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One-Pot Preparation of D-Amino Acids Through Biocatalytic Deracemization Using Alanine Dehydrogenase and ω-Transaminase

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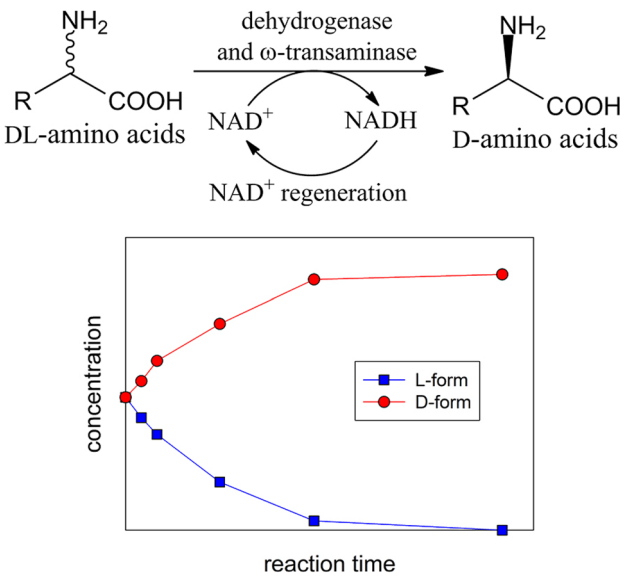
Abstract

D-Amino acids are pharmaceutically important building blocks, leading to a great deal of research efforts to develop cost-effective synthetic methods. Preparation of D-amino acids by deracemization has been conceptually attractive owing to facile synthesis of racemic amino acids by Strecker synthesis. Here, we demonstrated biocatalytic deracemization of aliphatic amino acids into D-enantiomers by running cascade reactions; (1) stereoinversion of L-amino acid to a D-form by amino acid dehydrogenase and ω-transaminase and (2) regeneration of NAD^+ by NADH oxidase. Under the cascade reaction conditions containing 100 mM isopropylamine and 1 mM NAD^+, complete deracemization of 100 mM DL-alanine was achieved after 24 h with 95% reaction yield of D-alanine (> 99% ee^D, 52% isolation yield).

Graphical Abstract



Keywords Cascade reaction · Deracemization · D-Amino acids · Alanine dehydrogenase · ω-Transaminase

1 Introduction

Twenty canonical amino acids possess chirality on their α-carbon, except glycine, and consequently exist in either of the two stereoisomers differing in optical rotation, i.e. levorotatory (L) and dextrorotatory (D). Although L-amino acids constitute building blocks of proteins and are major

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players in nature, D-amino acids also participate in a number of biological functions and therefore have been considered as one of the important target compounds in chemical industry [1–3]. Indeed, D-amino acids have gained growing attention in food, cosmetics and pharmaceutical industries [4, 5]. For example, D-alanine is an important building block of pharmaceutical drugs [6, 7] and an artificial sweetener [8].

In addition to chemocatalytic methods [9], several biocatalytic approaches to prepare D-amino acids have been developed [4, 10, 11]. D-Amino acids can be enzymatically synthesized from keto acids by reductive amination using D-amino acid dehydrogenase [12], D-amino acid transaminase [10, 13] and D-selective (or *R*-selective) ω-transaminase (ω-TA) [14–16]. However, high prices of the keto acid precursors have been a major drawback of the asymmetric synthesis approach. In contrast, kinetic resolution employing racemic amino acid substrates is often economically more feasible because the racemic amino acids can be readily prepared by Strecker synthesis [17]. Therefore, a number of kinetic resolution processes have been developed using acylase [18, 19], amidase [20], oxidase [21] and hydantoinase/carbamoylase [22]. A possible drawback of the kinetic resolution processes is that a theoretical maximum yield is only 50% unless an unwanted amino acid enantiomer is recycled by racemization. In this regard, deracemization is an attractive alternative since the maximum yield could reach 100% as well described in a review paper [23].

