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Deracemization and Stereoinversion to Aromatic D-Amino Acid Derivatives with Ancestral L-Amino Acid Oxidase

Shogo Nakano[a,] *, Yuki Minamino[a], Fumihito Hasebe and Sohei Ito*

Graduate School of Integrated Pharmaceutical and Nutritional Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan

Correspondence: *snakano@u-shizuoka-ken.ac.jp., *itosohei@u-shizuoka-ken.ac.jp

[a]*These authors contributed equally to this work.*

ABSTRACT

Enantiomerically pure amino acid derivatives could be foundational compounds for peptide drugs. Deracemization to L-amino acid derivatives from racemates can be achieved through the reaction of evolved D-amino acid oxidase (DAAO) and chemical reductants, whereas deracemization to D-amino acid derivatives has not progressed due to the difficulty associated with heterologous expression of L-amino acid oxidase (LAAO). In this study, we succeeded in developing an ancestral LAAO (AncLAAO) bearing broad substrate selectivity (13 L-amino acids) and high productivity through an *E. coli* expression system (~50.7 mg/L). AncLAAO can be applied to perform deracemization to D-amino acids in a similar way to deracemization to L-amino acids. In fact, full conversion (>99% ee, D-form) could be achieved for 16 racemates, including nine D,L-Phe derivatives, seven D,L-Trp derivatives, and a D,L-phenylglycine. Taken together, we believe that AncLAAO could be a key enzyme to obtain optically pure D-amino acid derivatives in the future.

Keywords: L-amino acid oxidase, deracemization, D-amino acid derivatives, ancestral sequence reconstruction, artificial protein

INTRODUCTION

Enantiomerically pure compounds could potentially be precursors in the synthesis of commercial products such as pesticides, pharmaceuticals, and antibiotics [1]. Dynamic kinetic resolution (DKR) and deracemization has previously been applied to enantiomerically pure compounds to obtain a theoretical 100% yield of chiral compounds from racemates [1]. To date, several enzymes have been used in this process as they are superior to conventional chemical catalysts. These enzymes are eco-friendly and can synthesize enantiomerically pure compounds under standard conditions [2-6]. The application of these enzymes requires that they be optimized in order to be suitable for use. Optimization of productivity, solubility, thermostability, reactivity, and broadening of substrate selectivity is required. Many protein engineering methods have been developed to perform this optimization and to enhance enzymatic functions. These methods can be categorized as sequence-based methods [7-9] and structure-based methods [10].

Deracemization to chiral amino acids from D,L-mixtures is a popular area of research at this time due to its potential application to commercial products [11]. Currently, many biocatalytic methods used to obtain chiral amino acids have been reported including: i) conversion of α-keto acid to D-amino acids utilizing engineered D-amino acid dehydrogenase and glucose dehydrogenase [12], ii) synthesis of L-amino acids with amination of cinnamic acid catalyzed by phenylalanine ammonia lyase [13, 14], iii) the hydantoinase process which utilizes racemase/hydantoinase/carbamoylase [15, 16], iv) *N*-acetyl amino acid

racemase/acylase biotransformation [17], v) transamination of α-keto acid [18], and vi) deracemization of racemic amino acids utilizing a combination of deaminase and oxidase and chemical reductants [19, 20]. L-amino acids can be obtained by chemoenzymatic reactions catalyzed by D-amino acid oxidase (DAAO) and chemical reductants (NH_3:BH_3, $NaBH_4$, etc.) [19, 21]. DAAO oxidizes the main chain amide group of D-amino acids [22] and produces imino acid, and this product is continuously reduced to the D,L-mixture by the reductant. Only L-amino acids are accumulated by repeating this process. In addition, DAAO generates H_2O_2 as a by-product. This H_2O_2 can be detected by the Trinder reaction. This creates a simple mode of detection to assess whether substrate candidates are oxidized by evolved DAAO produced by directed evolution and rational design [2, 3]. As an example, *R*-selective amine oxidase was designed from DAAO using a directed evolution approach; the evolved DAAO (Y228L/R283G mutant) bearing amine oxidase activity was screened by detecting the generation of H_2O_2 through the addition of (*R*)-phenylethylamine [23, 24].

