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Biocatalytic cascade reactions for asymmetric synthesis of aliphatic amino acids in a biphasic reaction system

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ABSTRACT

Enantiopure aliphatic amino acids, including l-3-hydroxyadamantylglycine (L-Hag), L-*tert*-leucine (L-Tle) and L-norvaline, are essential chiral building blocks for a number of pharmaceutical drugs. Here, we developed cascade enzyme reactions in an extractive biphasic system using a branched-chain amino acid transaminase (BCTA) and an (S)-selective ω-transaminase (ω-TA) for asymmetric synthesis of the aliphatic amino acids from achiral α-keto acid precursors. The extractive cascade reactions enabled equilibrium shift of the BCTA reaction by recycling an amino acid cosubstrate as well as acceleration of the ω-TA reaction by removing an inhibitory ketone product from an aqueous phase. Starting with 20 mM α-keto acid, 4 mM *rac*-homoalanine and 50 mM rac-α-methylbenzylamine (rac-α-MBA), the biphasic cascade reactions afforded synthesis of four unnatural amino acids (i.e., L-Tle, L-Hag, L-norvaline and L-norleucine) and two natural amino acids (i.e., L-valine and L-Leucine) with >92% conversion yield and >99.9% ee. To demonstrate the industrial feasibility of the extractive cascade reaction, preparative-scale synthesis of L-Hag was performed in a reaction mixture consisting of 300 mL hexane and 50 mL aqueous solution (50 mM phosphate buffer, pH 7.0) charged with 50 mM keto acid substrate, 5 mM L-homoalanine, 120 mM rac-α-MBA, 2 U/mL BCTA and 16 U/mL ω-TA. Conversion yield of L-Hag reached 92% with >99.9% ee at 70 h. Product isolation led to 0.32 g white solid of L-Hag (62 % isolation yield).

1. Introduction

Optically pure amino acids are increasingly employed as important chiral motifs in various pharmaceutical drugs [1,2]. For example, l-3-hydroxyadamantylglycine (L-Hag) and L-*tert*-leucine (L-Tle), which are unnatural amino acids carrying branched-chain aliphatic groups, are essential components of a type-2 diabetes drug (i.e., saxagliptin) [3] and a HIV-protease inhibitor [4], respectively. In addition, L-norvaline carrying a linear aliphatic side chain is a key intermediate of Perindopril (i.e., an ACE inhibitor) [5]. Growing industrial demands for the aliphatic amino acids have spurred massive research efforts to develop preparative methods via biocatalysis owing to the lack of the fermentative methods yet to access the unnatural amino acids. Several biocatalytic strategies have been developed by kinetic resolution of racemic amino acid derivatives using acylase, lipase and protease [6–8] or asymmetric amination of keto acids using transaminase and dehydrogenase [9–11]. Because of a two-fold higher maximum yield, the asymmetric synthesis is often favored over the kinetic resolution [12,13].

Branched-chain amino acid transaminase (BCTA) is a major metabolic producer of the natural branched-chain amino acids such as L-valine, L-Leucine and L-isoleucine [14]. BCTA displays a perfect match in the substrate specificity for a broad range of aliphatic α-keto acids, which enables production of the unnatural amino acids such as L-Tle and L-Hag [11]. Moreover, compared to the amino acid dehydrogenases [9,10], no requirement of an expensive external cofactor such as NADH renders BCTA promising for industrial applications in the asymmetric synthesis of the aliphatic amino acids. However, a crucial setback in the scale-up of the BCTA reaction is a neutral equilibrium position of the most BCTA reactions (i.e., equilibrium constant ≈1) [14]. To overcome the equilibrium problem, it was demonstrated that removal of a keto acid coproduct by spontaneous decarboxylation or enzymatic conversion using dehydrogenases could facilitate an equilibrium shift [11].

In the previous report, we demonstrated that the thermodynamically neutral BCTA reactions can be driven to completion by coupling a ω-transaminase (ω-TA) reaction as an equilibrium shifter [15]. Note that ω-TA is capable of employing primary amines as an amino donor and thereby the resulting ω-TA reaction between the primary amine and the keto acid is thermodynamically favor-

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able [16]. To couple the BCTA and ω-TA reactions, L-homoalanine was used as a shuttling substrate. Isopropylamine was used as an amino donor for the ω-TA from *Ochrobactrum anthropi* because isopropylamine was regarded as an ideal amino donor due to easy removal of the volatile deamination product (i.e., acetone) [17]. However, this strategy cannot be generalized to other ω-TAs because isopropylamine is rather a poor substrate for most ω-TAs. For example, two typical ω-TAs, i.e., an (S)-selective ω-TA from *Vibrio fluvialis* JS17 and an (*R*)-selective ω-TA form *Arthrobacter* sp., are known to show negligible activities for isopropylamine [18,19]. Note that the ω-TA from *O. anthropi* used in the previous study shows exceptionally high activity for isopropylamine (i.e., 43% activity relative to that for (S)-α-methylbenzylamine ((S)-α-MBA)) [20]. In this study, we seek to develop a generally applicable strategy in which an amine substrate of choice is reactive toward most ω-TAs (i.e., benzylamine and α-MBA) and thereby any ω-TA displaying desirable enzymatic properties for a manufacturing setting, including high operational stability and low product inhibition, can be used for a process scale-up.

2. Materials and methods

2.1. Chemicals

Pyridoxal 5′-phosphate (PLP) was purchased from Sigma–Aldrich Co. (St. Louis, USA). Pyruvic acid was obtained from Kanto Chemical Co. (Tokyo, Japan). Trimethylpyruvate was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). 2-(3-Hydroxy-1-adamantyl)-2-oxoethanoic acid was purchased from Hi-Tech Chemical Co. (Chongqing, China). *n*-Hexane was purchased from Duksan Chemical Co. (Ansan, South Korea). Materials used for culture media including yeast extract, tryptone and agar were purchased from Difco (Spark, USA). All other chemicals were purchased from Sigma–Aldrich Co. (St. Louis, USA) and of the highest grade available.

