Discovery and molecular and biocatalytic properties of hydroxynitrile lyase from an invasive millipede, *Chamberlinius hualienensis*

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Hydroxynitrile lyase (HNL) catalyzes the degradation of cyanohydrins and causes the release of hydrogen cyanide (cyanogenesis). HNL can enantioselectively produce cyanohydrins, which are valuable building blocks for the synthesis of fine chemicals and pharmaceuticals, and is used as an important biocatalyst in industrial biotechnology. Currently, HNLs are isolated from plants and bacteria. Because industrial biotechnology requires more efficient and stable enzymes for sustainable development, we must continuously explore other potential enzyme sources for the desired HNLs. Despite the abundance of cyanogenic millipedes in the world, there has been no precise study of the HNLs from these arthropods. Here we report the isolation of HNL from the cyanide-emitting invasive millipede *Chamberlinius hualienensis*, along with its molecular properties and application in biocatalysis. The purified enzyme displays a very high specific activity in the synthesis of mandelonitrile. It is a glycosylated homodimer protein and shows no apparent sequence identity or homology with proteins in the known databases. It shows biocatalytic activity for the condensation of various aromatic aldehydes with potassium cyanide to produce cyanohydrins and has high stability over a wide range of temperatures and pH values. It catalyzes the synthesis of (*R*)-mandelonitrile from benzaldehyde with a 99% enantiomeric excess, without using any organic solvents. Arthropod fauna comprise 80% of terrestrial animals. We propose that these animals can be valuable resources for exploring not only HNLs but also diverse, efficient, and stable biocatalysts in industrial biotechnology.

millipede hydroxynitrile lyase | bioresource exploration | biocatalysis | white biotechnology | arthropod

Enzymes are very specific and efficient catalysts. They are able to catalyze reactions without extreme conditions, such as high temperature and pressure, that are often required in chemical synthetic processes. Industrial biotechnologies using these biocatalysts have the advantages of higher reaction rates, efficiencies, and enantioselectivities. This technology also reduces energy consumption and decreases the production of hazardous chemical waste. Conventional industrial chemistry is gradually being replaced by industrial biotechnology. For example, nitrile hydratase is used for the synthesis of acrylamide from acrylonitrile. This strategy produces more than 600,000 tons of acrylamide worldwide every year (1).

Hydroxynitrile lyase (HNL) is found as a molecular component in plant defense systems and plays a role in the decomposition of stored cyanogenic glycosides into hydrogen cyanide and aldehydes (2). The enzyme's reverse activity is used industrially in the enantioselective production of cyanohydrins. Cyanohydrins are valuable building blocks for the synthesis of fine chemicals, pharmaceuticals, and agrochemicals such as denopamine (β1-adrenergic receptor agonist) (3, 4), clopidogrel (platelet aggregation inhibitor) (5), pyrethroids (insecticides) (6), and (*R*)-pantolactone [starting compound for synthesis of (*R*)-pantothenic acid, (*R*)-panthenol, and (*R*)-pantetheine] (7). The enantioselective condensation of benzaldehyde with hydrogen cyanide in the asymmetric synthesis of mandelonitrile using emulsin from the almond, *Prunus amygdalus*, as an HNL source (PaHNL), has been demonstrated (8). Versatile HNLs, homologous to FAD-dependent oxidoreductase, α/β-hydrolase, carboxypeptidase, a Zn^{2+}-dependent alcohol dehydrogenase, and a Mn^{2+}-dependent cupin structure have all been identified from plants and bacteria (9–12). Because PaHNL shows the highest specific activity in the synthesis of mandelonitrile among known HNLs (13), along with a wide substrate specificity and high stability, it serves as a valuable catalyst in industrial biotechnology. Although many scientists have identified HNLs in the last two decades (14, 15), further screening for better enzyme(s) with high reaction rates and stabilities from unexplored bioresources is necessary to enrich the current tool box for sustainable industrial development.

