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# Asymmetric synthesis of L-*tert*-leucine and L-3-hydroxyadamantylglycine using branched chain aminotransferase

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ABSTRACT

L-*Tert*-leucine (Tle) and L-3-hydroxyadamantylglycine of (HAG) are important intermediates for a variety of pharmaceutical classes. They were asymmetrically produced from corresponding keto acids using branched-chain aminotransferase (BCAT) with L-glutamate (Glu) as an amino donor. For the production of L-Tle and L-HAG, BCAT from *Enterobacter* sp. TL3 (BCAT*en*) and BCAT from *Escherichia coli* K12 (ilvE, newly named as BCAT*es*) were used, respectively. In our current study, we characterized the basic properties of BCAT*en* and BCAT*es* such as substrate specificity, enantioselectivity, and kinetic parameters. The activities of BCAT*en* and BCAT*es* were inhibited severely by α-ketoglutarate which is a deaminated product of L-Glu. In the presence of 10 mM α-ketoglutarate, both enzymes activities were reduced up to 80%. In order to overcome product inhibition by α-ketoglutarate and the problem of equilibrium of the transamination reaction, coupling reactions were carried out with L-glutamate dehydrogenase (GDH)/formate dehydrogenase (FDH) and AspAT. The coupling reaction dramatically increased the yields of both target compounds. 135 mM of L-Tle (>99% *ee*) was produced from 150 mM corresponding keto acid in BCAT*en*/GDH/FDH coupling reaction with 90% conversion. In addition, 90.5 mM L-HAG (>99% *ee*) was produced from 100 mM corresponding keto acid in BCAT*es*/AspAT coupling reaction using recombinant whole-cells.

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## 1. Introduction

Nonproteinogenic amino acids are important intermediates for a variety of pharmaceutical classes, most notably in peptidomimetics with antiviral and ontological applications [1]. Because of the great usage and versatility of these compounds, many optical active nonproteinogenic amino acids have been the targets for asymmetric synthesis and chemical or enzymatic resolution [2]. Among them, L-*tert*-leucine (Tle) and L-3-hydroxyadamantylglycine (2-(3-hydroxy-1-adamantyl)-(2S)-amino ethanoic acid: HAG) are very important precursors for the synthesis of pharmaceuticals (Fig. 1). L-Tle itself is a component of several pharmaceutical development projects as tumor-fighting agents or HIV protease inhibitors against several diseases such as tumors, rheumatic arthritis and AIDS [3]. L-HAG can be used as a precursor for the synthesis of Saxagliptin which is a dipeptidyl peptidase IV inhibitor [4]. Dipeptidyl peptidase-IV's role in blood glucose regulation is thought to be through degradation of GIP and the degradation of GLP-1. Thus, Saxagliptin is now under development by Bristol-Myers Squibb for treatment of type 2 diabetes [5,6].

There are numerous methods for the synthesis of L-Tle including chemical resolution, stereoselective synthesis and enzymatic approaches [7–10]. Kragl el al. [7] reported continuous production of L-Tle in series of two enzyme–membrane reactors by reductive amination of trimethylpyruvate with leucine dehydrogenase. Recently, Penicillin G acylase (PGA) from *Kluyvera citrophila* immobilized on Amberzyml was used for enantioselective hydrolysis of N-phenylacetylated-DL-*tert*-leucine to produce L-Tle [8]. Among them, the Degussa AG (now Evonik AG)-mediated process, utilizing leucine dehydrogenase-catalyzed reductive amination of trimethylpyruvate, has been successfully operated on a large scale [8]. In case of synthesizing L-HAG compound, it was originally prepared using an asymmetric Stecker amino acid synthesis [4]. After this research, approaches to enzymatically synthesize Saxagliptin's key intermediate L-HAG have been studied using modified form of a recombinant phenylalanine dehydrogenase cloned from *Thermoactinomyces intermedius* and expressed in *Pichia pastoris* or *Escherichia coli* [11].

Transaminases (TAs) play important roles in amino acid metabolism and are ubiquitous in microorganisms and eukaryotic cells. TAs have been studied extensively, due to its potential to

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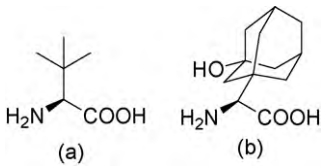


**Fig. 1.** The target unnatural amino acids by trasaminase reaction. (A) *Tert*-leucine (Tle); (B) L-3-hydroxyadamantylglycine (2-(3-hydroxy-1-adamantyl)-(2S)-amino ethanoic acid: HAG).

produce chiral amino acids and amines in biosystems, Till now various examples of using the transaminases for the production of natural and non-natural D- and L-amino acids as well as chiral amines have been published [12–15]. Despite their many advantages such as broad substrate specificity and high enantioselectivity, their industrial use have been limited mainly due to a low equilibrium constant of the TA reaction and/or severe inhibition by the product [16]. To over-come these problems, many attempts have been done to shift the equilibrium by stripping out or removing the coproduct [17,18].

