Rational Design of the Soluble Variant of L-Pipecolic Acid Hydroxylase using the α-Helix Rule and the Hydropathy Contradiction Rule

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ABSTRACT: The production of recombinant proteins in Escherichia coli is an important application of biotechnology. 2-Oxoglutarate-dependent L-pipecolic acid hydroxylase derived from Xenorhabdus doucetiae (XdPH) is an excellent biocatalyst that catalyzes the hydroxylation of L-pipecolic acid to produce cis-5-hydroxy-L-pipecolic acid. However, the enzyme tends to form aggregates in the E. coli expression system. Our group established two rules, namely, the “α-helix rule” and the “hydropathy contradiction rule,” to select residues to be altered for improving the heterologous recombinant production of proteins, by analyzing their primary structure. We rationally designed XdPH variants that are expressed in highly soluble and active forms in the E. coli expression system using these hotspot prediction methods, and the L142R variant showed a remarkably high soluble expression level compared to the wild-type XdPH. Further mutations were introduced into the L142R gene by site-directed mutagenesis. Moreover, the I28P/L142R and C76Y/L142R double variants displayed improved soluble expression levels compared to the single variants. These variants were also more thermostable than the wild-type XdPH. To analyze the effect of the alteration on one of the hotspots, L142 was replaced with various hydrophilic and positively charged residues. The remarkable increase in soluble protein expression caused by the alterations suggests that the decrease in the hydrophobicity of the protein surface and the enhancement of the interaction between nearby residues are important factors determining the solubility of the protein. Overall, this study demonstrated the effectiveness of our protocol in identifying aggregation hotspots for recombinant protein production and in basic biochemical research.

INTRODUCTION

Heterologous expression systems using Escherichia coli are widely used in basic and applied research in biochemistry. E. coli is advantageous as it is inexpensive, fast, and gives high yields of the recombinant protein. However, many limitations, such as the formation of aggregates called “inclusion bodies” caused by incorrect protein folding, are also associated with their use. Several strategies have been investigated to overcome these limitations. One of the most widely used methods is the optimization of the culture conditions of the transformant by lowering the cultivation temperature and changing the medium composition. Other strategies include codon optimization, coexpression with chaperones, and the use of promoters with different strengths to control the rate of protein synthesis. However, these strategies are time-consuming and do not always yield positive results.

We established two rules, namely, the α-helix rule and the hydropathy contradiction rule to identify residues (called aggregation hotspots) to improve solubility of the recombinant proteins by analyzing their primary structure. Replacing the amino acid residues at the hotspot with appropriate residues via directed evolution leads to more efficient protein folding, resulting in higher expression levels of the genes. To date, we have been successful in improving the production levels of the soluble forms of various proteins in E. coli by alteration of their residues using the α-helix and hydropathy contradiction rules.

In this study, we targeted 2-oxoglutarate-dependent L-pipecolic acid hydroxylase (EC1.14.11.4) derived from Xenorhabdus doucetiae (XdPH) for use as a biocatalyst for the hydroxylation of L-pipecolic acid. Hydroxy-pipecolic acids (HyPips) are naturally occurring six-membered heterocyclic hydroxy amino acids that are components of some peptide antibiotics, terpenoids, and alkaloids. HyPips have been used as chiral building blocks in the synthesis of pharmaceuticals. For example, cis-4-hydroxy-L-pipecolic acid is a component of...
palinavir, an HIV protease inhibitor, and cis-5-hydroxy-L-pipeolic acid (cis-5-HyPip) is a precursor for the synthesis of the β-lactamase inhibitor, MK-7655. In previous studies, proline hydroxylases belonging to the Fe(II)/2-oxoglutarate-dependent dioxygenase superfamily were shown to catalyze the hydroxylation of L-pipeolic acid (L-Pip). L-Proline trans-4-hydroxylase of the Dactylosporangium sp. strain RH1 converts L-Pip into trans-5-HyPip, while L-proline cis-3-hydroxylase of the Streptomyces sp. strain TH1 converts L-Pip into cis-3-HyPip. XdPH has been reported to be useful for catalyzing the production of cis-5-HyPip from L-Pip (Scheme 1).