The millipede *Chamberlinius hualienensis* (16) (Movie S1), originally from Hualien, Taiwan, invaded Okinawa Island, Japan in 1983 and has been expanding its habitat in Kyushu, Japan. Large swarms of the millipedes enter houses and sometimes cause train delays (17). The animal secretes mandelonitrile, benzaldehyde, and hazardous hydrogen cyanide as defense chemicals, as previously reported for polydesmid millipede species (18, 19). In the millipede species *Apheloria corrugate*, a predicted enzyme is thought to play a role in the decomposition of mandelonitrile and the release of hydrogen cyanide and benzaldehyde

Significance

Hydroxynitrile lyase (HNL) has been isolated from plants and bacteria and is a valuable tool in the chiral-specific synthesis of cyanohydrins, which are important building blocks of fine chemicals and pharmaceuticals. To discover more efficient and stable HNLs, we focused on the invasive cyanogenic millipede as a bioresource. The HNL identified from the millipede showed not only the highest specific activity toward benzaldehyde among known HNLs, including the almond HNL in industrial use, along with wide temperature and pH stabilities, but also high enantioselectivity in the synthesis of various cyanohydrins. These properties make it suitable as an industrial biocatalyst. Arthropods are likely to be valuable sources of potential biocatalysts for the next generation of industrial biotechnology.

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(20). If the enzyme derived from *C. hualienensis* is an HNL, the large population of invasive millipedes could be used as an important resource for HNL purification. Here we identify HNL from the invasive cyanogenic millipede, characterize its physicochemical properties and inhibitor susceptibility, and demonstrate its substrate specificity, kinetic parameters, and gene expression. We describe the potential of the millipede HNL to be widely used as a biocatalyst for the enantioselective synthesis of cyanohydrins, building