Among them, the most widely studied transaminase is α-transaminase, such as aspartate transaminase, tyrosine transaminase, branched-chain amino acid transaminase (BCAT), and valine–alanine transaminase of *E. coli* K-12 [19–22]. Although branched-chain amino acid transaminase shows reactivity towards various aliphatic amino acids such as leucine and iso-leucine, few attempts have been undertaken to produce L-Tle and HAG from the corresponding keto acids using transaminase. In order to produce L-Tle, we isolated *Enterobacter* sp. TL3 showing BCAT activity toward L-Tle from soil samples using enrichment culture method [23]. The present study illustrates the isolation of *Enterobacter* sp. TL3, molecular cloning, and expression of the gene encoding the new aminotransferase (BCAT*en*). Using the cloned BCAT from *E. coli* (BCAT*es*) and BCAT*en*, enantiopure L-HAG and L-Tle were produced in quantitative yield and high enantiomeric purity (*ee* > 99%).

## 2. Materials and methods

### 2.1. Material

Trimethylpyruvate (TMP) was obtained from the CKD Research Institute (Cheonan, Korea). Keto acid 2-(3-hydroxy-1-adamantyl)-2-oxoethanoic acid (HAOE) was purchased from Hi@Tech (Chongqing, China). Racemic L-3-hydroxyadamantylglycine and L-3-hydroxyadamantylglycine was obtained from the Samchully Pharm. Co. (Gyeonggi-do, Korea). Formate dehydrogenase (FDH) and L-glutamate dehydrogenase (GLDH), isopropyl-β-D-thiogalactopyranoside (IPTG), 2,3,4,6-tetra-O-acetyl-D-glucopyranosyl isothiocyanate (GITC) and all other chemicals were purchased from Aldrich chemical Co. (St Louis, MO). HPLC grade water and acetonitrile were obtained from Duksan chemical Co. (Ansan, Korea).

### 2.2. Enrichment culture

A spoonful of soil samples collected from various domestic sites were incubated in 50 mL of Luria–Bertani (LB) broth medium for 1 h. After the incubation, 0.1 mL of culture broth was transferred to 10 mL minimal medium containing 10 mM L-Tle as sole nitrogen source [23]. Enrichment culture was carried out by repeated dilution of culture broth with fresh minimal medium (20–30-folds) at 37 °C. After the enrichment, the diluted culture broth was spread out on the same minimal agar plates. Each colony on the plates was inoculated into 4 mL LB media. After the over-night incubation at 37°C, the harvested cells by centrifugation were washed with 1 mL of 50 mM phosphate buffer. After centrifugation, cells were added to 0.5 mL of reaction solution composed of 50 mM phosphate buffer (pH 8.0), 10 mM trimethylpyruvate and 50 mM L-glutamate (Glu) at 37 °C, and the specific activity and enantioselectivity were measured.

### 2.3. Genetic analysis

DNA manipulations, including preparation of plasmids, restriction enzyme digestion and ligation, transformation of *E. coli* were followed the methods by Sambrook, etc. Multiple alignments of the amino acid sequences among the homologous proteins were performed with CLUSTAL multiple sequence alignment program.

### 2.4. Preparation of enzymes

To express BCAT*en* without excessive flanking parts, the coding region of BCAT*en* was amplified from genomic DNA of *Enterobacter* sp. by PCR using P1 (5′-ATCATGGAATTCATGACCACGAAGAAAGCT-3′) and P2 (5′-AAAAAACTCGAGTTATTGATTAACTTGATCTAACCA-3′) primers. To express C-terminal fusion BCAT*en* with a His-tagged polypeptide, the coding region of BCAT*en* was amplified by PCR using P3 (5′-ATATATGGATCCATGACCACGAAGAAAGCT-3′) and P4 (5′-ATATATCTCGAGTTGATTAACTTGATC-3′) primers. The PCR products were digested with restriction enzymes (*EcoR*I/*Xho*I, or *BamH*I/*Xho*I site), and inserted into the vector pET24ma [24]. Since DNA sequence of BCAT*es* is highly homologues to that of BCAT*en*, the same primer sets are used to construct plasmids (pET24ma) for the expression of BCAT*es* from *E. coli* K-12 (accession number U00096). AspAT gene was amplified with P5 (5′-ATATATGGATCCATGTTTGAGAACATTACC-3′) and P6 (5′-ATATATCTCGAGTTACAGCACTGCCACAAT-3′) primers using genomic DNA of *E. coli* K12 as template, and was inserted into pET23b (+) containing *BamH*I/*Xho*I restriction sites.