Quantum mechanical/molecular mechanical (QM/MM) studies on the catalytic mechanism of Fe(II)/2-oxoglutarate-dependent enzymes have been reported. Various enzymes have been produced at high levels for industrial applications, using recombinant gene technology with E. coli as a host. However, the insolubility of the enzymes in E. coli has been a limiting factor in the functional analysis, protein structure analysis, and the XdPH-catalyzed synthesis of cis-5-HyPip for industrial applications.

In this study, we introduced rational alterations to XdPH using hotspot prediction methods developed in our laboratory, to improve the soluble expression of the enzyme in E. coli. The soluble expression of XdPH was achieved by introducing single or double mutations. These variants showed not only improved soluble expression but also higher thermostability. Biochemical analysis indicated that the L142R variant was highly soluble in the E. coli expression system compared to the wild-type protein. Saturation mutagenesis and bioinformatic analysis of the L142R variant suggested that the decrease in hydrophobicity of the protein surface and the enhancement of the interaction with nearby residues are important factors for the soluble expression of XdPH. This study demonstrated that the two aforementioned rules can be used to improve soluble expression of recombinant proteins in the field of biochemistry.

## RESULTS AND DISCUSSION

### Heterologous Expression and 2-Oxoglutarate-Dependent L-Pipeolic Acid Hydroxylase Activity of Wild-Type XdPH

In this study, the pET-28a (+) vector and E. coli BL21 (DE3) were used as expression systems for the XdPH gene, producing an N-terminal hexahistidine tag-fused recombinant XdPH. After gene expression using an auto-induction medium, the expression of the proteins induced by wild-type XdPH was confirmed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the enzyme activity assay described in the Experimental Section. The hydroxylation of L-Pip by the crude extract of wild-type XdPH was assayed by determining the conversion of L-Pip to cis-5-HyPip. The product was labeled with Na(5-(fluoro-2,4-dinitrophenyl)-L-leucinamide (L-FDLA) and analyzed by high-performance liquid chromatography (UPLC) (Figure S2). SDS-PAGE revealed the presence of recombinant XdPH in the insoluble fraction (Figure 1). The activity of XdPH was determined by UPLC analysis, and 1.4 mU/mg soluble protein was obtained (Figure S3). Based on these results, recombinant XdPH was considered to form inclusion bodies after the heterologous expression of the gene.

### Prediction of the Aggregation Hotspots in XdPH by the α-Helix rule and the Hydropathy Contradiction Rule

Aggregation hotspots were predicted by the α-helix rule and the hydropathy contradiction rule based on the amino acid sequence of XdPH. Hotspot residues were identified by the hydropathy contradiction rule based on the consensus design method, which improves protein function by replacing certain residues of the target protein with residues that are highly conserved in proteins of the same family. In this study, highly conserved residues were selected based on appearance rates, which were calculated for each residue through multiple sequence alignment with homologous proteins in a database. The HiSol scores were calculated for each residue of the target protein. The score was negative if the residue of the target protein was hydrophilic and the residue of the consensus protein was hydrophobic, and the score was positive if it was the other way around. Thus, the score identified residues with contradictory hydrophobicity by comparing them with the consensus amino acid residues. Using this method, appropriate amino acid residues can be altered for increasing the soluble expression of the recombinant protein. The hydropathy contradiction rule predicts aggregation hotspots according to the following three criteria:

1. High absolute value of HiSol score.
2. Highly conserved residues different from those of the target protein.
3. Alteration of the hydropathy index from a negative value to a positive value, or vice versa.

The appearance rate and HiSol score of each amino acid residue were calculated using the INTMSAlign_HiSol program, based on the amino acid sequences of XdPH and 112 other proteins obtained by a BLAST sequence similarity search (Supporting Data S1). However, hotspot residues can be identified by the α-helix rule by converting a hydrophobic amino acid present in the hydrophilic region of the α-helix in...
the protein into a hydrophilic amino acid, or by converting a hydrophilic amino acid existing in the hydrophobic region into a hydrophobic amino acid (Figure S4). Based on the aggregation hotspot prediction, 19 candidate residues were identified among a total of 294 residues (Table 1). Out of these, 14 candidate residues were identified by the hydropathy contradiction rule and five were identified by both the α-helix rule and the hydropathy contradiction rule (Figure 2). Twelve candidate residues on the α-helix and six candidate residues on the coil structures were displayed in the homology model of XdPH. The candidate residues were located on the protein surface (Figure 3). On the basis of these results, we prepared 19 variants to improve the solubility of XdPH for heterologous expression.