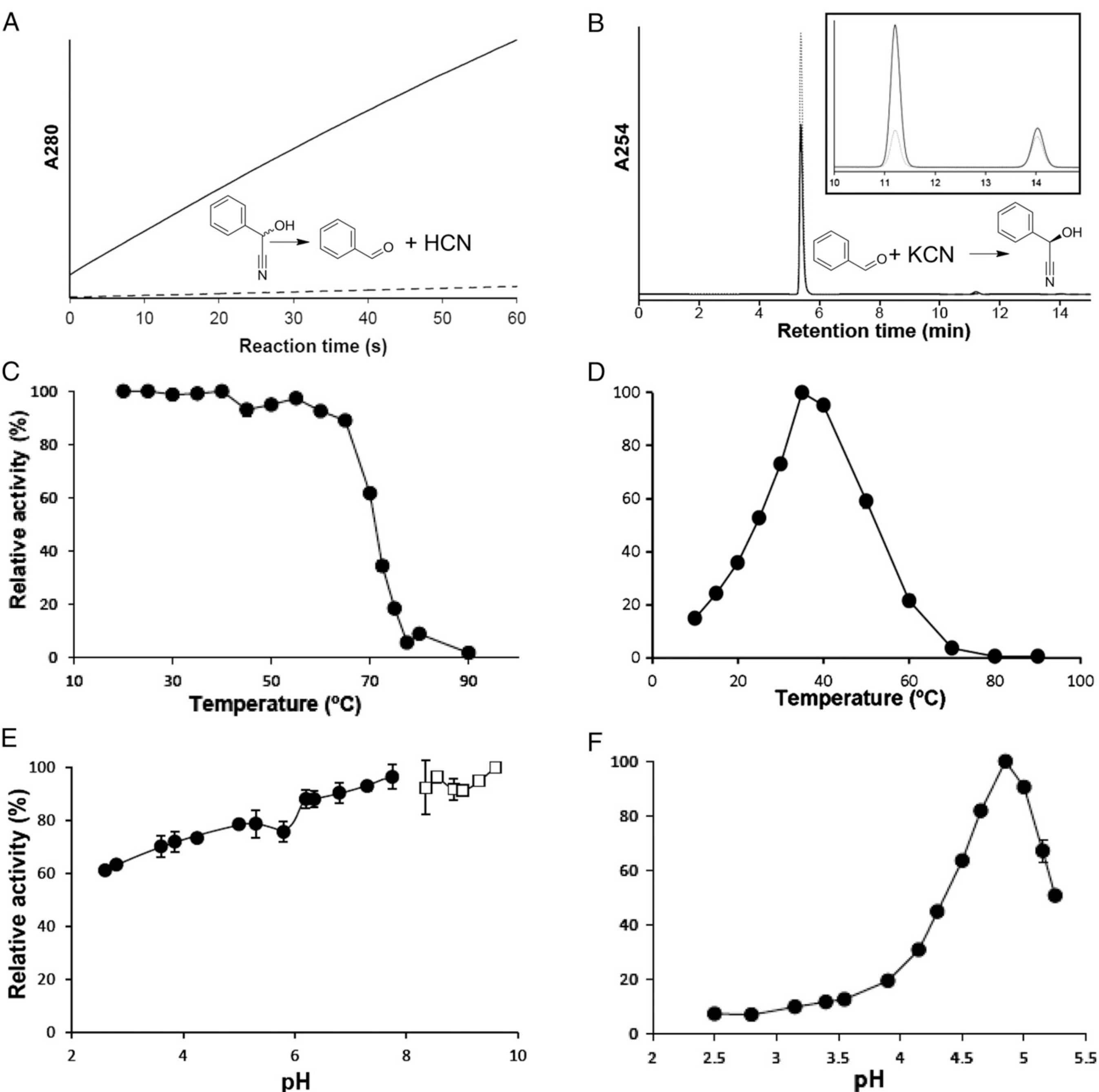

Fig. 1. Characterization of ChuaHNL. (*A*) Cleavage reaction of *racemic* mandelonitrile by ChuaHNL. Production of benzaldehyde in 100 mM citrate buffer, pH 5.5, at 22 °C was measured by monitoring the absorbance at 280 nm. Solid and dotted lines indicate absorbance with and without the enzyme, respectively. (*B*) Synthesis of (*R*)-mandelonitrile from 50 mM benzaldehyde in 400 mM citrate buffer, pH 4.2, at 22 °C. ChuaHNL specifically produces (*R*)-mandelonitrile, whereas the chemical reaction produces a scant amount of (*R*)- and (*S*)-mandelonitrile. Production of mandelonitrile was measured by monitoring the absorbance at 254 nm using an HPLC equipped with a chiral column. Retention times of (*R*)- and (*S*)-mandelonitrile were 11.4 min and 14.3 min, respectively. (*Inset*) Magnified view in the range of 10–15 min. Solid and dotted lines indicate the synthetic reaction of mandelonitrile from benzaldehyde and potassium cyanide with and without ChuaHNL, respectively. (*C*) Temperature stability. Enzyme samples were incubated at each temperature for 1 h and assayed for the remaining HNL activity (as shown in *B*). (*D*) Optimum temperature. Enzyme samples were assayed at each temperature (as shown in *B*). (*E*) pH stability. Enzyme samples were incubated at each temperature for 1 h and assayed for the remaining HNL activity (as shown in *B*). ●, enzymatic activity in a citrate-phosphate buffer; □, enzymatic activity in a glycine-sodium hydroxide buffer. (*F*) Optimum pH. Enzyme samples were assayed at each pH value (as shown in *B*). Values are the mean ± SD; $n = 3$.

Table 1. Purification of ChuaHNL from the millipede *C. hualienensis*

Purification step	Activity, U*	Protein, mg	Specific activity, U·mg^{-1}	Yield, %	Purification fold
Body crude homogenate	26,600	4,390	6.10	100	1.00
Saturated ammonium sulfate fractionation (50–70%)	19,600	1,000	19.6	73.7	3.20
DEAE-Toyopearl	11,600	180	64.5	43.8	10.9
Butyl-Toyopearl	5,160	14.0	369	19.4	61.5
Q Sepharose Fast Flow	1,900	1.40	1,360	7.14	226
Mono Q	1,270	0.46	2,760	4.77	450
Superdex 75	890	0.12	7,420	3.35	1,230

*Synthesis of (*R*)-mandelonitrile from benzaldehyde was monitored using an HPLC equipped with a chiral column.

blocks for fine chemicals and pharmaceuticals. Furthermore, we demonstrate the environmentally friendly synthesis of (*R*)-mandelonitrile from benzaldehyde and potassium cyanide using this HNL with 99.0% enantiomeric excess (ee) in 5 min without the use of organic solvents.