Recently, we reported deracemization of amino acids into either L or D-form using two enantiocomplementary transaminases [24]. In this method, mandatory use of an expensive keto acid cosubstrate (i.e., α-ketoglutarate) remained a problem. Here, we developed a new deracemization method for production of D-amino acids using L-alanine dehydrogenase (AlaDH), D-selective ω-TA and NADH oxidase (NOX). Unwanted L-amino acid in the racemic mixture is converted to a D-form after two consecutive reactions catalyzed by AlaDH and ω-TA, leading to complete deracemization or enantioenrichment depending on the substrate specificity of the two enzymes.

2 Materials and Methods

2.1 Chemicals

L-Alanine was obtained from Acros Organics (Geel, Belgium) and DL-alanine was purchased from Hayashi Pure Chemical Ind. (Osaka, Japan). Isopropylamine was purchased from Junsei Chemical Co. (Tokyo, Japan). All other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, Missouri, USA) and of the highest purity available.

2.2 Molecular Cloning

Genes encoding AlaDH and NOX were cloned into a pET28a(+) expression vector (Novagen) using an In-Fusion® HD Cloning Kit (Clontech) according to an instruction manual. The AlaDH gene was amplified by colony PCR of *Bacillus subtilis* str168 (KCTC2217, Korean Collection for Type Cultures, South Korea) using forward and reverse primers, 5′-AGGAGATATACCATG ATCATAGGGGTTCCTAAAG-3′ and 5′-GGTGGTGGT GCTCGAGAGCACCCGCCACAGATGA-3′, respectively. The NOX gene was amplified by colony PCR of *Lactobacillus brevis* (KCTC3498) using forward and reverse primers, 5′-TAAGAAGGAGATATACCATGAAAGTCA CAGTTGTTGGT-3′ and 5′-GTGGTGGTGGTGGTGCTC GAGAGCGTTAACTGATTGTTC-3′, respectively. The PCR products were ligated with a linearized pET28a(+) vector digested by *Nco*I and *Xho*I. Cloning was confirmed by gel electrophoresis and DNA sequencing.

2.3 Expression and Purification of Enzymes

For expression of His-tagged enzymes, *Escherichia coli* BL21(DE3) cells transformed with the expression vector harboring AlaDH, ω-TA or NOX were cultivated in a LB medium (typically 1 L) containing 50 μg mL^{-1} kanamycin at 37 °C. In the case of ω-TA, a previously constructed pET28a vector carrying a codon-optimized gene of an engineered D-selective ω-TA from *Arthrobacter* sp. ($ARTA_{mut}$) was used [15]. When OD_{600} reached 0.4, IPTG was added to induce protein expression and then cells were allowed to grow for 15 h. The culture broth was centrifuged for 20 min at 10,000×*g* and 4 °C. The resulting cell pellet was disrupted by a sonicator after being resuspended in a 15 mL suspension buffer [50 mM sodium chloride, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.1 mM PMSF in 50 mM Tris duffer (pH 8.0)], followed by centrifugation for 1 h at 10,000×*g* and 4 °C to remove cell debris.

Purification of the His-tagged enzymes was carried out on an FPLC system (ÄKTAprime plus, GE Healthcare). In brief, the cell-free extract was loaded to a HisTrap HP column (GE Healthcare) pre-equilibrated with a buffer (20 mM imidazole, 500 mM sodium chloride and 20 mM sodium phosphate, pH 7.4). Elution of the bound protein was done by increasing imidazole concentration with a linear gradient from 20 to 500 mM. Removal of imidazole from the protein solution was done on a HiTrap desalting column (GE Healthcare) using a desalting buffer (150 mM sodium chloride and 50 mM sodium phosphate, pH 7). Protein concentrations of the purified enzyme solutions were determined by measuring UV absorbance at

280 nm using a molar extinction coefficient (ε) obtained by a protein extinction coefficient calculator (http://www.biomol.net/en/tools/proteinextinction.htm). The ε values of AlaDH (homohexamer), $ARTA_{mut}$ (homodimer) and NOX (homotetramer) were 131,718, 102,135 and 146,572 M^{-1} cm^{-1}, respectively.

