Biochemical and structural characterization of a highly active branched-chain amino acid aminotransferase from *Pseudomonas* sp. for efficient biosynthesis of chiral amino acids

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Abstract

Aminotransferases (ATs) are important biocatalysts for the synthesis of chiral amines because of their capability of introducing amino group into ketones or keto acids as well as their high enantioselectivity, high regioselectivity and no requirement of external addition of cofactor. Among all ATs, branched-chain amino acid aminotransferase (BCAT) can reversibly catalyse branched-chain amino acids (BCAAs), including L-valine, L-leucine, and L-isoleucine, with α -ketoglutaric acid to form the corresponding ketonic acids and L-glutamic acid. Alternatively, BCATs have been used for the biosynthesis of unnatural amino acids, such as L-tert-leucine. In the present study, the BCAT from *Pseudomonas* sp. (PsBCAT) was cloned and expressed in *Escherichia coli* for biochemical and structural analyses. The optimal reaction temperature and pH of PsBCAT were 40 °C and 8.5, respectively. PsBCAT exhibited a comparatively broader substrate spectrum, and showed remarkably high activity with L-leucine, L-valine, L-isoleucine and L-methionine with activities of 105 U/mg, 127 U/mg, 115 U/mg and 98 U/mg, respectively. Additionally, PsBCAT had activities with aromatic L-amino acids, L-histidine,

L-lysine, and L-threonine. To analyse the catalytic mechanism of *PsBCAT* with the broad substrate spectrum, the crystal structure of *PsBCAT* was also determined. Finally, conjugated with the ornithine aminotransferase (OrnAT) from *Bacillus subtilis*, the coupled system was applied to the preparation of L-tert-leucine with 83% conversion, which provided an approximately 2.7-fold higher yield than the single BCAT reaction.

IMPORTANCE: Despite the enormous potential of BCATs, the vast majority of enzymes still lack suitably broad substrate scope and activity, thus new sources and novel enzymes are currently being investigated. Here, we described a previously uncharacterized *PsBCAT*, which showed a surprisingly wide substrate range and was more active towards BCAAs. This substrate promiscuity is unique for the BCAT family and could prove useful in industrial applications. Based on the determined crystal structure, we found some differences in the organization of the substrate binding cavity, which may influence the substrate specificity of the enzyme. Moreover, we demonstrated efficient biocatalytic asymmetric synthesis of L-tert-leucine using a coupling system, which can be used to remove the inhibitory by-product, and to shift the reaction equilibrium towards the product formation. In summary, the structural and functional characteristics of *PsBCAT* were analysed in detail, and this information will play an important role in the synthesis of chiral amino acids and will be conducive to industrial production of enantiopure chiral amines by aminotransferase.

1. Introduction

Enantiopure chiral amines are important building blocks in the synthesis of optically active pharmaceuticals, fine chemicals, agrochemicals and bioactive natural products (1-3) due to their structural diversity and functional versatility. The impressive number of applications of chiral amines has stimulated a great deal of innovation in synthetic methodologies for their preparation (4). Within the expanding biocatalytic toolbox for amine synthesis, ATs represent an attractive alternative with excellent optical purity, quantitative yields and high enantioselectivity (5). In general, ATs catalyse the amino

group transfer from an amino donor to a ketone or aldehyde amine acceptor with pyridoxal 5'-phosphate (PLP) as cofactor. The first step of the reaction is the transfer of the amino group to the cofactor covalently bound to an active site lysine via a Schiff base, resulting in the formation of pyridoxamine-5'-phosphate (PMP) and release of the amine donor as a ketone or aldehyde. The second step is the regeneration of PLP with the transfer of the amino group to the amine acceptor, which is released as a chiral amino if the acceptor is a ketone or a primary amine if the acceptor is an aldehyde (6).

PLP-dependent enzymes of aminotransferases can be grouped into four subgroups: α family, β family, D-alanine transaminase family, and alanine racemase family (7). Among the four subgroups, all ATs belong to the α family except for BCATs and D-amino acid aminotransferases (DATs), which belong to the D-alanine aminotransferase family (8). Structural similarity and sequence alignment analysis conducted by the Protein Family Database (Pfam) suggest that ATs are divided into six subgroups or classes, with classes I and II including L-aspartate aminotransferases (AspATs) and L-aromatic amino acid aminotransferases (AroATs), class III ω -transaminases (ω -ATs), class IV BCATs and DATs, class V L-serine aminotransferases, and class VI sugar aminotransferases (8, 9).

Among all ATs, BCATs are specific for proteinogenic BCAAs - leucine, isoleucine and valine, to α -ketoglutaric acid to form the respective branched-chain α -ketonic acids and glutamic acid (10). Alternatively, BCATs have been used for the biosynthesis of unnatural amino acids, including L-tert-leucine (4). Incorporation of unnatural amino acids into peptide drug serves an important strategy in the synthesis of biologically active compounds with better bioavailability and enhanced potency of the modified target molecules (11). The crucial amino acids in this regard, L-tert-leucine, is extensively used for templates in asymmetric synthesis based on its oxazolidine moiety. In addition, L-tert-leucine itself is an ingredient of several pharmaceutical developmental products, such as anti-HIV protease inhibitors or tumour-fighting agents (12, 13).