Theoretically, deracemization to D-amino acids from D,L-racemates could be achieved by replacing DAAO with an L-amino acid oxidase (LAAO) exhibiting broad substrate selectivity. LAAO would catalyze the oxidation of L-amino acids through a reaction mechanism similar to that of DAAO [25]. However, the deracemization to D-amino acids has been slow to progress due to the difficulty associated with heterologous overexpression of LAAOs in prokaryotic hosts, such as *E. coli*. These difficulties have been recognized by many research groups [2, 25]. As an alternative to LAAOs, L-amino acid deaminases (LAADs) could be applied in the

deracemization to D-amino acids [11, 20, 26]. However, unlike LAAOs, LAADs require an exogenous electron transfer system to oxidize the $FADH_2$ generated by the reaction. Thus, the reaction is often carried out using an entire cell expressing LAAD or through the addition of an artificial electron acceptor [2, 27]. Furthermore, LAADs generate very little H_2O_2 [11], indicating that the approach which is utilized in the design of evolved DAAOs cannot be applied to the design of LAADs. LAAOs can complement these points, and therefore we believe that the development and design of novel LAAOs will contribute to the ability to perform deracemization of D,L-amino acids to D-amino acids.

RESULTS & DISCUSSION

To find novel LAAOs, we focused on our previous study that followed the evolutionary track of orphan L-arginine oxidase (AROD) through the design and functional analysis of ancestral AROD (AncAROD) [28]. This study suggested that native AROD from *Oceanobacter kriegii* (OkAROD) exhibits high specificity toward L-Arg, whereas AncAROD acquired broad substrate selectivity towards 5 L-amino acids. Here,

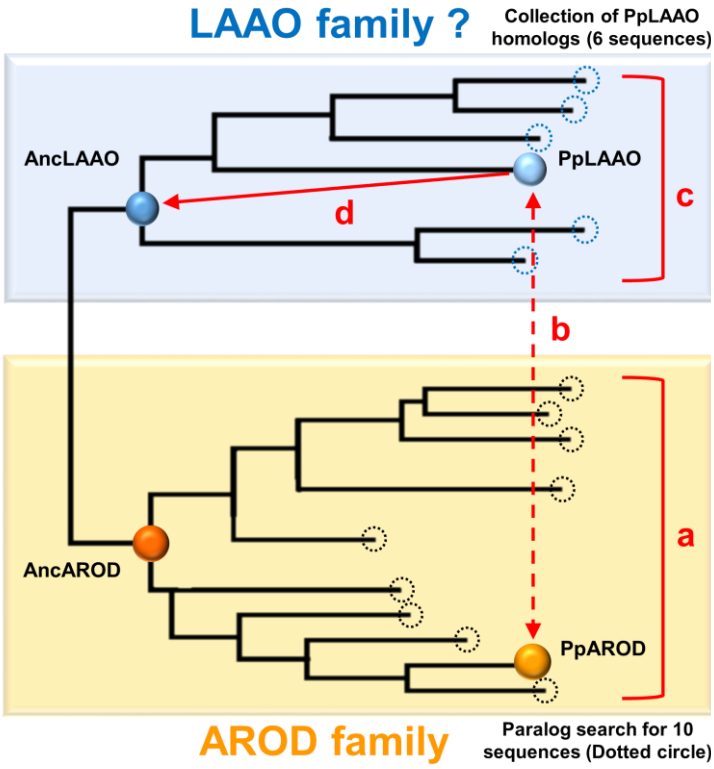


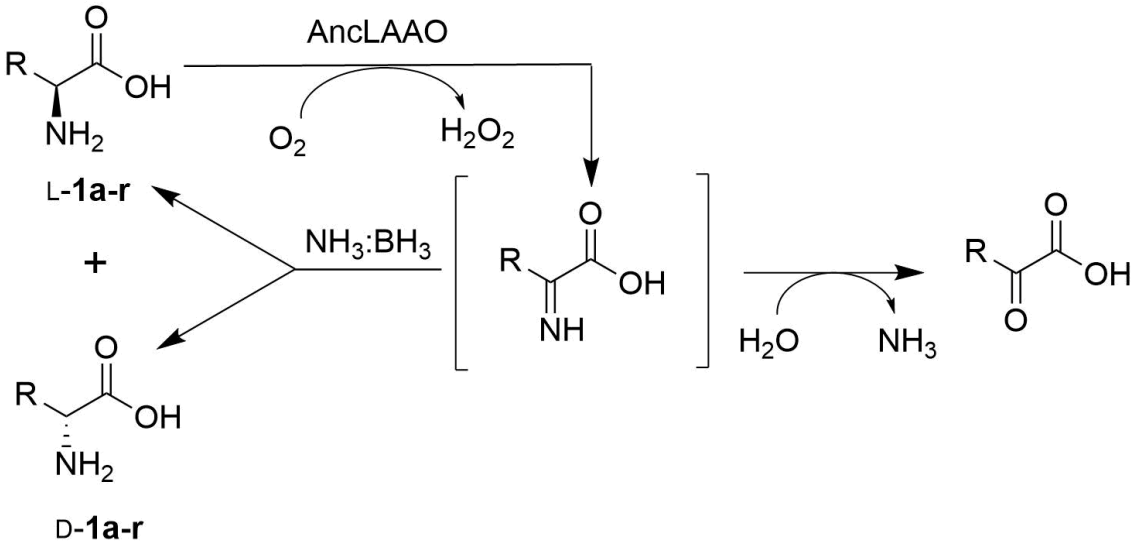
FIGURE 1. Schematic model showing how to design AncLAAO from AncAROD. The design was performed using the following procedure: paralog search (a), finding PpLAAO sequence (b), collection of six LAAO candidate sequences (Table S1) including PpLAAO (c), and design of AncLAAO (Table S2) with ASR (d).