Recombinant *E. coli* was grown at 37 °C in 1 L LB broth containing 50 μg mL$^{-1}$ of appropriate antibiotic. When the OD600 of the culture reached 0.5, 1 mM IPTG was added to the culture broth. After a 5 h induction, the cells were harvested. The harvested cells were suspended in 50 mL of 10 mM Tris–HCl (pH 7.2) containing 20 μM pyridoxal 5′-phosphate, 2 mM EDTA, 1 mM PMSF, and 0.01% (v/v) 2-mercaptoethanol and ultrasonically disrupted for 25 min at 4 °C. The samples were centrifuged (17,000 × *g*, 20 min), and the supernatants were dialyzed against 10 mM Tris–HCl buffer (pH 7.2) containing 20 μM pyridoxal 5′-phosphate, 0.2 mM EDTA, 1 μM PMSF, and 0.001% (v/v) 2-mercaptoethanol. The dialyzed cell extract was stored at −70 °C for further study.

The $His_6$-tagged fusion protein was purified with Ni-NTA agarose resin from Quiagen (Hilden, Germany) at 4 °C. The crude extract was passed directly over a column containing 10 mL of Ni-NTA agarose resin. After the column was washed with 50 mL of phosphate buffer (pH 8.0) containing 20 mM imidazole, the C-terminal $His_6$-tagged BCATs was eluted with 20 mL of phosphate buffer (pH 8.0) containing 200 mM imidazole buffer. The elution solution containing partially purified $His_6$-tagged BCAT was concentrated by ultra-filtration using Centriplus YM-30 (Millipore, Bedford, MA) with molecular mass cut-off of 10 kDa.

### 2.5. Enzyme assays

The activity assay of crude-extract was performed in 50 mM phosphate buffer (pH 8.0) containing 10 mM keto acid, 10 mM L-Glu at 37 °C, and the product was measured by HPLC. One unit of the enzyme activity represents 1 μmol of product formation per minute in 10 mM L-Glu.

Determination of the kinetic parameters was performed in 200 μL assay solution (50 mM phosphate buffer (pH 8.0)) contain-

ing the purified enzyme and 10 mM L-Glu. The concentration of keto acid (amino acceptor) was varied from 0.6 to 20 mM. BCAT was added to the reaction mixture to initiate the reaction. After 30 min incubation, the reaction was stopped by heating the mixture at 96 °C for 15 min. Initial reaction rate was determined by analysis of the production of L-HAG and L-Tle.

Quantitative analysis and enantiomeric purities of L-HAG and L-Tle were performed using Waters HPLC system with 3.9 (radius) × 150 mm $C_{18}$ Symmetry reverse phase column (Waters, Milford, USA) at 254 nm. In order to analyze chiral compounds, the racemic acids were derivatized with GITC [25].

Separation of derivatized HAG enantiomers was achieved with an elution of water/acetonitrile/trifluoacetic acid (70/30/0.1, v/v%) at a flow rate 1.0 mL $min^{-1}$ at 25 °C. Retention times for L-HAG derivative and D-HAG derivative were 9.7 and 11.3 min, respectively. Separation of derivatized Tle enantiomers was achieved with an elution of water/acetonitrile/trifluoacetic acid (65/35/0.1, v/v%) at a flow rate 1.0 mL $min^{-1}$ at 25 °C. Retention times for L-Tle derivative and D-Tle derivative were 4.8 and 5.2 min, respectively.

For the whole cell reaction of L-HAG production, the prepared recombinant *E. coli* BL21 over-expressing BCAT*es* (6 mg of wet cell weight) was added to the 0.5 mL reaction mixture consisting of 100 mM HAOE, 500 mM L-Glu, 300 mM L-Asp, 20 μM PLP and 200 mM phosphate buffer (pH 8.0) at 37 °C. Reaction products were quantitatively analyzed by HPLC after specific time intervals.

## 3. Results

### 3.1. Screening of microorganisms producing optically pure L-Tle from trimethylpyruvate with L-Glu

After several rounds of enrichment culture, a bacteria (TL3) later identified as *Enterobacter* sp. by 16s rRNA analysis (Korea Research Institute of Bioscience and Biotechnology, Korea) showed the highest reaction rate and L-specific stereo-selectivity. When whole cell reaction was carried out with 10 mM TMP and 20 mM L-Glu, the enantiomeric excess of the produced L-Tle was above 99%. To investigate whether or not the enzyme activity for producing L-Tle is really an aminotransferase, the enzyme activity was examined in the presence of typical inhibitors of PLP-dependent enzyme using the crude extract of *Enterobacter* sp. TL3. It was strongly inhibited by gabaculine, hydroxylamine and aminooxyacetic acid [26]. In addition, almost equivalent amounts of L-Tle were produced according to the consumed L-Glu. Therefore, the enzyme involved in the enzymatic production of L-TLe was confirmed to be a kind of a BCAT.