Results and Discussion

Identification and Purification of HNL from the Invasive Cyanogenic Millipede *C. hualienensis*. To confirm whether the millipede enzyme(s) can catalyze the decomposition of mandelonitrile into benzaldehyde and hydrogen cyanide, we measured the cleavage activity in millipede body extract. The extract decomposed *racemic* mandelonitrile into benzaldehyde in a time-dependent manner, with unambiguously higher reaction than the control (Fig. 1*A*). These results indicate that *C. hualienensis* has HNL in its body. On the other hand, the millipede extract enantioselectively catalyzed the synthesis of (*R*)-mandelonitrile from benzaldehyde and potassium cyanide (Fig. 1*B*). Thus, we designated this enzyme(s) from *C. hualienensis* as ChuaHNL and considered the invasive millipedes as a potential source for the purification of HNL.

Using ∼29 kg of collected millipedes in preliminary experiments, we optimized each purification step. After fractionation by 50–70% saturated ammonium sulfate precipitation to remove some recalcitrant impurities, ChuaHNL was purified 1,230-fold from 1 kg of frozen millipedes by a combination of ion exchange chromatography (Toyopearl DEAE-650M, Q Sepharose Fast Flow, and Mono Q 5/50 GL), hydrophobic interaction chromatography (Toyopearl Butyl-650M), and gel filtration (Superdex 75 10/300 GL). The specific activity in the synthesis of (*R*)-mandelonitrile from benzaldehyde and potassium cyanide was 7,420 U·mg^{-1} (Table 1). This value is superior not only to the 31.5, 136, and 220 U·mg^{-1} specific activities of HNLs extracted, respectively, from loquats (*Eriobotrya japonica*) (21), passion fruit (*Passiflora edulis*) (22), and Japanese apricots (*Prunus mume*) (23) but also to the 1,450 U·mg^{-1} specific activity of HNL from almonds (*Prunus amygdalus*) (13), which is used in industry.

Characterization of Enzymatic and Physicochemical Properties of ChuaHNL. Next, we assessed the inhibitor susceptibility and physicochemical properties of ChuaHNL in the synthesis of mandelonitrile from benzaldehyde and potassium cyanide. Most of the enzyme inhibitors tested did not obviously affect the HNL activity, except sulfhydryl reagents, iodoacetic acid, and iodoacetamide (18% and 28% remaining activity, respectively) (Table S1). The inhibitor profile is similar to FAD-containing HNLs from *E. japonica* (loquats) (21), *P. mume* (Japanese apricot) (23), and *P. amygdalus* (almond) (24). These results suggest that cysteine and/or serine residue(s) are likely to be important for the enzymatic activity. ChuaHNL has a molecular mass of 47,300 Da, as determined by gel filtration, and 25,000 Da, as determined from SDS/PAGE (Fig. S1*A*), suggesting that it is a homodimer consisting of two identical subunits. Periodic acid-Schiff (PAS) staining identified the enzyme as a glycoprotein (Fig. S1*B*). ChuaHNL is stable up to 65 °C after 1 h of incubation and is inactive at higher temperatures (Fig. 1*C*); the enzyme can catalyze the synthesis of (*R*)-mandelonitrile in a wide temperature range of 15–60 °C, and the optimum temperature is 35 °C (Fig. 1*D*). It is stable in the pH range of 2.6–9.6 (Fig. 1*E*). The optimum pH is 4.9 (Fig. 1*F*). The temperature and pH stabilities are comparable to those of PaHNL (25). Based on these properties, this enzyme is a good candidate biocatalyst for industrial applications.