sequence identity between AncAROD and OkAROD was about 60% [28], suggesting that a 40% displacement of the native AROD sequence could broaden the selectivity. Based on these results, we hypothesized that a paralogous gene of the native AROD may have different selectivity in a similar way to AncAROD. To prove this point, we attempted to find new paralogs of AROD via the procedure described in Figure 1. A total of 10 AROD candidates were assigned in the previous study and homology searches were performed for all of these AROD candidates in their respective genomes (**a** in Figure 1). A paralog

sequence bearing moderate sequence similarity to AROD from *Pseudoalteromonas piscicida* (PpAROD, NCBI Reference Number: WP_045988413.1) was found only in a genus of *Pseudoalteromonas* (**b** in Figure 1); the E-value calculated by Blastp was < $1.0E^{-25}$. The existence of LAAOs in this genus has been implicated in previous studies [25, 29]. Based on these analyses, we predicted that the paralog had the potential to oxidize certain L-amino acids, and it was therefore named PpLAAO. However, we did not confirm LAAO activity of PpLAAO at this time for multiple reasons including lower expression and solubility, and inappropriate usage of the expression host. Give these restrictions, we chose to perform experiments utilizing homologous sequences of PpLAAO. Ancestral sequence reconstruction (ASR) is one of the options that could be used to obtain the homologous sequences. To undertake this task, we attempted to redesign the sequence using ASR as many groups have reported that protein functions such as solubility and productivity can be improved through ASR without losing native activity [30-32]. After collecting sequences of six LAAO candidates bearing >60% genetic resemblance to PpLAAO (**c** in Figure 1), ancestral LAAO (AncLAAO) was designed by following the procedure described in the Materials & Methods (**d** in Figure 1). Here, no enzymatic functions of the other five LAAO candidates (Table S1) are estimated by experimental approaches, and only sequence data are currently available. Multiple sequence alignments amongst the six AROD, AncLAAO, and AncAROD sequences suggested that AncLAAO shares 27% sequence identity with AncAROD (Figure S1). Phylogenetic analysis indicated that AncLAAO was positioned on the ancestral node of

the LAAO family (Figure S2). This approach can clearly achieve an improvement in overexpression of LAAO. Up to 50 mg (672 units) of AncLAAO per 1 L simple batch cultivation can be obtained after purification with Ni affinity chromatography utilizing the BL21(DE3) expression system (Table S3).

Enzymatic properties of AncLAAO were characterized by biochemical analysis, and the results indicated that AncLAAO is an FAD-dependent LAAO bearing broad substrate selectivity. All of the enzymatic reactions were performed at a fixed oxygen concentration. Thus, the kinetic values obtained in this study are all apparent values. Analysis of the UV-Vis spectrum showed that two characteristic peaks derived from oxidized FAD (390 and 450



Scheme 1. Deracemization reaction cascades to obtain enantiomerically pure D-**1**.

nm) could be confirmed for the purified AncLAAO sample, and the peak disappeared with the addition of the substrate L-Met (Figure S3A) to the sample. This disappearance occured because oxidized FAD converted to the reduced form with the progression of the reaction. The thermostability of AncLAAO is not high as the relative activity decreases to <50% on

incubation at 40 °C (Figure S3B). The loss of AncLAAO activity at 30 °C and 35 °C was negligible (Figure S3B). The optimal pH value was 7.0 (Figure S3C). Substrate selectivity was estimated by utilizing 20 L- and D-amino acids as substrates. The results indicated that AncLAAO can react with 13 L-amino acids that are evenly distributed between hydrophilic and hydrophobic residues, whereas there is no activity towards any D-amino acids (Table 1). Enzyme kinetic parameters of AncLAAO were calculated for the six L-amino acids (L-Gln, L-Met, L-Leu, L-Glu, L-Phe, and L-Trp) which exhibited the highest activity (Figure S4). The measured parameters indicated that the k_{cat} values ranged from 6.0 to 33.0/s (Table 2). These values were comparable to the ones derived from other LAAOs. The k_{cat} and k_{cat}/K_m values of L-Met were the highest in the tested L-amino acids (Table 2).