2.4 Gel Electrophoresis

SDS-PAGE was carried out using standard protocols. Proteins were separated on 12% polyacrylamide gel in the presence of 0.1% SDS. Proteins were visualized by Coomassie blue staining.

2.5 Enzyme Assay

Unless otherwise specified, all enzyme assays were performed at 37 °C and pH 7 (50 mM Tris buffer). One unit of $ARTA_{mut}$ activity was defined as the enzyme amount required for formation of 1 µmol acetophenone in 1 min at 10 mM (*R*)-α-methylbenzylamine [(*R*)-α-MBA] and 10 mM sodium pyruvate. Acetophenone produced was monitored by a UV-spectrophotometer (UV-1650PC, Shimadzu Co.) at 285 nm as described elsewhere [25]. One unit of AlaDH was defined as the enzyme amount that produces 1 µmol pyruvate in 1 min at 10 mM L-alanine and 2 mM NAD^+. One unit of NOX was defined as the amount of the enzyme that oxidized 1 µmol NADH in 1 min at 0.2 mM NADH. Reaction progresses of the AlaDH and NOX assays were monitored by the UV-spectrophotometer at 340 nm using $\varepsilon_{NADH} = 6220$ M^{-1} cm^{-1}. In all the enzyme assays, initial rate measurements were carried out by linear curve fitting of the reaction progress data showing a time-dependent linear change in the UV absorbance.

2.6 Cascade Enzyme Reactions

Enzyme reactions were carried out at 37 °C in water bath without stirring. Reaction volume was 0.2 mL and the reactions were carried out in 50 mM Tris buffer (pH 7.0). 10-µL aliquots of the reaction mixture were taken at predetermined reaction times and diluted fivefold with water, followed by addition of 10 µL of 5 N HCl to stop the reaction. The resulting mixture was centrifuged at 13,000 rpm for 15 min and then the supernatant was used for quantitative chiral HPLC analysis of amino acid.

2.7 Preparative-Scale Deracemization

Deracemization of DL-**1a** in a preparative-scale was carried out in a 25 mL reaction mixture (50 mM Tris buffer, pH 7.0) charged with 0.22 g DL-**1a** (2.5 mmol), 0.15 g **3** (2.5 mmol), 17 mg NAD^+, 1750 U AlaDH, 40 U $ARTA_{mut}$ and 250 U NOX. Reaction was carried under magnetic stirring at 37 °C. Aliquots (typically 10 µL) of the reaction mixture were taken at predetermined reaction time and mixed with 40 µL of 5 N HCl after 20-fold dilution with water. Quantitative chiral analysis of DL-**1a** was performed by HPLC for determination of ee^D. When ee^D of D-**1a** exceeded 99%, reaction was stopped and then product isolation was performed.

The pH of the reaction mixture was adjusted to 1.0 by adding 5 N HCl and then filtered using a glass-fritted filter funnel to remove protein precipitates. The filtrate was loaded on a glass column packed with Dowex 50WX8 cation-exchange resin (40 g), followed by washing with 0.1 N HCl (100 mL) and then water (50 mL). Elution was performed by 10% (v/v) aqueous ammonia solution (150 mL) and then the eluate was evaporated at 70 °C and 0.25 bar. The resulting white solid was washed with ethanol (20 mL) and dried overnight at 37 °C.