The biosynthesis of L-tert-leucine has been achieved by coupling BCAT with several enzymes, including ornithine- δ -aminotransferase (OAT) (14), AspAT/pyruvate decarboxylase (PDC) (15), ω -AT (16), and GluDH/FDH (17). However, the crucial

setbacks in the scale-up of the most BCTA coupling reaction include low catalytic efficiency, cumbersome steps and high cost. It is apparent that the pool of enzymes effectively suitable for these biotransformations is limited, and new sources and novel enzymes are currently being investigated.

Here, we successfully expressed and purified BCAT from *Pseudomonas* sp. (*PsBCAT*) and described enzymatic properties, including optimal reaction pH and optimal reaction temperature. The substrate specificity and kinetic parameters of several typical specific substrates were also determined. To further study its structure-function feature, the three-dimensional crystal structure combined with PLP was determined. In addition, compared with *BsBCAT*, *CgBCAT* and *EcBCAT*, *PsBCAT* exhibited highest activities in biosynthesis *L*-tert-leucine, and the feasibility of *L*-tert-leucine synthesis was also verified.

2. Materials and methods

Sequence similarity networks (SSN) analysis

A sequence similarity network of the BCAT family proteins [57,443 sequences, PFam01063 from Pfam release 31.0] (18) was created using EFI-EST web tools (19). In the 1st round, 57,443 sequences from Pf01063 were clustered by 90% identity to 30,927 sequences for alignment, and a network with an E-value cut-off 10^{-190} was created and visualized using the default layout in Cytoscape 3.7.1 (20). To filter out highly conservative sequences and mine sequences with different substrates specificity, nodes with multiple InterPro (21) annotations containing more than 40 sequences from at least 2 prokaryotic genus and not grouped in networks were chosen as input sequences for the next round of analysis. The 2nd network was built with an E-value cut-off 10^{-75} to exclude ungrouped nodes. The results were displayed in Cytoscape using 50% identity RepNodes to obtain higher resolution. The 2nd network grouped the RepNodes into 3 clusters. Sequences in 3 groups were retrieved for the 3rd analysis. The third network had an E-value cut-off 10^{-3} and was coloured by family. Families with less than 3 nodes were deleted.

Cloning, expression and purification of BCATs and *BsOrnAT*

The *ilvE* gene of *PsBCAT* (NCBI Accession NO. WP_015095447) was

118 codon-optimized and synthesized with a N-terminal His-tag by General Biosystems. The
 119 *PsBCAT* gene was inserted into NdeI and XhoI sites of the pET-28a expression vector.
 120 The *ilvE* gene encoding *Bacillus subtilis* BCAT (*BsBCAT*, Genebank Accession NO.
 121 CP017112), *Corynebacterium glutamicum* BCAT (*CgBCAT*, Genebank Accession NO.
 122 CP025533) and *Escherichia coli* BCAT (*EcBCAT*, Genebank Accession NO. LR134248)
 123 were amplified using corresponding primers from the genomes, and the *rocD* gene
 124 encoding *Bacillus subtilis* OAT (*BsOrnAT*, Genebank Accession NO. CP019662) was
 125 amplified. The amplified products were assembled into the corresponding position of
 126 pET-28a using the Gibson assembly method and then transformed into *E. coli* Top10 for
 127 screening. All primers used above are presented in Table 1. Finally, the target plasmids
 128 were verified by sequencing (Tsingke, Beijing, China).

129 Table 1. Primers used for gene cloning

Primers	Sequence
PET28-F	5'-caaagcccgaaaggaagctgagttg-3'
PET28-R	5'-gccgctgctgtgatgatgatga-3'
<i>BsBCAT</i> -F	5'- catcatcacagcagcgcatgactaaacaaacgattcg-3'
<i>BsBCAT</i> -R	5'- gcttcctttcgggctttgttacttgctttcagtcagcg-3'
<i>CgBCAT</i> -F	5'-catcatcacagcagcgcatgacgtacctcgtgtggga-3'
<i>CgBCAT</i> -R	5'-gcttcctttcgggctttgtcacggaaaaatggtgggc-3'
<i>EcBCAT</i> -F	5'-atcatcatcacagcagcgcatgaccacgaagaaagctga-3'
<i>EcBCAT</i> -R	5'-cagcttcctttcgggctttgtattgattaacttgatctaaccagccc-3'
<i>BsOrnAT</i> -F	5'-catcatcacagcagcgcatgacagctttatctaaatcca-3'
<i>BsOrnAT</i> -R	5'-gcttcctttcgggctttgtatgcgtttcgagcacgt-3'

