Discovery and Structural Analysis to Improve the Enantioselectivity of Hydroxynitrile Lyase from *Parafontaria laminata* Millipedes for (R)-2-Chloromandelonitrile Synthesis

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**ABSTRACT:** Hydroxynitrile lyase (HNL) catalyzes the reversible synthesis and degradation of cyanohydrins, which are important synthetic intermediates for fine chemical and pharmaceutical industries. Here, we report the discovery of HNL from *Parafontaria laminata* (PlamHNL) millipedes, purification of the HNL to homogeneity, expression of the gene for the enzyme in heterologous expression hosts, and increase in the reaction rate and enantioselectivity in the synthesis of 2-chloromandelonitrile by protein engineering. The recombinant PlamHNL expressed in *Pichia pastoris* is glycosylated and has a higher thermostability and pH stability than the nonglycosylated HNL expressed in *Escherichia coli*. PlamHNL showed a unique wide substrate specificity among other millipede HNLs acting on various cyanohydrins, including 2-chloromandelonitrile, a key intermediate for the antithrombotic agent clopidogrel. We solved the X-ray crystal structure of the PlamHNL and found that the catalytic residues were almost identical to those of HNL from *Chamberlinius hualienensis*, although the forming binding cavity was different. In order to improve the catalytic activity and stereoselectivity, a computational structure-guided directed evolution approach was performed by an enzyme–substrate docking simulation at all of the residues that were exposed on the surface of the active site. The PlamHNL-N85Y mutant showed higher conversion (91% conversion with 98.2% ee of the product) than the wild type (76% conversion with 90% ee of the product) at pH 3.5 and 25 °C for 30 min of incubation. This study shows the diversity of millipede HNLs and reveals the molecular basis for improvement of the activity and stereoselectivity of the wild-type HNL to increase the reaction rate and enantioselectivity in the synthesis of 2-chloromandelonitrile.

**INTRODUCTION**

The discovery of new sources of novel and industrially useful enzymes is one of the most strong key research pursuits in enzyme biotechnology. Accordingly, the production of chiral intermediate compounds using biocatalysts has become increasingly common in the pharmaceutical and fine chemical industries. Enzyme-catalyzed reactions have many advantages, not only giving high regio- and enantioselectivities but also enabling the reactions under mild conditions with less energy and waste.1 Enantiomerically pure cyanohydrins are important chiral intermediates for the synthesis of many industrial products, such as food additives, vitamins, crop-protecting agents, veterinary products, and pharmaceuticals.2 They can be synthesized by the enantioselective addition of HCN to prochiral aldehydes or ketones by the action of hydroxynitrile lyases (HNLs; EC 4.1.2.10, 4.1.2.11, 4.1.2.46, and 4.1.2.47) as biocatalysts.3–6 We intensively screened new HNLs from plants in botanic gardens or commercial fruits using a method similar to microbial screening.7–9 We recently discovered new HNLs from millipedes, such as *Chamberlinius hualienensis*, that have the highest specificity activity among known HNLs.10 These HNLs have been categorized based on structure analyses into seven groups, including glucose–methanol–choline (GMC) oxidoreductase,11 α/β-hydrolases,12,13 serine carboxypeptidase,14 cupin,15 Bet v1,16 dimeric α + β barrel folds,17 and the newly classified *C. hualienensis* millipede HNL belonging to the lipocalin super family (Motojima et al.).41 It has been reported that (R)-mandelonitrile is synthesized and accumulated in the storage chamber as a substrate for the enzyme reaction whereby (R)-mandelonitrile is degraded to release benzaldehyde, benzoyl cyanide, mandelonitrile benzoate, and HCN as defensive chemicals.18–20 Recently, two enzymes have been identified to be involved in cyanohydrin
degradation in *C. hualienensis*. The first is HNL (ChuaHNL), which acts on mandelonitrile in the reaction chamber to release benzaldehyde and HCN through ozopores. ChuaHNL showed a wide substrate spectrum by yielding products with high enantiomeric excess (ee) in the synthetic reaction from various aldehydes and cyanide with stability even at low pH and high temperature. Another enzyme is mandelonitrile oxidase (ChuaMOX), which acts on *(R)-mandelonitrile to produce benzoyl cyanide and hydrogen peroxide.* Based on our knowledge, polydesmid millipedes can be a target for further discovery and identification of biocatalysts and their respective functions.

An outbreak of *Parafontaria laminata* millipedes (Diplopoda: Xystodesmidae) has occurred during autumn in the mountainous area of central Japan every eight years. These millipedes belong to the order Polydesmida and are known as cyanogenic millipedes, which have a similar but species-specific mixture of defense compounds. We expected that these millipedes could be a valuable source for a new HNL. In this study, we purified the HNL (designated as PlamHNL) from *P. laminata* and identified the gene from the cDNA by the rapid amplification of the cDNA end (RACE) method. The gene for PlamHNLs was expressed in *Escherichia coli* and *Pichia pastoris*, the enzymes were purified, and their catalytic properties, glycosylation, and enzyme stability were characterized to clarify the effect of glycosylation on the enzyme produced in *P. pastoris*. The wild-type PlamHNL showed high enantioselectivity in the synthesis of various cyanohydrins, including 2-chloromandelonitrile, a key intermediate for the antithrombotic agent clopidogrel. This characteristic distinguishes PlamHNL from other millipede HNLs, which do not act on the compound. A few reports appeared on the wild-type HNL from plants, which catalyzes the efficient and highly enantioselective synthesis of *(R)-2-chloromandelonitrile.* Thus, PlamHNL was chosen as a millipede HNL model for engineering to yield *(R)-2-chloromandelonitrile with much higher enantiomeric excess than the wild-type enzyme by a computer-aided substrate docking simulation.