Expression and Localization of the Gene Encoding ChuaHNL in the Body of the Millipede. *ChuaHNL* transcript and ChuaHNL were accumulated in the paraterga of the millipede (Fig. 2 *A* and *B*). Because Western blotting did not detect this enzyme in the hemolymph (Fig. 2*B*), the enzyme is likely to be localized in defensive secretory gland(s) in the paraterga. There are two types of paraterga. One houses a reaction chamber and a storage chamber, whereas the other does not (arrowhead and arrow in Fig. 2*C*). The millipede specifically expressed *ChuaHNL* in the paraterga housing the reaction chamber and the storage chamber (Fig. 2*C*) and exuded the gene product at the ozopore through a duct of the reaction chamber (Fig. 2*D*). In *A. corrugate*,

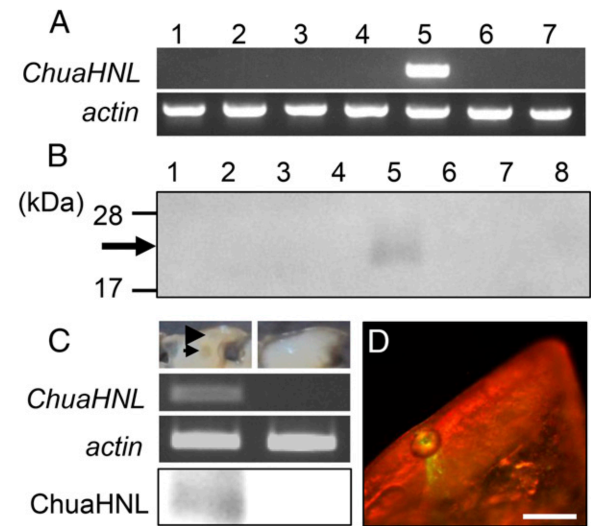

Fig. 2. Expression and localization of ChuaHNL. (*A*) *ChuaHNL* expression. RT-PCR detected *ChuaHNL* transcript in the paraterga; *actin* expression was used as an internal control. (*B*) Localization of ChuaHNL. Western blotting detected an immunoreactive material with a molecular mass of 25,000 Da in the paraterga. 1, antenna; 2, leg; 3, head; 4, integument; 5, paraterga; 6, fat body; 7, gut; 8, hemolymph. (*C*) *ChuaHNL* transcript and ChuaHNL were specifically accumulated in the paraterga. The arrowhead and arrow indicate the storage chamber and the reaction chamber, respectively. (*D*) Immunohistochemical localization. ChuaHNL was transferred through a duct of the reaction chamber and released from the ozopore. (Scale bar, 200 μm.)

Table 2. Steady-state kinetic parameters of ChuaHNL for aldehydes in the synthesis reaction of cyanohydrins

Substrate	Structure	K_m, mM	k_{cat}, s^{-1}	k_{cat}/K_m, $mM^{-1} \cdot s^{-1}$	V_{max}, μmol·min^{-1}·mg^{-1}
Benzaldehyde		3.0 ± 0.2	3,390 ± 71.0	1,100	8,186 ± 171
4-Bromo benzaldehyde	Br	10.0 ± 0.7	8,610 ± 190	860	20,778 ± 470
3-Methyl benzaldehyde		10.5 ± 2.5	6,890 ± 620	660	16,639 ± 1,491
4-Methyl benzaldehyde		5.4 ± 0.9	5,520 ± 250	1,000	13,338 ± 611
3-Methoxy benzaldehyde		7.7 ± 1.3	1,680 ± 87	220	4,060 ± 211
4-Methoxy benzaldehyde		7.5 ± 1.1	3,480 ± 15	460	8,394 ± 373
2,4-Dimethyl benzaldehyde		1.3 ± 0.3	300 ± 16	230	730 ± 38
2-Thiophene carboxaldehyde	S	18.0 ± 2.1	1,490 ± 85	83	3,605 ± 204
4-Biphenyl carboxaldehyde		10.3 ± 2.1	49.0 ± 3.5	4.7	118.7 ± 8.4

ChuaHNL (2.5 U) was used for the reactions. Values are the mean ± SD; $n = 3$.

a "factor E" involving the decomposition of mandelonitrile to benzaldehyde and hydrogen cyanide is thought to be stored in a smaller compartment of a gland (20). The factor E in the millipede seems likely to be an HNL, much like the one characterized in this study.