130
 131 *E. coli* BL21(DE3) carrying the recombinant plasmid was cultivated at 30 °C in 1 L
 132 auto-induction cultures (10 g/L tryptone, 5 g/L yeast extract, 3.3 g/L ammonium sulfate,
 133 6.8 g/L monopotassium phosphate, 17.9 g/L disodium hydrogen phosphate , 0.55 g/L
 134 glucose, 2.1 g/L lactose, 0.49 g/L magnesium sulfate, 2.0 mL/L glycerol) containing 50
 135 µg/mL kanamycin for the expression of recombinant proteins. After 20 h of induction, the
 136 cells were harvested, resuspended in 20 mM phosphate buffer (pH=7.5) containing 0.5 M

sodium chloride and 20 mM imidazole, and disrupted by sonication. The crude cell extract was centrifuged for 60 min at 12 000 rpm, and supernatants were prepared for further purification. Crude enzyme solution was loaded into Ni-NTA obtained from GE and washed with 50 mL solution buffer (pH=7.5) containing 20 mM imidazole, 20 mM phosphate and 0.5 M sodium chloride. Then, the proteins were eluted with elution buffer (pH=7.5) containing 0.5 M imidazole, 20 mM phosphate, and 0.5 M sodium chloride and desalted with a phosphate buffer (pH=7.5) containing 20 mM phosphate. Finally, the molecular weight of *PsBCAT* was analysed by 12% SDS-PAGE. Purified enzymes were stored in a -20 °C refrigerator for further research. All chemicals, including antibiotics, medium material, and imidazole, were obtained from Sigma-Aldrich.

Enzyme activity assay

The activity of recombinant *PsBCAT* was determined using L-valine as an amino donor and α -ketoglutaric acid as the amino receptor. The reaction was performed at 37 °C and pH 7.5 (20 mM phosphate buffer) with 10 mM L-valine, 4 mM α -ketoglutaric acid, 10 μ M PLP and an appropriate amount purified enzyme in a total volume of 1 mL. Reactions were terminated by 1 M HCl, and the concentration of α -ketoisovaleric acid was analysed using a C₁₈ column with a Shimadzu HPLC system at 210 nm. Separation of the target component was achieved through an isocratic elution with a mixture of 5% acetonitrile and 95% water (0.1% formic acid) at a flow rate of 1.0 mL/min. One unit of enzyme activity was defined as the amount of enzyme that can produce 1 μ mol of α -ketoisovaleric acid per min. All experiments were performed in triplicate.

Effect of pH, temperature and PLP on *PsBCAT* activity

The optimum pH for the reaction with 10 mM L-valine, 4 mM α -ketoglutaric acid and 10 μ M PLP was determined at 37 °C as described above using the following buffers: 50 mM Na₂HPO₄- citric acid buffer (pH 5.0-7.0), 50 mM Tris-HCl buffer (pH 7.0-9.0) and 50 mM Gly-NaOH buffer (pH 9.0-10.5). The optimal temperature was estimated in 50 mM Tris-HCl buffer (pH 8.5) with 10 mM L-valine, 4 mM α -ketoglutaric acid and 10 μ M PLP under different temperature (25~55 °C). The effect of PLP concentration on activity was tested at 37 °C by performing 10 mM L-valine and 4 mM α -ketoglutaric acid reactions in 50 mM Tris-HCl buffer (pH 8.5) (22).

167

168 **Substrate specificity**

169 The substrate specificity of the *PsBCAT* for amino acids or amines as amino donors
170 was investigated using α -ketoglutaric acid as the amino acceptor. The reaction mixture
171 consists of 10 mM amino donors, 4 mM α -ketoglutaric acid, and 10 μ M PLP in 50 mM
172 Tris-HCl (pH 8.5) at 37 °C. Reactions were terminated by 1 M HCl, and the reduction of
173 α -ketoisovaleric acid was analysed by HPLC.

174 **Kinetic analysis**

175 For steady-state kinetic analysis, apparent kinetic constants of *PsBCAT* for L -valine,
176 L -leucine, L -isoleucine and L -tert-leucine ranging from 0 to 50 mM were determined at a
177 fixed concentration of α -ketoglutaric acid in 1-mL reaction mixtures containing 50 mM
178 Tris-HCl (pH 8.5) and 10 μ M PLP at room temperature. For L - glutamic acid, kinetic
179 constants were measured simultaneously but with a fixed concentration of
180 α -ketoisovaleric acid as amino acceptor. In the reverse direction, apparent kinetic
181 constants of α -ketonic acids were measured as well. Apparent K_m and V_{max} values were
182 calculated by fitting the Michaelis–Menten equation to the experimental data using the
183 non-linear least-squares fit mode of Origin 9.0.

184 **Crystallization, data collection and structure determination**

185 Crystals of *PsBCAT* were obtained using the hanging-drop vapour-diffusion method at
186 16°C by mixing equal volumes of protein (12 mg/mL) and reservoir solution that
187 contained 0.25 M ammonium citrate dibasic and 20% (w/v) PEG 3350. The crystals were
188 soaked in reservoir solution supplied with an additional 20% (v/v) glycerol and then
189 flash-frozen in liquid nitrogen. The diffraction datasets were collected at the Shanghai
190 Synchrotron Radiation Facility (SSRF, China) on a beamline BL19U1 with a wavelength
191 of 0.9789 Å at 100 K. The data were processed with XDS (23) and CCP4 (24). Initial
192 phases were obtained by molecular replacement with PHASER (25) using the structure of
193 branched-chain aminotransferase from *Deinococcus radiodurans* (PDB entry 3UYY) (28)
194 as a search model. The model of *PsBCAT* was automatically and manually built by
195 PHENIX.AUTOBUILD (26) and COOT (27), respectively, and refined with PHENIX
196 (26). The data collection and refinement statistics are summarized in Table 2. The

197 coordinates and structure factors for *PsBCAT* have been deposited in the Protein Data
198 Bank under accession 6JIF.