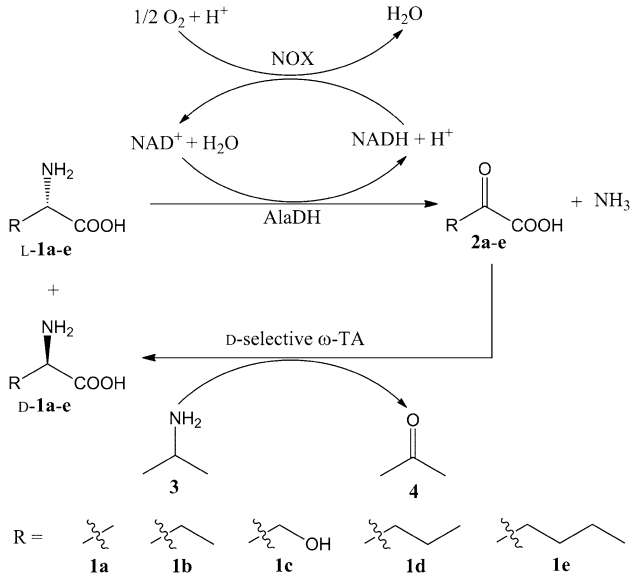
2.8 HPLC Analysis

All the HPLC analyses were carried out using a Waters HPLC system or an Agilent 1260 Infinity HPLC. Analysis of pyruvate was performed with an Aminex HPX-87H column (Bio-Rad) under an isocratic elution of 5 mM H_2SO_4 solution at 0.4 mL min^{-1}. Column oven temperature was set to 40 °C and UV detection was done at 210 nm. Quantitative chiral analysis of amino acids were carried out using a Symmetry C18 column (Waters) after derivatization with Marfey's reagent [26]. Details of the HPLC analyses are described in the Supplementary Materials.

3 Results and Discussion

3.1 Reaction Design of Deracemization

We envisioned that deracemization of racemic amino acid into D-enantiomer could be implemented by stereoinversion of L-amino acid while keeping D-amino acid intact (Scheme 1). The stereoinversion of unwanted L-enantiomer was designed to proceed through two-step enzyme reactions; (1) oxidative deamination of L-amino acid (L-**1a**–**e**) by L-alanine dehydrogenase (AlaDH) at the expense of NAD^+ and (2) reductive amination of the resulting keto acid (**2a**–**e**) back to the amino acid in an enantiomerically opposite form by D-selective ω-transaminase (ω-TA) using isopropylamine (**3**) as an amino donor cosubstrate. Note that **3** is one of the preferred amino donors for ω-TA reactions owing to easy removal of **4** [27]. In our previous study for deracemization using enantiocomplementary transaminases, the oxidative deamination step was carried out by α-transaminase such as branched-chain transaminase and D-amino acid transaminase [24]. It is notable that substitution of α-transaminase



Scheme 1 Deracemization cascade to produce D-amino acids

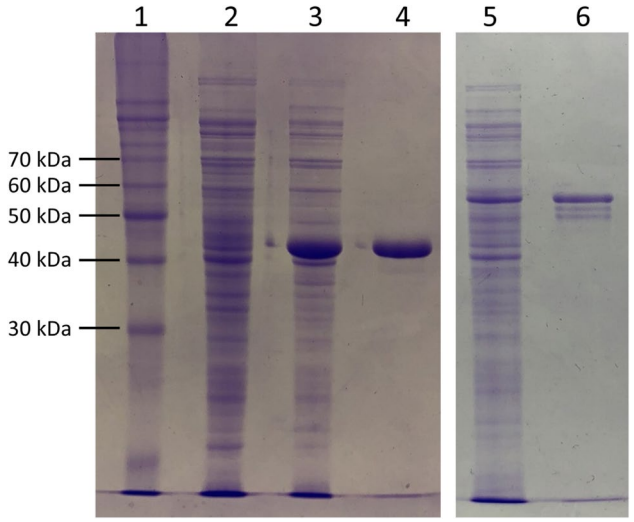


Fig. 1 SDS-PAGE analysis of AlaDH and NOX. Lanes: *1* marker, *2* cell-free extract control prepared with *E. coli* BL21(DE3) cells not carrying an expression vector, *3* cell-free extract prepared with cells expressing AlaDH, *4* purified AlaDH, *5* cell-free extract prepared with cells expressing NOX, *6* purified NOX

with AlaDH eliminates the need for an expensive keto acid cosubstrate. The oxidative deamination of amino acid can be also carried out using amino acid oxidase as demonstrated elsewhere [28]. However, generation of chemically reactive H_2O_2 as a coproduct necessitated an auxiliary enzyme, such as catalase, to neutralize the toxic coproduct.