### 3.2. Cloning and DNA sequencing of BCATen

*Enterobacter* sp. TL3 belongs to gammaproteobacter in proteobacteria. In order to clone the gene coding the BCAT*en*, the degenerative primers were designed from highly conserved regions. We collected 13 amino acid sequences of BCAT from gammaproteobacter using Gen-Bank Database (*E. coli* K12, *Haemophilus influenzae* Rd, *Pseudomonas putida* KT2440, *Salmonella enterica* subsp., *Shigella flexneri* 2a str, *Shewanella oneidensis* MR-1, *Salmonella typhimurium* LT2, *Xanthomonas axonopodis* pv, *Xanthomonas campestris* pv, *Xylella fastidiosa*, *Yersinia pestis*, *Pseudomonas aeruginosa*). The multiple alignments of the selected bacterial BCAT amino acid sequences revealed two highly conserved regions; LHYGT(Q,M)S(G,E,Q)V(I,C)FEG (30th–39th amino acids based on BCAT*es* from *E. coli*) and GTAAE(V)I(V)TP (257th–264th amino acids based on BCAT*es* from *E. coli*) which were long enough for designing the degenerative primers. Degenerate PCR primers were designed based upon the two consensus amino acid sequences to clone the gene. Pr1f (5′-CTGCACTAYGGIMMGIMGRTITTYGAAGG-3′) and Pr2r (5′-AYYGGIGTRAYTWCIGCCGC-IGTWCC-3′) were synthesized, where I, R, S, and Y indicate inosine, A or G, C or G, and C or T, respectively. PCR was performed using genomic DNA of *Enterobacter* sp. TL3 as a template. When Prlf/Pr2r was used as primers, about 700 bp gene fragments were obtained. This size matches to the expected size. The amplified fragments were cloned in the pGEM-T vector and sequenced. BLAST results showed that the cloned fragments had high homology to bacterial BCATs, especially showing 86% identity of DNA sequence with *E. coli*. In order to clone the BCAT*en* gene, PCR primers were designed based upon nucleotide sequences of BCAT*es* from *E. coli* due to highly homologous DNA sequences; prf (5′-ATCATGGAATTCA-TGACCACGAAGAAAGCT) and prr (5′-CTCGAGTTATTGATTAACTTGATCTAACCA) were designed. 918 bp fragment was obtained from genomic DNA of *Enterobacter* sp. TL3 by PCR with primers (prf and prr). This DNA sequence encodes a protein of 309 amino acid residues and identity with *E. coli* was 87% when the nucleotide sequences were compared (Fig. 2).

### 3.3. Over-expression and purification of the BCATs in E. coli BL21

The recombinant BCAT*en* and C-terminal His-tagged BCAT*en* were over-expressed in *E. coli* BL21. The relative quantities of the recombinant proteins were almost equal in SDS-PAGE (data not shown). The specific activities for L-Tle using crude extract of the native enzyme and the His-tagged BcAT*en* were 17 and 10 U $mg^{-1}$, respectively. The recombinant *E. coli* BL21 expressing BCAT*en* showed 45-fold higher activity compared to the wild-type *Enterobacter* sp. TL3. The specific activities of the recombinant *E. coli* over-expressing $His_6$-tagged BCAT*es* were 8.2 U $mg^{-1}$. The purified BCAT*en* and BCAT*es* gave a single protein band on SDS-PAGE, respectively.

### 3.4. Characterization of the his-tagged BCATs from E. coli BL21

The enzyme activity versus the pH was determined within pH range of 6.0–9.0 in the presence of 10 mM trimethylpyruvate and 10 mM L-glutamate. Three kinds of reaction buffer, 100 mM citrate (pH 6.0), 100 mM potassium phosphate (pH 6.0–8.0), and 100 mM boric acid (pH 8.0–9.0) were used for the enzyme assay. The pH-activity profile of the enzyme for BCAT*en* and BCAT*es* was both bell-shaped, showing a maximum value at pH 7.5. The enzyme activities at pH 6.0 and 9.0 were 58 and 78% of that at pH 7.5, respectively.

The amino acceptor specificity of the BCAT from *E. coli* and *Enterobacter* sp. was examined in the presence of 10 mM L-Glu, and the enzyme showed high activity toward various aliphatic keto acids such as 4-methyl-2-oxovaleric acid (keto form of leucine), 3-methyl-2-oxobutyric acid (keto form of valine) and 3-methyl-2-oxovaleric acid (keto form of iso-leucine). This result corresponds to the enzyme activity that had been reported [27]. Synthesizing our target unnatural amino acids, trimethylpyruvate; TMP (keto form of Tle) and 2-(3-hydroxy-1-adamantyl)-2-oxoethanoic acid: HAOE (keto form of 3-hydroxyadamantylglycine) also showed high activity (Table 1). Whereas, aromatic α-keto acid like phenylpyruvate was inert. The amino donor specificity of the enzyme was examined in the presence of 10 mM TMP and 10 mM HAOE. Among Glu, Asp and Ala, L-Glu was the most reactive amino donor.