199 Table 2. Statistics of crystal diffraction and structural refinement of *PsBCAT*

Crystal diffraction statistics	Value
Wavelength	0.98
Resolution range	47.86-1.70 (1.76-1.70)
Space group	P 21 21 21
Unit cell	71.83 95.72 109.82 90 90 90
Total reflections	1071694 (75160)
Unique reflections	83772(6091)
Multiplicity	12.8 (12.3)
Completeness (%)	99.9 (99.0)
Mean I/sigma (I)	16.57 (2.86)
Wilson B-factor	20.87
R-merge	0.11 (0.982)
R-meas	0.11 (1.067)
R-pim	0.03134 (0.29)
CC1/2	0.999 (0.84)
CC*	1 (0.96)
Reflections used in refinement	83754 (8230)
Reflections used for R-free	2000 (197)
R-work	0.19 (0.28)
R-free	0.23 (0.33)
CC (work)	0.96 (0.82)
CC (free)	0.93 (0.71)
Number of non-hydrogen atoms	5927
macromolecules	5158
ligands	30
solvent	739
Protein residues	667
RMS (bonds)	0.01
RMS (angles)	1.05
Ramachandran favored (%)	97.44
Ramachandran allowed (%)	2.26
Ramachandran outliers (%)	0.30
Rotamer outliers (%)	0.00
Clashscore	3.42
Average B-factor	25.53
macromolecules	24.16
ligands	19.06
solvent	35.36
Number of TLS groups	13

200 Statistics for the highest-resolution shell are shown in parentheses.

201

202 Biosynthesis of *L*-tert-leucine

203 To investigate the thermodynamic equilibrium of *L*-tert-leucine formation catalysed by
204 *PsBCAT*, reactions were performed with various concentrations of *L*-glutamic acid (20

205 mM, 50 mM, 100 mM) in 1 mL of 50 mM Tris-HCl buffer (pH 8.5) containing 20 mM
 206 trimethylpyruvic acid and 10 μ M PLP and appropriate amount purified enzyme of
 207 *PsBCAT*. To shift the unfavourable equilibrium, *PsBCAT* coupling to *BsOrnAT* was
 208 applied to synthesise L-tert-leucine. The reaction mixture contained 0.5 M Tris-HCl buffer
 209 (pH 8.5), 0.1 M of trimethylpyruvic acid, 20 mM sodium glutamate, 80 mM L-ornithine
 210 monohydrochloride, 20 μ M PLP and appropriate amounts of purified *PsBCAT* and
 211 *BsOrnAT* enzyme in a total volume of 5 mL at 30 °C. The concentration of L-tert-leucine
 212 was analysed by HPLC after derivatized using dinitrofluorobenzene (DNFB).

213 **3. Results**

214 **Cloning, expression and purification of BCATs**

215 To discover novel BCATs with high activities, BCAT sequences with large sequence
 216 diversity were analysed. Three iterative analyses were performed to remove highly
 217 conserved BCAT sequences and filter out sequences with low occurrence. After the 3rd
 218 round of analysis, a 788-node network was obtained (Figure 1). The representative
 219 sequences of the most concentrated clusters (the BCATs from *E. coli*, *Bacillus subtilis* and
 220 *Pseudomonas* sp.) were chosen for further analysis. In addition, the well-studied BCAT
 221 from *Corynebacterium glutamicum* was also selected. A homology search was conducted
 222 based on BCAT from *E. coli* using the blast program against the GenBank database. The
 223 BCAT sequences from *Bacillus subtilis*, *Corynebacterium glutamicum* and *Pseudomonas*
 224 sp. exhibit 25%, 13% and 30% similarities with that of *E. coli*, representatively.

225

226 Figure 1. Representation of the BCATs as a sequence similarity network. In this network, nodes
 227 represent individual sequences, and edges represent the alignment score from pairwise alignments.
 228 Longer edges represent lower scores. Three sequences were selected for further investigation from
 229 each cluster. Selected sequences are displayed in yellow with a black border.

230 The BCAT proteins were produced primarily as soluble protein. The expression of
 231 *PsBCAT* was better than other BCATs. *BsBCAT*, *EcBCAT* followed, and *CgBCAT* was
 232 the least. The purified BCATs of *BsBCAT*, *CgBCAT*, *EcBCAT* and *PsBCAT* were
 233 electrophoretically homogenous as shown on SDS-PAGE with apparent molecular
 234 weights of approximately 40.3 kDa, 28.2 kDa, 35.5 kDa, and 37.3 kDa (Figure 2),
 235 respectively. Molecular weights of *PsBCAT* and *EcBCAT* are similar with the *PsBCAT*
 236 homologous BCATs, such as *DrBCAT* (28), *SmIlvE* (29) and *MtIlvE* (30) at 38~39 kDa,
 237 but higher than that from *Methanococcus aeolicus* (31) with molecular masses of 31.8
 238 kDa for each monomer of the hexamers. The molecular weight of *BsBCAT* is similar with
 239 that of mammalian BCATs, which are commonly found as dimers with molecular masses
 240 of 41.3 and 43 kDa for the hBCATm (32) and hBCATc (33) monomers, respectively.
 241 Among the four BCATs, *PsBCAT* exhibits highest activity towards L-tert-leucine at 7 ± 0.7
 242 U/mg at 37 °C and pH 8.5. The activities of *BsBCAT*, *CgBCAT*, and *EcBCAT* were 1 ± 0.1
 243 U/mg, 2 ± 0.3 U/mg, and 4 ± 0.3 U/mg, respectively. Therefore, *PsBCAT* was chosen for
 244 further analysis.

