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PAPER

Deracemization of unnatural amino acid: homoalanine using D-amino acid oxidase and ω-transaminase†

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A deracemization method was developed to generate optically pure L-homoalanine from racemic homoalanine using D-amino acid oxidase and ω-transaminase. A whole cell reaction using a biphasic system converted 500 mM racemic homoalanine to 485 mM L-homoalanine (>99% ee).

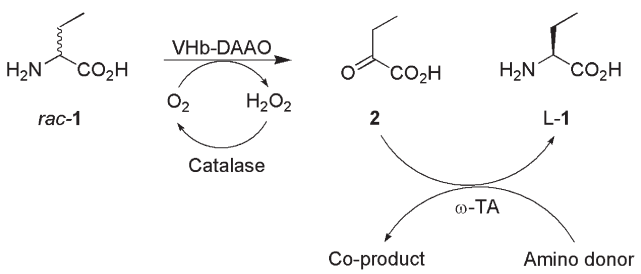
Introduction

The production of unnatural amino acids is important in the chemical industry due to an increase in demand for optically pure non-proteinogenic amino acids as building blocks for pharmaceutical compounds.[1,2] Given the significance of the unnatural amino acid, its efficient synthesis in optically pure form has become an attractive challenge to organic chemists and biologists.[3] Natural amino acids are traditionally obtained through extraction and fermentation methods. However, the production of unnatural amino acids through these traditional methods has not been well established yet.[4] Among the different unnatural amino acids, L-homoalanine, L-**1** is of high pharmacological significance as it is used as a key chiral intermediate for the synthesis of several important drugs such as brivaracetam, levetiracetam and ethambutol.[5] L-**1** has been previously produced by transaminase (TA) reaction with 2-ketobutyrate, **2** using tyrosine TA,[6] aspartate TA[7] and ω-TA.[1] L-Threonine was converted to **2** using threonine deaminase and later it was converted to L-**1** by TA reaction in a one-pot reaction.[8,9] It was also produced from an amino acid ester by a protease catalyzed reaction[10] and from an *N*-acetyl derivative by an aminoacylase catalyzed reaction.[5] More recently, L-**1** was generated directly from glucose using metabolically engineered *Escherichia coli* where glutamate dehydrogenase was evolved to fix ammonia directly onto **2**.[11]

The traditional application of enzymes in enantioselective synthesis can be classified into two methods: the kinetic resolution of racemates (*e.g.* protease and acylase) and asymmetric synthesis from pro-chiral compounds (*e.g.* TA and dehydrogenase reaction). Asymmetric synthesis is usually preferred as it generates a maximum theoretical yield of 100% which is twice than that of the kinetic resolution. However, prochiral ketoacid is more expensive compared to *rac*-amino acid.[6] Recently, the deracemization process has gained considerable attention due to its ability to produce enantiomerically pure amino acids from racemate with a maximum theoretical yield of 100%.[12] Ingenza developed a deracemization method to produce L-**1** from *rac*-**1** using whole cell D-amino acid oxidase (DAAO) and heterogeneous metal catalysts (Pd/C).[13] This method generated 99% ee with 95% conversion for 100 mM of substrate loading. The Ingenza process is economically more efficient when compared to other deracemization processes which employ amine-boranes.[12d] However in the Ingenza reaction system, the concentration of non-selective reductant must be high, in order to avoid the hydrolysis of imino acid to form **2**.[14] Moreover, the metal catalyst may tend to bind with metal ions and thereby hamper the reaction. In this study, we propose an alternative deracemization method for the efficient production of L-**1** using VHb-DAAO (*vide infra*) and ω-TA (Scheme 1).

Results and discussion

The flavo enzyme DAAO which contains FAD as cofactor, catalyzes stereospecific oxidation of D-**1** to α-imino acids, which are



Scheme 1 Deracemization of *rac*-**1** using VHb-DAAO and ω-TA.