## RESULTS AND DISCUSSION

### Purification of PlamHNL from *P. laminata* Millipedes.

In nature, millipedes excrete defensive compounds when they are near enemies. Cyanogenesis proceeds by mixing *(R)-mandelonitrile in the reservoir of defensive grinds with HNL*, resulting in external benzaldehyde and hydrogen cyanide secretion through ozopores as defensive compounds. The crude extract from *P. laminata* showed a specific activity of 40 U mg\(^{-1}\) and was purified to 33-fold with a 4.7% recovery yield, showing a specific activity equivalent to 1320 U mg\(^{-1}\) protein toward benzaldehyde as a model substrate in a cyanohydrin synthesis reaction. The native molecular mass of purified PlamHNL-N was determined to be 43 kDa using a gel filtration column. The enzyme gave a single band in denatured and reduced form on SDS-PAGE (Figure S1A) with a calculated molecular mass of 21 kDa. These results indicate that PlamHNL-N is likely to be active as a homodimer. The purified enzyme was stained with the periodic acid–Schiff (PAS) reagent and showed a pink color, indicating that the PlamHNL from millipedes was glycosylated (Figure S1A). Dadashipour et al. purified the ChuaHNL from several kilograms of frozen *C. hualienensis* millipedes through a combination of column chromatography and column chromatography. ChuaHNL has a molecular mass of 47.3 kDa with glycosylational modification and nearly six times higher specific activity (7420 U mg\(^{-1}\) protein) than PlamHNL-N. The specific activity of PlamHNL is almost equal to that of almond (*Prunus amygdalus*) but higher than those of known HNLs from plants, such as rubber (*Hevea brasiliensis*), yellow passion fruit (*Passiflora edulis*), loquats (*Eriobotrya japonica*), and sorghum (*Sorghum bicolor*).

### cDNA Cloning and Nucleotide Sequencing.

Since the transcription of the gene for HNL and the accumulation of the enzyme were localized in defensive secretory glands of the paraterga, we cloned the full-length cDNA encoding PlamHNL from the paraterga of an adult millipede by rapid amplification of cDNA ends. Degenerate primers corresponding to conserved regions were designed by our laboratory and based on the internal amino acid sequence to obtain the internal peptide of the HNL fragment. The 552 bp cDNA sequence encoded 183 amino acid residues, including a 20 amino acid-long signal peptide. The predicted internal peptide sequences from the cDNA were identical to those of purified PlamHNL from millipedes (Figure S3). The predicted mature protein (no signal peptide) had a molecular mass of 20,855 Da and an isoelectric point of 5.13.

Recently, we discovered and identified HNLs from 10 cyanogenic millipedes that could be categorized into two families. As shown by the phylogenetic analysis based on the amino acid sequence of known HNLs from millipedes (Figure S4), PlamHNL belongs to the Xystodesmidae family and is shown to have more than 80% identity with HNLs from *Parafontaria falcifera* (PlfHNL), *P. tonominea* species complexes 1, 2, and 3 (*Pton1HNL, Pton2HNL*, and *Pton3HNL*, respectively), and *P. tokaiensis* (*PtokHNL*) as well as 67% identity with HNLs from *Riukia semicircularis* (RshHNL) and *Riukia sp.* (RshHNL) in the same family. The other family is Paradoxosomatidae, which exhibited 49, 49, 48, and 49% identity with NtmHNL (*Nedyopus tambanus maugeinus*), NthHNL (*Nedyopus tambanus tambanus*), OgraHNL (*Oxidus gracilis*), and ChuaHNL, respectively. PlamHNL was clustered together in *Parafontaria* genus, indicating that genes encoding HNL may have evolved from one ancestral gene during the evolution of polydesmid millipedes. Moreover, the predicted mature PlamHNL had one potential N-glycosylation site on the asparagine residue at 69.

### Expression of PlamHNL Gene in *E. coli* and *P. pastoris*.

Although the HNL from *P. laminata* millipedes (PlamHNL-N) has a high specific activity and shows high potential as a biocatalyst, it is impractical to purify the enzyme from millipedes for applications. Molecular cloning of the gene for the enzyme and its expression in microorganisms, such as *E. coli*, enables an easier supply of the enzyme. In our previous report, HNLs from millipedes have characteristic eight conserved Cys residues that functionally form disulfide bonds that play important roles in the folding, activity, and stability of the millipede HNLs. Therefore, it was considered that the gene for the HNLs from millipedes could be better expressed in *E. coli* SHuffle T7 because they express disulfide bond isomerase DsbC, which promotes the correction of misoxidized proteins into their correct form. Therefore, in this study, we chose *E. coli* SHuffle T7 (PlamHNL-E) and *P. pastoris* GS115 (PlamHNL-P) as expression hosts for a comparison of the effect of glycosylation on HNL. As expected, the genes for PlamHNL were successfully expressed in the two hosts, as summarized in Table S1. However, the expression
level of PlamHNL in the E. coli host was low (1.75 mg L$^{-1}$ culture), and most of the target protein was in an insoluble form in the inclusion bodies (Figure S2). The soluble part of PlamHNL-E was easily purified by affinity chromatography using Ni Sepharose Fast Flow, and the final step resulted in 115-fold purification with 55.7% recovery. The specific activity of purified PlamHNL-E was 2190 U mg$^{-1}$, which is slightly higher than that of PlamHNL-P. The production of PlamHNL-E (6,890 U L$^{-1}$) in E. coli was 20 times higher than that in PlamHNL-P (351 U L$^{-1}$) produced in P. pastoris. PlamHNL gene could be expressed much better (data not shown) when the codon was optimized and coexpressed with the protein disulfide bond isomerase in the P. pastoris system following our previous report.32 PlamHNL-E and PlamHNL-P had molecular masses of 20,000 and 24,000 Da, respectively (Figure S1B). The molecular masses varied depending on the expression host because the PlamHNL-P was glycosylated by cellular organelles. In contrast, PlamHNL-E from prokaryotic cells lacked such modifications, but it was folded and showed activity similar to those produced in eukaryotic cells.

Glycosylation Analysis. To determine the glycosylation and nonglycosylation profiles of the wild-type and recombinant PlamHNLS, the purified enzymes were stained with the protein disulfide bond isomerase in the P. pastoris system following our previous report.32 PlamHNL-E and PlamHNL-P had molecular masses of 20,000 and 24,000 Da, respectively (Figure S1B). The molecular masses varied depending on the expression host because the PlamHNL-P was glycosylated by cellular organelles. In contrast, PlamHNL-E from prokaryotic cells lacked such modifications, but it was folded and showed activity similar to those produced in eukaryotic cells.