Substrate Specificity and Kinetic Studies in the Synthesis of Cyanohydrins Using ChuaHNL. Because millipedes exude defensive secretions of aromatic compounds, such as mandelonitrile and benzaldehyde (19), millipede HNLs seem likely to have substrate specificity toward aromatic compounds. Thus, we sought to synthesize cyanohydrins using ChuaHNL in a citrate buffer, using the aromatic aldehydes in our chemical library (26, 27) as starting materials, and evaluated the chiral configuration and ee, which are important indices for fine chemical syntheses. ChuaHNL catalyzed the synthesis of cyanohydrins from monosubstituted, disubstituted, biphenyl, heteroaromatic, and bicyclic aldehydes with an almost exclusive *R* configuration and an ee range of 7.5–90% (Table S2). Overall, ChuaHNL displayed wide substrate specificity in cyanohydrin synthesis in an aqueous system at a mild temperature (22 °C) and was shown to be a potent biocatalyst. Although other HNLs from almond or Japanese apricot showed a wide substrate spectrum, they required biphasic systems containing organic solvents (13, 26). Catalyzing reactions in aqueous medium (citrate buffer in this research) is closer to a green chemistry concept (28). ChuaHNL catalyzes the synthesis of many cyanohydrins including mandelonitrile, an intermediate in the synthesis of several chemicals such as (*S*)-amphetamines (nervous system drug) (29) and (*R*)-mandelic acid (anti-bacterial agent) (30). *para*-Methoxybenzaldehyde cyanohydrin has applications as a synthetic intermediate (e.g., in natural hydroxyl amides with insecticidal and adrenaline activity) and it is present in the structure of (−)-tempamide, an antiemetic, and (−)-denopamine, a cardiac drug used in the treatment of congestive heart failure and angina (4). ChuaHNL synthesizes 4-bromomandelonitrile (and some other cyanohydrins of benzaldehyde) as precursors of 2,3-disubstituted transaziridines (naturally occurring mitosanes). The aziridine ring is essential for antitumor activity (31). Based on the preliminary screening (Table S2), we selected the substrates with high ee and determined steady-state kinetic parameters for the synthesis of cyanohydrins. The Michaelis–Menten constants (K_m) for benzaldehyde, 4-bromobenzaldehyde, 3-methylbenzaldehyde, 4-methylbenzaldehyde, 3-methoxybenzaldehyde, 4-methoxybenzaldehyde, 2,4-dimethylbenzaldehyde, 2-thiophenecarboxaldehyde, and 4-biphenylcarboxaldehyde are in the range of 3.0–18 mM. In contrast, the catalytic efficiency (k_{cat}/K_m) of these compounds is in the wider range of 4.7–1,100 $mM^{-1} \cdot s^{-1}$ (Table 2).

The highest catalytic efficiency belongs to mandelonitrile synthesis at 22 °C. The enzyme not only accepts benzaldehyde and its monosubstitutes but also exhibits remarkable activity toward bulkier substrates such as 2-thiophene carboxaldehyde. ChuaHNL exhibited a decreased k_{cat}/K_m toward bulkier substrates possessing an additional ring, such as 4-biphenylcarboxaldehyde, which has a flexible, nonplanar structure and exhibits a low k_{cat}. The enzyme showed as high a catalytic efficiency in the synthesis of the sterically demanding 4-methylbenzaldehyde cyanohydrin as in mandelonitrile synthesis. More structure–function relationships will emerge upon solving the crystal structure of this glycoprotein.

To summarize, ChuaHNL can broadly catalyze the condensation of various aromatic aldehydes with potassium cyanide in the synthesis of cyanohydrins.