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then hydrolyzed spontaneously to **2** and ammonia. Subsequently, the produced **2** is converted to L-**1** by ω-TA using benzylamine as the amino donor. The catalase enzyme catalyzes the decomposition of hydrogen peroxide, the by-product of DAAO into water and oxygen. Vitreoscilla hemoglobin (VHb) which extracts oxygen molecules from water, and releases the captive oxygen for the cellular activities of the organism, was fused with DAAO from *Rhodotorula gracilis* to create a fusion protein (VHb-DAAO).[15] This recombinant protein was earlier reported to significantly enhance the DAAO activity and stability in the bioconversion process of cephalosporin C[16] and *rac*-3-fluoroalanine.[17]

Initially, we compared the activity of both the enzymes. The genes of DAAO and VHb-DAAO were cloned and expressed effectively in *E. coli*. The enzyme was then purified on a Ni-NTA affinity column (Fig. S1, ESI†). The K_m values of VHb-DAAO and DAAO towards D-**1** were 1.33 and 1.23 mM respectively. Nonetheless, the existence of VHb in the fusion protein did not have much affect towards the affinity of the enzyme on D-**1**. VHb-DAAO also had a substantially higher k_{cat} value (82.5 s^{-1}) compared to that of DAAO (31.0 s^{-1}). The catalytic efficiency of VHb-DAAO (62.0 s^{-1} mM^{-1}) was 2.5-fold higher than that of DAAO (25.2 s^{-1} mM^{-1}). The optimal pH of DAAO and VHb-DAAO towards D-**1** was 8.0, which corresponds well with the reported pH 8.0 for D-Ala.[17]

The kinetic resolution of 100 mM *rac*-**1** was carried out in 1 mL of 100 mM phosphate buffer (pH 8.0) containing VHb-DAAO (0.04 mg mL^{-1}), 1 μM FAD and catalase (0.05 mg mL^{-1}). The reaction vial was loosely capped, and shook with 200 rpm at 37 °C without any additional oxygen supply. L-**1** reached an ee >99% with a conversion of 52.4% and enantioselectivity >100 after 11 h (Fig. 1).

The successful kinetic resolution of *rac*-**1** further allowed us to deracemize *rac*-**1** to L-**1** using VHb-DAAO and ω-TA reactions.[18] ω-TAs have recently gained a lot of attention due to their ability to produce a wide range of optically pure amine compounds. It belongs to the pyridoxal-5′-phosphate (PLP) dependent enzymes which transfer an amino group from an amino donor onto a carbonyl moiety of an amino acceptor, in which at least one of the two substances is not an α-amino acid or an α-keto acid.[19] For deracemization, ω-TA from *Vibrio fluvialis* JS17 was cloned and expressed in *E. coli*. The enzyme was later purified on a Ni-NTA affinity column (Fig. S2, ESI†). The excellent reactivity of ω-TA towards benzylamine makes it an ideal amino donor for ω-TA reaction.[1,20] Moreover, benzylamine is economical and the benzaldehyde byproduct formed in the reaction can be easily extracted. Initially, as depicted in Scheme 1, we conducted the reaction with low concentration (10 mM) using 0.02 mg mL^{-1} of VHb-DAAO, 0.18 mg mL^{-1} of ω-TA, 1 μM FAD and 0.05 mg mL^{-1} catalase. To ensure the complete amination of **2**, the concentration of benzylamine (20 mM) was kept 2-fold higher than that of *rac*-**1**. The entire reaction took 8 h for the conversion of 10 mM *rac*-**1** to 7.5 mM L-**1** with ee >99% (Fig. 2).

Enzyme inhibition by benzaldehyde was the major hurdle in carrying out the ω-TA reaction efficiently. ω-TA activity in the reaction between **2** and benzylamine was almost lost when benzaldehyde exceeded 3 mM. To overcome the product inhibition, we used a biphasic reaction system in which an organic solvent was employed to extract the inhibitory benzaldehyde from an aqueous system. Based on the partition coefficients of benzaldehyde with various organic solvents, isooctane was selected as an ideal solvent for the organic–aqueous biphasic system.[1] In the biphasic reaction, **1**, benzylamine and **2** existed in the aqueous solution due to their electric charges at neutral pH. 1 mL of isooctane was added to the aqueous solution to overcome product inhibition. To suppress emulsion formation that might cause interfacial enzyme inactivation, the biphasic mixture was gently shook in a shaking incubator with 250 rpm at 37 °C. The removal of benzaldehyde using the biphasic system substantially boosted the conversion compared to that of the aqueous medium (Fig. 3). It took 8 h for the conversion of *rac*-**1** to reach above 99% conversion with >99% ee. Later, the deracemization reaction was conducted with 100 mM *rac*-**1** and 200 mM benzylamine in a biphasic system using 0.04 mg mL^{-1} of VHb-DAAO and 0.36 mg mL^{-1} of ω-TA. After 24 h of the reaction, L-**1** was generated with a conversion of 98.8% and >99% ee (Fig. S3, ESI†).

