Journal of Molecular Catalysis B: Enzymatic 100 (2014) 32–39

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Substrate profile of an ω-transaminase from *Burkholderia vietnamiensis* and its potential for the production of optically pure amines and unnatural amino acids

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ARTICLE INFO

Article history:
Received 23 July 2013
Received in revised form 6 November 2013
Accepted 16 November 2013
Available online 25 November 2013

Keywords:
Biocatalysis
ω-Transaminase
Chiral amine
Unnatural amino acid
β-Keto ester

ABSTRACT

A new (*S*)-enantioselective ω-transaminase (ω-TA) gene from *Burkholderia vietnamiensis* G4 was functionally expressed in *Escherichia coli* BL21 (DE3), and the purified recombinant N-terminal His-tagged ω-TA (HBV-ω-TA) had a dimeric structure with optimum pH and temperature of 8.4 and 40 °C, respectively. The enzyme showed higher activities toward aromatic amines than aliphatic amines and (*S*)-1-methylbenzylamine ((*S*)-α-MBA) was the most active amino donor. For amino acceptor, keto acids, keto esters and aldehydes were more reactive than ketones with pyruvate ethyl ester being most active. Several chiral amines and unnatural amino acids or esters were synthesized using HBV-ω-TA as the catalyst and isopropylamine or (*S*)-α-MBA as amino donor. Notably, HBV-ω-TA catalyzed the amino transfer to β-keto esters to give optically pure β-amino acid esters. In addition, glyoxylate was used as amino acceptor for the first time in the kinetic resolution of racemic amines and optically pure amines, such as (*R*)-1-methylbenzylamine, (*R*)-1-phenylpropylamine, (*R*)-2-amino-4-phenylbutane and (*R*)-1-aminotetraline, were obtained.

1. Introduction

Chiral amines and unnatural amino acids, including β-amino acids have been widely used in diverse sectors such as the pharmaceutical, chemical, cosmetic, food, and agricultural industries [1–3]. For example, β-amino acids are key building blocks in many natural and synthetic drugs such as taxol and cispentacin, which are used for their antitumor and antifungal activities, respectively [3,4]. The peptides containing unnatural amino acids usually show higher stability against peptidases than natural peptides and such mixed α/β-peptides often retain their biological activity [5–9], thus providing useful chemical building blocks for new drugs that are not degraded or rejected by the human body [10–13].

Given the significance of chiral amines and unnatural amino acids, efficient synthesis of these compounds in optically pure form has become an attractive challenge. However, unlike natural amino acids which are usually produced using fermentation method [14], chemical and biocatalytic methods have been explored for the production of enantiomerically pure amines and unnatural amino acids [15–18]. Among these methods, ω-TA-catalyzed amino transfer reaction to carbonyl compounds is one of the most prominent approaches because of its superior features compared to other enzymatic and chemical methods [19–22]. ω-TAs show high stereoselectivity, rapid reaction rate, broad substrate specificity, and no requirement for external cofactor. Moreover, ω-TAs can be applied in both the kinetic resolution of racemic amines [23–26] and the asymmetric synthesis of optically pure amines from the corresponding prochiral carbonyl compounds [24,27,28] (Fig. 1). Although some excellent examples have demonstrated the success of transaminases in the production of important chiral amines and unnatural amino acids [25,28,29], the full potential of this group of enzymes for industrial application is yet to be realized. Present challenges include the unfavorable thermodynamic equilibrium and severe product and substrate inhibitions shown by various transaminases [19,20,24]. In this study, we take the approach of discovering a new omega-transaminase making use of the microbial genome database as a source of new biocatalysts better known as genome mining. The new ω-TA gene was cloned and overexpressed in *Escherichia coli* BL21. The recombinant enzyme was then characterized with respect to its enzymatic properties, substrate spectrum and its potential for the production of optically pure amines and unnatural amino acids.

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http://dx.doi.org/10.1016/j.molcatb.2013.11.013

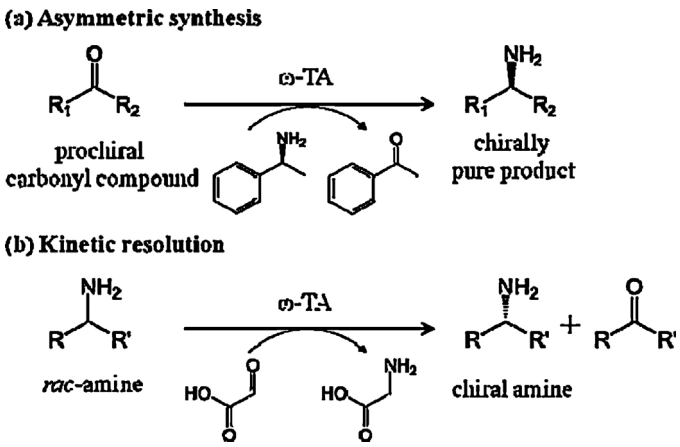

Fig. 1. ω-TA catalyzed reactions. (a) Asymmetric synthesis of chirally pure unnatural amino acids using (*S*)-α-MBA as amino donor. (b) Kinetic resolution of racemic amines using glyoxylate as amino accepter.

2. Materials and methods

2.1. Chemicals

Most of the chemicals were of the highest grade available and obtained from commercial sources such as Alfa Aesar and Sigma–Aldrich Chemical Co. 4-Aminovaleric acid was prepared chemically according to reported methods [30]. Materials used for culture media including peptone, yeast extract and agar were purchased from Becton, Dickinson and Company (BDX).