First Screening of the Soluble Variants of XdPH with Single Amino Acid Alterations. XdPH variants were generated using site-directed mutagenesis. The genes encoding wild-type XdPH and its variants were expressed in E. coli BL21 (DE3). Enzyme activity in the soluble fraction of the proteins was subsequently measured to compare the soluble expression of wild-type XdPH with that of its variants (Figure 4A). A crude enzyme extract was used in the activity assay. The soluble fractions of the wild-type, V31E, V62D, C76Y, and L142R variants showed activities of 1.4, 2.53, 2.51, 7.36, and 20.5 mU/mg soluble protein, respectively. Increased enzyme activity was observed in V31E, V62D, C76Y, and L142R variants. In particular, the activity of the L142R variant was more than 14.6-fold higher per unit of soluble protein relative to that of the wild-type XdPH. The soluble fractions of the proteins were analyzed by SDS-PAGE to compare the solubility of the wild-type XdPH with that of its variants (Figure 4B). Higher levels of C76Y and L142R variants were observed compared to those of the wild-type XdPH (Figure 4B).

Second Screening of the Soluble XdPH Variants with Double Alterations in Amino Acids. To further improve these, 14 candidate residues were identified by the hydropathy contradiction rule and five were identified by both the α-helix rule and the hydropathy contradiction rule (Figure 2). Twelve candidate residues on the α-helix and six candidate residues on the coil structures were displayed in the homology model of XdPH. The candidate residues were located on the protein surface (Figure 3). On the basis of these results, we prepared 19 variants to improve the solubility of XdPH for heterologous expression.

Figure 2. Helical wheel depiction of the α-helix regions in XdPH that contributed to the improvement in soluble expression. Helical wheels are depicted for the following α-helix regions of XdPH: residues 75–84 (DCINQLIRNN) (A), residues 206–218 (TSLRDSLHIAEH) (B), and residues 243–255 (REYFQLLDECFSR) (C). Hydrophobic and hydrophilic residues are shown in black and white letters, respectively. The numbers represent the amino acid residues. The alterations in the residues are indicated as asterisks (*), whose polar properties are opposite to those of the hydrophobic or hydrophilic region. The hydrophobic residues, C76 (A) and L248 (C), in XdPH were located in the hydrophilic regions of the α-helix. However, the hydrophilic residues, R209 (B) and Y245 (C), were located in the hydrophobic regions of the same secondary structure. Residues to be modified are presented according to the hydropathy contradiction rule.

Figure 3. Location of the aggregation hotspots in the homology model of XdPH. Predicted hotspots in the homology models of XdPH are indicated as cyan spheres, which are located on the surface of the protein (A). I28, V31, V62, C76, and L142 are indicated as red spheres (B).

| target residue | HiSol score | conserved residue | appearance rate (%) | position | prediction method |
|----------------|-------------|------------------|---------------------|----------|------------------|
| Glu15          | −1.103      | Ala              | 9 → 18.4            | helix    | HiSol            |
| Ile28          | 2.042       | Pro              | 1.4 → 13.6          | coil     | HiSol            |
| Val31          | 2.035       | Glu              | 3.6 → 34.5          | coil     | HiSol            |
| Val62          | 2.24        | Asp              | 0 → 29.1            | coil     | HiSol            |
| Cys76          | 1.06        | Tyr              | 0 → 54.8            | helix    | HiSol + α       |
| Leu89          | 2.021       | Arg              | 5.5 → 25.6          | helix    | HiSol            |
| Ile108         | 2.487       | Arg              | 0.2 → 64.2          | coil     | HiSol            |
| Leu142         | 2.228       | Arg              | 1.2 → 53.1          | coil     | HiSol            |
| Cys189         | 1.132       | Asp              | 0 → 11.5            | coil     | HiSol            |
| Leu208         | 1.63        | Glu              | 12.7 → 26.6         | helix    | HiSol + α       |
| Arg209         | -1.355      | Leu              | 24.8 → 18.4         | helix    | HiSol + α       |
| His218         | -1.784      | Val              | 0.5 → 24            | helix    | HiSol            |
| Arg243         | -1.205      | Gly              | 3.8 → 21.2          | helix    | HiSol            |
| Glu244         | -1.077      | Ala              | 14.8 → 19.5         | helix    | HiSol            |
| Tyr245         | -1.181      | Val              | 0 → 26.1            | helix    | HiSol + α       |
| Phe246         | 1.051       | Tyr              | 18.9 → 49.9         | helix    | HiSol            |
| Leu248         | 1.244       | Trp              | 18.8 → 45.9         | helix    | HiSol + α       |
| Arg262         | -2.271      | Val              | 3.6 → 28.7          | helix    | HiSol            |
| Ile271         | 1.682       | Val              | 0 → 37.5            | helix    | HiSol            |

