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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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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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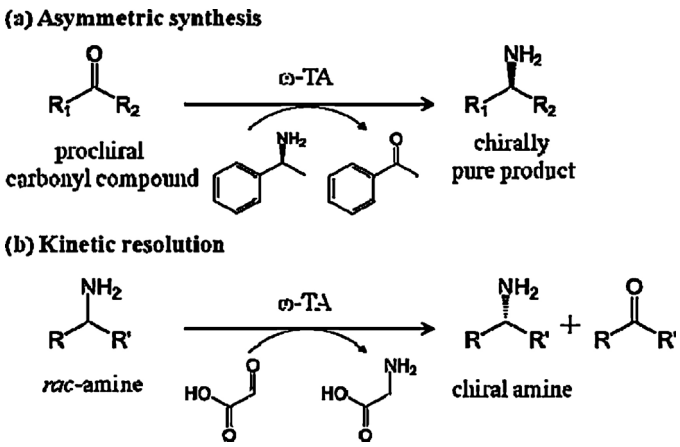


Fig. 1. ω-TA catalyzed reactions. (a) Asymmetric synthesis of chirally pure unnatural amino acids using (*S*)-α-MBA as amino donor. (b) Kinetic resolution of racemic amines using glyoxylate as amino accepter.

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.

Table 1
Amino donor specificity of HBV-ω-TA.

Amino donor	Relative activity (%)[a]
(*S*)-1-Methylbenzylamine (D1)	100
(*R*)-1-Methylbenzylamine (D2)	0
(*S*)-1-Phenylpropylamine (D3)	25.7
(*S*)-1-Phenylbutylamine (D4)	6.2
(*R*,*S*)-2-Amino-4-phenylbutane (D5)	17.3
(*S*)-1-Aminoindane (D6)	32.3
(*S*)-1-Aminotetraline (D7)	32.9
Benzylamine (D8)	27
Cyclopentylamine (D9)	5.5
Cyclohexylamine (D10)	5.7
Isopropylamine (D11)	4.9
(*R*,*S*)-2-Pentanamine (D12)	5.6
(*S*)-2-Aminohexane (D13)	6.5
DL-3-Aminobutyric acid ethyl ester (D14)	9.4
β-Alanine (D15)	2.6
DL-β-Phenylalanine (D16)	0
L-α-Leucine (D17)	1.6

[a] The activity for (*S*)-α-MBA (34 U/mg) was defined as 100%. Relative activity of less than 1% was expressed as zero. One unit of the enzyme activity was defined as the amount of the enzyme that catalyzed the formation of 1 μmol acetophenone from 10 mM (*S*)-α-MBA and 10 mM pyruvate in 1 min.

After the reaction, the amount of produced acetophenone was analyzed and the *ee* values of the produced amines were determined according to Section 2.10.

2.8. Asymmetric synthesis of enantiomerically pure amine compounds

In a typical procedure, (*S*)-α-MBA (10 mM) or isopropylamine (100 mM) were mixed with the carbonyl compounds (10 mM, Table 3) in 1 ml of phosphate buffer (100 mM, pH 7.4) containing PLP (20 μM). The reaction was started by adding 0.8 mg/ml of HBV-ω-TA, and the mixture was incubated at 37 °C for 12 h. Then the residual (*S*)-α-MBA, acetophenone and 4-phenyl-2-butanone and the *ee* values of the products were detected according to Section 2.10.

Isopropylamine (100 mM) and ethyl propionylacetate (10 mM) were mixed in 100 ml of phosphate buffer (100 mM, pH 7.4) containing PLP (20 μM). The reaction was started by adding 0.8 mg/ml of HBV-ω-TA, and the mixture was shaken at 37 °C for 12 h. After that, the pH was adjusted to about 4.0 with HCl solution (1 M), and the reaction mixture was extracted with ethyl acetate (50 ml 3×). After separation of the organic phase, the pH of the aqueous phase was adjusted to about 9.0 with saturated sodium carbonate solution and the mixture was extracted with ethyl acetate (50 ml 3×). The organic extract was dried over anhydrous sodium sulfate and removal of the solvent gave the product, 3-aminopentanoic acid ethyl ester (13 mg, 9% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.98 (t, 3H, J = 8 Hz), 1.27 (t, 3H, J = 8 Hz), 1.60 (m, 2H), 2.47–2.51 (dd, J = 16 Hz, 8 Hz, 1H), 2.58–2.62 (dd, J = 16 Hz, 8 Hz, 1H), 3.29 (m, 2H), 4.17 (q, 3H). HRMS: calcd for $C_7H_{16}NO_2$ (MH^+) 146.1181, found 146.1162.