2.2. Expression and purification of TAs

Overexpression of His_6-tagged TAs was carried out as described previously with minor modifications [21]. *Escherichia coli* BL21(DE3) cells carrying the expression vectors (i.e., pET28a(+) harboring the TA gene) were cultivated in LB medium (typically 1 L) containing 50 μg/mL kanamycin. Protein expression was induced by IPTG at 0.4 OD_{600} and the cells were allowed to grow for 10 h. The culture broth was centrifuged and the resulting cell suspension was subjected to ultrasonic disruption. Protein purification was carried out as described previously [21]. Molar concentrations of the purified TAs were determined by measuring UV absorbance at 280 nm.

2.3. Enzyme assay

Typical enzyme assays were carried out at 37 °C in 50 mM phosphate buffer (pH 7). One unit of BCTA is defined as the enzyme amount catalyzing formation of 1 μmole 2-oxobutyrate in 1 min at 20 mM trimethylpyruvate and 20 mM L-homoalanine. One unit of ω-TA from *Paracoccus denitrificans* (PDTA) is defined as the enzyme amount catalyzing formation of 1 μmole acetophenone in 1 min at 20 mM pyruvate and 20 mM (S)-α-MBA. After 10 min reaction, the enzyme reaction (100 μL reaction volume) was stopped by adding 600 μL acetonitrile. For the initial rate measurements, 2-oxobutyrate and acetophenone for BCTA and PDTA, respectively, were analyzed by HPLC.

2.4. Cascade enzyme reactions to produce aliphatic amino acids

Unless otherwise specified, the cascade reactions were performed in 50 mM phosphate buffer (pH 7) at 37 °C. In the biphasic reactions, 3 or 6 mL of hexane was carefully overlaid on the aqueous reaction mixture (1 mL) and the reactions were carried out without agitation. Aliquots of the aqueous and organic phases (30 μL each) were taken at predetermined reaction times and mixed with 180 μL acetonitrile. The reaction progresses were monitored by HPLC analysis of the keto acid substrate or the amino acid product.

2.5. Preparative-scale synthesis and isolation of L-Hag

Preparative-scale extractive cascade reaction for asymmetric synthesis of L-Hag was carried out at 37 °C under magnetic stirring in an oven-dried beaker charged with 50 mL reaction mixture containing 2-(3-hydroxy-1-adamantyl)-2-oxoethanoic acid (2.5 mmol), L-homoalanine (0.25 mmol), rac-α-MBA (6 mmol), PLP (5 μmol), BCTA (0.18 μmol), PDTA (0.7 μmol) and potassium phosphate (50 mM, pH 7.0). Hexane (300 mL) was added to the aqueous reaction mixture. When the conversion exceeded 90%, the aqueous reaction mixture was subjected to product isolation.

The aqueous reaction mixture (50 mL) was separated from the organic phase using a separation funnel and the pH was adjusted to 1.0 by adding 5 N HCl (4.5 mL). Protein precipitate was removed by filtration through a glass-fritted filter funnel. The pH of the filtrate solution was adjusted to 7.0 by adding 5 N NaOH solution (6 mL). The resulting solution was evaporated at 30 °C and 0.1 bar until the final volume reached around 2 mL. The precipitate formed during the evaporation was obtained by filtration, washed by 10 mL EtOH and then dried overnight in a drying oven (75 °C). The resulting white solid of L-Hag was subjected to mass spectrometric analysis and elemental analysis as described in the Supplementary data.

2.6. HPLC analysis

Analysis of acetophenone was performed on a Waters HPLC system (Milford, USA) using a Symmetry C18 column (Waters Co.,) with isocratic elution of 60% methanol/40% water, both containing 0.1% trifluoroacetic acid, at 1 mL/min. Detection was done with a UV detector tuned at 254 nm. Retention time of acetophenone was 3.8 min.

α-Keto acids were analyzed using an Aminex HPX-87H column (Bio-Rad, Hercules, USA) with isocratic elution of 5 mM H_2SO_4 solution at 0.5 mL/min. UV detection was carried out at 210 nm. Column oven temperature was set to 40 °C. Retention times of the keto acids were 12.0 (2-oxobutyrate), 14.2 (2-oxopentanoic acid), 11.9 (3-methyl-2-oxobutyrate), 17.7 (2-oxohexanoic acid), 15.3 (4-methyl-2-oxopentanoic acid), 9.7 (trimethylpyruvate) and 11.7 min (2-(3-hydroxy-1-adamantyl)-2-oxoethanoic acid).

For determination of amino acid concentration and enantiomeric excess, 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) was used for amino acid derivatization [22]. The GITC-derivatives of the amino acids were resolved on the Symmetry column at a flow rate of 1 mL/min with UV detection tuned at 254 nm. Retention time of l/d-enantiomers of homoalanine was 26.9/28.7 min at isocratic elution of 40% methanol (A)/60% water (B), both containing 0.1% trifluoroacetic acid,. Retention time of l/d-enantiomers of Hag was 21.1/34.4 min at isocratic elution of 45% A/55% B. Retention time of l/d-enantiomers of norvaline, valine, leucine, Tle were 10.1/14.9, 8.8/13.3, 15.2/23.4 and 11.6/19.0 min, respectively, at isocratic elution of 50% A/50% B. Retention time of l/d-enantiomers of norleucine was 6.0/7.9 min at isocratic elution of 60% A/40% B.