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# Enzymatic Synthesis of L-*tert*-Leucine with Branched Chain Aminotransferase

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**In this study, we demonstrated the asymmetric synthesis of L-*tert*-leucine from trimethylpyruvate using branched-chain aminotransferase (BCAT) from *Escherichia coli* in the presence of L-glutamate as an amino donor. Since BCAT was severely inhibited by 2-ketoglutarate, in order to overcome this here, we developed a BCAT/aspartate aminotransferase (AspAT) and BCAT/AspAT/pyruvate decarboxylase (PDC) coupling reaction. In the BCAT/AspAT/PDC coupling reaction, 89.2 mM L-*tert*-leucine (ee >99%) was asymmetrically synthesized from 100 mM trimethylpyruvate.**

**Keywords:** Aminotransferase, branched-chain aminotransferase, coupling reaction, L-*tert*-leucine, unnatural amino acid

Optically active amino acids are fundamental building blocks for the preparation of agrochemical target molecules such as peptides, proteins, and many other natural products [9]. Amino acids are extensively used as chiral starting materials, auxiliaries, and catalysts in modern organic synthesis [2]. Incorporation of unnatural amino acids into peptide has become an important strategy in the synthesis of biologically active compounds owing to the resistance to enzymatic degradation [1]. One of the more interesting amino acids in this regard is L-*tert*-leucine, which has a sterically demanding side-chain. L-*tert*-Leucine can be used for templates in asymmetric synthesis and as building blocks for pharmaceutically active compounds. L-*tert*-Leucine itself is a component of several pharmaceutical development projects as tumor fighting agents or HIV protease inhibitors. A whole range of ligands for asymmetric catalysts has also been developed with L-*tert*-leucine, mostly based on the oxazolidine moiety [3].

Given the significance of L-*tert*-leucine, its efficient synthesis in optically pure form has become an attractive challenge to organic chemists in the recent years and biocatalytic methods for the production of L-*tert*-leucine have been reported. L-*tert*-Leucine was prepared by lipase-catalyzed kinetic resolution of racemic 2-phenyl-4-*tert*-butyloxazolin-5(4*H*)-one and subsequent two-step hydrolysis [14]. In earlier, reports L-*tert*-leucine prepared by kinetic resolution of *N*-phenylacetylated-D,L-*tert*-leucine with Penicillin G acylase from *Kluyvera citrophila* [7, 8] had a drawback of theoretical yield that could never exceed 50%. Therefore, the asymmetric synthesis is usually preferred as the theoretical maximum yield is 100%, which is two times higher than that of the kinetic resolution. The Degussa AG-mediated process, utilizing leucine dehydrogenase-catalyzed reductive amination of trimethylpyruvate, has been successfully operated on a large scale [10] but had a drawback of requirement of NADH recycling.

Aminotransferases have been extensively studied for the production of chiral amino acids owing to their rapid reaction rates, broad substrate specificity, and high enantioselectivity [4, 12, 16]. Among them, the branched-chain aminotransferae (BCAT) shows reactivity towards various aliphatic amino acids such as leucine and iso-leucine, and has been used to prepare L-*tert*-leucine [5, 6, 13]. In the BCAT reaction for the production of L-*tert*-leucine, enzyme inhibition by 2-ketoglutarate was the major hurdle to overcome and carry out the efficient BCAT reaction. To overcome product inhibition by 2-ketoglutarate, a BCAT and ornithine-δ-aminotransferase coupling reaction was carried out in which L-ornithine δ-aminotransferase transferred the δ-amino group from L-ornithine to 2-ketoglutarate, and produced L-glutamate-γ-semialdehyde and L-glutamic acid [6]. The formation of L-Glu from 2-ketoglutarate was strongly favored by the spontaneous cyclization of L-glutamate-γ-semialdehyde to form $\Delta^1$-pyrroline-5-carboxylate and drove the reaction to formation of L-*tert*-leucine [6]. Recently, L-*tert*-leucine was asymmetrically synthesized using novel BCAT from *Enterobacter* sp., and to overcome the product inhibition by 2-ketoglutarate, the BCAT reaction was coupled with L-glutamate dehydrogenase and formate dehydrogenase. L-