The use of whole cells as the biocatalyst in an enzymatic process has the advantage of a simple and economical preparation as expenses for cell lysis and/or purification are saved.[21] Another advantage of whole cell reaction system is that it can be performed effectively even in the absence of expensive cofactor

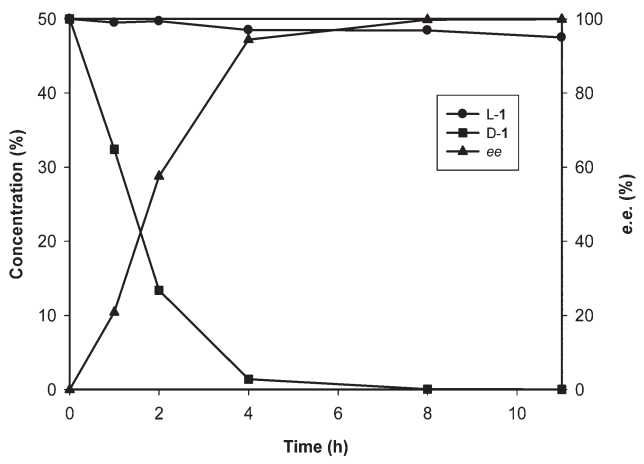


Fig. 1 Kinetic resolution of *rac*-**1** with VHb-DAAO.

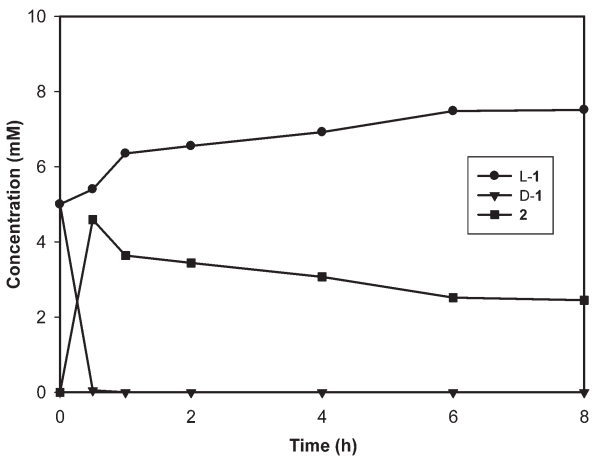


Fig. 2 Deracemization of 10 mM *rac*-**1** using VHb-DAAO and ω-TA in aqueous reaction system.

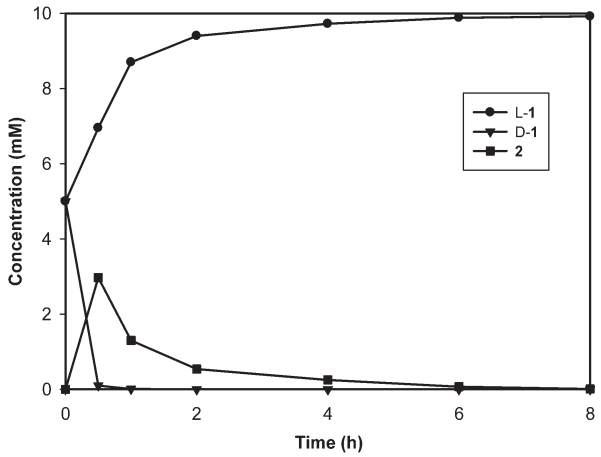
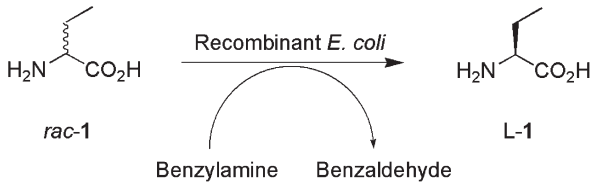


Fig. 3 Deracemization of 10 mM *rac*-**1** using VHb-DAAO and ω-TA in biphasic reaction system.



Scheme 2 Deracemization of *rac*-**1** using whole cell reaction.