2.9. Kinetic resolution of racemic amines

Racemic amine (10 mM, Table 4) was mixed with glyoxylate (10 mM) in 1 ml of phosphate buffer (100 mM, pH 7.4) containing PLP (20 μM). The reaction was started by adding 0.8 mg/ml of HBV-ω-TA, and the mixture was incubated at 37 °C for 12 h. The amount and *ee* value of the residual amino donor were determined according to Section 2.10.

2.10. Analytical methods

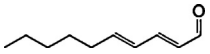
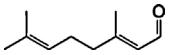
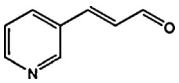
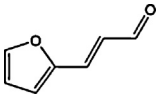
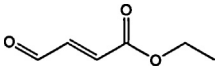
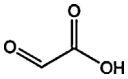
The concentrations of acetophenone, 4-formylbenzoic acid, 4-phenyl-2-butanone, 1-indanone and amines in the samples were determined by HPLC analysis on an Eclipse XDB-C18 column (4.6 mm × 150 mm, Agilent, USA) with isocratic elution of acetonitrile/water (50/50, v/v) at 1 ml/min. Similarly, the concentration of pyruvate was analyzed on an Aminex HPX-87H column (Bio-Rad, USA) at 45 °C. An aqueous solution of H_2SO_4 (5 mM) was used as eluent at a flow rate of 0.6 ml/min. All UV detections were carried out at 205 nm.

Chiral analysis of alanine was performed on PIRKLE (S,S) WHELK-O1 column (REGIS, USA) with isocratic elution (0.6 ml/min) of isopropanol/hexane (20/80, v/v) and UV detection at 254 nm after derivatization with *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu). The derivatization procedure was as follows. Fmoc-OSu was added to the reaction mixture at pH 9.0 and incubated at 37 °C for 12 h. After centrifugation, the supernatant was extracted with *tert*-butyl methyl ether. The pH of the aqueous phase was adjusted to 3.0 and the mixture was extracted with ethyl acetate. The extract was analyzed to measure the *ee* values of the derivatized product. Chiral analysis of 1-methylbenzylamine, 1-phenylpropylamine, 1-aminoindane and 1-aminotetraline were performed on CROWNPAK CR(+) column (Daicel Chemical Industries, Japan) with

Table 2
Amino acceptor specificity of HBV-ω-TA.

Amino acceptor	Relative activity (%)[a]	*ee* (%)
Pyruvate (A1)	100	>99 (*S*)
Pyruvate ethyl ester (A2)	127.7	>99 (*S*)
Ethyl acetoacetate (A3)	5	>99 (*S*)
Ethyl propionylacetate (A4)	1.4	>99 (*S*)
Levulinic acid (A5)	1.1	>99 (*S*)
α-Ketoglutarate (A6)	0	n/a[b]
1-Indanone (A7)	1.7	>99 (*S*)
1-Tetralone (A8)	2.4	>99 (*S*)
1-Phenylpropanone (A9)	1.8	>99 (*S*)
4-Phenyl-2-butanone (A10)	2.7	>99 (*S*)
2-Hexanone (A11)	0	n/a
Benzaldehyde (A12)	100.6	n/a
4-(Trifluoromethyl)benzaldehyde (A13)	24.8	n/a
4-Cyanobenzaldehyde (A14)	89.6	n/a
4-Formylbenzoic acid (A15)	68.1	n/a
Salicylaldehyde (A16)	119.3	n/a
Caproaldehyde (A17)	30.9	n/a
1-Nonanal (A18)	8.1	n/a
2,6-Dimethyl-5-heptenal (A19)	17.2	n/a
(*E*,*E*)-2,4-Hexadienal (A20)	15.4	n/a