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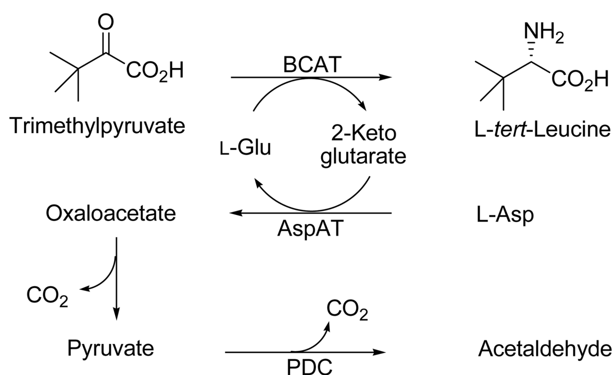


**Fig. 1.** The asymmetric synthesis of L-*tert*-leucine by BCAT.
To overcome the product inhibition of 2-ketoglutarate, AspAT was coupled with BCAT; here the produced 2-ketoglutarate is converted back to L-Glu using L-Asp as the amino donor by AspAT, and spontaneous decarboxylation of oxaloacetate forms pyruvate. Thus AspAT drives the reaction beyond equilibrium. Pyruvate decarboxylase (PDC) performs the non-oxidative thiaminediphosphate-mediated decarboxylation of pyruvate to acetaldehyde, which can further shift the equilibrium of the reaction.

Glutamate dehydrogenase catalyzes the NADPH-dependent amination of 2-ketoglutarate to yield L-Glu, and formate dehydrogenase regenerates NADH using formate. In this study, we asymmetrically synthesized L-*tert*-leucine from trimethylpyruvate using BCAT from *Escherichia coli*. To overcome the product inhibition by 2-ketoglutarate and to drive the reaction equilibrium towards the forward direction, BCAT was coupled with aspartate aminotransferase (AspAT) and pyruvate decarboxylase (PDC) in which AspAT converted 2-ketoglutarate to L-Glu using L-Asp and PDC converted pyruvate into acetaldehyde (Fig. 1).

To express BCAT in *E. coli*, the BCAT gene was amplified from the genomic DNA of *E. coli* K12 by PCR using primers 5'-AAAAAAGGATCCATGACCACGAAG AAAGCT-3' and 5'-AAAAAACTCGAGTTGATTAACT TGATC-3'. The PCR product was digested with *Bam*HI and *Xho*I, and inserted into pET24ma. The plasmid was then introduced into the *E. coli* (BL21) and transformants were grown in 1 l LB broth containing 50 µg/ml of kanamycin at 37°C. When the $OD_{600}$ reached 0.25, IPTG was added to a final concentration of 0.5 mM. After 6 h of induction, the cells were harvested and washed twice with 50 mM phosphate buffer (pH 8.0). After centrifugation, the cell pellet was resuspended in 20 ml of 50 mM phosphate buffer (pH 7.0) containing 20 µM pyridoxal 5'-phosphate (PLP), 1 mM EDTA, and 1 mM PMSF, and the cell was disrupted by ultrasonication. The enzyme was purified on a Ni-NTA affinity column, as described elsewhere [5]. The corresponding size of the overexpressed protein band was well matched with the molecular mass (36.5 kDa), and the purified enzyme appeared as a single protein band on SDS−PAGE. The purified enzyme was stored in 25% glycerol solution at −20°C for further study.