**Effects of pH, Temperature, and Metal Ions on the Activity and Stability of Recombinant PlamHNLS.** The effects of pH and temperature on the activities of both purified recombinant PlamHNLS were compared with the same enzyme activity (15 U mL$^{-1}$). The optimal pH optimum for (R)-mandelonitrile synthesis shown by PlamHNL-E and PlamHNL-P was 4.0, which exhibited the maximum initial activity (120–130 μmol h$^{-1}$) with a high enantiomeric excess of 99–100% of the product (Figure 1A,B). However, the activity was significantly decreased at pH levels higher than 5.0 due to a rapidly increasing nonenzymatic reaction, yielding the racemic mixture containing both of the enantiomers (Figure 1A,B). To suppress the nonenzymatic reaction, a low pH condition is needed for chiral cyanohydrin synthesis. In the pH range of 2.5–3.5, PlamHNL-P was more stable than the one expressed in E. coli (55–60% retaining activity), displaying 80% remaining activity after 1 h of incubation time. PlamHNL-
P showed stable activity compared to PlamHNL-E in the pH range of 4.0–5.5 (Figure 1C). The effect of pH on the stability of PlamHNL-P may have been caused by post-translational glycosylation of the enzyme that is lacking in the E. coli system. This result corresponds to the glycosylation of recombinant HNL from almonds (PaHNL5) expressed in P. pastoris and provides a reason for a greater stability under low pH condition than is observed with the nonglycosylated form.34 At the optimal pH (pH 4.0), the ideal temperature of both recombinant PlamHNLs was estimated to be 25–30°C, which resulted in an initial activity for (R)-mandelonitrile synthesis and enantiomeric excess up to 120–140 μmol h⁻¹ and 99–100%, respectively (Figure 1D,E). PlamHNL-P exhibited a slightly higher initial velocity than PlamHNL-E. At an elevated temperature from 35 to 50°C, the synthetic velocity linearly increased up to 200–240 μmol h⁻¹. However, it also accelerates the nonenzymatic reaction, which lowers the enantiomeric purity of the product below 90% ee (Figure 1E). Thus, 30°C was selected as the optimum temperature for (R)-mandelonitrile synthesis, which enabled the yield of products with the highest % ee.

To evaluate the thermostability of recombinant PlamHNLs, both enzymes were incubated at a temperature of 30 to 100°C for 1 h. All of the PlamHNLs showed 100% remaining activity at a temperature of 30 to 55°C after 1 h of preincubation. In direct comparisons at 80°C, PlamHNL-P exhibited 100% remaining activity and displayed more stability than PlamHNL-E, which lost 50% of its activity under these conditions. These results suggest that the glycosylation contributes to the thermostability of PlamHNL-P. According to our previous work, the HNL from P. edulis, a plant enzyme produced in P. pastoris, showed similar properties with the expressed HNL. They are more thermostable than that produced in E. coli because of the glycosylation.35 However, even the nonglycosylated PlamHNL-E was still quite stable at temperatures up to 70°C because it has Cys residues, which are known to make the protein more stable by forming disulfide bond(s) (Figure 1F).36

The effects of ions, inhibitors, and various additives on the recombinant PlamHNLs were determined. The metal ion or inhibitor sensitivity of nonglycosylated PlamHNL-E was not significantly different from that of glycosylated PlamHNL-P. The (R)-mandelonitrile synthesis activity of recombinant PlamHNLs was slightly inhibited by 1 mM MgSO₄, MnSO₄, ZnSO₄, CdCl₂, CuSO₄, KCl, FeCl₃, LiCl, CoCl₂, and NaF, causing 5–30% inhibition (Table S2). The activity of PlamHNL-E and PlamHNL-P was dramatically inhibited with cysteine- or histidine-modifying reagents, such as iodoacetamide (26.5 and 30.2% remaining activity, respectively), iodoacetic acid (18.3 and 21.3% remaining activity, respectively), and diethylpyrocarbonate (68.4 and 62.5% remaining activity, respectively). These results indicate that cysteine and histidine are required for PlamHNL and might be involved in binding or maintaining the fold. Similar results to the cysteine- or histidine-modifying reagents have been observed in HNLs from C. hualienensis,10 E. japonica,37 Prunus mume,38 Phlebodium aureum,39 and Ximenia americana.40

### Table 1. Kinetic Parameters of PlamHNL for Aldehyde in the Synthesis Reaction of Cyanohydrins

| Substrate               | Structure | e.e. (%) | Kₘ (mM) | kₐ, (s⁻¹) | kₐ/Kₘ (s⁻¹ mM⁻¹) | Vₙₐₓ (μmol min⁻¹ mg⁻¹) |
|------------------------|-----------|----------|---------|-----------|------------------|------------------------|
| Benzaldehyde           | ![Structure](image) | 95       | 2.3±0.2 | 694±19    | 298±1.9          | 2660±12                |
| 2-Chlorobenzaldehyde   | ![Structure](image) | 76       | 59.3±2.6| 378±19    | 6.38±2.2         | 909±35                 |
| 4-Bromo benzaldehyde   | ![Structure](image) | 99       | 36.7±1.2| 416±25    | 11.4±0.4         | 1000±90                |
| 2-Methyl benzaldehyde  | ![Structure](image) | 86       | 15.8±0.5| 520±0.6   | 32.8±1.1         | 1250±67                |
| 3-Methyl benzaldehyde  | ![Structure](image) | 94       | 15.3±1.1| 417±7.3   | 27.6±2.3         | 1000±125               |
| 4-Methyl benzaldehyde  | ![Structure](image) | 99       | 32.1±0.8| 320±22    | 10±0.6           | 770±48                 |
| 2,4-Dimethyl benzaldehyde | ![Structure](image) | 99       | 13.6±1.0| 85±2.3    | 6.2±1.1          | 204±22                 |
| 3-Methoxy benzaldehyde | ![Structure](image) | 91       | 1.6±0.1 | 1380±125  | 8680±0.5         | 33300±475              |
| 1-Naphthaldehyde       | ![Structure](image) | 88       | 2.2±0.2 | 68.3±9.3  | 31.3±0.6         | 164±36                 |
| 2-Naphthaldehyde       | ![Structure](image) | 90       | 12.1±1.1| 220±20    | 18.2±1.2         | 526±8.8                |
| p-Anisaldehyde         | ![Structure](image) | 99       | 66±0.8  | 378±14    | 5.7±0.1          | 999±63                 |
| 4-Hydroxybenzaldehyde  | ![Structure](image) | 86       | 2±0.1   | 83±45     | 416±2.6          | 2000±5.2               |

