectoine and 5-hydroxyectoine were determined by the Chromaster HPLC system (Hitachi High-Technologies, Tokyo, Japan) equipped with an Inertsil ODS-3 column (4.6 × 150 mm; GL Science, Tokyo, Japan) kept at 40 °C using a mobile phase consisting of 0.1% (v/v) perchloric acid, at a flow rate of 0.5 mL min^{-1} flow, and a detection wavelength of 210 nm. 2-OG and succinate were determined by the same system equipped with an Ultron PS-80H column (Shinwa Chemical Industries, Kyoto, Japan) kept at 60 °C using mobile phase consisting of 0.1% (v/v) perchloric acid at the 1 mL min^{-1} flow rate and 210 nm detection wavelength. FDAA-derivatized amino acids were detected by ESI-MS analysis in positive mode using an LCQ-Fleet system (Thermo Fisher Scientific, Waltham, MA, USA).

Results

Gene expression and purification of EctDs

The five candidate genes encoding HeEctD, ScaEctD, SerEctD, SavEctD, and ScoEctD were amplified by PCR and inserted into a pET-21a(+) vector at each compatible site between *Nde*I with *Xho*I or *Hin*dIII under the T7 promoter. The resulting plasmids were introduced into *E. coli* Rosetta 2(DE3). After IPTG-induced gene expression, SDS-PAGE analysis revealed that HeEctD, ScaEctD, SerEctD, and SavEctD were expressed in soluble forms as carboxy-

terminal His$_6$-tagged fusion proteins. These proteins were purified to homogeneity by Ni^{2+} affinity chromatography followed by gel filtration chromatography (Fig. 3). The molecular masses of the purified enzymes estimated by SDS-PAGE analysis were nearly identical to those of the calculated values, including His$_6$-tag. However, ScoEctD was not entirely expressed in *E. coli* as either soluble or insoluble forms.

Substrate specificity of EctDs

The substrate specificity of EctDs, except for ScoEctD, was assessed using the purified enzymes. When the physiological substrate ectoine was subjected to the hydroxylation reaction, all enzymes showed ectoine hydroxylation activity. The formation of 5-hydroxyectoine was clearly confirmed by HPLC analysis (Fig. 4a), and the reactions were linear up to 1 h. Subsequently, an enzymatic L-Pro hydroxylation reaction was conducted under the same conditions, and a dose-dependent decrease in L-Pro corresponding with an increase of an unidentified product was clearly confirmed in HeEctD and ScaEctD. According to chiral HPLC analysis, the retention time of the enzymatically produced substance was entirely identical to that of the authentic standard of *trans*-3-Hyp, and no other Hyp isomers were detected (Fig. 4b). Furthermore, we isolated the product and confirmed the molecular mass by electrospray ionization-mass spectrometry (ESI-MS) analysis, and the corresponding protonated ion of FDAA-derivatized *trans*-3-Hyp was detected (observed, 383.79; calculated, 384.11). Slight L-Pro hydroxylation activity was also detected under the standard conditions using SerEctD and SavEctD, whereas they had sufficient capacity for ectoine hydroxylation.

To investigate the mechanism of L-Pro acceptance in EctD, molecular simulation was performed to predict the potential interactions between EctD and *trans*-3-Hyp (Fig. S1). The docking model showed that the hydroxy group on the C-3
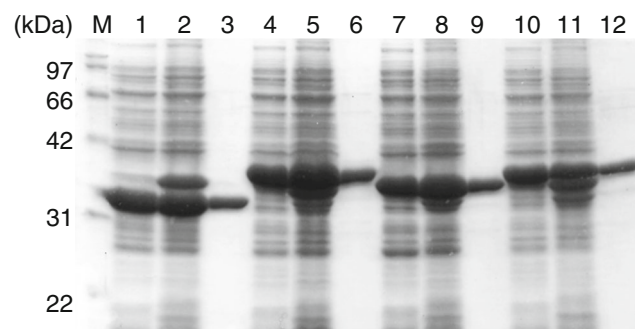

Fig. 3 SDS-PAGE analysis of recombinant ectoine hydroxylases. Lane M, molecular standard marker; lanes 1–3, ScaEctD; lanes 4–6, SavEctD; lanes 7–9, SerEctD; lanes 10–12, HeEctD. Lanes 1, 4, 7, and 10, soluble proteins of cell extracts after centrifugation; lanes 2, 5, 8, and 11, insoluble proteins of disrupted cells; lanes 3, 6, 9, and 12, purified enzymes