245

246 Figure 2. SDS-PAGE of purified BCATs. Lane 1, protein marker; lane 2, 3, 4, 5, and 6 purified
247 *BsBCAT*, *CgBCAT*, *EcBCAT*, *PsBCAT* and *BsOrnAT*, respectively.

248 Effect of pH and temperature on *PsBCAT* activity

249 The effect of pH on *PsBCAT* activity was examined over pH values ranging from 5.0 to
250 10.5 at 37 °C (Figure 3A). The highest activity of *PsBCAT* for *L*-valine was observed at
251 pH 8.5 in 50 mM Tris-HCl buffer, while it decreased significantly with pH values less
252 than 7.5 or greater than 10.0 with only approximately 10 % activity or even less. The
253 results were consistent with that of most BCATs, including the thermal BCATs from
254 *Vulcanisaeta moutnovskia* (34) and *Thermoproteus uzoniensis* (35, 36), the optimum pH
255 of which were observed at pH 8.5.

256 In terms of temperature, *PsBCAT* has an optimal temperature typical of most of BCATs
257 at 40 °C. As shown in Figure 3B, the *PsBCAT* activity decreased very quickly to
258 approximately 30 % at 50 °C, indicating that it was unstable at high temperatures.
259 However, the temperature preference of *PsBCAT* exhibited significant differences
260 compared with thermal BCATs from *Thermoproteus uzoniensis* (35, 36), *Thermococcus*
261 *sp.* CKU-1 (37) and *Vulcanisaeta moutnovskia* (34), which showed maximum activity at >
262 90 °C.

263 Investigation of PLP requirement of *PsBCAT*

264 The purified *PsBCAT* showed low activity when assayed in the absence of PLP but was
265 significantly activated in the presence of PLP (Figure 3C). The effect of PLP

concentration on *PsBCAT* activity was investigated using L-valine as the substrate, and the Michaelis constant was 3.4 μ M. The maximum activity of *PsBCAT* was observed when the PLP concentration reached 20 μ M, and no significant activity increase was noted at higher PLP concentrations.

Figure 3. Effects of pH, temperature and PLP on the activities of the *PsBCAT*. (A) The optimal reaction pH of *PsBCAT* was 8.5. (B) The optimal reaction temperature of *PsBCAT* was 40 °C. (C) The PLP concentration was 20 at the peak of enzyme activity.

Substrate specificity and kinetic analysis of *PsBCAT*

To evaluate the substrate specificity of *PsBCAT*, we determined its reactivity toward a series of amine donors (Figure 4). *PsBCAT* exhibited maximum activity toward L-leucine followed by L-valine, L-isoleucine and L-methionine with specific activities of 127 U/mg, 115 U/mg, 105 U/mg and 98 U/mg, respectively. Despite the considerably lower activity, *PsBCAT* also showed activities toward L-phenylalanine, L-alanine and L-tert-leucine, but it was inert to L-tryptophan, L-tyrosine, L-threonine, L-histidine, L-lysine, L-theanine and prebalin. Moreover, *PsBCAT* exhibited no activity for amines in propylamine, aniline, and phenylethylamine.

Figure 4. Substrate specificity of PsBCAT

The results demonstrate that L-leucine, L-isoleucine, L-valine, L-glutamic acid, and L-tert-leucine served as suitable substrates. The kinetic parameters were determined for these reactions in two-side directions (Table 3). The effects of the concentration of amino donors on the initial rate followed a typical Michaelis-Menten equation. The k_{cat}/K_m value of L-leucine was much greater than any others, suggesting that the active site of PsBCAT has evolved to catalyse the transamination reaction for L-leucine most effectively. For L-tert-leucine transamination, the K_m value (11.50 mM) was approximately 5-fold higher than that for L-leucine, and the k_{cat}/K_m value was much lower ($0.90 \text{ s}^{-1} \text{ mM}^{-1}$). Since previous reports have shown that the enzyme activities of aminotransferases were affected by the product or substrate concentrations, product or substrate inhibitions extensively exist among BCATs (10). Kinetics experiments demonstrated that substrate inhibition by L-leucine, L-isoleucine, L-valine, L-glutamic acid, and L-tert-leucine were not observed up

297 to 50 mM. On the contrary, the ketonic acids exhibit significant inhibition of *PsBCAT*.
 298 For example, the reaction rate increased as the concentration of α -ketoglutaric acid
 299 increased up to 5 mM. However, when α -ketoglutaric acid exceeded 5 mM, the reaction
 300 rate was decreased. The reaction rate with 10 mM was 55.4% of that with 5 mM
 301 α -ketoglutaric acid. The ketonic acid inhibition by α -ketoisovaleric acid was almost the
 302 same. The reaction rate with 25 mM was 66.7% of that with 15 mM α -ketoisovaleric acid.
 303 As a result, the apparent kinetic parameters of amino acceptors were not obtained.