The stereoinversion reaction requires two substrates (i.e., L-**1a–e** and **3**) and a NAD^+ cofactor. Because the AlaDH reaction consumes NAD^+, produced NADH should be converted back to NAD^+. We used NADH oxidase (NOX) from *L. brevis* for the NAD^+ recycling because NOX does not require an externally added cosubstrate for oxidation of NADH. Note that the NOX from *L. brevis* does not form H_2O_2 but produce H_2O [29]. Exploitation of the H_2O-forming NOX eliminates the need for catalase. As a result, the overall deracemization consists of the stereoinversion reaction performed by AlaDH and D-selective ω-TA and the cofactor regeneration reaction by NOX.

3.2 Cloning and Functional Expression of AlaDH and NOX

To carry out the deracemization reaction, three enzymes (i.e., AlaDH, ω-TA and NOX) were required to be purified. In the case of D-selective ω-TA, we used an engineered D-selective (or *R*-selective) ω-TA from *Arthrobacter* sp. ($ARTA_{mut}$) which was cloned and expressed in a previous study [15]. For preparation of the other two enzymes, we cloned AlaDH from *B. subtilis* (*ald*, Gene ID: 936557) and NOX from *L. brevis* (*nox*, GenBank accession number: AF536177) in a pET28a(+) expression vector. His-tagged AlaDH and NOX were purified to homogeneity (Fig. 1). Protein mobility of AlaDH in SDS–PAGE analysis was in agreement with the molecular weight deduced from the amino acid sequence (i.e., 39.7 kDa) while that of NOX was a little lower than the deduced molecular weight (i.e., 50 kDa).

To examine functional expression of AlaDH and NOX, the purified enzymes were subjected to activity assays by real-time monitoring of changes in NADH concentration at 340 nm (Fig. S1). Initial rates were determined by linear curve fitting of Abs_{340} data showing a linear increase with respect to reaction time, i.e. up to 2 min for both enzymes. Based on the initial rate measurements, specific activities of AlaDH and NOX were 4 and 20.5 U mg^{-1}, respectively.

3.3 Substrate Range for Deracemization

Feasibility of the stereoinversion reaction is dependent on enzyme activities of AlaDH and ω-TA for L-amino acids and its keto acids, respectively. Substrate specificity of $ARTA_{mut}$ for various keto acids were examined in the previous study [15]. Owing to a steric constraint in a small substrate binding pocket which is a general property for all the ω-TAs known to date, activities of $ARTA_{mut}$ showed an inverse correlation with the size of side chains of keto acid substrates (Table 1). The steric constraint for substrate was found to be more striking for AlaDH, which led to less than 1% enzyme activity, relative to L-**1a**, for L-amino acids carrying a side chain larger than a methyl substituent (i.e., L-**1b–e**). Eventually, activity for L-**1e** carrying a *n*-butyl substituent was below a detection limit. These results indicate that stereoinversion

Table 1 Substrate specificity of AlaDH for amino acids and $ARTA_{mut}$ for their cognate keto acids

Substrates (AlaDH/$ARTA_{mut}$)	Relative activity (%)[a]	
	AlaDH[b]	$ARTA_{mut}$[c]
1a/2a	100	100
1b/2b	0.9	33
1c/2c	0.7	6
1d/2d	0.05	2
1e/2e	n.d.	2

n.d. not detectable

[a]Relative activities of AlaDH and $ARTA_{mut}$ represent initial rates normalized by those for **1a** and **2a**, respectively. Specific initial rate of AlaDH for **1a** was 0.51 ± 0.02 Mm^{-1} min^{-1} μM^{-1}-enzyme

[b]Reaction conditions: 10 mM DL-**1a–e** and 2 mM NAD^+ in 50 mM Tris buffer (pH 7.0) at 37 °C

[c]Relative activities were taken from a previous report where initial rates were measured at 20 mM **2a–e**, 20 mM (*R*)-α-MBA [15]

employing AlaDH and $ARTA_{mut}$ should be optimal for L-**1a** and much slower reaction progresses would be observed for L-**1b–e**.