Table 2 (*Continued*)

Amino acceptor		Relative activity (%)[a]	*ee* (%)
2-*trans*-4-*trans*-Decadienal (A21)		7.1	n/a
Citral (A22)		3.6	n/a
trans-3-(3-Pyridyl) acrolein (A23)		27.1	n/a
2-Furanacrolein (A24)		11.4	n/a
Ethyl *trans*-4-oxo-2-butenoate (A25)		32.6	n/a
Glyoxylate (A26)		98.4	n/a

[a] The activity for pyruvate (34 U/mg) was defined as 100%. The relative activity of less than 1% was expressed as zero. One unit of the enzyme activity was defined as the amount of the enzyme that catalyzed the formation of 1 μmol acetophenone from 10 mM (*S*)-α-MBA and 10 mM pyruvate in 1 min.
[b] n/a, not applicable.

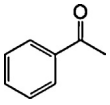
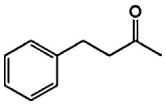
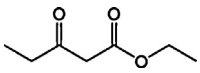
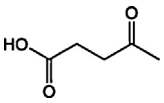
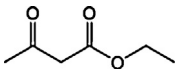
isocratic elution (0.5 ml/min) of perchloric acid solution (pH 1.5) containing 10% of methanol at 30 °C and UV detection at 200 nm. Chiral analysis of α-alanine ethyl ester, 3-aminobutyric acid ethyl ester and 3-aminopentanoic acid ethyl ester were performed by GC on Chirasil-DEX CB column (25 m × 0.25 mm × 0.25 μm, Varian, USA) after acetylation, which was performed by mixing acetic anhydride, pyridine and the product (3:1.2:1) in tetrahydrofuran. GC conditions were as follows: for α-alanine ethyl ester, column temperature was 115 °C, isothermal, the split ratio was 20:1, flow rate of the carrier gas (helium) was 2 ml/min, FID detector (220 °C); for 3-aminobutyric acid ethyl ester and 3-aminopentanoic acid ethyl ester, column temperature was 140 °C, isothermal, the split ratio was 20:1, flow rate of the carrier gas (helium) was 2 ml/min, FID detector (220 °C) [32]. Chiral analyses of 2-amino-4-phenylbutane and 4-aminovaleric acid were performed on C18 column with isocratic elution of phosphoric acid–triethylamine (50 mM, pH 3.0)/acetonitrile and UV detection at 340 nm after FDAA derivatization. For 2-amino-4-phenylbutane, the ratio of phosphoric acid–triethylamine to acetonitrile was 55/45 (v/v) and the flow rate was 1 ml/min, while those for 4-aminovaleric acid were 70/30 (v/v) and 0.5 ml/min, respectively. FDAA derivatizations were performed according to the instruction manual of FDAA (Thermo Fisher Scientific, USA). The retention times refer to Table S1 (Supplemental material).

3. Results and discussion

3.1. Overexpression, purification and molecular mass of the ω-transaminase

A putative ω-transaminase gene from *B. vietnamiensis* G4 was overexpressed in *E. coli* BL21 (DE3), and the purified HBV-ω-TA

Table 3
Asymmetric synthesis of enantiomerically pure amine compounds by HBV-ω-TA.

Substrate		Concentration (mM)	Conversion (%)	*ee* (%)
Acetophenone[a]		10	16	>99 (*S*)
4-Phenyl-2-butanone[a]		10	30	>99 (*S*)
Ethyl propionylacetate[b]		10	17	>99 (*S*)
Levulinic acid[b]		10	66	>99 (*S*)
Ethyl acetoacetate[b]		10	89	>99 (*S*)

[a] 100 mM isopropylamine was used as the amino donor.
[b] 10 mM (*S*)-α-MBA was used as the amino donor.

Table 4
Kinetic resolution of amines with glyoxylate as amino acceptor.