One unit of BCAT was defined as the amount of enzyme catalyzing the formation of 1 µmol of L-*tert*-leucine per minute in 100 mM phosphate buffer (pH 7.5) containing 10 mM trimethylpyruvate and 10 mM Glu at 37°C. The specific activity of the purified BCAT was 7.8 U/mg. Chiral analysis of *tert*-leucine was performed using a $C_{18}$ Symmetry column (Waters, MA, USA) with a Waters HPLC system at 254 nm after the derivatization of the sample with 3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) [11]. Separation of each enantiomer was achieved through an isocratic elution with a mixture of 50% methanol and 50% water (0.1% TFA) at a flow rate of 1.0 ml/min. To quantify each enantiomer, an appropriate amount of valine (an internal standard) was added to the stop solution, and the mixture was derivatized with GITC. After derivatization, the retention times for L-Val, L-*tert*-leucine, and D-*tert*-leucine were observed to be 12.1, 15.9, and 27.0 min, respectively. To determine the optimal pH, the initial reaction rate was measured in the presence of 10 mM trimethylpyruvate and 10 mM Glu within a pH range of 6.0 to 10 [100 mM phosphate buffer (pH 6.0 to 8.0) and 100 mM Tris/HCl buffer (pH 7.5 to 10.0)]. The enzyme showed the highest activity at pH 7.5. When the asymmetric synthesis was carried out with 10 mM trimethylpyruvate, 10 mM L-Glu, and BCAT (0.5 U/ml) in 1 ml of 100 mM phosphate buffer (pH 7.5) at 37°C, 4.6 mM L-*tert*-leucine (ee >99%) was produced.

Since the enzyme activity of aminotransferase is affected by the product and substrate concentrations, product and

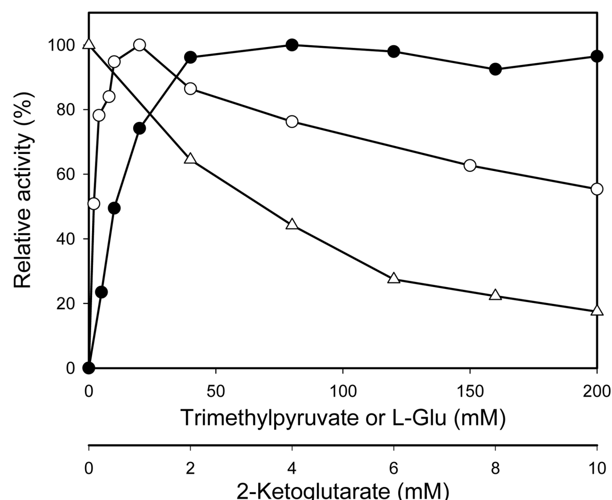


**Fig. 2.** Substrate and product inhibition of BCAT.
The reaction was carried out with 1 ml of 100 mM phosphate buffer (pH 7.5) containing BCAT (0.05 U/ml) at 37°C. The substrate inhibition by L-Glu (○), reaction condition: 20 mM trimethylpyruvate, L-Glu (0−200 mM); the substrate inhibition by trimethylpyruvate (●), reaction condition: 20 mM L-Glu and trimethylpyruvate (0−200 mM); the product inhibition by 2-ketoglutarate (△), reaction condition: 20 mM trimethylpyruvate, 20 mM L-Glu and 2-ketoglutarate (0−10 mM).

substrate inhibitions of BCAT were examined. To measure the substrate inhibition by L-Glu, the initial rate of the enzyme was analyzed with 20 mM trimethylpyruvate and various concentrations of L-Glu (0–200 mM) (Fig. 2). The substrate inhibition by L-Glu was not observed up to 200 mM. The substrate inhibition by trimethylpyruvate was examined with 20 mM L-Glu and various concentrations of trimethylpyruvate (0–200 mM). The reaction rate increased as the concentration of trimethylpyruvate was increased up to 20 mM. However, when trimethylpyruvate exceeded 20 mM, the reaction rate was decreased, and the reaction rate with 200 mM trimethylpyruvate was 55.4% of that with 20 mM trimethylpyruvate. The product inhibition by 2-ketoglutarate was examined with 20 mM L-Glu and 20 mM trimethylpyruvate and various concentrations of 2-ketoglutarate (0–10 mM). The enzyme lost 82.5% of its activity in the presence of 10 mM 2-ketoglutarate. The product inhibition of BCAT by 2-ketoglutarate was already well reported [4–6, 13]. Those results indicated that the removal of the 2-ketoglutarate would be a key factor for the efficient production of L-*tert*-leucine using BCAT.