3.4 In Situ NAD^+ Regeneration

To examine whether the proposed deracemization worked as we intended, we first carried out a cascade reaction without NOX. Starting with 10 mM DL-**1a**, 10 mM **3** and 1 mM NAD^+, supplementation of the reaction mixture with AlaDH and $ARTA_{mut}$ enabled time-dependent decreases in the concentration of L-**1a** with increases in the concentration of D-**1a** by stoichiometric amounts of the L-**1a** depletion (Fig. S2). However, the absence of NAD^+ regeneration resulted in only 10% conversion of L-**1a** to D-**1a** after 12-h reaction. Enantiomeric excess was only 9.7% ee^D at 12 h, corresponding to partial enantioenrichment rather than decent deracemization. Therefore, the reaction mixture was additionally charged with NOX, so NAD^+ could be regenerated in situ and consequently conversion exceeded beyond the stoichiometric amount of the NAD^+ dose (Fig. 2). Indeed, we achieved complete deracemization at 12 h with > 99% ee^D and 96% reaction yield of D-**1a**. Taken together, these results clearly indicate that stereoinversion from L to D-amino acid using AlaDH and $ARTA_{mut}$ can lead to complete deracemization contingent upon NAD^+ recycling.

3.5 One-Pot Deracemization

The complete deracemization of 10 mM DL-**1a** using the cascade reactions led us to move on to use of a higher substrate concentration with the activity levels of enzyme components elevated (Fig. 3). Under the cascade reaction conditions, 50 mM DL-**1a** was completely deracemized after

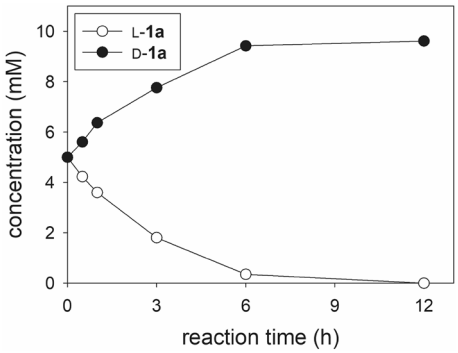


Fig. 2 Proof-of-concept of the deracemization strategy. Reaction conditions: 10 mM DL-**1a**, 10 mM **3**, 1 mM NAD^+, 0.14 U mL^{-1} AlaDH, 1 U mL^{-1} $ARTA_{mut}$ and 5 U mL^{-1} NOX in 50 mM Tris buffer (pH 7.0) at 37 °C

6 h with > 99% ee^D and 89% reaction yield of D-**1a**. **2a** was kept no higher than 0.7 mM throughout the reaction, indicating that reductive amination of **2a** by $ARTA_{mut}$ occurred as efficiently as the oxidative deamination of L-**1a** by AlaDH.

To expand the applicability of the proposed method, we tried deracemization of 50 mM DL-**1b–d** under the same reaction conditions as those for Fig. 3 (Table 2; entries 1, 3 and 5). No activity of AlaDH for L-**1e** led us to exclude DL-**1e** from the substrate list. Due to the low reactivities of L-**1b–d** for AlaDH as well as **2b–d** for $ARTA_{mut}$, complete deracemization was not achieved for any of the three amino acids. After 28-h reaction, ee^D of the resulting D-**1b** and D-**1c** reached 82 and 80%, respectively, and that of D-**1d** was only 19%. To achieve complete deracemization of these amino acids, increase in the enzyme levels or use of other enzymes showing matching substrate specificity would be required. Therefore, we tried the cascade reactions under modified reaction conditions where concentration of AlaDH was tenfold increased because of the too low activities for L-**1b–d**