304 Table 3. Kinetic analysis of *PsBCAT*

Substrate	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m ($s^{-1} \text{ mM}^{-1}$)
L-valine	9.16±1.37	220.13±11.97	24.41±2.59
L-leucine	2.27±0.36	165.10±10.37	74.13±9.24
L- isoleucine	2.85±0.77	175.57±23.95	63.91±10.83
L-tert-leucine	11.50±2.94	9.74±0.30	0.90±0.22
L- glutamic acid	18.70±0.64	10.53±0.82	0.56±0.03

305 Overall structure of *PsBCAT*

306 To analyse the catalytic mechanism of *PsBCAT* with a broad substrate spectrum, the
 307 crystal structure of *PsBCAT* was determined at a resolution of 1.9 Å (Figure 5A).
 308 Structural alignments of *PsBCAT* with type IV class of PLP dependent enzymes, such as
 309 *DrBCAT* (28) (BCAT from *Deinococcus radiodurans*, PDB entry 3UYY), *SmIlvE* (29)
 310 (BCAT from *Streptococcus mutans*, PDB entry 4DQN) and *MtIlvE* (30) (BCAT from
 311 *Mycobacterium tuberculosis*, PDB entry 3HT5) for all C α atoms are 0.46 Å, 0.50 Å and
 312 0.93 Å, respectively, which indicated that *PsBCAT* shared typical folds of the type IV
 313 class of PLP-dependent enzymes.

314 The asymmetric unit contains one *PsBCAT* homodimer (Figure 5A). Each *PsBCAT*
 315 subunit is built up by two separate domains. The first 7 amino acids at the N-terminal
 316 region were disordered and invisible in the density map. The small domain (Asn8 to
 317 Pro154 and Ile337 to Val340) displays a Rossmann fold and consists of seven β -strands
 318 (β 1- β 7) and three α -helices (α 1- α 3). Seven β -strands of this domain run in an

up-and-down fashion to form a core antiparallel β -sheet with α 1- α 3 helices on both sides. The large domain (His166 to Pro312) of *PsBCAT* consists of nine β -sheets (β 8- β 16), which are surrounded by three α -helices (α 4- α 6). These two domains are linked by α 7 helix (residues 313-336) and an interdomain loop (Val155 to Pro165). The interdomain helix is expected to provide a rigid framework for stabilization of the two interacting domains, whereas the interdomain loop is proposed to provide flexibility near the active-site entrance and allow both substrate and product access into and out of the substrate-binding pocket.

Figure 5. Crystal structures of *PsBCAT*. (A) The *PsBCAT* dimer is shown from an orientation almost perpendicular to the 2-fold axis. The PLP (green stick) is located at the bottom of the active site. (B) The PLP-bound monomer structure of *PsBCAT* is shown with six α -helices (blue) and 16 β -strands (yellow). The monomer structure contains small and large domains linked by the interdomain loop (Val155 to Pro165, shown in orange). The cofactor PLP (green) is located between the two domains. (C) The highly conserved residues combined with PLP in the binding pocket of *PsBCAT*. (D) Interactions among residues, PLP, and the L-glutamate molecules. (E) Interactions among residues, PLP,

335 and L -tert-leucine molecules.

336 **Active site of *PsBCAT***

337 The highly conserved residues confer crucial interactions with PLP in the binding
338 pocket of *PsBCAT*, including Arg83, Lys185, Tyr190, Glu221, Ile248, Thr249 and Thr286
339 (Figure 5C), whereas the corresponding residues are Arg101, Lys204, Tyr209, Glu240,
340 Ile271, Thr272 and Thr314 in *MtIlvE* (30); Arg100, Lys202, Tyr207, Glu238, Ile266,
341 Thr237 and Thr304 in *DrBCAT* (28); and Arg81, Lys184, Tyr189, Glu220, Val247,
342 Thr248 and Thr285 in *SmIlvE* (29). In addition, this positively charged active site pocket
343 is surrounded by residues Phe59, Glu60, Gly61, Tyr126, Arg128, Arg175, Ser224, Ala225,
344 Asn226, Leu245, Gly247 and Gly285.

345 After docking the ligands into *PsBCAT*, the enzyme-ligand complexes reveal several
346 essential residues for the ligand binding, and some of these residues are conserved in all
347 *BCAT* enzymes from various species. In the *PsBCAT*- L -glutamate complex, Arg128 and
348 Tyr54 confer hydrogen bonds with the γ -carboxylate oxygen atom of L -glutamate,
349 whereas Tyr126 and the backbone H atom of Ala287 make hydrogen bonds with the
350 α -carboxylate oxygen atoms. Ser224 and Tyr190 accept hydrogen bonds from the amide
351 hydrogens of L -glutamate. The binding mode of L -glutamate is similar with that in
352 *EcBCAT* crystal structure (38). In the *PsBCAT*- L -tert-leucine complex, Tyr126 and Ala287
353 also form hydrogen bonds with the α -carboxylate oxygen atom of L -tert-leucine, and the
354 side chain OG atom of Tyr190 and the backbone O atom of Ser224 confer
355 hydrogen-bonding with the amide hydrogens of L -glutamate. Remarkably, in both
356 *PsBCAT*-amino acid complexes, Tyr54, Tyr126 and Lys128 make a hydrogen-bonding
357 network to stabilize the carboxylate oxygen atom of the amino acids. The detailed
358 interactions among residues, PLP, and the L -glutamate/ L -tert-leucine molecules are shown
359 in Figure 5D, 5E.