When asymmetric synthesis were carried out with 20, 50, and 100 mM trimethylpyrvate in 1 ml of 100 mM phosphate buffer (pH 7.5) containing 100 mM Glu and BCAT (1.0 U/ml), the yields of L-*tert*-leucine (>99%) were 64.6%, 42.4%, and 23.4%, respectively (Fig. 3). The amount of produced L-*tert*-leucine (21.2 mM) from 50 mM trimethylpyruvate was similar to that (23.4 mM) from 100 mM trimethylpyruvate. These results are mainly due to the product inhibition by 2-ketoglutarate, and to overcome it, aspartate AT (AspAT) was coupled with BCAT. Here, the produced 2-ketoglutarate is converted back to L-Glu using L-Asp as the amino donor by AspAT, and spontaneous decarboxylation of oxaloacetate forms pyruvate. Thus, AspAT drives the reaction beyond equilibrium. Pyruvate decarboxylase (PDC), which can further shift the equilibrium of the reaction, was also additionally introduced into the BCAT/AspAT coupling reaction (Fig. 1). PDC performs the non-oxidative thiamine diphosphate-mediated decarboxylation of pyruvate to acetaldehyde [15]. The AspAT gene was amplified from the genomic DNA of *E. coli* K12 by PCR using primers (5'-AAAAAACATATGTTTGAGAACATTACCG CCGCT-3' and 5'-AAAAAAGAATTCTTACAGCACTG CCACAATCGC-3'), digested with *Nde*I and *Eco*RI, and inserted into pET22b(+) (Novagene, USA). The PDC gene was amplified from genomic DNA of *Zymomonas mobilis* by PCR using primers (5'-AAAAAACATATGAGTTATAC TGTCGGTACCTATTTAG-3' and 5'-AAAAAAGGATCCCT AGA-GGAGCTTGTTAACAGGCTTAC-3'), digested with *Nde*I and *Bam*HI, and inserted into the pET24ma vector. The crude extracts of recombinant *E. coli* were prepared by ultrasonication of the cell pellet as described elsewhere [15]. The AspAT activity of crude extract of recombinant *E. coli* expressing AspAT was measured in 100 mM phosphate buffer (pH 7.0) containing 10 mM 2-ketoglutarate and 10 mM L-Asp at 25°C, and observed to be 35 U/mg. The PDC activity of the crude extract of recombinant *E. coli* expressing PDC was measured in 100 mM phosphate buffer (pH 6.5) containing 10 mM pyruvate, 5 mM $MgSO_4$, and 0.1 mM thiamine pyrophosphate at 25°C, and determined to be 8.5 U/mg. The pyruvate (retention time, 15.5 min) and 2-ketoglutarate (retention time, 15.6 min) were analyzed on an Aminex HPX-87H HPLC column (Bio-Rad, CA, USA) with an isocratic elution of 5 mM $H_2SO_4$ at 0.6 ml/min. The crude extracts were directly used for the coupling reaction without a further purification step.

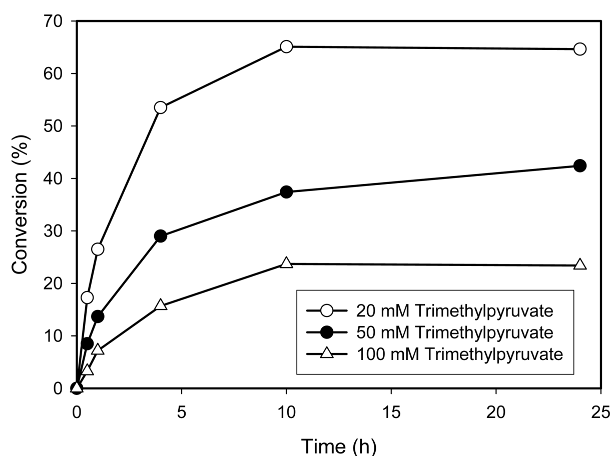


**Fig. 3.** The asymmetric synthesis of L-*tert*-leucine.
The reaction was carried out in 1 ml of 100 mM phosphate buffer (pH 7.5) with 100 mM L-Glu, BCAT (1.5 U/ml), and different concentrations of trimethylpyruvate (20, 50, and 100 mM).