### Substrate Specificity and Kinetic Parameters
According to our previous work, we found that the millipede HNL preferred aromatic aldehydes, analogues of benzaldehyde,
which is the degraded product of the defensive secretion compound (R)-mandelonitrile. Aromatic aldehydes were tested as substrates in kinetic analyses of recombinant PlamHNL in the synthetic reaction with a reaction time of 5 min at 25 °C. The Michaelis–Menten constants ($K_m$) of the purified enzyme toward benzaldehyde, 2-chlorobenzaldehyde, 4-bromobenzaldehyde, 2-methylbenzaldehyde, 3-methylbenzaldehyde, 4-methylbenzaldehyde, 2,4-dimethylbenzaldehyde, 3-methoxybenzaldehyde, 4-biphenylcarboxaldehyde, 2-naphthaldehyde, $p$-anisaldehyde, and 1-naphthaldehyde were calculated to be in the range of 1.6–66 mM. The enzyme activities varied widely with different aldehydes with the $V_{max}$ value in the range of 164–33,300 μmol min$^{-1}$ mg$^{-1}$. The maximum $V_{max}$, $k_{cat}$, and $k_{cat}/K_m$ values of PlamHNL toward 3-methoxybenzaldehyde were calculated to be 33,300 μmol min$^{-1}$ mg$^{-1}$, 13,800 s$^{-1}$, and 8680 s$^{-1}$ mM$^{-1}$, respectively (Table 1). In contrast, the $K_m$ value toward 3-methoxybenzaldehyde was the lowest among the aromatic aldehyde derivatives tested. Although the benzene aromatic substrate specificity profile of PlamHNL was similar to ChuaHNL from C. hualienensis, PlamHNL acted on the benzaldehyde substituted at the ortho position much better than ChuaHNL, and the ee of the products was higher. For example, the ee values of the cyanohydrin products of PlamHNL catalysis from 2-chlorobenzaldehyde and 2-methylbenzaldehyde were 76 and 86%, respectively, which were much higher than the corresponding products synthesized by ChuaHNL with ee values of 11 and 44.9%, respectively. Interestingly, PlamHNL catalyzed the synthesis of (R)-2-chloromandelonitrile from 2-chlorobenzaldehyde. The compound is used as a precursor for (R)-2-mandelic acid, which is a key intermediate for a potent oral anticoagulant. We used this compound as a model for further study to improve the activity and enantioselectivity of PlamHNL based on structural analyses.

**Overall PlamHNL Structure and Reaction Mechanism.** The X-ray crystal structure of recombinant PlamHNL-E was obtained after removing the His tag, and the amino acid residues were numbered from the N-terminal Met residues with the signal peptide and beginning at amino acid 21 (Lys21) of the mature protein. The ligand-free PlamHNL structure was determined by the molecular replacement method with the initial model ChuaHNL (PDB ID: 6JHC) and refined at 1.42 Å resolution. The PlamHNL crystal belongs to $P_2_1$ and is a homodimer composed of two α-helices and eight β-sheets in each subunit connected with three intramolecular and two intermolecular disulfide bonds (Figure 2A). The overall structure is almost identical to that of ChuaHNL (Figure 2B), which shows high similarity to the folding pattern of the lipocalins. Lipocalins are secreted proteins that occur in animals, plants, and bacteria and play roles in different functions such as retinol transport, lipid transport, cryptic colorization, facilitation of feeding for hemetophasous insects, and the enzymatic synthesis of prostaglandins (lipocalin-type prostaglandin D synthase; LPGDS). The structural features of the lipocalin fold can also be seen in the tertiary structure of millipede HNLs, such as a large cup-shaped cavity, which is composed of structurally conserved regions (SCRs). Their tertiary structure consists of six-or eight-stranded β-barrels stabilized by one, two, or three sulfide bridges. Although PlamHNL showed low identity of overall SCR motif sequences with other lipocalins (Figure S5A), its tertiary structure is closely related to human retinol binding protein 4 (RBP4), walleye sandercyanin fluorescent protein, and LPGDS (Figure S5B). LPGDS is one of a few lipocalins with enzyme activity, which form two large central cavities that separate the binding pocket (surrounded by aliphatic hydrophobic side chain) and a catalytic pocket connecting with the narrow tunnel between two pockets. In the case of PlamHNL structures, the ligand-binding cavity and

![Figure 2](https://dx.doi.org/10.1021/acsomega.0c03070)
catalytic residues were located in the same pocket and formed by many hydrophobic aromatic residues (such as F44, Y60, A74, F87, A96, L98, W109, F111, Y124, and A126) identical to those of ChuaHNL (Figure 2C). This deep hydrophobic cavity might facilitate the acceptance of various bulky cyanohydrins as observed previously.10 There were two distinct corresponding amino acid residues between PlamHNL and ChuaHNL, one in the entrance region (I89 in PlamHNL and G90 in ChuaHNL) and another at the bottom of the ligand cavity (N85 in PlamHNL and Y86 in ChuaHNL) on the β4-barrel (Figure 2C). The longer aliphatic side chain I89 of PlamHNL made the binding cavity deeper than that of ChuaHNL (G90), and the lower part at N85 of PlamHNL created a small tunnel (Figure 2D). PlamHNL was different from ChuaHNL, which formed a more compact pocket (Figure 2E). This observation seems to be important to the effectiveness of the shape of the binding cavities and might be reflected in the substrate specificity of each millipede HNL.

The residues important for catalysis have been confirmed in our laboratory by mutagenesis and kinetic analyses.41 Furthermore, since we found that the residues (R58, Y124, Y60, D76, and K138) are conserved in all of the known HNLs from millipedes (Figure 3A), we propose that the mechanism of PlamHNL is identical. We successfully determined the structure of the complex of PlamHNL with benzaldehyde (Figure 3B) and benzaldehyde with thiocyanate (by cocrystallization with benzaldehyde in a buffer containing potassium thiocyanate (0.2 M), sodium acetate (0.1 M, pH 5.5), polyethylene glycol (PEG) 8000 (10% w/v), and PEG 1000 (10% w/v)) (Figure 3C). Although we performed many soaking experiments with various substrates, such as rac-mandelonitrile and rac-2-chloromandelonitrile, we could not obtain a structure of substrate-bound PlamHNL. In the ligand-free form, two water molecules were observed in the active site. In the complex with benzaldehyde (Figure 3B), the polar part of the carbonyl group formed a hydrogen bond with the amine group of K138 (distance, 2.71 Å), and one water formed a hydrogen bond with the OH group of Y124 (2.79 Å) and the amine group of R58 (3.0 Å). In the complex with benzaldehyde and thiocyanate, which occupies the water position, the anion thiocyanate group interacted with the guanidinium group of R58 (Figure 3C). This observation of the negatively charged thiocyanate complex appears to be analogous to the cyanide source of mandelonitrile synthesis in PlamHNL, although it could not be utilized for the condensation of benzaldehyde to yield mandelonitrile. It is reasonable to speculate that the hydrogen bond length in the R58 and Y124 dyad in this complex might increase the basicity of R58 to deprotonate the proton of HCN. Subsequently, the negatively charged carbon of the cyanide ion acts as a nucleophile and attacks the carbonyl carbon of benzaldehyde. The carbonyl oxygen receives an electron and a proton from...
cyanide and RS8, respectively, to release (R)-mandelonitrile out of the active site of PlamHNL (Figure S6). Motojima et al. reported that the proposed catalytic mechanism of millipede HNLs, in which RS8 and K138 are conserved and directly involved in the catalysis reaction, has been considered to be most likely by docking simulation with (R)-mandelonitrile and site-directed mutagenesis with results showing that the enzyme activities of mutations on these sites caused a complete loss of activity.41