As discussed above, a large amount of AncLAAO can be obtained to perform deracemization to D-amino acids from D,L-racemates. Thus, deracemization was performed by using the combination of AncLAAO and the chemical reductant, NH_3:BH_3 (scheme 1). Firstly, stereoinversion of two L-amino acids, L-Phe (L-1a) and L-Trp (L-1k), to D-amino acids was performed. Chiral HPLC analysis indicated that both L-1a (red line, Figure 2A) and L-1k (Figure 2B) can be completely converted into a D-enantiomer (> 99% ee) with the addition

of 0.6 mg of AncLAAO (blue line in Figure 2A, B). The time course of D-**1a** and D-**1b** production from the D,L-mixture was monitored as shown in Figure 2C and 2D, respectively. The results suggest that both D,L-mixtures are completely converted into D-amino acids and the times required to complete their conversions are dependent on the compounds and the addition of 0.6 mg of AncLAAO. In fact,

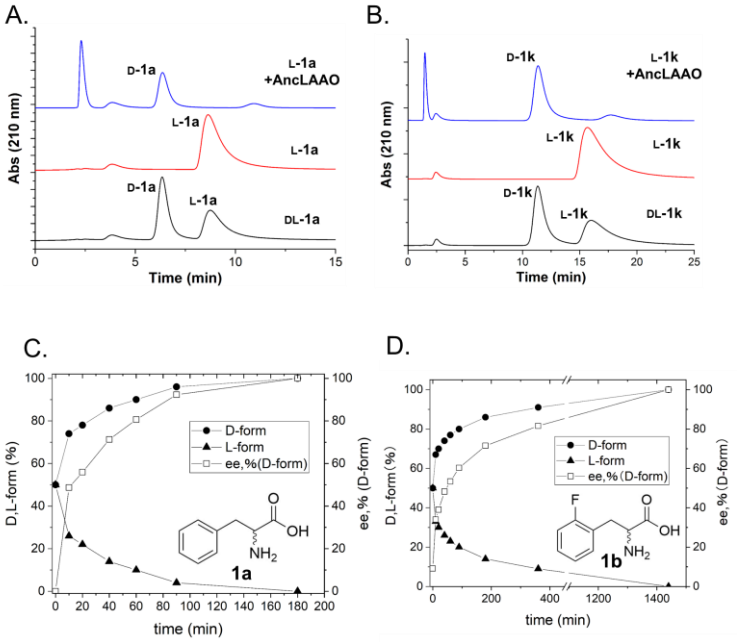


FIGURE 2. Chiral HPLC chromatograms of compounds before (red line) and following stereoinversion (blue) by AncLAAO (A, B). The stereoinversions for L-1a and L-1k are represented in A and B, respectively. In both cases, HPLC chromatograms were collected for racemates (black line). Through the addition of AncLAAO to L-1a and 1k (red line), the peak for the L-enantiomer disappeared, whereas the peak for the D-enantiomer appeared after the stereoinversion reaction (blue line). **Time course of D-1a (C) and D-1b (D) production from D,L-mixture by deracemization reaction.**

D,L-**1a** completely converted within 3 h (Figure 2C), whereas the conversion of D,L-**1b** required approximately 24 h (Figure 2D). AncLAAO was stable during the deracemization process in spite of its low thermostability.

The deracemization reaction was applied to a total of 18 D,L-amino acid derivatives (Figure 3). Chiral HPLC chromatograms for each compound are represented in Figure S5. For D,L-Phe derivatives (D,L-**1a** to **1j**), 9 of 10 D,L-mixtures were completely converted to D-enantiomers (>99% ee) within 24 h. Specific activity of D,L-**1g** (0.33 U/mg, Figure 3) was

more than one order lower than those of other D,L-Phe derivatives; and this may be one of the reasons for the incomplete deracemization in this instance. For D,L-Trp derivatives (D,L-**1k** to **1q**), six of the seven tested D,L-mixtures were completely converted to D-enantiomers (> 99% ee). Specific activity ranged from 0.63 to 12.6 U/mg, and the addition of the chemical group at the 7‘ position of the indole ring appeared to decrease the activity. In fact, the activity of D,L-**1o** (0.63 U/mg) was approximately one order lower than that of D,L-**1k** (6.4 U/mg, Figure 3). D,L-phenylglycine (D,L-**1r**) was also completely converted to the D-enantiomer despite lower specific activity (0.21 U/mg). This is remarkable for AncLAAO because the activities