Table 1. Predicted Aggregation Hotspots, HiSol Scores, and Conserved Residues Corresponding to Residues in XdPH^a^

^aα: α-helix rule; HiSol: hydropathy contradiction rule.
The soluble expression of the protein, four double variants (I28P/L142R, V31E/L142R, V62D/L142R, and C76Y/L142R), each containing a unique second alteration in addition to L142R, were constructed using site-directed mutagenesis. The gene encoding the variants was expressed by induction at 15°C for 24 h. Amounts of the proteins in the soluble fraction were analyzed by SDS-PAGE, and enzyme activities were measured to compare the solubilities of the L142R variant and variants containing the additional alterations at different sites. Enzyme activity was measured in the crude enzyme extracts. SDS-PAGE analysis showed that the soluble expression levels of the L142R variant and double variants at 15°C were identical (Figure 5A). Variants I28P, C76Y, L142R, I28P/L142R, V31E/L142R, V62D/L142R, and C76Y/L142R had activities of 30.1, 28.7, 23.3, and 30.3 mU/mg soluble protein, respectively (Figure 5C).

Because the double variants showed improved soluble expression, they were further studied at an elevated temperature of 30°C for 12 h. SDS-PAGE analysis revealed that the soluble expression of the L142R and double variants increased at 30°C compared with that at 15°C (Figure 5A,B). Variants I28P, C76Y, L142R, I28P/L142R, V31E/L142R, V62D/L142R,
L142R, and C76Y/L142R had activities of 1.39, 2.45, 46.2, 79.6, 49.6, 48.5, and 64.8 mU/mg soluble protein, respectively (Figure 5C). The enzyme activity of the L142R variant was 2-fold higher at 30 °C than that at 15 °C. Furthermore, the enzymatic activities of the I28P and C76Y variants were detected at 30 °C. The I28P/L142R and C76Y/L142R double variants showed a 1.7-fold and 1.4-fold increase in activity, respectively, compared to the L142R variant. These results indicate a significant improvement in the thermostability of the L142R and double variants, as the enzyme activity of the wild-type XdPH was not detected at 30 °C. After induction at 30 °C for 12 h, the whole-cell lysate, soluble fraction, and insoluble fraction were analyzed by SDS-PAGE to compare the solubilities of the wild-type XdPH, L142R, I28P/L142R, and C76Y/L142R variants (Figure 6B). The results indicated that more soluble variants could be obtained using any combination of more than one mutation. These results suggest that the soluble expression level was improved by the cumulative effects of the alterations, in addition to the effect of L142R alteration alone.

**Temperature—Stability Relationship of XdPH and Its Soluble Variants.** As the temperature—stability relationship of the L142R variant and double variants was markedly improved, that of the wild-type and the double variants with improved soluble expression was investigated. Enzyme activity was measured under the same conditions as those of the standard enzyme assay after incubation of the enzyme at 4, 20, and 30 °C for 60 min. The enzyme activity of the wild-type XdPH was between 4 °C and 20 °C; however, a significant decrease in activity was observed at temperatures above 30 °C (Figure 7). L142R and the double variants showed activity at temperatures between 4 and 30 °C. When the enzyme activity at 4 °C was set to 100%, the relative activity of the C76Y/L142R variant at 30 °C was 80%, whereas that of the other variants was 20%. Hence, the C76Y/L142R variant exhibited improved stability at high temperatures. These results indicate that the C76Y alteration may improve thermostability through the substitution of free cysteine on the protein surface.18