Substrate	Concentration (mM)	Conversion (%)	*ee* value (%)
(*R*,*S*)-1-Methylbenzylamine	10	50	>99 (*R*)
(*R*,*S*)-1-Phenylpropylamine	10	50	>99 (*R*)
(*R*,*S*)-2-Amino-4-phenylbutane	10	53	>99 (*R*)
(*R*,*S*)-1-Aminotetraline	10	51	>99 (*R*)

showed a single band of about 45 kDa on SDS-PAGE (Fig. 2), consistent with the predicted value of a 461-amino acid polypeptide (50,279 Da). The molecular mass of the native HBV-ω-TA was estimated to be 100 kDa by gel filtration chromatography, suggesting that the enzyme has a dimeric structure of the enzyme.

3.2. Effects of pH, temperature and substrate concentrations

The effects of temperature and pH on the enzyme activity are shown in Fig. 3. The optimum reaction temperature was between 42 °C and 45 °C, and the activity of HBV-ω-TA decreased 20% when the temperature was 50 °C. HBV-ω-TA was more active under weak alkaline conditions with the optimum pH being about pH 8.4. This weak alkaline pH optimum for the enzyme activity was likely due to the increase in the effective concentration of the deprotonated amino donor that enhances the formation of an external aldimine, which was also observed for the known transaminases from *Mesorhizobium* sp. Strain LUK (pH 7.6) [27], *Caulobaceter crescentus* (pH 8.5) [33], *Arthrobacter* sp. KNK168 (pH 8.0–9.0) [34], *Alcaligenes denitrificans* Y2k-2 (pH 9.0) [26], and *V. fluvialis* JS17 (pH 9.2) [35].

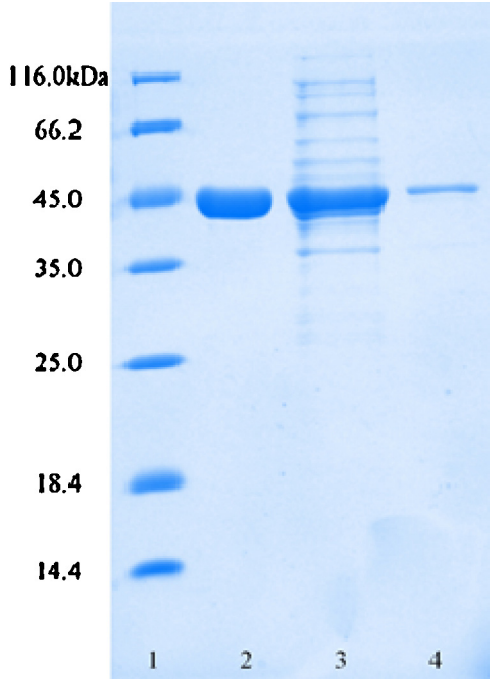


Fig. 2. SDS-PAGE of HBV-ω-TA. Lanes: (1) protein marker (molecular mass 116.0, 66.2, 45.0, 35.0, 25.0, 18.4, 14.4 kDa); (2) purified enzyme; (3) soluble fraction of cell extract; (4) precipitate fraction of cell extract.

The effects of temperature and pH on the enzyme stability are shown in Fig. 4. When HBV-ω-TA was incubated in phosphate buffer (100 mM, pH 7.4) at various temperatures for 20 min, the enzyme activity remained nearly unchanged up to 50 °C, but dropped sharply above 50 °C. The activity was completely lost at 60 °C. The enzyme was incubated in various pH buffers for 72 h and the remaining activity of the enzyme was measured. HBV-ω-TA was relatively stable between pH 5.0 and 9.0. Its activity decreased above pH 9.0 and 60% activity was lost after incubation at pH 10.0 for 72 h.

The effects of the substrate concentration of (*S*)-α-MBA and pyruvate on the enzyme activity were examined. Using the nonlinear fit method of Origin software, the apparent K_m for (*S*)-α-MBA and pyruvate were calculated to be 14.67 ± 1.02 mM and 1.65 ± 0.22 mM, respectively. The V_{max} for (*S*)-α-MBA and pyruvate were calculated to be 82.98 ± 2.52 U/mg and 77.80 ± 2.90 U/mg, respectively. The apparent K_m values for pyruvate were 2.29, 3.9 and 11 mM for the reported transaminases from *Arthrobacter* sp. KNK168, *Mesorhizobium* sp. Strain LUK and *A. denitrificans* Y2k-2, respectively [26,27,34].