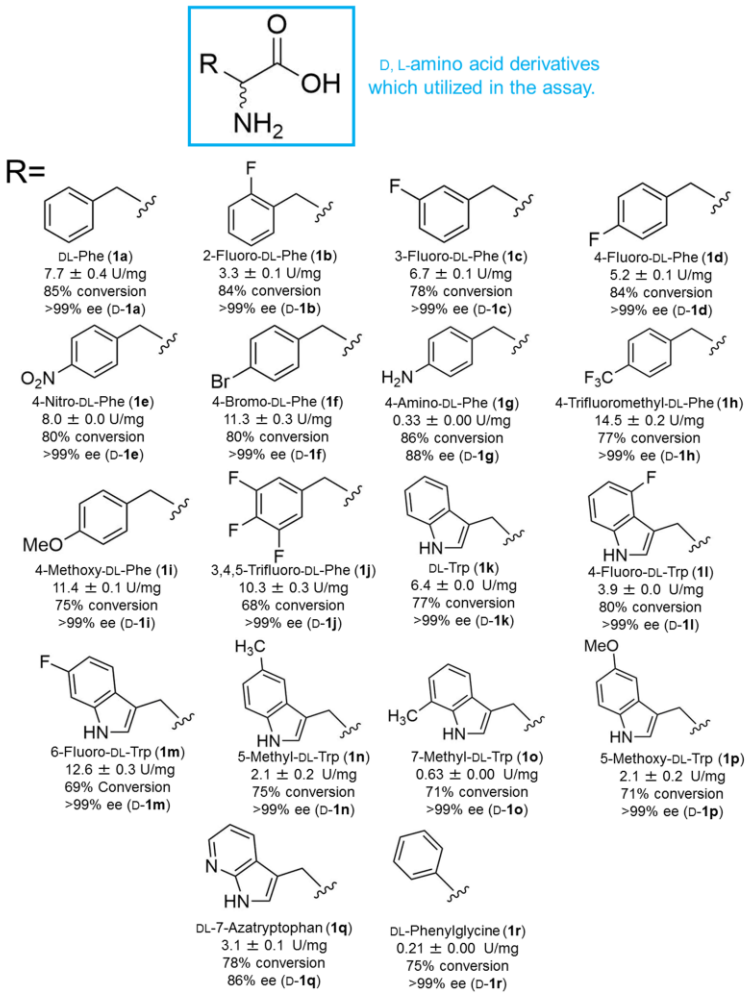


FIGURE 3. Deracemization to D-1a-r amino acid derivatives from D,L-mixtures. The “R” in the figure represents amino acid side chain. Reaction conditions for the deracemization are as follows: 100 mM KPB (pH 8.0), 5 mM substrate, 150 mM NH_3:BH_3, 200 U catalase (only for **1g-1r**), 10 U (**1a-1q**), or 20 U (**1r**) AncLAAO, 30 °C, 150 rpm. Conversion rate and enantio excess were detemined as per the equation in the supporting information. Chromatography conditions and retention time for the compounds are listed in Table S4. Summarizing the results, 6.9-8.6 μM of the products, D-amino acid derivatives, were produced in 24 h by the addition of 0.6 mg (D-**1a-q**) and 1.1 mg (D-**1r**) purified AncLAAO.

of the reported LAADs toward D,L-**1r** were too weak to apply to the deracemization process [20].

In order to prove that AncLAAO can be applied to deracemize aromatic D,L-amino acids to D-amino acid derivatives at preparative scale, the following five D,L-amino acid derivatives were deracemized following the methods of the previous study [20]: D,L-2-fluoro phenylalanine (183 mg, 10 mM, 100 mL), D,L-3-fluoro phenylalanine (183 mg, 10 mM, 100 mL), D,L-4-nitro phenylalanine (210 mg, 10 mM, 100 mL), D,L-3,4,5-trifluoro phenylalanine (219 mg, 10 mM, 100 mL), and D,L-phenylglycine (151 mg, 10 mM, 100 mL). In the presence of 150 mM NH_3:BH_3, a total of 7 mg of AncLAAO was utilized to deracemize four D,L-phenylalanine derivatives, whereas a total of 70 mg of AncLAAO was utilized to deracemize D,L-phenylglycine. Enantiopure D-forms (> 99% ee) were obtained for the four phenylalanine derivatives after a 24 h-reaction time (Figure S6A-D), whereas the D-form of phenylglycine was obtained at 84% ee after 48 h (Figure S6E). This indicated that improvement of AncLAAO reactivity toward L-phenylglycine is required to obtain enantiopure D-phenylglycine at preparative scale. The conversion to the D-forms was evaluated by HPLC and LC-HRMS analysis (Figure S6).