2.2. Selection of the ω-TA gene

The *Vibrio fluvialis* JS17 ω-transaminase, which has been found to use pyruvate methyl ester and pyruvate ethyl ester as amino acceptor and ethyl β-aminobutyrate as amino donor [31], was used as a template for BLASTP search in NCBI at default settings (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify new ω-transaminase. A putative ω-transaminase from *Burkholderia vietnamiensis* G4, which showed 53% identity and 71% similarity to the *V. fluvialis* enzyme, was selected (Fig. S1 in Supplemental material).

2.3. Overexpression and purification of the ω-transaminase

The putative transaminase gene from *B. vietnamiensis* G4 (Accession No. YP_001110355.1; Supplemental material) was codon-optimized and synthesized with a His-tag coding sequence at N-terminus by Shanghai Xuguan Biotechnological Development Co., Ltd. and ligated into the pET-32a expression vector at *Nde* I/*Bam*H I restriction sites. The vector was then transformed into *E. coli* BL21 (DE3). The transformant was grown at 37 °C in 500 ml Luria-Bertani broth supplemented with ampicillin (100 μg/ml). When the OD_{600} reached approximately 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1 mM) was added. After induction at 25 °C for 12 h, the cells were harvested and washed twice with 200 ml of phosphate buffer (50 mM, pH 7.0). The cells were re-suspended in 50 ml of lysis buffer [50 mM phosphate buffer, pH 7.0, 20 μM pyridoxal 5′-phosphate (PLP), 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.01% (v/v) β-mercaptoethanol, 30 mM imidazole and 500 mM NaCl] and disrupted by high pressure homogenizer. Cell debris was removed by centrifugation at 14,000 × *g* for 20 min at 4 °C. Supernatant was then applied on a 10 ml Ni-NTA affinity column and the eluted solution containing HBV-ω-TA was collected, and dialyzed against phosphate buffer (50 mM, pH 7.0) containing 20 μM PLP and 0.01% β-mercaptoethanol. The purified enzyme solution was then stored at 4 °C for further experiments.

2.4. Protein determination and molecular mass measurement

Protein purity was analyzed by SDS-PAGE according to standard procedure using 12.5% polyacrylamide gels. The protein bands were visualized by Coomassie blue staining. Protein concentration was determined with BCA protein assay kit (CWBIO, China).

The apparent molecular mass of HBV-ω-TA was estimated by gel filtration on a Superdex 200 HR 10/30 column (GE, USA) equilibrated and eluted with 50 mM phosphate buffer (pH 7.2) containing 150 mM NaCl at a flow rate of 0.4 ml/min.

2.5. Enzyme assays

Unless otherwise specified, enzyme assays were carried out at 37 °C in phosphate buffer (100 mM, pH 7.4) containing PLP (20 μM), (*S*)-α-MBA (10 mM) and pyruvate (10 mM). The typical reaction volume was 1 ml, and the reaction was initiated by adding purified enzyme (6.5×10^{-3} mg) to the reaction mixture. After 5 min, the reaction was stopped by adding 375 μl of 16% (v/v) perchloric acid. The produced acetophenone was analyzed by HPLC according to Section 2.10.

To study the effect of temperature on enzyme activity, reactions were carried out at various temperatures from 25 °C to 50 °C. To investigate the thermostability of HBV-ω-TA, the enzyme was incubated in phosphate buffer (100 mM, pH 7.4) at the specific temperature (30, 35, 40, 45, 50, 55, 60 °C) for 20 min and the remaining activity was assayed as described above.

To study the effect of pH on enzyme activity, reactions were carried out at various pH from 5.0 to 10.0. For the effect of pH on enzyme stability, the enzyme was incubated in the specific pH buffer (100 mM, pH = 5.0, 6.0, 7.0, 8.0, 9.0, 10.0) at 4 °C for 72 h and then the remaining activity was assayed as described above. The buffers used were sodium acetate (pH 3.8–5.6), sodium phosphate (pH 5.8–7.6), boric acid–borax (pH 7.8–9.2) and borax sodium hydroxide (pH 9.3–10.1).

2.6. Measurement of kinetic parameters

To determine the kinetic parameters, initial rates (i.e. conversion <5%) were measured at varying concentrations of (*S*)-α-MBA and pyruvate by following the activity assay procedure described above. The produced acetophenone was analyzed by HPLC according to Section 2.10. For the kinetic constants of HBV-ω-TA toward (*S*)-α-MBA, pyruvate (50 mM) was used as the amino acceptor and the concentration of (*S*)-α-MBA was as follows: 1, 2, 4, 6, 8, 10, 15, 20, 30 and 50 mM. For the kinetic constants of HBV-ω-TA toward pyruvate, (*S*)-α-MBA (50 mM) was used as the amino donor and the concentration of pyruvate was as follows: 1, 2, 3, 4, 5, 6, 8 and 10 mM. With the help of software Origin (OriginLab, USA), the kinetic constants for (*S*)-α-MBA and pyruvate were calculated.

2.7. Substrate specificity and enantioselectivity

Amino donor specificity was assayed by following the similar procedure of Section 2.5. The amino donor substrates (10 mM) listed in Table 1 reacted with pyruvate (10 mM) as amino acceptor. For racemic amino donor, the concentration was 20 mM. After the reaction, residual pyruvate was analyzed by HPLC according to Section 2.10.

Amino acceptor specificity was assayed by following the similar procedure of Section 2.5. The amino acceptor substrates (10 mM) listed in Table 2 reacted with (*S*)-α-MBA (10 mM) as amino donor.