3.3. Substrate specificity and enantioselectivity

The amino donor specificity of HBV-ω-TA was examined in terms of initial rate with pyruvate as the amino acceptor [31]. It can be seen from Table 1 that HBV-ω-TA was active toward a wide range of amino donors. Similar to the *V. fluvialis* transaminase [31], HBV-ω-TA showed high activity toward aromatic (*S*)-amines such as (*S*)-1-methylbenzylamine (D1, the most effective one), (*S*)-1-aminoindane (D6) and (*S*)-1-aminotetraline (D7), while no activity was detected for (*R*)-1-methylbenzylamine (D2). The high enantioselectivity toward the amino donor was beneficial for preparation of enantiopure amines via kinetic resolution. The initial activity decreased in the order of (*S*)-1-methylbenzylamine (D1), (*S*)-1-phenylpropylamine (D3) and (*S*)-1-phenylbutylamine (D4). This was consistent with the two-binding pocket model for the conserved substrate recognition mechanism of ω-transaminase [31]. The two substrate binding pockets were located at subunit interface and constituted active site together with PLP binding

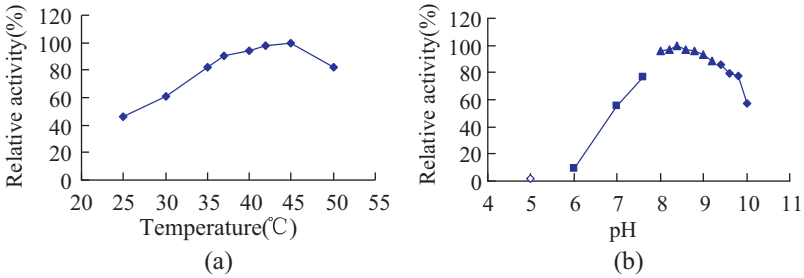


Fig. 3. Effects of temperature (a) and pH (b) on the enzyme activity. Reactions were carried out at various temperatures from 25 °C to 50 °C (a) and various pHs from 5.0 to 10.0 (b), respectively. The buffers for different pHs were sodium acetate (pH 3.8–5.6), sodium phosphate (pH 5.8–7.6), boric acid–borax (pH 7.8–9.2) and borax sodium hydroxide (pH 9.3–10.1).

pocket (including catalytic lysine residue) [36]. Several key active site residues in the binding pockets were identified using the crystal structure of ω-transaminase from *Paracoccus denitrificans* [36]. The large pockets suitably accommodated the aryl group, while the small pocket suitably accommodated methyl group [31]. In addition, the steric constraint in small pocket was studied using free energy analysis method with structurally similar substrates such as (*S*)-1-methylbenzylamine and (*S*)-1-phenylpropylamine [37]. In this study, the enzyme activity decreased when the methyl group was replaced with ethyl and propyl groups because of the increasing steric constraint in the small binding pocket of HBV-ω-TA. Most of the aliphatic amines showed relatively low activity compared with aromatic amines. Short-chain β-amino acid esters showed considerable activity (1.7 U/mg for DL-3-aminobutyric acid ethyl ester (D14)), whereas aromatic β-amino acids such as DL-β-phenylalanine (D16) was inert. β-alanine (D15), a typical substrate of ω-transaminases, showed low activity. Overall, amino acids were less reactive substrates than amines.