**Saturation Mutagenesis and Bioinformatic Analysis of the Positive Variants of the Residue at Position 142.** To investigate the effect of alterations of residues on soluble expression, residue L142 of XdPH was replaced by 19 different residues using site-directed mutagenesis. All genes encoding the variants were expressed in E. coli BL21(DE3). The enzymatic activity of the soluble fractions was measured. Nine variants, namely, L142R, L142K, L142N, L142Q, L142H, L142W, L142S, L142A, and L142C, were expressed in a soluble form, whereas the other variants did not show any increase in solubility compared to wild-type XdPH (Figure 8A). In particular, substitution with negatively charged amino acids decreases enzyme activity. The amino acid sequence of XdPH was compared with those of proteins with high sequence homology (Figure S5). The protein with the highest sequence identity between XdPH and _L._proline _cis-4-hydroxylase_ from the _Mesorhizobium japonicum_ strain LMG 29417 (PDB: 4P7W) was employed to model the wild-type XdPH and the L142R variants, whose soluble expression had improved. The sequence identity between XdPH and _L._proline _cis-4-hydroxylase_ from the _M._japonicum strain LMG 29417 is 30.3%. The
L142R variant model was constructed by replacing L142 using the SWISS-MODEL.

The major factors causing inclusion body formation in *E. coli* are the protein’s amino acid composition, its hydrophobicity, and overall net charge. In this study, we demonstrated that the decrease in hydrophobicity through replacement of hydrophobic L142 with hydrophilic residues improves its soluble expression. Hydrophobicity analysis of the surface and hydrophobic patch of XdPH by homology modeling indicated a decrease in the surface hydrophobicity of the L142R variant (Figure 9A,B). Replacement with a hydrophilic residue may have reduced hydrophobicity of the hydrophobic site, resulting in improved solubility. These results suggest that the hydrophilicity of amino acid residues on the surface of the protein is an important factor determining the soluble expression of XdPH. When L142 was replaced with arginine, R142 was predicted to interact with residues D191 and E194 (Figure 9C). The bonds between R142 and the surrounding residues were analyzed using Ring 2.0 and Cytoscape. R142 showed increased hydrogen bonding with E194 (Figure S6). These results suggest that stabilization of the structure by strengthening the interaction between L142 and its surrounding residues is also an important factor for the soluble expression of XdPH.

**CONCLUSIONS**

Recombinant gene technology using *E. coli* as a host has been successfully used for basic research and large-scale industrial production of proteins. However, many recombinant proteins often fail to fold correctly, resulting in aggregates known as inclusion bodies.
improve the solubility of recombinant proteins in the structures, rationally selected mutations can be introduced to the hydropathy contradiction rule. By analyzing the target inclusion bodies. We established two rules: the \( \alpha \)-helix rule and the hydropathy contradiction rule. For rational mutagenesis, 19 aggregation hotspots were predicted using the \( \alpha \)-helix and hydropathy contradiction rules. Site-directed mutagenesis of the aggregation hotspots on XdPH was used to produce the V31E, V62D, C76Y, and L142R variants, which showed considerable improvements in the solubility of the enzyme in the \( \text{E. coli} \) expression system compared with the wild-type XdPH. The enzyme activity per unit of soluble protein displayed by the L142R variant was 14.6-fold higher than that of the wild-type XdPH. Furthermore, double mutations were introduced into the gene encoding the enzyme by site-directed mutagenesis, and the resulting double variants showed significantly improved soluble expression levels compared to the single variants. This indicates that the synergistic effects of these alterations increased the soluble expression of XdPH. The temperature—stability relationship between the wild-type XdPH and soluble XdPH variants was investigated. The XdPH variants with improved soluble expression retained their enzyme activity, whereas the wild-type showed no activity at 30 °C. Among the soluble XdPH variants, C76Y/L142R exhibited improved thermostability.

To investigate the effect of these alterations on soluble expression, L142 in XdPH was altered by site-directed mutagenesis. Replacement of L142 with hydrophilic residues improved its soluble expression; however, replacement of L142 with negatively charged residues resulted in a decrease in soluble protein expression. Homology modeling analysis also suggested that the hydrophilicity of the residue at position 142 might determine the solubility of XdPH when expressed in \( \text{E. coli} \). Moreover, when L142 was replaced with arginine, homology modeling indicated that R142 interacted with the residues D191 and E194. The bonds between the residues surrounding L142 were examined, and the L142R variant was found to possess more hydrogen bonds than the wild-type XdPH. These results suggest that the stabilization of the protein structure by interaction between the residue at position 142 and its surrounding residues is an important factor determining the soluble expression of XdPH.