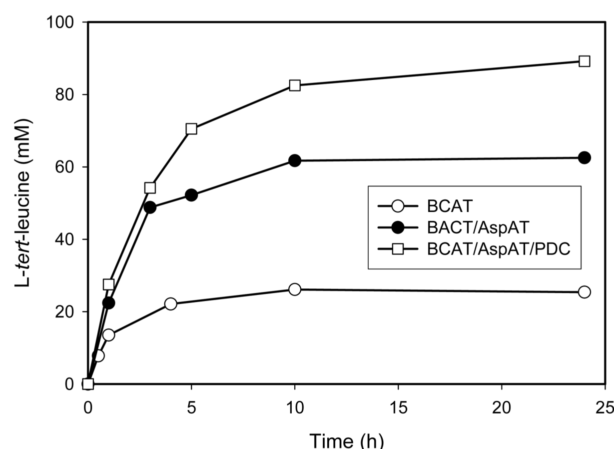


**Fig. 4.** The asymmetric synthesis of L-*tert*-leucine using a coupling reaction.
The reaction was carried out in 1 ml of 100 mM phosphate buffer (pH 7.5). BCAT reaction condition: 100 mM trimethylpyruvate, 100 mM L-Glu, BCAT (1.5 U/ml). BCAT/AspAT reaction condition: 100 mM trimethylpyruvate, 50 mM L-Glu, 200 mM L-Asp, BCAT (1.5 U/ml), and AspAT (2.0 U/ml). BCAT/AspAT/PDC reaction condition: 100 mM trimethylpyruvate, 50 mM L-Glu, 200 mM L-Asp, BCAT (1.5 U/ml), AspAT (2.0 U/ml), and PDC (2.0 U/ml).

When the asymmetric synthesis was carried out in 100 mM phosphate buffer (pH 7.5) with 100 mM trimethylpyruvate, 100 mM L-Glu, and BCAT (1.5 U/ml), 25.4 mM L-*tert*-leucine (>99%) was produced for 24 h (Fig. 4). The BCAT/AspAT and BCAT/AspAT/PDC coupling reactions produced 62.5 and 89.2 mM L-*tert*-leucine (>99%) from 100 mM trimethylpyruvate for 24 h, respectively (Fig. 4). The BCAT/AspAT coupling reaction gave about 2.5-fold higher yield than the single BCAT reaction, and BCAT/AspAT/PDC gave 3.5-fold higher yield than the BCAT reaction. It is notable that pyruvate itself did not show any significant inhibitory effect on the BCAT reaction (data not shown). The enhanced yield of BCAT/AspAT/PDC might be due to the equilibrium shift. When the whole-cell reaction was carried out with recombinant *E. coli* expressing BCAT, AspAT, and PDC, there was also an advantage of the BCAT/AspAT/PDC coupling system compared with that of the BCAT/AspAT coupling system. In the BCAT/AspAT system, the final by-product pyruvate will be converted into L-Ala by endogenous ATs, which will disrupt the isolation of L-*tert*-leucine [6]. However, in the BCAT/AspAT/PDC coupling system, L-Ala formation will be reduced by converting the pyruvate to acetaldehyde. Therefore, the target product can be easily purified from the reaction mixture. The development of asymmetric synthesis of L-*tert*-leucine by a whole-cell reaction with recombinant *E. coli* co-expressing BCAT, AspAT, and PDC is currently being investigated in our laboratory.

In summary, we successfully developed an efficient BCAT-catalyzed asymmetric synthesis of L-*tert*-leucine using a coupling system with AspAT and PDC, which can be used to remove the inhibitory by-product, and to shift the reaction equilibrium towards the product formation.

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