Structure-Guided Engineering to Improve the Activity toward 2-(R)-Chlorobenzaldehyde. To improve the catalytic activity and enantioselectivity of (R)-2-chloromandelonitrile synthesis of PlamHNL, a computational approach was used for enzyme engineering. The (R)-2-chloromandelonitrile was docked into the substrate-binding site of the PlamHNL crystal structure (PDB ID: 7BOW) using the Molecular Operating Environment program (MOE). Based on the catalytic mechanism (Figure S6), the model of the wild type giving the most prominent affinity score ($S = -5.32$ kcal mol$^{-1}$) is shown in Figure 4A. The hydroxyl of (R)-2-chloromandelonitrile interacts with RS8 and K138 with bond lengths of 2.45 and 2.3 Å, respectively. The nitrile group forms a hydrogen bond with Y124 with a bond length of 2.9 Å. This simulation of the ligand was used to generate the mutants using the MOE program in the function of alanine and residue scanning around 14 amino acid residues at the binding pocket of PlamHNL in order to identify the critical residues for substrate affinity. The lowest scores of the affinity variants are shown in Table 2. From 13 preliminary selected hits, the catalytic activities of six variants N85H, N85Y, N85E, N85Q, T95A, and W109H, generated by alterations at three positions N85, T95, and W109, were detected with 2-chloromandelonitrile as a substrate. In contrast, the mutant at positions RS8, Y60, L98, and Y124 could not be detected due to these residues being involved in the activity mechanism and substrate binding of PlamHNL (Figure S6). The best variant obtained from alanine scanning functionality T95A exhibited an approximately 1.5 times higher activity than the wild type, whereas N85H, N85Y, W109H, N85E, and N85Q showed lower activity than the wild type. These results indicate that the mutation at T95 might contribute to improving the enzyme activity toward 2-chlorobenzaldehyde. In order to demonstrate which variant can improve the enantiomeric excess of (R)-2-chloromandelonitrile, the synthesis reaction from 2-chlorobenzaldehyde and KCN was carried out with 1.2 U of all purified variants. Interestingly, N85Y increased to 92% enantiomeric excess of (R)-2-chloromandelonitrile compared to 76% of the wild type. Thus, the mutation at N85 to tyrosine contributes to increasing enantiomeric excess in the

Figure 4. (A, B) Docking model of the (A) wild type and (B) PlamHNL-N85Y mutant complexed with (R)-2-chloromandelonitrile (magenta). The crystal structures of wild type and mutant are colored in blue. The catalytic residues (RS8, Y60, D76, Y124, and K138) are shown in yellow. The hydrogen bonds are shown as black dotted lines, and their distances are labeled. The CH−π is shown as light green dotted lines. N85 and N85Y are colored in green.

Table 2. HNL Productivity, Activity, and Enantiomeric Excess of Selected PlamHNL Variants44

| variants          | $dA$ affinity$^b$ (kcal mol$^{-1}$) | productivity $^c$ (U L$^{-1}$) | specific activity$^d$ (U mg$^{-1}$) | % ee$^e$ |
|-------------------|-----------------------------------|-------------------------------|-----------------------------------|---------|
| N85H              | −0.9664                           | 477                           | 402 ± 36                          | 85      |
| L98W              | −0.7173                           | N.D.                          | N.D.                             | N.D.    |
| L98E              | −0.6709                           | N.D.                          | N.D.                             | N.D.    |
| N85Y              | −0.6488                           | 233                           | 304 ± 18.2                       | 92      |
| R58W              | −0.5245                           | N.D.                          | N.D.                             | N.D.    |
| W109H             | −0.4386                           | 160                           | 205 ± 14.2                       | 22      |
| A74W              | −0.4163                           | N.D.                          | N.D.                             | N.D.    |
| N85E              | −0.3178                           | 382                           | 290 ± 7.6                        | 53      |
| R58K              | −0.2335                           | N.D.                          | N.D.                             | N.D.    |
| N85Q              | −0.2192                           | 180                           | 165 ± 26.7                       | 15      |
| Y60A              | −0.1977                           | N.D.                          | N.D.                             | N.D.    |
| Y124A             | −0.1061                           | N.D.                          | N.D.                             | N.D.    |
| T95A              | −0.0494                           | 580                           | 695 ± 42                         | 86      |
| N85H/T95A         | 450                               | 420 ± 8.6                     | 81      |
| N85Y/T95A         | 457                               | 401 ± 8.5                     | 88      |
| wild-type         | 420                               | 506 ± 7.8                     | 76      |