In comparison between BCAT from *E. coli* and *Enterobacter* sp., BCAT*en* from *Enterobacter* sp. showed slightly higher activity toward branched chain amino acids including TMP. Thus, BCAT*en* was chosen for further studies for the production of L-Tle. On the other hand, the result indicates that BCAT*es* from *E. coli* is more suitable for the synthesis of bulky amino acid, L-HAG, therefore, BCAT*es*

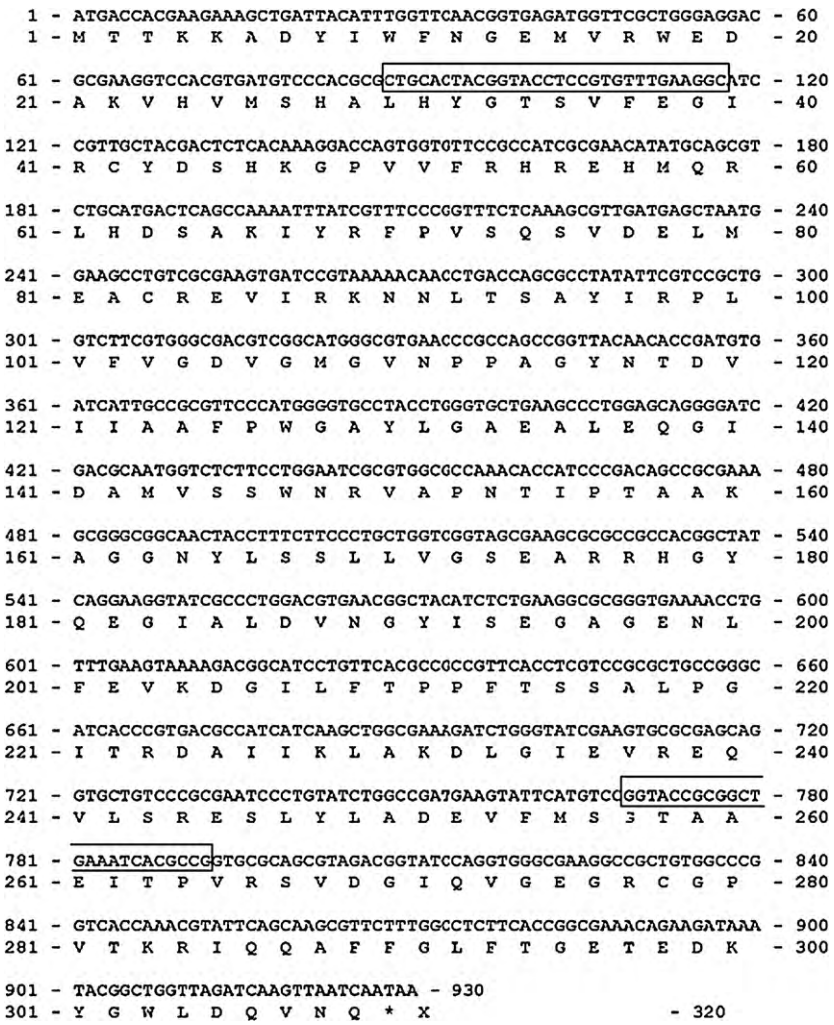


**Fig. 2.** DNA and amino acid sequences of BCAT*en* from *Entrerobacter* sp. The boxes indicate the consensus amino acid sequences used to design the degenerate primers.

was selected for further studies for the asymmetric synthesis of L-HAG.

### 3.5. Substrate and product inhibition of the enzymes for asymmetric synthesis

Since the reaction rate of aminotransferase is greatly influenced by the product and substrate concentrations, product and substrate inhibitions of BcAT*en* and BCAT*es* were examined. The substrate inhibitions of the BCAT*en* and BCAT*es* by L-Glu were determined in the presence of 20 mM TMP and HAOE, respectively. Both enzymes did not show substrate inhibition by L-Glu up to 200 mM. The substrate inhibition of BCAT*en* by TMP was not observed until 20 mM. However, as the concentration of TMP exceeded 20 mM, the initial rate decreased, and about 44% of the initial rate at 20 mM was lost at 200 mM TMP (Fig. 3). BCAT*es* also showed substrate inhibition by HAOE. The enzyme activity was maximal at 80 mM and then decreased as the concentration increased. Judging from the substrate inhibition by keto acids, the control of concentrations of the keto acids in the reaction mixture would be a key factor maintaining the maximum aminotransferase activity of these enzymes.

The activities of BCAT*en* and BCAT*es* were inhibited severely by α-ketoglutarate (deaminated product of L-Glu). In the presence of 10 mM α-ketoglutarate, only 20% of the enzyme activity remained (Fig. 3B). This result indicates that the removal of the α-ketoglutarate would be essential for the successful enzymatic production of L-Tle and L-HAG using BCATs. Indeed, when enzyme reaction was carried out in 1 mL of 100 mM phosphate buffer (pH 8.0) containing 20 or 50 mM TMP, 100 mM L-Glu and BCAT*en* (7 U $mL^{-1}$), the amounts of produced L-Tle did not exceed 17 mM.