In this study, we demonstrated the effectiveness of the \( \alpha \)-helix rule and the hydropathy contradiction rule in the rational design of soluble biocatalysts. Solubility was found to be an important factor determining the efficiency of biocatalytic production, from basic research to industrial applications.

## EXPERIMENTAL SECTION

**Chemicals and Bacterial Strains.** \( \text{cis-S-HyPiP}, \ \text{l-Pip}, \text{and l-FDLA} \) were “special grade” and purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All other chemicals were also “special grade” and purchased from Kanto Chemical Co., Inc. (Tokyo, Japan) or Nacalai Tesque Co., Inc. (Kyoto, Japan), unless otherwise stated. \( \text{E. coli DH5}\alpha \) and BL21(DE3) were purchased from Nippon Gene Co., Ltd. (Tokyo, Japan). The pET-28a(+) vector was purchased from Novagen (Darmstadt, Germany).

**Prediction of the Aggregation Hotspots in XdPH by the \( \alpha \)-Helix Rule and the Hydropathy Contradiction Rule.** Based on the amino acid sequences of XdPH (accession no. CDG16639), aggregation hotspots were predicted according to the \( \alpha \)-helix rule and the hydropathy contradiction rule. These calculations were performed using the INTMSAlign_HiSol program according to a previous study. The library file for the analysis using the INTMSAlign_HiSol was created by collecting amino acid sequences similar to that of XdPH using a BLAST database search. Prediction of the secondary structure of XdPH by homology modeling was performed using the SWISS-MODEL.

**Construction of the Expression Plasmid and Site-Directed Mutagenesis.** The gene encoding XdPH was codon-optimized, synthesized, and cloned into pJexpress411 by a service provider (ATUM, DNA2.0, CA). Based on the sequences of the XdPH genes, a primer pair was designed to construct the expression plasmid (Table S1). The XdPH gene was amplified by PCR, and a plasmid containing the synthesized gene was used as the template (Supporting Method S1). The DNA fragments were purified and ligated into a pET-28a(+) vector pretreated with NdeI and XhoI to obtain pET28-NHXdPH. In this study, the gene was expressed with an \( \text{N-terminal} \) hexahistidine tag fused to the recombinant protein. The insertion of the gene was confirmed by DNA sequencing using an ABI PRISM 310 genetic analyzer.

Site-directed mutagenesis of the XdPH gene was performed using the megaprimer PCR method. The primers used for site-directed mutagenesis were designed based on the sequence of the XdPH gene (Table S1). The site-directed mutagenesis methods are described in the Supporting Method S2.

**Gene Expression and Analysis of Soluble Proteins.** \( \text{E. coli BL21(DE3)} \) cells were transformed with the constructed plasmid to express the enzyme. The experimental procedure for gene expression and soluble protein analysis is described in Figure S1. A glycerol stock for inoculation was prepared using a Luria-Bertani (LB) medium containing 50 \( \mu \)g/mL kanamycin. Glycerol stocks (30 \( \mu \)L) were inoculated into 3 mL of LB-Autoinduction medium (Overnight Express Autoinduction System1, Merck) containing 50 \( \mu \)g/mL kanamycin and cultivated at 30 °C with shaking at 250 rpm. The bacteria were grown to an OD600 of ~0.6 to 0.8. Protein expression was induced at 15 °C for 24 h or at 30 °C for 12 h, with shaking at 250 rpm. After induction, 2 mL of the cultures was centrifuged at 10000 g for 5 min at 4 °C. The cell pellets were resuspended in 500 \( \mu \)L of 100 mM 2-morpholinoethanesulfonic acid (MES) buffer (pH 6.5). Whole-cell lysates were prepared by disrupting the cell suspension via ultrasonication for 10 min on ice using a Bioruptor UCD-250 (TOSHO DENKI, Japan). Soluble and insoluble fractions were prepared from the whole-cell lysate by centrifugation at 20 000 g for 20 min at 4 °C. The supernatant was collected as the soluble fraction, and the precipitate was resuspended in the same volume of MES buffer as the supernatant (insoluble fraction). Each fraction (2.5 \( \mu \)L) was analyzed by SDS-PAGE using a 10% (w/v) polyacrylamide gel.