FAD. Firstly, ω-TA gene and VHb-DAAO was cloned and well expressed in separate pET24ma systems. Later, in order to develop a recombinant *E. coli* system expressing both the enzymes in single cell, the ω-TA gene was cloned in a pBAD/HisA vector. This vector was later introduced into an *E. coli* system bearing pET24ma vector containing VHb-DAAO. Initially, when ω-TA was individually expressed, it showed a high level of yield in the pBAD/HisA vector but its expression level got drastically reduced when it was co-expressed with VHb-DAAO in the recombinant *E. coli* system. Nevertheless, VHb-DAAO continued to show a high level of expression (Fig. S4, ESI†). Therefore, it was decided to carry out the whole cell reaction using *E. coli* cell with the enzymes expressed in separate pET24ma systems. Subsequently, the deracemization was successfully carried out employing whole cell catalysts in a biphasic system with 100 mM *rac*-**1** and 200 mM benzylamine in a biphasic system (Scheme 2). Using 0.82 mg mL^{-1} of VHb-DAAO whole cell and 0.36 mg mL^{-1} of ω-TA whole cell, the reaction gave 99.1% conversion (>99% ee) in 10 h (Fig. S5, ESI†). It was observed that even in the absence of catalase and FAD, the deracemization reaction was successful. D-**1** was completely removed within 4 h of the whole cell reaction. Later, for the 500 mM *rac*-**1** reaction, 10 mL of isooctane was carefully added to 10 mL of 100 mM phosphate buffer solution (pH 8.0) containing 500 mM *rac*-**1**, 500 mM benzylamine, 1.64 mg L^{-1} VHb-DAAO whole cell and 0.9 mg L^{-1} ω-TA whole cell. The reaction generated 97% conversion (>99% ee) in 36 h (Fig. 4).

Conclusions

In summary, we have developed a new deracemization method to efficiently produce optically pure L-homoalanine at an industrial scale (in terms of substrate concentration) by combining VHb-DAAO with ω-TA. DAAO enables the oxidation of D-amino acid to its corresponding keto acid while ω-TA is used to convert the keto acid into optically pure L-amino acid. The relaxed substrate specificity of DAAO and ω-TA makes it capable of generating a wide range of optically pure unnatural amino acids. This reaction scheme can also be used to generate D-amino acids by employing L-amino acid oxidase along with (*R*)-ω-TA.

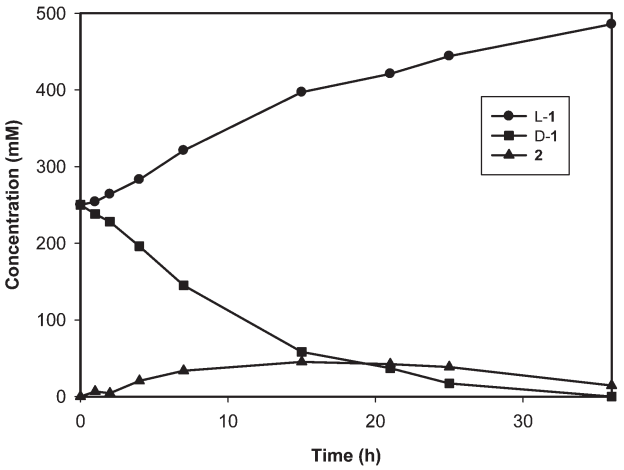


Fig. 4 Deracemization of 500 mM *rac*-**1** using VHb-DAAO and ω-TA in a whole cell biphasic system.

Experimental

Isopropyl-β-D-thiogalactopyranoside (IPTG), 2,3,4,6-tetra-*o*-acetyl-α-D-glucopyranosyl isothiocyanate (GITC), benzylamine, benzaldehyde, **1**, **2**, *rac*-**1** and D-**1** were from Sigma-Aldrich, Korea. The rest of the chemicals were of analytical or reagent grade.

Quantitative chiral analysis of **1** was performed using a C18 symmetry column (Waters, MA) with a Waters HPLC system at 254 nm after the derivatization of the sample with GITC.[17] Separation of each enantiomer was achieved through an isocratic elution with a mixture of 50% methanol and 50% water (0.1% TFA) at a flow rate of 1.0 mL min^{-1}. To quantify each enantiomer, an appropriate amount of L-glutamate (an internal standard) was added to the stop solution (acetonitrile), and the mixture was derivatized with GITC. After derivatization, the retention times for L-glutamate, L-**1**, and D-**1** were observed to be 5.18, 8.04, and 10.95 min respectively (Fig. S6, ESI†). To analyze **2**, Aminex HPX-87H HPLC column (Bio-Rad, CA) with an elution of 5 mM sulfuric acid solution at UV 210 nm was used. Its retention time was 9.61 min (Fig. S7, ESI†).