Taken together, we succeeded in the design of AncLAAO, which is a notable development given its broad substrate selectivity and higher productivity in the *E. coli* expression system (~50.7 mg/L). Phylogenetic tree analysis for homologs of AncLAAO in the genus of *Pseudoalteromonas* indicated that AncLAAO is located near the ancestor of three gene

clusters (red-filled circle in blue-squared region, Figure S7). AncLAAO can be applied to deracemize several aromatic D,L-amino acids to D-amino acids at preparative scale as well as previously reported enzymes [20]. LAAOs from several fungi have been used to attempt expression in the prokaryotic system, however, the expression levels of native LAAOs are quite low and cleavage of the MBP tag and SDS treatment were required for their activation [33, 34]. In contrast, no protein fusion tag or activation process was required for AncLAAO, which makes it highly preferable. Recently, several groups have synthesized enantiopure D-amino acids using the multienzyme reaction system, and LAADs and specific LAAOs were incorporated into this system [35, 36]. LAADs are especially favorable to synthesize enantiopure D-tryptophan derivatives at preparative scale [35]. Along with these enzymes, we believe that in the future, AncLAAO could be an important contributing component in the process of deracemization to D-amino acids.

Materials & Methods

Reconstruction of ancestral L-amino acid oxidase

After the assignment of paralog sequences of L-arginine oxidase from *Pseudoalteromonas piscicida* (PpAROD), we selected the six homolog sequences of the paralog (listed in Table S1). With reference to the selection procedure indicated in a previous study [28], we selected the sequences bearing >60% identity to each other. The sequences were aligned with MAFFT software [37]; the aligned sequences were represented in Figure S1. The aligned sequences were analyzed by MEGA6 [38], and the phylogenetic tree was generated by the maximum-likelihood method. The aligned sequences and phylogenetic data were submitted to the FastML web server [39]; JTT empirical model was adopted for the analysis. Finally, we were able to obtain one ancestral L-amino acid oxidase (AncLAAO); the sequence is indicated in Table S2. Posterior probabilities (PPs) would be a criterion for whether the designed ancestral sequence provides a plausible estimation of ancestral proteins [31, 40]. The average PPs for AncLAAO was 0.92, suggesting that the value was not inferior to other ancestral proteins [31, 40].

Overexpression and purification of AncLAAO

Genes encoding AncLAAO were synthesized and cloned into the pET28a vector via the *Nco*I and *Xho*I sites by ordering in GeneWiz. The plasmids were transformed into the BL21(DE3) strain. The strain was cultivated in 1 L of LB broth at 37 ºC. The temperature

was decreased to 23 ºC when the OD_{600} reached to 0.5–0.8, and then IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.5 mM. After the cells were grown overnight, they were harvested by centrifugation. The cells were suspended in buffer A (20 mM Tris-HCl [8.0] and 10 mM NaCl). After sonication of the cells, the supernatant was collected by centrifugation at 11000 *g* for 35 min. The supernatant was applied to the HisTrap HP column (GE Healthcare, Uppsala, Sweden), and the column was washed with 30 mL of buffer A containing 10 mM imidazole. The samples were eluted utilizing 15 mL of buffer A containing 70 mM and 300 mM imidazole, and the latter samples were utilized for further purification. The eluted samples were concentrated and applied to the Superdex 200 Increase column (GE Healthcare, Uppsala, Sweden), equilibrated by buffer A. AncLAAO purity was determined by SDS-PAGE. The production of AncLAAO in the *E. coli* expression system was quantified by measuring specific activity of the cell-free extract and sample towards L-Met, which applied to the His-tagged purification system (Table S3). The purified AncLAAO was utilized in subsequent biochemical assays.