Unique Amino Acid Sequence of ChuaHNL. Information regarding the cDNA sequence is indispensable to obtain an unlimited recombinant protein for industrial uses. Based on the N-terminal and internal amino acid sequences determined from the purified ChuaHNL, we designed primers and applied RACE. The cDNA sequence of 783 bp encoded 183 aa residues, including a 21-aa-long signal peptide. The deduced protein included the three previously determined amino acid sequences, indicating that the cloned cDNA encodes ChuaHNL. The calculated molecular mass and pI were 18,225 Da and 4.97, respectively. The protein had one predicted *O*-glycosylation site at position 2 and three predicted *N*-glycosylation sites at positions 99, 109, and 123 (Fig. S2). This prediction is in agreement with the results of the PAS staining (Fig. S1*B*), suggesting that ChuaHNL most likely consists of 27% oligosaccharides. It is noteworthy that ChuaHNL shares no amino acid sequence identity with any of the HNLs previously reported in the blastp search. The deduced protein comprised two α-helices and eight β-structures. The ChuaHNL sequence contains no FAD-binding domain (Fig. S3*A*). This information is in agreement with the UV-visible (UV-Vis) spectrum of the purified millipede HNL at the range of 200–600 nm (Fig. S4*A*), distinguishing ChuaHNL from the HNLs of *P. amygdalus* (almond) (FAD-dependent oxidoreductase type) and *Manihot esculenta* (α/β-hydrolase type) (refs. 32 and 33 and Fig. S3 *B* and *C*). The CD spectra of ChuaHNL (Fig. S4*B*) suggests that the enzyme contains little α-helix (222 nm) and more random structures, based on a decrease in mean residue ellipticity at 200–210 nm (34). CD spectral analysis using K2D2 (35) also suggests 10.3% and 30.7% for α-helices and β-strands, respectively. DichroWeb (36) predicted 9.1% α-helices, 26% β-strands, 7% turns, and 58% unordered structures.

An Excellent EE of the ChuaHNL in the Synthesis of Mandelonitrile in Aqueous Medium. Industrial chemistry requires chirally pure chemicals not only to allow simple synthetic steps without additional separation but also to decrease the amount of hazardous waste. Therefore, we examined the (*R*)-mandelonitrile synthesis from benzaldehyde and cyanide as a "model reaction" using purified recombinant ChuaHNL from *Pichia pastoris* and evaluated the ee values under various experimental conditions without organic solvents. ChuaHNL (20 $U{\cdot}mL^{-1}$) catalyzed the synthesis of (*R*)-mandelonitrile with a maximum of 88.9% ee (Fig. 3*A*). It was reported that a low pH suppresses the spontaneous chemical reaction producing *racemic* mandelonitrile (9). Thus, we exposed 20 $U{\cdot}mL^{-1}$of ChuaHNL to benzaldehyde in various pH conditions. ChuaHNL still had enzymatic activity at a pH of 2.7, as presented in Fig. 1 *E* and *F*, and gave a maximum ee of 93.0% (Fig. 3*B*). Furthermore, we measured the amount of (*R*)-mandelonitrile produced by the enzyme (20–150 $U{\cdot}mL^{-1}$) in the same pH condition as in Fig. 3*B*. ChuaHNL (150 $U{\cdot}mL^{-1}$) gave a maximum ee of 99.0% in 5 min (Fig. 3*C*), which is sufficient for industrial use. We have shown that the enzyme catalyzes cyanohydrin synthesis with high efficiency and enantiopurity at room temperature (22 °C) and in a citrate buffer system, unlike other HNLs, which normally catalyze these reactions in biphasic systems containing organic solvents.