360 **Application of *PsBCAT* for L -tert-leucine biosynthesis**

361 As an important drug intermediate, L -tert-leucine is widely used in the synthesis of
362 several pharmaceuticals, such as anti-HIV protease inhibitors or tumour-fighting agents.
363 To measure the chemical reaction equilibrium, syntheses were carried out with various

concentrations of L-glutamic acid (20 mM, 50 mM, 100 mM) in 1 mL of 50 mM Tris-HCl buffer (pH 8.5) containing 20 mM trimethylpyruvate, 10 μ M PLP and appropriate amounts of purified *PsBCAT*. The yields of L-tert-leucine were 41%, 52%, and 68%, respectively (Figure 6A, 6B).

Figure 6. Synthesis of L-tert-leucine. (A) The synthesis of L-tert-leucine with *PsBCAT*. (B) The reaction was performed in 1 mL of 50 mM Tris-HCl buffer (pH 8.5) with 20 mM trimethylpyruvate, *PsBCAT* and different concentrations of L-glutamic acid (20 mM, 50 mM, and 100 mM). (C) The synthesis of the L-tert-leucine with *PsBCAT* coupling *BsOrnAT*. (D) The reaction was performed in 5 mL of 0.5 M Tris-HCl buffer (pH 8.5) with 100 mM trimethylpyruvate, *PsBCAT*, *BsOrnAT*; 20 mM L-glutamic acid; or 80 mM L-ornithine.

Although the concentration of L-glutamic acid was up to five times than that of trimethylpyruvate, the substrate cannot be completely transformed. To overcome the product inhibition by α -ketoglutaric acid and shift the unfavourable thermodynamic equilibrium to the synthesis of L-tert-leucine, a *PsBCAT/BsOrnAT* coupling reaction was performed in which L-ornithine δ -aminotransferase transferred the δ -amino group from L-ornithine to α -ketoglutaric acid, producing L-glutamate- γ -semialdehyde and L-glutamic

acid. The formation of L-glutamic acid from α -ketoglutarate was strongly favoured by the spontaneous cyclization of L-glutamate- γ -semialdehyde to form Δ^1 -pyrroline-5-carboxylate and drove the reaction to the formation of L-tert-leucine (Figure 6C). The synthesis of L-tert-leucine were performed with 0.1 M trimethylpyruvate in 5 mL of 0.5 M phosphate buffer (pH 8.5) containing 20 mM L-glutamic acid, 80 mM L-Ornithine, 20 μ M PLP and appropriate amounts of purified *PsBCAT* and *BsOrnAT* with a yield of 83% at 42 h (Figure 6D).

Discussion

Aminotransferases are pyridoxal PLP-dependent enzymes that catalyse reversible transamination reactions between amino acids and α -ketoglutaric acid. This enzyme is important for the conversion between inorganic nitrogen and organic nitrogen in living organisms (4).

PsBCAT (BCAT from *Pseudomonas* sp.), comprising 340 amino acids, shows many features typical of bacterial BCATs or BCATs from eukaryotes: a comparable molecular weight, similarities in the secondary, tertiary (fold type IV) and quaternary structure, optimal pH value, activity toward BCAAs and L-glutamic acid, and inhibition by high concentrations of α -ketonic acids. Meanwhile, *PsBCAT* displays broad but atypical substrate specificity that catalysed the overall transamination reaction of various branched chain amino acids and hydrophobic amino acids when α -ketoglutaric acid was used as an amino acceptor. The enzyme has remarkably high activity with L-leucine, L-valine, L-isoleucine and L-methionine (127, 115, 105 and 98 U/mg, respectively), whereas other bacterial BCATs only show approximately 10-40 U/mg activities at 37 °C. Additionally, *PsBCAT* shows activities with aromatic L-amino acids, L-histidine, L-lysine, and L-threonine, thus displaying broad amino donor specificity.

The molecular mechanism of substrate recognition for BCATs was proposed by Goto based on the analysis of the data on the substrate specificity of *EcBCAT* and structural data (38). The torsional angle of C3-C4-C4'-N of PLP with Lys185 in the *PsBCAT* crystal structure is 17°, indicating that the internal aldimine bond (Schiff base, C4'=N) is

409 essentially coplanar with the PLP ring to make a conjugated π -system of the PLP-Schiff
 410 base. The C4'=N double bond is fixed by the phosphate group, the pyridine hydroxyl
 411 group and the pyridine N atom group (Figure 5A). The phosphate group forms hydrogen
 412 bonds and salt bridges with Arg83, Ile248, Thr249 and Thr286 and water molecules. The
 413 oxygen atom of the PLP pyridine hydroxyl group accepts hydrogen bonds with Tyr190.