The amino acceptor specificity of HBV-ω-TA was examined by measuring the initial rate with (*S*)-α-MBA as the amino donor [31]. As shown in Table 2, HBV-ω-TA accepted a wide range of amino acceptors. Pyruvate ethyl ester (A2) was the most active amino acceptor among the tested carbonyl compounds. In addition, pyruvate (A1), benzaldehyde (A12), salicylaldehyde (A16) and glyoxylate (A26) also showed good activity, whereas α-ketoglutarate (A6) was inert. Glyoxylate is a cheaper amine acceptor than pyruvate and may be used in the large-scale kinetic resolution [38]. It is noteworthy that the enzyme showed distinct activity toward ethyl acetoacetate (1.7 U/mg) (A3), ethyl propionylacetate (0.5 U/mg) (A4) and levulinic acid (0.4 U/mg) (A5). The *ee* values of all the corresponding products exceeded 99%. To the best of our knowledge, there was no reported method to produce (*S*)-3-aminopentanoic acid ethyl ester and (*S*)-4-aminovaleric acid using transaminase as catalyst, suggesting that HBV-ω-TA might have potential for use in the asymmetric synthesis of some valuable enantiomerically pure unnatural amino acids. Relatively high activity was observed for most of the tested aldehydes, while all the ketones showed poor activity. In addition, this enzyme showed considerable activity toward α,β-unsaturated aldehydes such as 2,6-dimethyl-5-heptenal (A19), trans-3-(3-pyridyl) acrolein (A23) and ethyl trans-4-oxo-2-butenoate (A25). These carbonyl compounds have not been reported as the substrates of ω-TA before. Overall, HBV-ω-TA showed higher activity toward keto acids, keto esters and aldehydes than ketones.

3.4. Asymmetric synthesis of enantiomerically pure amine compounds

The feasibility of HBV-ω-TA for the synthesis of optically pure amine compounds by asymmetric amino transfer reaction was tested with different carbonyl compounds as the amino acceptor (Fig. 1a). The results are listed in Table 3. All the prochiral carbonyl compounds were aminated with excellent enantioselectivity.

4-Phenyl-2-butanone and acetophenone were aminated with low conversions which might be caused by the unfavorable thermodynamic equilibrium, poor solubility and severe inhibitions of ketone substrate [28,39,40].

ω-Transaminase is an attractive alternative biocatalyst for the production of optically pure unnatural amino acids. Levulinic acid was aminated by HBV-ω-TA to give (*S*)-4-aminovaleric acid with 66% conversion and high *ee* value (99%). This is the first report that optically pure (*S*)-4-aminovaleric acid was produced with transaminases as biocatalyst. It has been reported that the transaminase-catalyzed amination of β-keto acids giving β-amino acids was hindered by their instability caused by spontaneous decarboxylation of the carboxyl moiety [21,27]. In this study, HBV-ω-TA can catalyzed the amino transfer to the stable β-keto esters (ethyl acetoacetate and ethyl propionylacetate) to give optically pure β-amino acid esters, which could be hydrolyzed to the corresponding β-amino acids, offering an alternative approach to access these compounds by transaminase [21]. It was the first report that optically pure (*S*)-3-aminopentanoic acid ethyl ester was produced

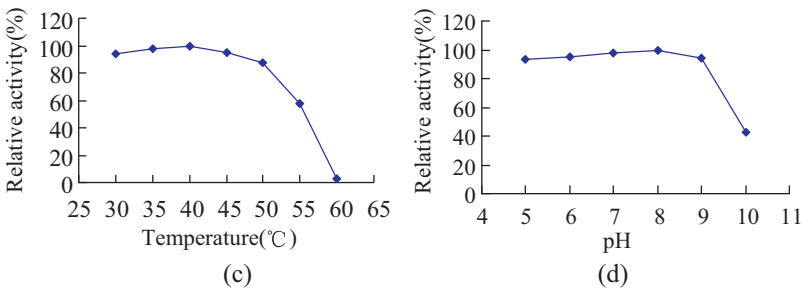


Fig. 4. Effects of temperature (c) and pH (d) on the enzyme stability. The remaining activity was assayed after 20 min incubation at certain temperatures (c) or 72 h incubation at 4 °C in the specific pH buffers (d).

with transaminases. The conversion for ethyl acetoacetate was 89%, while that for ethyl propionylacetate was only 17%. The relatively low conversion of ethyl propionylacetate may be due to the lower activity caused by the steric hindrance in small pocket of the ω-TA [31]. Christopher et al. [29] enlarged the substrate binding pockets of the ω-TA from *Arthrobacter* sp. KNK168 by protein engineering methods and endowed a new enzyme with extraordinary ability to asymmetrically synthesize sitagliptin using high concentration of isopropylamine as amino donor. The small pocket amino acid residues are mainly engaged in the binding step but not significantly involved in the catalytic step [37], indicating that the small pocket of HBV-ω-TA may be enlarged to relieve the steric constraint toward bulky substituents by protein engineering in the future.