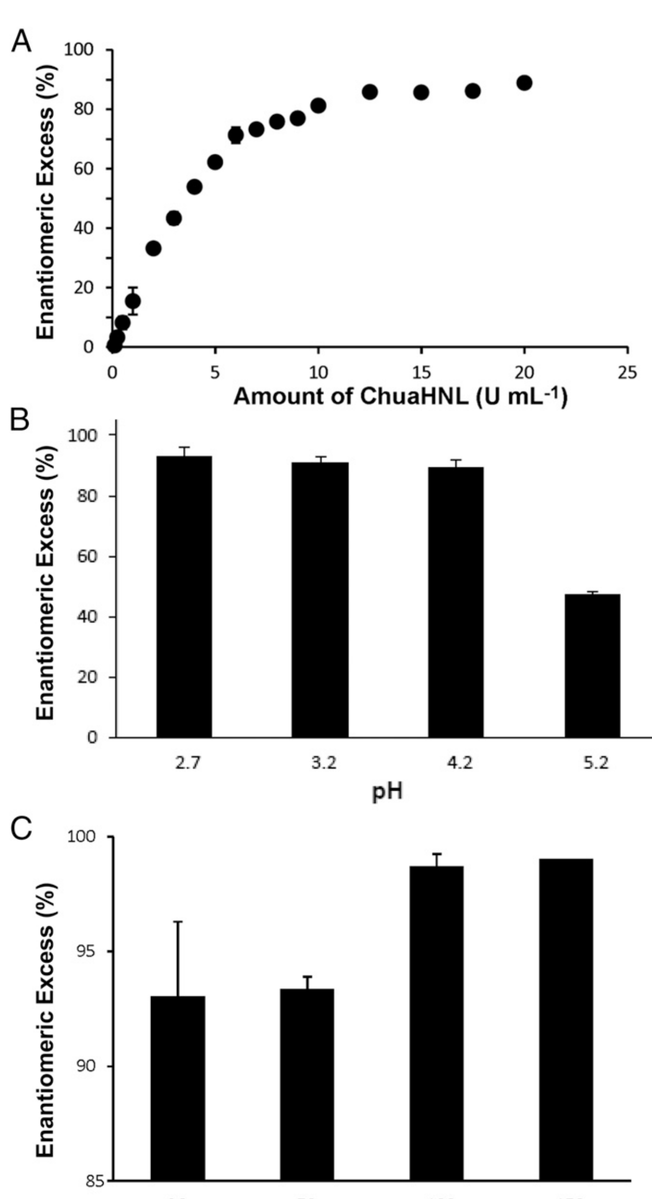

Fig. 3. (*R*)-Mandelonitrile synthesis with an excellent ee value using ChuaHNL in aqueous solution. (*A*) Relationship between the amount of ChuaHNL (0–20 $U{\cdot}mL^{-1}$) and ee (%). The reaction was performed in 400 mM citrate buffer, pH 4.2, at 22 °C for 5 min. (*B*) Relationship between pH and ee. ChuaHNL (20 $U{\cdot}mL^{-1}$) was reacted with the 50 mM substrate benzaldehyde at 22 °C and at various pH values. (*C*) (*R*)-mandelonitrile synthesis under optimized conditions. ChuaHNL (20–150 $U{\cdot}mL^{-1}$) was reacted with substrate in 400 mM citrate buffer, pH 2.7.

Conclusion

ChuaHNL is derived from an arthropod. Because the global arthropod species richness is at least 5 million species (37), the animal kingdom is likely to be the next bioresource for desired enzymes. This millipede HNL, which shows no homology with reported HNLs, exhibits high temperature and pH stability and wide substrate specificity toward aromatic carbonyl aldehydes, and it enables the production of enantioselectively pure mandelonitrile. In addition, in the synthesis of (*R*)-mandelonitrile from benzaldehyde and

potassium cyanide, the enzyme shows a fivefold higher specific activity compared with PaHNL (the already industrialized and highly active almond enzyme), effectively saving materials and energy previously needed for the reaction. In HNL research, cycles of enzyme identification, physicochemical property characterization, and rational protein engineering have been repeated for the sustainable development of industrial biotechnology for a century (38). In this respect, the discovery of millipede HNL can serve as a template for the isolation of promising and efficient enzymes and the design of tailor-made enzymes by rational protein engineering, which are needed to spur the industrial synthesis of fine chemicals and pharmaceuticals in the next generation.

Materials and Methods

Materials and methods are described completely in *SI Materials and Methods*.

Nucleotide Sequence Accession Number. The nucleotide sequence data of ChuaHNL cDNA have been deposited in the DNA Data Bank of Japan (accession no. LC004755).

Description of *SI Materials and Methods*. The materials used in this study and the methods of millipede collection, enzyme assays, enzyme purification, protein sequencing, detection of sugar molecules in the HNL, UV-Vis, and CD spectra of the purified millipede HNL, cDNA cloning, recombinant gene expression, tissue collection, RT-PCR, Western-blot analysis, immunohistochemistry, and NMR data for the synthesized cyanohydrins are described in *SI Materials and Methods*.

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