Fig. 4 Identification of hydroxylated products by HPLC analysis. Arrows indicate the positions where authentic compounds eluted. **a** Ectoine hydroxylation, **b** l-Pro hydroxylation
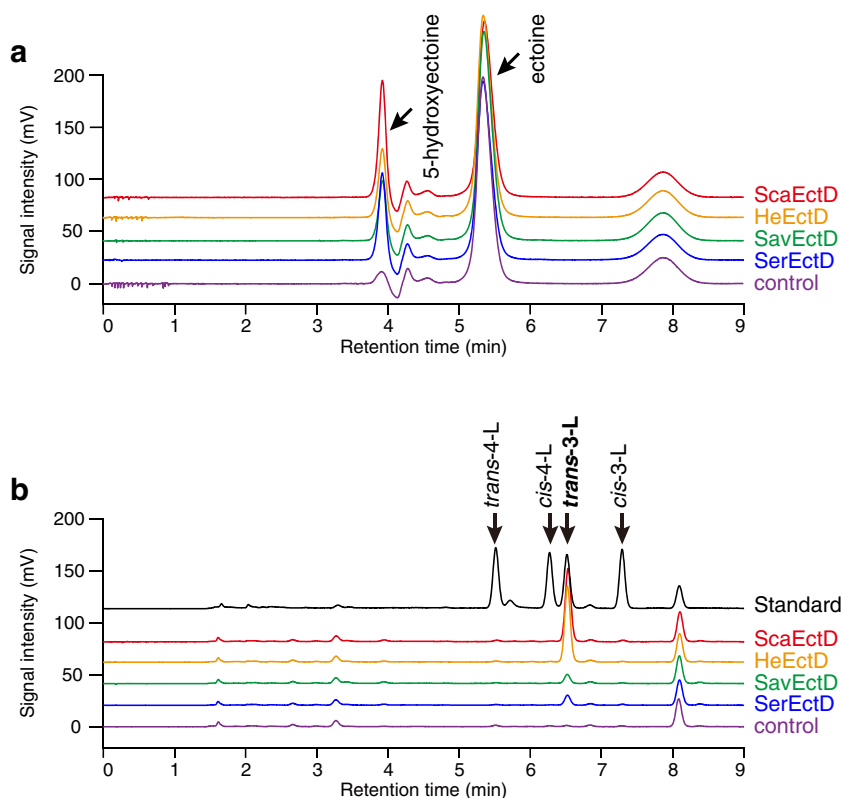
carbon of *trans*-3-Hyp was apparently adjacent to the Fe^{3+} atom at a distance of 2.81 Å, allowing the coordination with other catalytic residues (H150, D152, and H251) and cosubstrate 2-OG.

In addition, other structurally relevant amino acids were tested using ScaEctD as a model, owing to its high catalytic activity. HPLC and ESI-MS analyses revealed that 3,4-dehydro-L-Pro, 2-methyl-L-Pro, and L-pipecolic acid were converted to putative oxygenated products (Fig. S2). The reaction product from 3,4-dehydro-L-Pro was confirmed by the comparison of authentic *cis*- and *trans*-3,4-epoxy-L-Pro synthesized in accordance with previous reports (Hara et al. 2014; Shibasaki et al. 1999b). Although it was difficult to isolate the products owing to extremely low yield, the protonated molecular masses of the FDAA-derivatized reaction products from 2-methyl-L-Pro and L-pipecolic acid were 398.13 and 398.12, respectively, which were greater than each substrate by 16, indicating the occurrence of ScaEctD-mediated hydroxylation.