414 BCAT active sites can accommodate two types of substrates with a hydrophobic side
 415 chain (branched chain or aromatic amino acids) and with an acidic side chain (acidic
 416 amino acids, 2-oxoglutarate) in almost the same positions in the substrate-binding pocket.
 417 Phe14, Phe59, Phe159, Tyr190, Thr286, Ala287 are suggested to confer hydrophobic
 418 interactions with the hydrophobic or aromatic side chains of the L-amino acids, whereas
 419 Arg128, Tyr54 and Tyr126 are responsible for the binding of acidic side chain. A
 420 comparison of *PsBCAT* and *DrBCAT* complex structures shows a distinct dynamic loop
 421 (Gly156 to Gly162), which is inferred to block the access of the solvent molecules to the
 422 active site, thereby preventing the side process of racemization that occurs in the
 423 PLP-dependent alanine racemase with the solvent-accessible active site. The Ala157
 424 residue in *PsBCAT*, corresponding to Pro157 in other BCATs, may significantly facilitate
 425 the flexibility of the interdomain loop. In addition, Glu197 in BCATs from *Escherichia*
 426 *coli* (38) (PDB ID: 1IYD) and *Thermus thermophilus* (unpublished work) (PDB ID:
 427 1WRV) is suggested to confer electrostatic interactions for substrate binding. However,
 428 the truncated side chain of the corresponding residue Ala225 in *PsBCAT* seems to enlarge
 429 the large pocket, which might aid in the entry of substrates with bulky moieties. Previous
 430 studies have reported L-tert-leucine synthesis using BCAT/AspAT/PDC, BCAT/ ω -AT, and
 431 BCAT/GluDH/FDH coupling systems. In the BCAT/AspAT/PDC coupling system, the
 432 formation of L-glutamic acid from α -ketoglutaric acid was favoured by the spontaneous
 433 decarboxylation of oxaloacetate to form pyruvate (16). Pyruvate decarboxylase (PDC)
 434 further performs the non-oxidative thiamine diphosphate-mediated decarboxylation of
 435 pyruvate to shift the equilibrium of the reaction (16). The BCAT/ ω -AT coupling reaction
 436 was also developed to eliminate the product inhibition by simultaneously performing an
 437 energetically favourable ω -AT reaction using a two-liquid phase reaction system (17).

438 Recently, for the efficient synthesis of L-tert-leucine, BCAT from *Enterobacter* sp.
439 coupling with L-glutamate dehydrogenase and formate dehydrogenase was applied. The
440 L-glutamate dehydrogenase catalyses the NADPH-dependent amination of α -ketoglutaric
441 acid to yield L-glutamic acid, and formate dehydrogenase regenerates NADH using
442 formate (18). Compared with these coupling systems, no requirement of complicated
443 cascade reactions or an expensive external cofactor, such as NADH, renders the
444 BCAT/OAT coupling system promising for industrial applications in the asymmetric
445 synthesis of L-tert-leucine. The formation of L-glutamic acid from α -ketoglutaric acid was
446 strongly favoured by the spontaneous cyclization of L-glutamate- γ -semialdehyde to form
447 Δ^1 -pyrroline-5-carboxylate and drove the reaction to the formation of L-tert-leucine.
448 Indeed, PsBCAT/OAT coupling reaction produced 83 mM L-tert-leucine from 100 mM
449 trimethylpyruvate for 42 h, which provided an approximately 2.7-fold higher yield than
450 the single BCAT reaction.

451 In conclusion, we determined the crystal structure of branched-chain amino acid
452 aminotransferase from *Pseudomonas* sp. The enzyme is active toward typical BCAT
453 substrates, including L-leucine, L-isoleucine and L-valine, and also shows significant
454 activity with aromatic L-amino acids, L-histidine, L-lysine, and L-threonine. The structure
455 of PsBCAT is similar to the known structures of bacterial BCATs. However, we found
456 some differences in the organization of the substrate binding cavity, which may influence
457 the substrate specificity of the enzyme. Taking into account a broad range of accepted
458 substrates and detected ability to catalyse asymmetric synthesis of L-tert-leucine, this
459 enzyme hold promises in biotechnological applications, and the structural fingerprint will
460 aid in further engineering to increase the activity with amines.

461 SUPPLEMENTAL MATERIAL

462 Supplementary material for this manuscript has been submitted.

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467

468 **Conflict of interest**

469 The authors declare that there are no conflicts of interest.

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1**2****3****4****5****6****116.0****86.2****45.0****35.0****25.0****18.4****14.4**

A

B

C

Amino donor Activity (U mg⁻¹)

L-arginine	n.d
L-histidine	$(7 \pm 0.8) \times 10^{-2}$
L-lysine	$(3 \pm 0.2) \times 10^{-2}$
L-aspartic acid	n.d
L-glutamic acid	$(2 \pm 0.2) \times 10^{-1}$
L-serine	n.d
L-threonine	$(7 \pm 0.3) \times 10^{-2}$
L-asparagine	n.d
L-glutamine	n.d
L-cysteine	n.d
L-glycine	n.d
L-proline	n.d
L-alanine	1 ± 0.1
L-valine	115 ± 2.1
L-leucine	127 ± 5.6
L-isoleucine	105 ± 4.7
L-methionine	98 ± 4.9
L-phenylalanine	4 ± 0.3
L-tyrosine	$(9 \pm 0.2) \times 10^{-2}$
L-tryptophan	$(1 \pm 0.1) \times 10^{-1}$

Amino donor Activity (U mg⁻¹)

L-tert-leucine	7 ± 0.7
L-theanine	n.d
pregabalin	n.d
propylamine	n.d
n-heptylamine	n.d
aniline	n.d
allylamine	n.d
ethanediamine	n.d
sec-butylamine	n.d
2-aminopentylamine	n.d
cyclohexylethylamine	n.d
phenylethylamine	n.d

A

B

C

D