**Table 1**
Kinetic parameters of BCATs from *E. coli* and *Enterobacter* sp.

| | BCAT (*E. coli*) | | | BCAT (*Enterobacter* sp.) | | |
|---|---|---|---|---|---|---|
| | Km (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}$/Km ($s^{-1}$ $mM^{-1}$) | Km (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}$/Km ($s^{-1}$ $mM^{-1}$) |
| 4-Methyl-2-oxovaleric acid | 0.42 | 6.61 | 15.74 | 0.97 | 17.32 | 17.85 |
| 3-Methyl-2-oxobutyric acid | 1.64 | 16.84 | 10.27 | 0.78 | 12.45 | 15.96 |
| 3-Methyl-2-oxovaleric acid | 0.99 | 13.75 | 13.89 | 0.63 | 12.15 | 19.29 |
| Trimethylpyruvate | 0.09 | 2.20 | 24.44 | 0.06 | 2.59 | 43.16 |
| 2-(3-Hydroxy-1-adamantyl)-2-oxoethanoic acid | 7.38 | 4.11 | 0.56 | 9.52 | 2.90 | 0.3 |

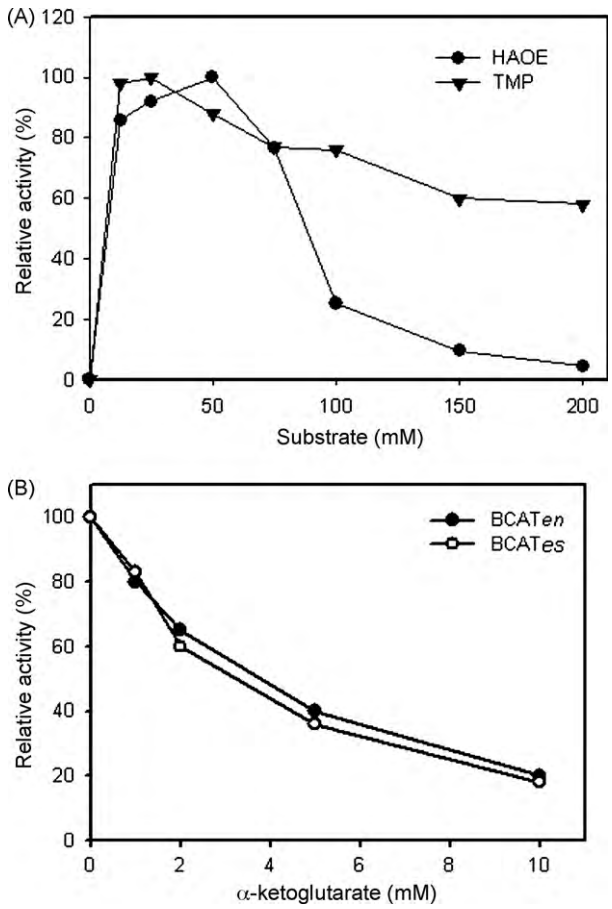


**Fig. 3.** Substrates and product inhibition of enzymes (A). Substrate inhibition of BCAT*en* by TMP (▼) and substrate inhibition of BCAT*es* by HAOE (●). For inhibition by TMP against the BCAT*es*, reaction mixture was comprised of as follows: 7 U BCAT*en*, 10 mM L-glutamate, the specific concentrations of TMP, and 100 mM phosphate buffer (pH 8.0). For inhibitions by HAOE against BCAT*es*, reaction mixtures were comprised of as follows: 0.7 U BCAT*es*, 10 mM L-glutamate, the specific concentrations of HAOE, and 100 mM phosphate buffer (pH 8.0). 100% corresponds to 0.21 mM $min^{-1}$ reaction rate; (B) α-ketoglutarate inhibition of BCAT*en* and BCAT*es*. Reaction mixtures were comprised of as follows: 0.7 U BCAT*es* or 0.7 U BCAT*en*, 10 mM L-glutamate, the specific concentrations of α-keto glutarate, and 100 mM phosphate buffer (pH 8.0). 100% corresponds to 0.07 mM $min^{-1}$ reaction rate for both enzymes.