Catalytic efficiency on ectoine and L-Pro

HeEctD and ScaEctD showed prominent hydroxylation activity toward L-Pro as well as ectoine (Fig. 4); thus, we performed further kinetic analysis for each substrate to obtain insights into their catalytic properties. The velocity was plotted against the substrate concentrations, and they were fitted to the Michaelis-Menten model to determine K_m and k_{cat} values (Fig. 5). As shown in Table 2, K_m value of ScaEctD for ectoine was three
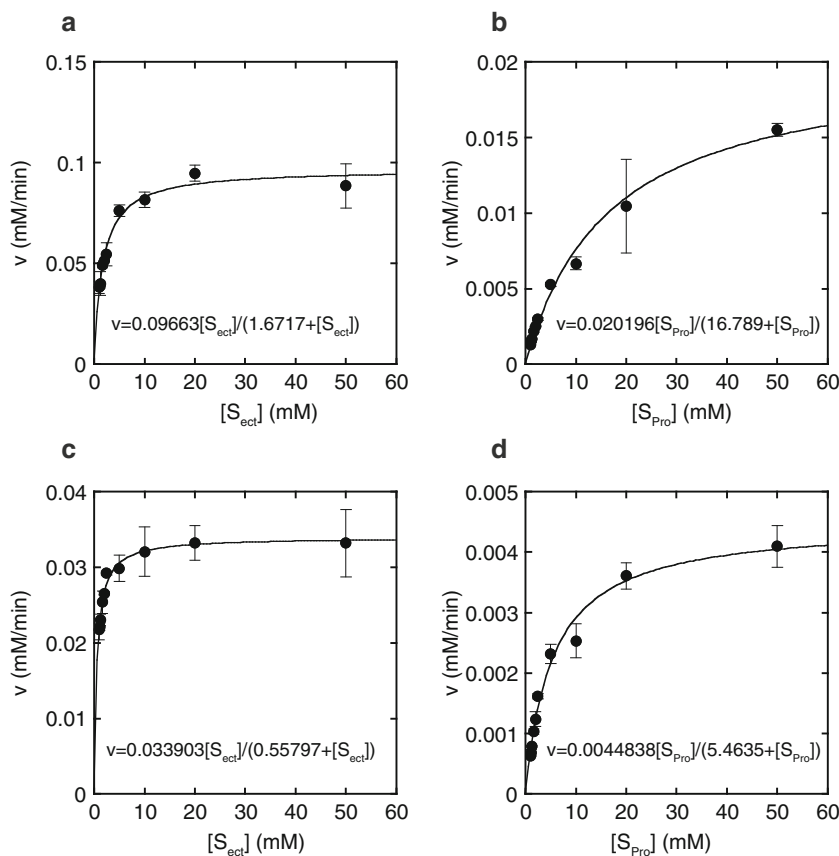
times higher than that of HeEctD, and the k_{cat} value was 2.5 times higher in ScaEctD than in HeEctD. In case of L-Pro, however, K_m values in both enzymes were 10 times higher than that for ectoine. k_{cat} values for L-Pro decreased in both enzymes when compared with those for ectoine. In addition, the comparison of catalytic efficiency (k_{cat}/K_m) demonstrated that HeEctD resulted in a slightly faster ectoine hydroxylation activity than ScaEctD, whereas ScaEctD exhibited slightly higher activity than HeEctD in L-Pro hydroxylation.

Effects of temperature, pH, and ligands

We determined the optimum reaction temperature, pH, metal ions, and salts of this enzyme. The highest activity was obtained at 30 °C, which significantly decreased at temperatures greater than 40 °C. The optimum pH was approximately neutral; the most suitable value was 7.5.

More than a 50% decrease in activity was observed in the presence of divalent metal ions, such as Mn^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, and Ni^{2+}, and the activity was entirely abolished by the addition of EDTA or 1,10-phenanthroline (Fig. S3). Several reported EctDs were affected by salts, such as KCl, NaCl, and potassium glutamate. In particular, KCl showed an activation effect (Widderich et al. 2014). Thus, we investigated the effect of KCl and NaCl. However, unlike the reported EctDs, salts did not facilitate enzyme activity in ScaEctD under the conditions used in this study (Fig. S3).