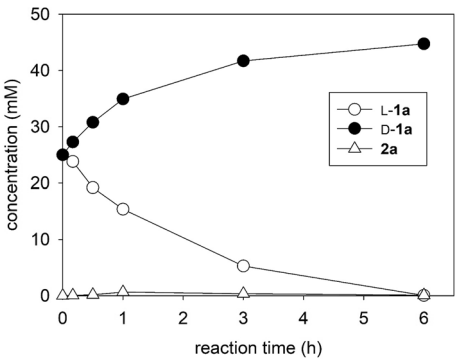


Fig. 3 One-pot production of D-**1a** by biocatalytic deracemization of DL-**1a**. Reaction conditions: 50 mM DL-**1a**, 50 mM **3**, 1 mM NAD^+, 14 U mL^{-1} AlaDH, 16 U mL^{-1} $ARTA_{mut}$ and 5 U mL^{-1} NOX in Tris buffer (50 mM, pH 7.0)

Table 2 One-pot deracemization of DL-**1b-d**

Entry	Substrate	Reaction conditions[a]	Reaction time (h)	Conversion[b] (%)	Reaction yield[c] (%)	ee^D (%)
1	DL-**1b**	A	28	82	90	82
2	DL-**1b**	B	9	> 99	93	> 99
3	DL-**1c**	A	28	81	85	80
4	DL-**1c**	B	20	> 99	95	> 99
5	DL-**1d**	A	28	10	66	19
6	DL-**1d**	B	48	88	90	88

[a]A: 50 mM DL-**1b–d**, 50 mM **3**, 1 mM NAD^+, 14 U mL^{-1} AlaDH, 16 U mL^{-1} $ARTA_{mut}$ and 5 U mL^{-1} NOX. B: 10 mM DL-**1b-d**, 50 mM **3**, 1 mM NAD^+, 140 U mL^{-1} AlaDH, 8 U mL^{-1} $ARTA_{mut}$ and 5 U mL^{-1} NOX

[b]Conversion represents a ratio of consumption of L-amino acid to the initial amount of L-amino acid, i.e. $([\text{L-amino acid}]_{t=0} - [\text{L-amino acid}]_t)/[\text{L-amino acid}]_{t=0}$

[c]Reaction yield = $[\text{D-amino acid}]_t/[\text{DL-amino acid}]_{t=0}$

(i.e. less than 1% activity relative to that of L-**1a**). Under the reaction conditions, 10 mM DL-**1b** and DL-**1c** were completely deracemized and led to 93 and 95% reaction yields of the desired D-amino acid products with > 99% ee^D after 9 and 20 h, respectively (Table 2; entries 2 and 4). However, L-**1d** was not completely converted to D-**1d** even after 48-h reaction and the resulting ee^D was 88% (entry 6).

For demonstration of practical applicability, we performed preparative-scale deracemization of DL-**1a** in a 25 mL reaction mixture under the reaction conditions of 100 mM DL-**1a**, 100 mM **3**, 1 mM NAD^+, 70 U mL^{-1} AlaDH (73 μM), 1.6 U mL^{-1} $ARTA_{mut}$ (200 μM) and 10 U mL^{-1} NOX (2.4 μM). Complete deracemization was achieved after 24 h, leading to 95% reaction yield and > 99% ee^D of D-**1a**. Product isolation using cation-exchange chromatography led to recovery of optically pure D-**1a** (0.11 g, 52% isolation yield). The purified D-**1a** was structurally characterized by ^1H NMR, ^{13}C NMR and MS (see Supplementary Materials).

4 Conclusions

In this study, we developed a deracemization cascade for preparation of D-amino acids using L-selective amino acid dehydrogenase, D-selective ω-TA and NOX. Compared to the previous deracemization processes using amino acid oxidase or α-transaminase for oxidative deamination of an unwanted amino acid enantiomer [24, 28], the proposed method benefits from no generation of hydrogen peroxide and no need for an expensive keto acid cosubstrate, respectively. In addition, the deracemization method might be modified for preparation of L-amino acids by switching enantioselectivity of amino acid dehydrogenase and ω-TA. However, future direction of the proposed strategy should be expansion of a product pipeline by exploiting diverse amino acid dehydrogenases and ω-TAs with a broad substrate range.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest to disclose.

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