3.5. Kinetic resolution of racemic amines

Given the excellent enantioselectivity of HBV-ω-TA, the feasibility of using this enzyme as a catalyst was studied for the kinetic resolution of several racemic amines (Fig. 1b). The results are summarized in Table 4. All of the racemic amines tested were successfully resolved and enantiomerically pure (*R*)-enantiomers were obtained with good conversions. As such, HBV-ω-TA showed potential in the production of enantiomerically pure amines by kinetic resolution. The results also indicated that glyoxylate was an effective and practical amino acceptor which can be used in the kinetic resolution of amines.

4. Conclusion

In this study, a putative ω-TA gene from *B. vietnamiensis* G4 was expressed in *E. coli* and the recombinant enzyme was biochemically characterized. The new enzyme exhibited broad substrate specificity and excellent enantioselectivity. It can serve as a useful catalyst both in the kinetic resolution of racemic amines and in the asymmetric synthesis of unnatural amino acids from the corresponding keto acids. Especially, HBV-ω-TA catalyzed the amino transfer to the stable β-keto esters to give optically pure β-amino acid esters, offering an alternative approach to access β-amino acids by transaminases. These results demonstrate that HBV-ω-TA is a valuable addition to the current transaminase tool-box and should be an attractive platform enzyme for further studies. Similar to the previously reported ω-TAs [23,26,27], HBV-ω-TA could not aminate aromatic β-keto esters. This may be caused by steric constraint in the binding pocket [31,37] which could be enlarged by protein engineering to accommodate aromatic β-keto esters as amino accepters to produce aromatic β-amino acids [29]. Directed evolution studies are currently carried out in our laboratory to possibly improve the activity and stability of HBV-ω-TA toward high concentration of isopropylamine, making it a preferred amino donor for HBV-ω-TA for further applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2013.11.013.