Fig. 5 Michaelis–Menten plot for the hydroxylation of ectoine and L-Pro. **a** Ectoine hydroxylation by ScaEctD; **b** L-Pro hydroxylation by ScaEctD; **c** ectoine hydroxylation by HeEctD; **d** L-Pro hydroxylation by HeEctD

$$v = 0.09663[S_{ect}]/(1.6717+[S_{ect}])$$

$$v = 0.020196[S_{Pro}]/(16.789+[S_{Pro}])$$

$$v = 0.033903[S_{ect}]/(0.55797+[S_{ect}])$$

$$v = 0.0044838[S_{Pro}]/(5.4635+[S_{Pro}])$$

Production of *trans*-3-Hyp using *ectD*-expressing whole-cell *E. coli*

As mentioned above, we identified EctD exhibiting L-Pro hydroxylation activity to form *trans*-3-Hyp in the purified enzyme reaction. To confirm the feasibility of the whole-cell biocatalyst, we investigated *trans*-3-Hyp production using *E. coli* expressing the *ScaEctD* gene as a whole-cell biocatalyst. Jar-fermentor-cultivated *E. coli* was freeze-thawed to improve substrate permeability, and the bioconversion of L-Pro into *trans*-3-Hyp was performed in a 200-mL batch reactor with a 50-mL working volume. Considering 2-OG degradation by the *E. coli* endogenous enzyme, a two-fold higher concentration of 2-OG over L-Pro was utilized in the reaction mixture.

In our preliminary experiments, low reaction temperatures were favorable for long-term *trans*-3-Hyp production rather than optimum conditions, probably owing to time-dependent enzyme inactivation. Subsequently, the bioconversion was terminated within 1 h; only 12.4 mM *trans*-3-Hyp was produced with a conversion of 41% from 30 mM L-Pro (Fig. 6a). This result was probably caused by the depletion of 2-OG, suggesting a rate-limiting factor for sufficient *trans*-3-Hyp productivity.

To overcome the limitation in the process, we searched for the enzyme involved in 2-OG degradation in *E. coli* based on

the genome sequence. As such, SucAB, which encodes the 2-OG dehydrogenase complex, appeared to serve as a major 2-OG degradation enzyme and a member of the tricarboxylic acid cycle. Indeed, we confirmed the loss of 2-OG degradation activity in the absence of L-Pro (Fig. S4). Thus, using SucAB-deficient *E. coli* instead of wild-type JM109, we repeated the same bioconversion of L-Pro. As expected, *trans*-3-Hyp was successfully produced at up to 26.8 mM with a conversion of 89%, and the consumed 2-OG was comparable with the produced *trans*-3-Hyp (Fig. 6b). Finally, when an equal concentration of 2-OG and L-Pro was supplied in the bioconversion, 25.1 mM *trans*-3-Hyp with a conversion of 84% was yielded from 30 mM L-Pro (Fig. 6c).

Discussion

Regio- and stereo-selective synthesis of *trans*-3-Hyp is the last remaining challenge in the synthesis of naturally occurring L-Hyps. In the present study, to achieve a practical process for *trans*-3-Hyp production, we screened for enzymes catalyzing selective hydroxylation at the C3 position of L-Pro. EctD catalyzes the conversion of ectoine into 5-hydroxyectoine for osmotolerance. Although the substrate specificity of the enzyme was highly limited to ectoine in previous reports, we discovered L-Pro as a new substrate for EctD. The reaction
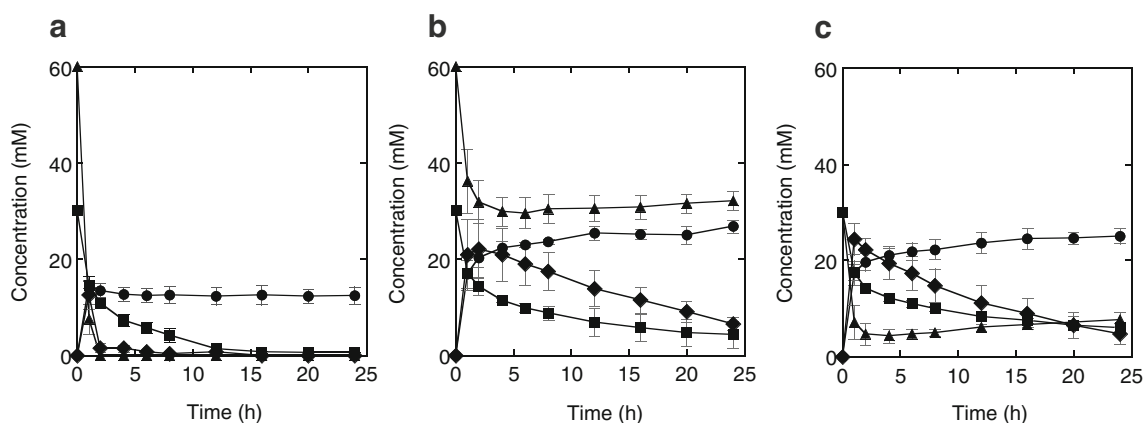
Fig. 6 Bioconversion of ʟ-Pro into *trans*-3-Hyp using whole cells of ScaEctD-expressing *E. coli*. Circles, squares, triangles, and diamonds indicate *trans*-3-Hyp, ʟ-Pro, 2-OG, and succinate, respectively. **a** 60 mM 2-OG in wild-type strain; **b** 60 mM 2-OG in *sucAB* disruptant; **c** 30 mM 2-OG in *sucAB* disruptant. The data represent the mean of three independent experiments, and error bars show the standard deviations