Estimation of thermal stability and pH dependency of AncLAAO

The thermal stability of AncLAAO was determined as follows. The enzyme solution was incubated at 30–80 ºC for 10 min without substrate. The heat-treatment samples were moved to an ice-bath. The remaining activity was measured by applying a procedure identical to that of the measurement of substrate specificity (Figure S3B). The pH

dependency of AncLAAO was determined under the same conditions, except that sodium acetate (pH 3.5 to 5.5), bis-Tris HCl (pH 6.0 to 7.0), HEPES-NaOH (pH 7.5 to 8.0), and CAPSO (pH 9.7 to 10.5) were used as buffers (Figure S3C).

Estimation of substrate selectivity and kinetic parameters for AncLAAO

AncLAAO activity was measured by quantifying the amount of H_2O_2 produced by enzymatic reaction using a color-developed method. The assay buffer was composed of the following reagents: 1.5 mM aminoantipyrine, 2 mM phenol, 50 U/mL horseradish peroxidase, and 100 mM bis-Tris HCl (pH 7.0). Specific activity of AncLAAO was measured by adding 10 mM of substrate (20 L-amino acids and 20 D-amino acids) and a purified AncLAAO sample to the assay buffer. The initial velocity of AncLAAO was calculated by monitoring the time-dependent absorption change at 505 nm which was derived from *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)aniline bearing ε_{505} = 12,700 M^{-1} cm^{-1} with a UV-Vis spectrometer (UV-2450, Shimazu). The relative activity of AncLAAO towards 20 L-amino acids was calculated relative to the activity of L-Met (Table 1).

The kinetic parameters for six L-amino acids (L-Met, L-Glu, L-Phe, L-Leu, L-Trp, and L-Gln) were measured by utilizing the following substrate concentrations: 0.1–10 mM L-Met (Figure S4A), 0.3–10 mM L-Leu (Figure S4B), 0.1–10 mM L-Phe (Figure S4C), 0.5–15 mM L-Trp (Figure S4D), 0.1–10 mM L-Gln (Figure S4E), and 0.5–13 mM L-Glu (Figure S4F). The procedure to determine the initial velocity was identical to that for the measurement of

substrate specificity. The parameters were estimated by fitting the initial velocity to the Michaelis-Menten equation and by applying the non-linear least-squares method (Table 2).

Deracemization and stereoinversion of D,L-amino acid derivatives to D-amino acids

Deracemization of D,L-amino acids (D,L-1a to 1r) was performed by the following procedure. We first prepared the reaction buffers composed of 100 mM KPB (pH 8.0), 150 mM NH_3:BH_3, 200 U/mL Catalase (only for the case of D,L-**1g** to **1r**), and 5 mM substrates (D,L-**1a** to **1r**). The deracemization reaction was started by adding 0.6 mg (D,L-**1a** to **1q,** 10 units) or 1.1 mg (D,L-**1r**, 20 units) of purified AncLAAO. The reactions progressed for 20 h at 30 ºC to determine the conversion rate and ee (%) value. After the reaction was complete, 80 μL of the reaction mixture were quenched by adding 720 μL of 1.15% (w/v) $HClO_4$ solution; the supernatant was applied to subsequent HPLC analyses. Stereoinversion of L-**1a** (Figure 2a) and L-**1k** (Figure 2b) was performed by applying the identical procedure.

The time course of deracemization of D,L-**1a** (Figure 2c) and D,L-**1b** (Figure 2d) was determined by sampling 100 μL of the reaction mixture after incubation for 0, 10, 20, 40, 60, 90, 180, and 360 min, and 24 h. In the measurement, the amount of AncLAAO was changed to 5 U/mL and other components of the reaction buffer were identical to that of the deracemization condition. The mixture was incubated at 90 ºC for 10 min to stop the reaction, and a total 40 μL of the mixture was dissolved by adding 360 μL of 1.15% (w/v) $HClO_4$ solution; the supernatant was applied to HPLC analyses.

HPLC analyses

Reverse phase HPLC analyses were performed on the Shimadzu apparatus (Prominence) equipped with a UV-Vis detector (SPD-20AV, Shimadzu) and CROWNPAK CR-I(+) column, length/internal diameter = 150/3.0 mm (DAICEL, Osaka, Japan). Elution of the compounds was monitored by detecting UV changes at 210 nm. Mobile phase condition was 1.15% (w/v) $HClO_4$, and column temperature was set to 30 ºC. Other conditions, such as retention time and flow rate for each of the compounds (1a-1r), are summarized in Table S4. HPLC chromatograms for all of the compounds are shown in Figure S5. Conversion rates and enantiomer excesses (ee(%)) were calculated by the following equation:

$$ee\ (\%) = {[(D_{area}) - (L_{area})]}\Big/{[(D_{area}) + (L_{area})]} \times 100 \quad \text{(After reaction)}$$

$$\text{Conversion rate}\ (\%) = {(D_{area})_{after\ reaction}}\Big/{[(D_{area}) + (L_{area})]_{before\ reaction}}$$

Here, D_{area} and L_{area} represent the peak areas of HPLC corresponding to the D- and L-enantiomers, respectively.