$^a$All of variants were selected based on a calculation of the alanine or residue scanning functionality around 14 amino acid residues in the binding pocket using the MOE program to identify critical residues for affinity. N.D. = not detectable, which indicates the case that the measuring enzyme activity was the same as omitting the enzyme reaction (control). $^b$dAffinity values refer to the relative binding affinity of the mutation to the wild-type protein. dAffinity is equal to the Boltzmann average of the relative affinities of the ensemble. A more negative value indicates a mutation with better affinity. $^c$Specific activity was assayed after 5 min in a reaction mixture containing 2-chlorobenzaldehyde (50 mM) and KCN (100 mM) at 25 °C with the same amount (0.64 μg) of purified PlamHNL variants. $^d$Enantiomeric excess was obtained for (R)-2-chloromandelonitrile synthesis by HPLC with the same concentration of purified PlamHNL variants (1.2 Unit).
production of (R)-2-chloromandelonitrile. The double mutation of N85H/T95A and N85Y/T95A was performed; both variants did not improve the enzyme activity but slightly improved the enantiomeric excess of (R)-2-chloromandelonitrile compared with the wild type. Next, N85 was replaced by single amino acid substitutions with 19 amino acids, and expression, purification, and the HNL activity of all mutants were performed. For the position N85, all variants exhibited a lower specificity toward 2-chlorobenzaldehyde than that of the wild type except N85C and N85S, which showed higher specificity. Some mutants, N85P and N85D, could not be expressed in the E. coli SHuffle T7 host (Figure S7A). At the same amount of enzyme (1.2 U), most expressible mutants of amino acids with a hydrophobic side chain at the position 85 showed a similar % ee value compared to the N85 wild type in the range of 75%–92%. The maximum ee of 92% was obtained with the N85Y mutant, whereas the mutants of amino acids with polar side chains N85K, N85R, N85E, and N85Q showed a loss of activity and stereoselectivity for (R)-2-chloromandelonitrile synthesis (Figure S7B). N85 is located at the bottom of the active pocket of PlamHNL that formed a large pocket and small opened tunnel, which is different from ChuaHNL (Figure 2D,E). Substitutions with a bulky amino acid side chain (especially tyrosine) at the position N85 might reform the shape of the cavity active site to close a small tunnel and make the cavity more compact than the wild type, similar to the structure obtained at the position Y86 in the ChuaHNL structure (Figure 2E). From the results of HPLC analysis, the production of (S)-2-chloromandelonitrile by PlamHNL-N85Y is lower than that of the wild type and the production of (R)-2-chloromandelonitrile of the PlamHNL-N85Y is higher than that of the wild type in the same reaction condition. This result indicates that the variant N85Y has better enantioselectivity than the wild type. This evidence was supported by docking simulation of (R)-2-chloromandelonitrile with the active site of variant PlamHNL-N85Y, the distance of hydroxyl of (R)-2-chloromandelonitrile interacting with R58 and K138, and the nitrile group forming a hydrogen bond with Y124, which have longer bond lengths than the wild type (3.3, 3.23, and 3.1 Å, respectively). Therefore, the estimated affinity of PlamHNL-N85Y to (R)-2-chloromandelonitrile was reduced (−4.40 kcal mol⁻¹), which showed the specific activity lower than that of the wild type (Figure 4B). On the other hand, the improvement of enantioselectivity of PlamHNL-N85Y could be explained by the newly generated cavity form and the CH–π interaction between F87 and the (R)-2-chloromandelonitrile was abolished upon substitution of asparagine with tyrosine at the position 85. The newly opposite CH–π interaction between (R)-2-chloromandelonitrile and Y60 of PlamHNL-N85Y was formed, which might preferentially recognize (R)-2-chloromandelonitrile versus the wild type (Figure 4A,B).

Enantioselectivity of PlamHNL Mutants. The best mutant candidate for improving enantioselectivity, N85Y, was further studied to optimize the conditions for (R)-2-chloromandelonitrile synthesis. The maximum enantiomeric excess of (R)-2-chloromandelonitrile produced by N85Y increased to 96.3% (E = 53) when the enzyme quantity was increased (4 U), as compared with the maximum ee of 87.0% (E = 14) produced under the same conditions by the wild-type enzyme (Figure 5A). Furthermore, the ee of 98.2% was obtained under low pH conditions (pH 3.5) by the N85Y mutant (Figure 5B). The production of (R)-2-chloromandelonitrile with purified enzyme N85Y showed higher conversion (91% conversion with 98.2% ee; E = 110) than the wild type (76% conversion with 90% ee; E = 19) at pH 3.5 and 25 °C for 30 min of incubation (Figure 5C,D). These results showed that the enantioselectivity of PlamHNL for (R)-2-chloromandelonitrile synthesis was remarkably improved by the Asn85 mutation.

Figure 5. Enantioselectivity for (R)-2-chloromandelonitrile synthesis of purified enzymes from PlamHNL wild type (black bar or circle open) and mutant N85Y (cross bar or triangle up solid). (A) Effect of enzyme quantity. (B) Effect of low pH conditions. (C, D) Time course of (C) total (R + S)-2-chloromandelonitrile production and (D) their enantiomeric excess in the reaction of citrate buffer (300 mM, pH 3.5) containing 2-chlorobenzaldehyde (50 mM), KCN (100 mM), and enzyme (4 U) at 25 °C.

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**CONCLUSIONS**

We showed that the millipede, *P. laminata*, collected from nature in Japan, has a high specific activity and stabilities against acidic condition and heat. The cDNA for PlamHNL was cloned and expressed in *E. coli*, and the enzyme was characterized. Recombinant wild-type PlamHNL showed a good specific activity on the synthesis of a drug intermediate (R)-2-chloromandelonitrile from unnatural substrate 2-chlorobenzaldehyde with moderate % ee. Since the gene for the enzyme was expressible in *E. coli*, further rational modification of the enzyme was possible in a short time. PlamHNL produced in *E. coli* was subjected to X-ray crystallography, and the results of the structural studies allowed us to improve the stereoselectivity for (R)-2-chloromandelonitrile synthesis using a computational-directed evolution approach. We showed the merits of PlamHNL to be applied for the synthesis of enantiomerically pure cyanohydrins because the enzyme is stable and the gene accepts further rational evolution in *E. coli* based on our structural studies.

**EXPERIMENTAL SECTION**

**Millipede Collection.** Living *P. laminata* millipedes, which are often found individually under disintegrating leaves or embedded in the soil after heavy rain, were manually collected in Takaoa Castle Park, Toyama, Japan from June to August 2015 and subsequently reared in the laboratory on leaf litter collected from the site under a natural photoperiod with high humidity (ca. 100% RH) at 20 °C.

**HNL Activity Assay.** The enzyme was assayed by measuring the amount of optically active (R)-mandelonitrile with benzaldehyde and potassium cyanide as the substrates. The reaction mixture (0.5 mL) was prepared in a microtube. Benzaldehyde (1.25 M in DMSO, 20 μL) was added to sodium citrate buffer (400 mM, pH 4.0) followed by the addition of enzyme solution and KCN solution (1.0 M, 50 μL). The reaction was monitored by taking an aliquot of the reaction mixture (100 μL) and extracting with 900 μL of organic solvent (85% n-hexane and 15% isopropanol by volume). The organic layer containing benzaldehyde and (R) - and (S) - mandelonitrile was obtained after centrifugation of the mixture at 15,000 g for 10 min at 4 °C. Aliquots (10 μL) of the organic phase were then analyzed by chiral HPLC as previously described. One unit of HNL activity was defined as the amount of enzyme that produced 1 mol of optically active (R)-mandelonitrile from benzaldehyde and KCN per min under the assay conditions. To determine the substrate specificity of PlamHNL, aldehyde substrates were used instead of benzaldehyde. The standards of cyanohydrins were prepared by chemical synthesis and described by Dadashipour et al.10