Enzyme expression, purification and preparation of whole cell reaction

DAAO and VHb-DAAO genes were amplified and cloned into the IPTG-inducible expression vector pET24ma.[17] These vectors were then transported to *E. coli* BL21 and cultured in 1 L of LB medium containing kanamycin (50 mg L^{-1}) at 30 °C in a 3 L

flask. When the optical density of cell culture reached 0.4–0.6 at 600 nm, 0.5 mM of IPTG was added. The C-terminal His6-tagged proteins were purified at 4 °C on a Ni-NTA agarose resin purchased from Qiagen (Hilden, Germany). The eluted solution, containing purified protein from Ni-NTA agarose, was dialyzed against 100 mM sodium phosphate buffer (pH 8.0) containing 7% glycerol, 2 mM EDTA, and 5 mM 2-mercaptoethanol, and concentrated using an Amicon ultrafiltration unit (Millipore, USA). Glycerol was added to the purified enzyme solution (25% glycerol) and stored at −20 °C for further studies. For the whole cell preparation of VHb-DAAO, the cell pellet was washed twice with 100 mM phosphate buffer (pH 8.0) after induction. Subsequently glycerol was added to the whole cell solution (25% glycerol) and stored at −70 °C for further study.

In the case of ω-TA, the coding region of ω-TAVf was amplified and cloned into pET24ma vector and was then introduced into *E. coli* BL21.[18] The transformant of *E. coli* BL21 were grown in 1 L of LB broth containing 50 mg L^{-1} of kanamycin at 37 °C. When the OD_{600} of transformants reached 0.4–0.6, 0.5 mM of IPTG was added. After 6 h of induction, the cells were harvested and washed twice with 50 mM phosphate buffer (pH 8.0). After centrifugation, the cell pellet was resuspended in 20 mL of 50 mM phosphate buffer (pH 8.0) containing 20 μM PLP, 2 mM EDTA, 1 mM PMSF, 1 mM DTT, and 10% glycerol. Crude extracts were obtained by cell disruption using sonication. The crude extract of the recombinant *E. coli* cell containing His-tagged ω-TAVf was applied directly to Ni-NTA agarose resin which was purchased from Qiagen (Hilden, Germany). The enzyme was then purified as described elsewhere.[18] The purified enzyme was then dialyzed against 50 mM potassium phosphate buffer (pH 7.0) containing 20 μM PLP and concentrated with an Amicon ultrafiltration unit (Millipore, USA). Glycerol was added to the purified enzyme solution (25% glycerol) and stored at −20 °C for further studies. For the whole cell preparation of ω-TA, the cell pellet was washed twice with 100 mM phosphate buffer (pH 8.0) after induction. Subsequently, glycerol was added to the whole cell solution (25% glycerol) and stored at −70 °C for further study.

Co-expression of ω-TA with VHb-DAAO

Initially, the coding region of the ω-TA enzyme was amplified by PCR using the primers A1 (5′- CCGCTCGAG ATGAACAAACCGCAAAGCTGG-3′) and A2 (5′- CCCAAG CTTTTATTAGGCAACCTCGGCAAAG-3′) from pET24-ma:ω-TA. The PCR product was then digested with XhoI and HindIII, and was subsequently inserted into the pBAD/HisA vector (Invitrogen, USA). The pBAD/HisA:ω-TA was then introduced into the *E. coli* (BL21) containing pET24ma:VHb-DAAO. Later these transformants were grown at 30 °C in 1L LB broth containing 50 mg L^{-1} of kanamycin and 50 mg L^{-1} of Amp. When the OD_{600} reached 0.2, IPTG (0.5 mM) and arabinose (0.02%) were added to culture media. After 6 h of induction, the cells were harvested and washed twice with 50 mM phosphate buffer (pH 8.0) and the expression level was analyzed. The single expression of ω-TA using *E. coli* BL21 carrying only pBAD/HisA:ω-TA was similar to that of the above mentioned procedure except for the introduction of only 50 mg L^{-1} of Amp to the cell culture and the addition of only arabinose (0.02%) to the culture media.

Acknowledgements

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