### 3.6. Asymmetric synthesis of tert-leucine with BCATen using coupling system

To overcome product inhibition of α-ketoglutarate, coupling reaction with GLDH or AspAT was applied to the BCAT reaction (Fig. 4). In BCAT/AspAT coupling system, keto-acid acceptor (TMP) is converted into L-Tle using BCAT with L-Glu as the amino donor. The corresponding α-ketoglutarate is then converted back to L-Glu using L-aspartic acid (L-Asp) as the amino donor by AspAT, accompanied by the formation of oxaloacetic acid. Therefore, the inhibitory α-ketoglutarate can be maintained at low concentration in the reaction mixture. In the BCAT/AspAT coupling reaction, enzyme reaction was carried out in 100 mM phosphate buffer (pH 8.0) containing 50 mM of TMP, 100 mM of L-Glu, 200 mM L-Asp, BCAT*en* (7 U $mL^{-1}$) and AspAT (15 U $mL^{-1}$). 37.5 mM L-Tle was produced with 75% yield. Although the reaction yield increased about 2.2-fold, compared to previous results without coupling system (17 mM L-Tle was produced), still the yield (75%) was not high enough. We examined inhibitory effects on the BCAT*en* reaction by pyruvate. The inhibitory effect by pyruvate was negligible up to 150 mM of pyruvate. It is not clear why reaction profile reach to plateau with 75% conversion. Taylor et al. [2] briefly mentioned that one of the byproduct, pyruvate, accumulation is often undesirable because it can undergo a competitive transamination to form L-alanine, and the presence of this additional byproduct can effect the reaction.

In case of GLDH coupling system, formate dehydrogenase (FDH) was used to regenerate NAD/NADH. Here ammonium formate supplies ammonium for GLDH and formate for FDH. The favorable equilibrium of FDH for NADH derives the overall reaction toward the production of L-Tle. Ammonium formate dehydrogenase takes the ammonium formate and releases carbon dioxide and the amine source. NADH can be regenerated on this account. Therefore, additional feeding of amine source to avoid biproduct formation by dehydrogenase itself is unnecessary. To minimize substrate inhibition by TMP (Fig. 3), fed-batch reaction was carried out by feeding 50 mM TMP. BCAT*en* (7 U $mL^{-1}$) was added to 10 mL of 100 mM phosphate buffer (pH 8.0) containing 50 mM of TMP, 30 mM of L-Glu, 200 mM ammonium formate, GLDH (15 U $mL^{-1}$) and FDH (20 U $mL^{-1}$). The solid-state substrate corresponding to 50 mM TMP was added to the reaction mixture intermittently and the course of reaction was monitored using HPLC analysis. The yield of L-Tle reached up to 90% with 99% *ee* in 150 mM high substrate concentration conditions (Fig. 5).

### 3.7. Asymmetric synthesis of L-3-hydroxyadamantylglycine in BCATes/AspAT coupling system

For the asymmetric synthesis of L-3-hydroxyadamantylglycine, the purified enzyme (0.7 U $mL^{-1}$ BACT*es*) was added to 0.2 mL of 100 mM phosphate buffer (pH 8.0) containing 20 mM of the HAOE, 100 mM L-Glu, 60 mM L-Asp at 37 °C for 15 h. 10 mM of HAOE was converted into 12.2 mM L-HAG with 61.2% conversion. When 0.7 U $mL^{-1}$ BACT*es* and crude extract of AspAT was added in this condition, the coupling system gave 20 mM L-HAG with 85.6% conversion, which is 1.4-folds higher conversion than BCTA*es* single enzyme reaction.

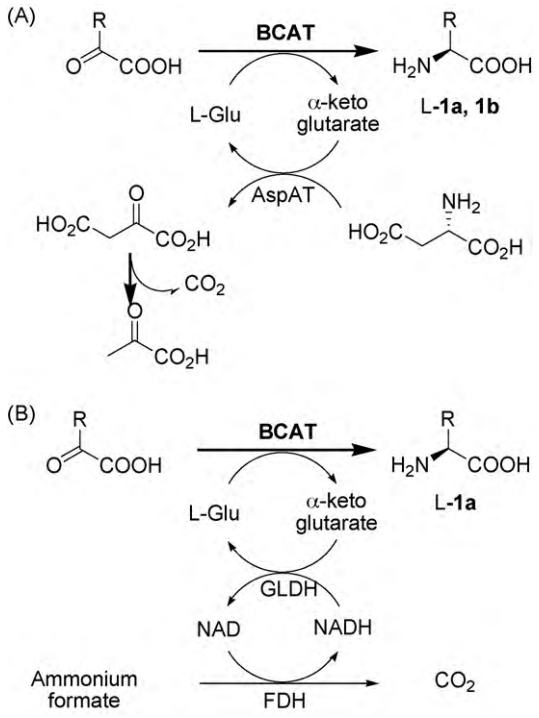


**Fig. 4.** The reaction schemes of enzyme coupling reaction. (A) BCAT/GLDH coupling reaction; (B) BCAT/AspAT coupling reaction.