product was identified as *trans*-3-Hyp. We also accomplished the bioconversion of ʟ-Pro into *trans*-3-Hyp using *E. coli* expressing the ectoine hydroxylase gene. These findings will create a new approach for practical *trans*-3-Hyp production, which is involved in the enzymatic production of all naturally occurring ʟ-Hyps.

To date, the following two studies have reported the substrate specificity of EctD. One study was conducted on EctD obtained from *Salibacillus salexigens* (Bursy et al. 2007). The native EctD of this strain was purified to homogeneity, and the substrate specificity was investigated in detail. Although this enzyme had comparative capacity to hydroxylate ectoine, no ʟ-Pro hydroxylation activity was detected. Moreover, the EctD did not act on ectoine analogs, not even the seven-membered ring analog (4*S*)-2-methyl-4,5,6,7-tetrahydro-1*H*-1,3-diazepine-4-carboxylic acid (homoectoine) or five-membered ring analog 4,5-dihydro-2-methylimidazole-4-carboxylic acid (DHMICA). The other investigation was also reported by the same study group. The native EctD from *S. coelicolor* A3(2) was purified to homogeneity, and the substrate specificity was assessed (Bursy et al. 2008). Similar to the other enzyme, this EctD was able to hydroxylate ectoine, but it did not act on ʟ-Pro. These reports suggested that the substrate specificities of EctDs were significantly narrow and strict. Generally, the known EctDs are highly specific to ectoine, and no such EctDs capable of hydroxylating ʟ-Pro have been reported. Thus, our results demonstrate a novel substrate specificity of EctD.

For the application to *trans*-3-Hyp bioproduction, we carried out bioconversion using whole-cell *E. coli* expressing ScaEctD. However, *trans*-3-Hyp accumulation was not sufficient owing to the lack of 2-OG (Fig. 6a). We proposed an improved strategy using gene disruption of the endogenous SucAB (Fig. 6b, c). It would be generally advantageous to the 2-OG-dependent bioconversion using a whole-cell biocatalyst, especially for high cell densities. ʟ-Pro was markedly

degraded only in wild-type strain. This was also affected by the depletion of 2-OG, resulting in degradation of residual substrate by proline dehydrogenase (PutA, EC 1.5.5.2). When sufficient 2-OG existed in the reaction mixture, ʟ-Pro hydroxylation occurred prior to degradation. In contrast, synthesized *trans*-3-Hyp did not decrease in all cases. This was attributed to the strict specificity of proline dehydrogenase (Ostrander et al. 2009). The productivity of *trans*-3-Hyp was still lower than industrial *trans*-4-Hyp production, probably due to low catalytic efficiency. ʟ-Pro may be an inappropriate substrate for EctD, because this enzyme essentially prefers ectoine to ʟ-Pro, as revealed by kinetic analysis. Although this should be overcome by enzyme engineering for better product yield in future industrial applications, our findings still elucidate the mechanisms of *trans*-3-selective hydroxylation of ʟ-Pro.

In conclusion, we demonstrated that HeEctD has the ability to hydroxylate ʟ-Pro as well as ectoine, and EctDs from actinomycetes exhibited the same characteristics to varying degrees. Among the EctDs, ScaEctD showed the highest ʟ-Pro hydroxylation activity. This enzyme was applicable to *trans*-3-Hyp production by whole-cell bioconversion with genetically modified *E. coli*. Hence, this process has great potential for use in industrial production of *trans*-3-Hyp.

Compliance with ethical standards

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