**Extraction and Purification of Hydroxynitrile Lyase from Millipedes.** Eight hundred living millipedes were extracted by hand shaking to induce the compounds and HNL for 2 min at room temperature before adding 10 mM phosphate-buffered saline (PBS) pH 7.4 buffer containing 0.133 M NaCl. After confirmation of the smell-like benzaldehyde, the crude extract was separated, and the supernatant was collected by centrifugation at 8000 g for 10 min at 4 °C to remove the insoluble material. Ammonium sulfate was added to the supernatant at 30% saturated concentration and loaded onto a TOYOPEARL butyl-650M (Tosoh) column chromatograph (10 × 2.8 cm). The enzyme was eluted with a stepwise gradient of 10 mM PBS buffer (pH 7.4) containing 30% saturated ammonium sulfate (30, 30 to 20, 20 to 10, and 10 to 0%, with 50 mL in each container) in the same buffer. The fractions containing the highest activity were pooled, dialyzed, concentrated, and loaded onto a Mono Q 5/50 GL anion-exchange column (GE Healthcare) and eluted with a linear gradient of sodium chloride from 0 to 500 mM in the same buffer at a flow rate of 0.5 mL min⁻¹. The active fractions were pooled, and ammonium sulfate was added up to 30% for application to the RESOURCE phenyl HR column (GE Healthcare) and eluted with a linear gradient from 30 to 0% saturated ammonium sulfate in the same buffer at a flow rate of 0.5 mL min⁻¹. All purification steps were performed at 4 °C.

**Protein Sequencing.** The internal amino acid sequencing was performed as previously described.11 Briefly, the purified PlamHNL-N was separated using 15% SDS-PAGE and stained with Coomassie bright blue. The visible band was excised and combined for in-gel trypsin digestion (digestion-grade modified trypsin; Promega, Madison, WI, USA). The internal peptides were analyzed using a nanoflow liquid chromatography–tandem mass spectrometry system. The digested peptides were loaded onto a nanoACQUITY UPLC symmetry C18 trap column (particle size: 5 μm; 180 μm i.d. × 20 mm; Waters, Milford, MA, USA) and an ACQUITY UPLC peptide CSH C18 nanoACQUITY column (particle size: 1.7 μm; 75 μm i.d. × 200 mm; Waters). Mobile phases A and B were water containing 0.1% formic acid and acetonitrile containing 0.1% formic acid, respectively. The elution profile was a linear gradient of B (10–80%) at a flow rate of 300 nL min⁻¹ for 90 min. Mass spectrometry was simultaneously performed in the positive electrospray ionization mode with a capillary voltage of 3 kV and cone voltage of 40 V using a SYNAPT G2-Si high-resolution quadrupole time-of-flight mass spectrometer (Waters). [Glu1]-fibrinopeptide B was used as a mass calibrant. The MS/MS spectra were analyzed using BioLynx (Waters).

**cDNA Cloning of PlamHNL.** The total RNA was prepared from dissected *P. laminata* paraterga immediately homogenized with the TRIzol reagent (Thermo Fisher Scientific). First-strand cDNA was synthesized directly from the total RNA by reverse transcription using a SMART RACE cDNA amplification kit (Clontech Laboratories) and SuperScript III (Life Technologies) as reverse transcriptases. Degenerate primers corresponding to the conserved regions were designed by our laboratory to obtain the internal peptide of HNL fragments with the following 1F and 1R primers (Table S4). The HNL fragments were extended in both the 5′ and 3′ directions using gene-specific primers (primers 2–7; Table S4) and the cDNA library as a PCR template. All resulting PCR were separated by agarose gel electrophoresis, purified with NucleoSpin (Macherey-Nagel, Germany), and ligated into a T-vector pMD20 (Clontech Laboratories) using a DNA Ligation Kit (Mighty Mix, Takara). DNA sequences were determined using a 3500 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The obtained sequences were assembled and analyzed using ATGC and GENETYX ver. 12 (GENETYX, Tokyo, Japan).

**Bioinformatics Analysis.** The amino acid sequence and secondary-based alignment of HNLs from millipedes were performed using PROMALS3D12 and illustrated using ESPript 3.0 (http://espiript.ibcp.fr).12 A phylogenetic analysis was performed for the millipede HNLs. Multiple sequence alignment of HNLs was performed using Clustal X with
default parameters. The phylogenetic tree was constructed by the neighbor-joining method using MEGA 6 software.54

**Construction of Expression Vectors of Recombinant PlamHNL in E. coli and P. pastoris.** The recombinant strain E. coli SHuffle T7 express (New England Biolabs, Ipswich, MA, USA) was used as an expression host and constructed in the pET28a(+) vector (Novagen; Darmstadt, Germany) as an expression vector. The DNA insert was PCR-amplified using primers 8F and 8R as gene-specific primers (Table S1) and PlamHNL cDNA as the template DNA. The purified insert DNA was ligated to the corresponding site of the pET28a(+) vector using a DNA Ligation Kit (Mighty Mix, Takara). The constructed vector was designated as pET28a-PlamHNL.

To express the recombinant gene for PlamHNL in P. pastoris GS115 cells, a gene insert was amplified using Tks Gflex DNA polymerase and the gene-specific primers 9F and 9R (Table S4). Meanwhile, pPICZαA was linearized by PCR amplification using an inverted PCR method (KOD-Plus-Mutagenesis Kit; Toyobo). After PCR purification with NucleoSpin (Macherey-Nagel, Germany), both amplified products were ligated with an In-Fusion HD Cloning Kit (Clontech Takara Bio USA, Inc.), generating pPICZαA-PlamHNL. Then, the coding sequence of 6× His tag at the N-terminus and TEV protease (primers 10 and 11) recognition sequence were introduced by a second inverted PCR method designated as pPICZαA-TEV-His PlamHNL. The constructed vector pPICZαA-TEV-His PlamHNL was linearized by digestion with SacI and electroporated into P. pastoris GS115 cells using an Easyselect Pichia Expression Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions.