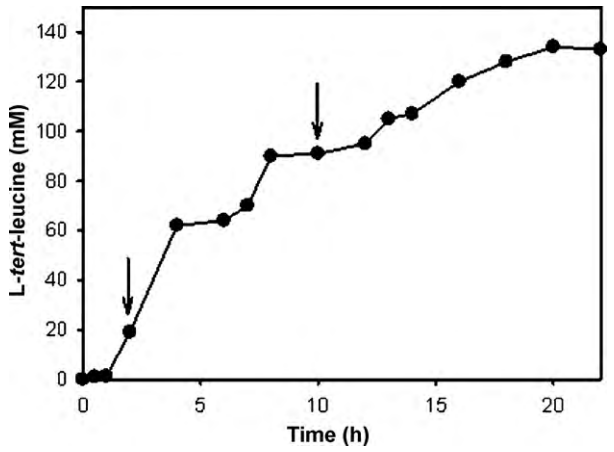


**Fig. 5.** The production of L-Tle using BCAT*en*/GLDH coupling reaction. The reaction started with the reaction mixture containing 100 mM phosphate buffer (pH 8.0), 50 mM of TMP, 30 mM of L-Glu, 200 mM ammonium formate, 7 U BCAT*en*, 15 U GDH and 20 U FDH in 10 mL. The arrows indicate when solid TMP (50 mM) was added.

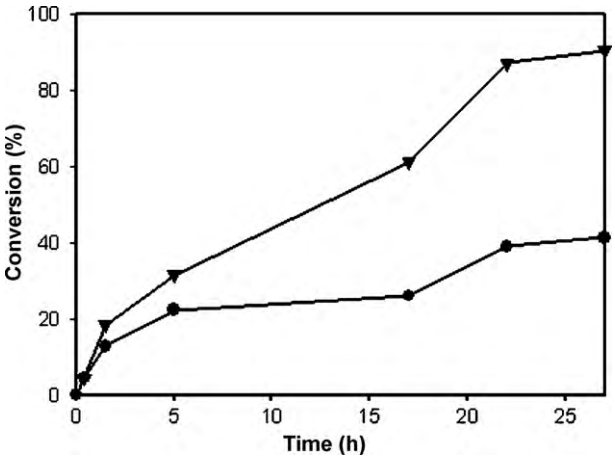


**Fig. 6.** The asymmetric synthesis of L-HAG using whole cells reaction. The reaction mixture (0.5 mL) contained 200 mM phosphate buffer (pH 8.0), 100 mM of HAOE, 500 mM L-Glu and 300 mM L-Asp using whole cell harboring BCAT (0.7 U $mL^{-1}$). Course of the conversion when only BCAT*es* (0.7 U $mL^{-1}$) was used (●). Course of the conversion when whole cell harboring BCAT*es* (0.7 U $mL^{-1}$) and AspTA (12 mg wet cell weight $mL^{-1}$) in *E. coli* BL21 was separately added (▼).

The use of whole cells as the biocatalyst in an enzymatic process has the advantage of simple and economical preparation as expenses for cell lysis and/or purification are saved. For the whole cell reaction, freshly prepared whole-cells were added to reaction mixture containing 100 mM of HAOE, 500 mM L-Glu and 300 mM L-Asp at 37 °C. The recombinant *E. coli* expressing BCAT*es* (12 mg wet cell weight $mL^{-1}$, which is corresponding to 0.7 U $mL^{-1}$) gave 41% conversion yield. When reaction was carried out using *E. coli* BL21 expressing BCAT*es* (12 mg wet cell weight $mL^{-1}$) and *E. coli* BL21 expressing AspTA (12 mg wet cell weight $mL^{-1}$), 90.5 mM L-HAG was produced with >99% *ee*, which was 2.2-fold higher than that of using BCAT alone (Fig. 6). When the same reaction conditions were executed using the crude extract of the enzymes in same units, 100 mM of HAOE was converted into 55.4 mM L-HAG with 55.4% conversion in case of only using BACT*es*, and the coupling system gave 78.5 mM L-HAG with 78.5% conversion. Although the substrate keto acid could be converted to hydroxy acid by the ketoreductase existing in the whole cell or the product could be metabolized, the whole-cell appeared to be less sensitive to the product inhibitions exerted by α-ketoglutrate and/or by-products because the enzyme was indirectly exposed to high substrate concentrations. In addition, *E. coli* may also maintain inhibitory by-products at low concentration by intracellular-metabolism.

## 4. Conclusion

In this study, the basic properties of BCAT*en* from *Enterobacter* sp. TL3 which is isolated by selective enrichment from soil samples were characterized in detail. This study also successfully demonstrated the production of L-TLe and L-HAG using BCAT/AspAT coupling and BCAT/GLDH/FDH coupling reactions which are designed to overcome product inhibition of the enzymes by α-ketoglutarate as well as the equilibrium problem of the transamination reaction. We were able to achieve 90% conversion with 99% *ee* in 150 mM high substrate concentration condition by BCAT*en*/GLDH/FDH coupling system. In the synthesis of L-HAG, The AspTA/BcAT coupling system using two recombinant whole cells achieved 90.5% conversion of 100 mM substrate with >99% *ee*. In this study, coupling system was proven to be successful for increasing the bioconversion of the transamianse reaction, giving almost full conversion for the synthesis of both L-Tle and L-HAG.

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