References

[1] M. Friedman, Chem. Biodivers. 7 (2010) 1491–1530.
[2] M. Friedman, C. Levin, Amino Acids 42 (2012) 1553–1582.
[3] J.S. Ma, Chim. Oggi 21 (2003) 65–68.
[4] I. Ojima, S. Lin, T. Wang, Curr. Med. Chem. 6 (1999) 927–954.
[5] M.I. Aguilar, A.W. Purcell, R. Devi, R. Lew, J. Rossjohn, A.I. Smith, P. Perlmutter, Org. Biomol. Chem. 5 (2007) 2884–2890.
[6] J. Frackenpohl, P.I. Arvidsson, J.V. Schreiber, D. Seebach, ChemBioChem 2 (2001) 445–455.
[7] W.S. Horne, L.M. Johnson, T.J. Ketas, P.J. Klasse, M. Lu, J.P. Moore, S.H. Gellman, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 14751–14756.
[8] D. Seebach, J. Gardiner, Acc. Chem. Res. 41 (2008) 1366–1375.
[9] D.L. Steer, R.A. Lew, P. Perlmutter, A.I. Smith, M.-I. Aguilar, Lett. Pept. Sci. 8 (2001) 241–246.
[10] A. Montero, J.M. Beierle, C.A. Olsen, M.R. Ghadiri, J. Am. Chem. Soc. 131 (2009) 3033–3041.
[11] S. Moradi, S. Soltani, A.M. Ansari, S. Sardari, Anti-Infect. Agents Med. Chem. 8 (2009) 327–344.
[12] D.L. Steer, R.A. Lew, P. Perlmutter, S.A. Ian, M.I. Aguilar, Curr. Med. Chem. 9 (2002) 811–822.
[13] O.M. Stephens, S. Kim, B.D. Welch, M.E. Hodsdon, M.S. Kay, A. Schepartz, J. Am. Chem. Soc. 127 (2005) 13126–13127.
[14] W. Leuchtenberger, K. Huthmacher, K. Drauz, Appl. Microbiol. Biotechnol. 69 (2005) 1–8.
[15] M. Höhne, U.T. Bornscheuer, ChemCatChem 1 (2009) 42–51.
[16] M. Liu, M.P. Sibi, Tetrahedron 58 (2002) 7991–8035.
[17] B. Weiner, W. Szymanski, D.B. Janssen, A.J. Minnaard, B.L. Feringa, Chem. Soc. Rev. 39 (2010) 1656–1691.
[18] D. Zhu, L. Hua, Biotechnol. J. 4 (2009) 1420–1431.
[19] M.S. Malik, E.-S. Park, J.-S. Shin, Appl. Microbiol. Biotechnol. 94 (2012) 1163–1171.
[20] S. Mathew, H. Yun, ACS Catal. 2 (2012) 993–1001.
[21] J. Rudat, B.R. Brucher, C. Syldatk, AMB Express 2 (2012) 11.
[22] B.-Y. Hwang, B.-K. Cho, H. Yun, K. Koteshwar, B.-G. Kim, J. Mol. Catal. B: Enzym. 37 (2005) 47–55.
[23] H.S. Bea, H.J. Park, S.H. Lee, H. Yun, Chem. Commun. 47 (2011) 5894–5896.
[24] D. Koszelewski, K. Tauber, K. Faber, W. Kroutil, Trends Biotechnol. 28 (2010) 324–332.
[25] D. Koszelewski, B. Grischek, S.M. Glueck, W. Kroutil, K. Faber, Chem. Eur. J. 17 (2011) 378–383.
[26] H. Yun, S. Lim, B.K. Cho, B.G. Kim, Appl. Environ. Microbiol. 70 (2004) 2529–2534.
[27] J. Kim, D. Kyung, H. Yun, B.K. Cho, J.H. Seo, M. Cha, B.G. Kim, Appl. Environ. Microbiol. 73 (2007) 1772–1782.
[28] D. Koszelewski, I. Lavandera, D. Clay, D. Rozzell, W. Kroutil, Adv. Synth. Catal. 350 (2008) 2761–2766.
[29] C.K. Savile, J.M. Janey, E.C. Mundorff, J.C. Moore, S. Tam, W.R. Jarvis, J.C. Colbeck, A. Krebber, F.J. Fleitz, J. Brands, P.N. Devine, G.W. Huisman, G.J. Hughes, Science 329 (2010) 305–309.
[30] M. Otsuka, T. Masuda, A. Haupt, M. Ohno, T. Shiraki, Y. Sugiura, K. Maeda, J. Am. Chem. Soc. 112 (1990) 838–845.
[31] J.S. Shin, B.G. Kim, J. Org. Chem. 67 (2002) 2848–2853.
[32] J. Wu, X. Chen, R. Guo, C.-h. Yeung, A.S. Chan, J. Org. Chem. 68 (2003) 2490–2493.
[33] B.Y. Hwang, H.Y. Park, B.S. Lee, J. Microbiol. Biotechnol. 18 (2008) 48–54.
[34] A. Iwasaki, K. Matsumoto, J. Hasegawa, Y. Yasohara, Appl. Microbiol. Biotechnol. 93 (2012) 1563–1573.
[35] J.S. Shin, H. Yun, J.W. Jang, I. Park, B.G. Kim, Appl. Microbiol. Biotechnol. 61 (2003) 463–471.
[36] C. Rausch, A. Lerchner, A. Schiefner, A. Skerra, Proteins: Struct. Funct. Bioinf. 81 (2013) 774–787.
[37] E.-S. Park, J.-S. Shin, Enzyme Microb. Technol. 49 (2011) 380–387.
[38] U. Kaulmann, K. Smithies, M.E.B. Smith, H.C. Hailes, J.M. Ward, Enzyme Microb. Technol. 41 (2007) 628–637.
[39] J.-S. Shin, B.-G. Kim, Biotechnol. Bioeng. 60 (1998) 534–540.
[40] P. Tufvesson, J. Lima-Ramos, J.S. Jensen, N. Al-Haque, W. Neto, J.M. Woodley, Biotechnol. Bioeng. 108 (2011) 1479–1493.