**Expression and Production of Recombinant PlamHNL in E. coli and P. pastoris.** A single colony of E. coli SHuffle T7 express harboring pET28a-PlamHNL was inoculated into 5 mL of Luria–Bertani (LB) broth containing kanamycin (80 μg mL⁻¹) and incubated overnight at a shaking rate of 300 rpm at 30 °C. The starter culture (5 mL) was transferred to the LB medium (500 mL) in 2 L Erlenmeyer flasks containing kanamycin (80 μg mL⁻¹) and cultured at a shaking rate of 150 rpm at 30 °C. After 12 h, isopropl-β-d-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM, and cells were cultured at 18 °C for 24 h with the same shaking rate. The cells were centrifuged (8500 g for 15 min) and resuspended in potassium phosphate buffer (KPB; 20 mM, pH 7.0) containing sodium chloride (0.5 M) and imidazole (25 mM). The resuspended cells were lysed by sonication, and the lysate was centrifuged (15,000 g for 15 min at 4 °C) to remove debris. The supernatant was loaded onto Ni Sepharose 6 Fast Flow (GE Healthcare, Little Chalfont, UK) columns (i.d. 25 mm; column volume, 20 mL), washed with 50 mM imidazole, and eluted with a linear gradient of 50–300 mM imidazole in KPB (20 mM, pH 7.0) containing sodium chloride (0.5 M) at a flow rate of 0.5 mL min⁻¹. The enzyme activity was measured by the method described above, and the active fractions were pooled, dialyzed, concentrated, and checked for purity by SDS-PAGE.55

**P. pastoris** transformants were inoculated into 5 mL of YPD (1% yeast extract, 2% peptone, and 2% dextrose) broth containing Zeocin (100 μg mL⁻¹). After overnight preculture at 30 °C with shaking at 300 rpm, the inoculum was transferred to a buffered minimal glycerol medium (BMGH; 100 mM potassium phosphate buffer (pH 6.0), 1.34% yeast nitrogen base without amino acid, 4 × 10⁻⁵% biotin, 0.004% histidine, and 1.0% glycerol) and grown at 30 °C with shaking at 200 rpm. After 48 h of incubation, cells were harvested by centrifugation and resuspended in 500 mL of expression medium, buffered minimal methanol medium (BMMH; the same as BMGH but 0.5% (v/v) methanol was added instead of 1% (v/v) glycerol), and 0.5% methanol as an inducer every 24 h. As the maximum extracellular HNL activity was observed after 6 days of cultivation, the supernatant was harvested by centrifugation at 3000 g for 10 min and concentrated 25-fold with a hollow fiber ultrafilter (Microza; Asahi Kasei Chemical). Ammonium sulfate (30% w/v) was added to the supernatant and then directly applied to a TOYOPEARL Butyl-650M column (Tosoh; 25 mm i.d.; column volume, 30 mL) previously equilibrated with equilibration buffer (20 mM KPB (pH 7.0) and ammonium sulfate (30% w/v)). The absorbed enzymes were washed with 10 volumes of equilibration buffer and eluted with a gradient of ammonium sulfate from 30 to 0% in the same buffer, and the active fraction was dialyzed against 20 mM KPB (pH 7.0). All purification steps were performed at 4 °C.

**Effects of pH and Temperature on the Activity and Stability.** The optimum pH and temperature for synthesis activity were assayed as described above at pH 2.5–6.0 and temperatures of 5.0–60.0 °C. To determine pH stability, the recombinant PlamHNLs were preincubated at 30 °C in the pH range of 2.5 to 11.0 for 1 h. The thermostability of PlamHNL was preincubated in the range of 30 to 80 °C for 1 h. The remaining activity of the enzyme was measured as described above.

**Effects of Additives and Inhibitors on Activity and Stability.** The effects of various additives and inhibitors of purified recombinant PlamHNLs were examined. After incubation with various additives and inhibitors in KPB (10 mM, pH 6.0) at 30 °C for 1 h, the remaining activity of the enzyme was measured as described above.

**Glycosylation Analysis.** Purified PlamHNL from millipedes (PlamHNL-N) and recombinant PlamHNLs were stained with periodic acid–Schiff (PAS) reagent using a GelCold Glycoprotein Staining Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. Enzymatic methods were also used to determine N-glycosylation, such as endoglycosidase H (Endo H) and peptide-N-glycosidase F (PNGase F) (BioLabs, New England, USA). To expose the glycosylation site before treating with deglycosylated enzymes, the purified enzymes (0.20 mg mL⁻¹) were boiled for 10 min in the optimized buffer for the deglycosylated enzymes and treated with Endo H or PNGase F at 37 °C overnight according to the manufacturer’s instructions.

**Crystallization and Structure Determination.** After cleavage by thrombin protease (GE Healthcare) to remove His-tagged protein, the recombinant PlamHNL-E was purified to homology using a HisTrap FF column (GE Healthcare). The purified PlamHNL-E without His tag was dialyzed against KPB (10 mM, pH 7.5) using PD-10 (GE Healthcare) and concentrated to 10 mg mL⁻¹ using an Amicon ultra centrifugal filter unit NMWL, 10 kDa (Merck Millipore, Billerica, MA, USA). The concentrated PlamHNL was used for crystal screening with commercial screening index HT (Hampton Research), PACT Suite (Qiagen), and Clear Strategy Screen I (Molecular Dimensions) by the vapor diffusion sitting drop method at 20 °C in 96-well Intelli-Plates (Art Robbins Instruments, CA, USA). Crystals appeared within 1 day in Clear Strategy Screen I under the following conditions: 0.2 M...
Droplets for crystallization were prepared by mixing 1.5 M potassium thiocyanate, 10% PEG 8000, and 8% PEG 1500 μL of 0.5 M Re at 100 K at the beamline BL-5A of KEK-PF (Tsukuba, Japan). The solution was also examined. The crystals were soaked in solution. The ligand-free crystal soaked in each ligand were soaked in cryoprotectant solutions containing 25% replacement with the program MOLREP using coordinates of ligand-free and ligand-bound ones were solved by molecular rounds of model modifications. Good electron density for the ligands. A summary of the ligand-free crystals; Table S3, statistics for data collection and refinement; Figure S1, SDS-PAGE analysis of purified PlamHNLs; Figure S2, SDS-PAGE analysis of PlamHNL-E production in E. coli SHuffle T7; Figure S3, cDNA and deduced amino acid sequences of PlamHNL; Figure S4, phylogenetic tree of HNLs from millipedes; Figure S5, secondary structure-based multiple sequence alignment among PlamHNL; Figure S6, proposed catalytic mechanism for PlamHNL; Figure S7, activity and % ee of mutants generated by saturation mutagenesis at Asn85; and Table S4, primers used for PCR (PDF).

ASSOCIATED CONTENT

Supporting Information

Table S1, purification summary of PlamHNLs; Table S2, effect of inhibitors and metal ions on synthesis activity of PlamHNLs; Table S3, statistics for data collection and refinement; Figure S1, SDS-PAGE analysis of purified PlamHNLs; Figure S2, SDS-PAGE analysis of PlamHNL-E production in E. coli SHuffle T7; Figure S3, cDNA and deduced amino acid sequences of PlamHNL; Figure S4, phylogenetic tree of HNLs from millipedes; Figure S5, secondary structure-based multiple sequence alignment among PlamHNL; Figure S6, proposed catalytic mechanism for PlamHNL; Figure S7, activity and % ee of mutants generated by saturation mutagenesis at Asn85; and Table S4, primers used for PCR (PDF).

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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