**Measurement of Enzymatic Activity.** The hydroxylation of \( \text{l-Pip} \) by the crude enzyme extract in the soluble fraction was assayed by determining the conversion of \( \text{l-Pip} \) to \( \text{cis-S-HyPiP} \) using UPLC (ACQUITY UPLC, Waters, MA) and comparing the results to those of authentic standards. To determine
whether the enzyme recognized L-Pip as a substrate, the hydroxylation was conducted as follows: a 50 \( \mu \)L reaction mixture containing 150 mM MES buffer (pH 6.5), 20 mM \( \alpha \)-ketoglutarate, 1 mM \( \alpha \)-ascorbate, 0.5 mM \( \text{FeSO}_4 \), and 1 mg/mL of each crude enzyme extract was incubated at 20 °C for 10 min. After incubation, the amount of cis-5-HyPip in the reaction mixture was analyzed by UPLC after derivatization with 1-FDLA.\(^{30}\) The analytical methods are described in the Supporting Method S3. The concentration of cis-5-HyPip was measured, and a peak was observed at 8.57 min (Figure S2). The amount of enzyme required to produce 1 \( \mu \)mol of cis-5-HyPip per minute, using L-Pip as a substrate, was set to 1 U. Protein concentrations were estimated by the Bradford method using bovine serum albumin standard.

**Structural Homology Modeling and Bioinformatic Analysis.** Structural models of the wild-type XdPH and the L142R variants of XdPH were constructed based on the crystal structure of \( \alpha \)-proline cis-4-hydroxylase from the *M. japonicum* strain LMG 29417, obtained from the Protein Data Bank (PDB accession code 4P7W). Amino acid replacement was performed using the SWISS-MODEL. The PyMOL program was used to display the protein structure,\(^{14,15}\) and the Molecular Operating Environment program (MOE version 2019, Montreal, Canada) was used for the hydrophobic patch analysis. The hydrogen bonds between adjacent ligands were analyzed to reveal the residue interaction network using Ring 2.0 and Cytoscape.\(^{25,26}\) Amino acid sequence-based alignment of XdPH and other hydroxylases was performed and illustrated using GENETYX ver.12 (GENETYX, Tokyo, Japan).

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c04247.

Construction of expression plasmid (Supporting Method S1); outline of the megaprimer PCR method (Supporting Method S2); product analysis (Supporting Method S3); experimental procedure of the protein solubility test in the *E. coli* expression system (Figure S1); UPLC chromatogram of 1-FDLA-derivatized cis-5-HyPip (Figure S2); UPLC analysis of enzyme reaction products by the XdPH wild-type (WT) and L142R variant (Figure S3); helical wheel depictions for \( \alpha \)-helix regions and mutations based on the \( \alpha \)-helix rule (Figure S4); comparison of the amino acid sequence of XdPH and other hydroxylases (Figure S5); network analyses and the comparison of XdPH wild type and L142R with Ring 2.0 and Cytoscape (Figure S6); list of primers (Table S1); and the amino acid sequences for the analysis using the INTMSAlign_HiSol program (Supporting Data S1) (PDF).

**Accession Codes**

XdPH: CDG16639, \( \alpha \)-proline cis-4-hydroxylase from the *Mesorhizobium japonicum* strain LMG 29417: Q989T9 and 4P7W.

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**Author Contributions**

S.S., R.M., and Y.A. designed the study. S.S., A.I., H.S., and R.M. performed the experiments. S.S., A.I., and H.K. performed data analysis. S.S., A.I., and Y.A. wrote the manuscript.

**Notes**

The authors declare no competing financial interest.

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**Abbreviations**

XdPH, 2-oxoglutarate-dependent \( \alpha \)-pipecolic acid hydroxylase from *Xenorhabdus doucetiae*; cis-5-HyPip, cis-5-hydroxy-\( \alpha \)-pip; L-Pip, \( \alpha \)-pip; 1-FDLA, \( \alpha \)-(5-fluoro-2,4-dinitrophenyl)-L-leucinamide

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