Qualitative analysis of products by LC-HRMS

LC-HRMS analysis (Figure S6) was carried out using Q Exactive (Thermo Fisher Scientific, MA, USA), equipped with an electrospray ionization module. To detect the products by LC-HRMS, the following columns which are jointed into the LC-HRMS system were utilized: a

UPLC column (XBridge® BEH Amide XP column [length, 2.1 x 50 mm; inner diameter (i.d.), 2.5 μm; Nihon Waters K.K., Tokyo, Japan]) equipped with a guard column (XBridge® BEH Amide XP VanGuard cartridge [length, 2.1 x 5 mm; i.d., 2.5 μm; Nihon Waters K.K., Tokyo, Japan]). The column was maintained at 40°C. 5 mM ammonium formate in 90% acetonitrile (solvent A) and 5 mM ammonium formate in 50% acetonitrile (solvent B) were used as mobile phases for the gradient elution of products at a flow rate of 0.4 mL/min. Products were eluted as follows: 0% B for 1 min, 0–100% B over 4 min, 100% B for 2 min, and 0% B for 5 min.

Supporting Information Available

Supporting information includes the following figures: Multiple sequence alignment of homologous sequences of PpLAAO; Phylogenetic analysis of the AncLAAO, AncAROD, and six homologous sequences; UV-Vis spectrum changes, thermal stability, and optimal pH of AncLAAO; Enzyme kinetics of AncLAAO toward six L-amino acids; Chiral HPLC chromatograms of compounds from D,L-1a to 1r; LC-HRMS analysis of D-amino acid derivatives deracemized at preparative scale; and Phylogenetic analysis for paralogs of AncLAAO in genus of *Pseudoalteromonas*. The Supporting Information contains the following tables: Sequence lists used to design AncLAAO; Sequence of AncLAAO; Summary of purification of AncLAAO from 1 L cultivation; and LC condition and retention time for amino acid derivatives. This information is available free of charge on the ACS Publications website.

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TABLES

Table 1. Relative activity of AncLAAO towards 20 L-amino acids[a]

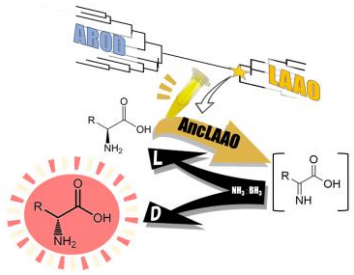
Substrate	Relative activity (%)
L-Glutamine	101.0±1.8
L-Methionine	100±2.1
L-Leucine	85.2±1.7
L-Glutamic Acid	65.9±1.4
L-Phenylalanine	52.9±0.3
L-Tryptophan	40.8±0.5
L-Tyrosine	25.8±1.1
L-Isoleucine	8.8±0.2
L-Arginine	5.5±0.2
L-Proline	1.5±0.2
L-Valine	1.3±0.4
L-Alanine	1.3±0.4
L-Lysine	0.8±0.6
L-Cysteine	< 0.0
L-Aspartic Acid	< 0.0
Glycine	< 0.0
L-Histidine	< 0.0
L-Asparagine	< 0.0
L-Serine	< 0.0
L-Threonine	< 0.0

[a] There is no activity toward any of the 20 D-amino acids.

Table 2. Enzyme kinetic parameters of AncLAAO towards L-Met, L-Leu, L-Phe, L-Trp, L-Gln, and L-Glu[a]

Compounds	k_{cat}	K_m	k_{cat}/K_m
	/s	mM	/(s·mM)
Hydrophobic amino acids			
L-Met	22.0 ± 1.1	0.4 ± 0.1	55.0
L-Leu	7.5 ± 0.5	0.6 ± 0.1	12.5
Aromatic amino acids			
L-Phe	12.6 ± 0.4	3.7 ± 0.3	3.4
L-Trp	6.4 ± 0.6	3.1 ± 0.6	2.1
Hydrophilic amino acids			
L-Gln	13.3 ± 0.2	1.5 ± 0.1	8.9
L-Glu	33.0 ± 3.5	11.5 ± 2.2	2.9

[a]The measurement of enzyme kinetic parameters was performed independently three times (n=3).



TOC Figure