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Biocatalytic cascade reactions for asymmetric synthesis of aliphatic amino acids in a biphasic reaction system

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Keywords: amino acids; biocatalysis; asymmetric synthesis; product inhibition; cascade reaction; transaminase

Highlights

• Extractive enzyme reaction for asymmetric synthesis of amino acids

• Cascade reactions by coupling two transaminases.

• Equilibrium shift by recycling a cosubstrate

• In situ selective extraction of an inhibitory ketone

• The biphasic reaction afforded synthesis of six amino acids with > 92 % yield and > 99.9 % *ee*.

Abstract

Enantiopure aliphatic amino acids, including L-3-hydroxyadamantylglycine (L-Hag), L-*tert*-leucine (L-Tle) and L-norvaline, are essential chiral building blocks for a number of pharmaceutical drugs. Here, we developed cascade enzyme reactions in an extractive biphasic system using a branched-chain amino acid transaminase (BCTA) and an (*S*)-selective ω-transaminase (ω-TA) for asymmetric synthesis of the aliphatic amino acids from achiral α-keto acid precursors. The extractive cascade reactions enabled equilibrium shift of the BCTA reaction by recycling an amino acid cosubstrate as well as acceleration of the ω-TA reaction by removing an inhibitory ketone product from an aqueous phase. Starting with 20 mM α-keto acid, 4 mM *rac*-homoalanine and 50 mM *rac*-α-methylbenzylamine (*rac*-α-MBA), the biphasic cascade reactions afforded synthesis of four unnatural amino acids (i.e. L-Tle, L-Hag, L-norvaline and L-norleucine) and two natural amino acids (i.e. L-valine and L-leucine) with > 92 % conversion yield and > 99.9 % *ee*. To demonstrate the industrial feasibility of the extractive cascade reaction, preparative-scale synthesis of L-Hag was performed in a reaction mixture consisting of 300 mL hexane and 50 mL aqueous solution (50 mM phosphate buffer, pH 7.0) charged with 50 mM keto acid substrate, 5 mM L-homoalanine, 120 mM *rac*-α-MBA, 2 U/mL BCTA and 16 U/mL ω-TA. Conversion yield of L-Hag reached 92 % with > 99.9 % *ee* at 70 h. Product isolation led to 0.32 g white solid of L-Hag (62 % isolation yield).

1. Introduction

Optically pure amino acids are increasingly employed as important chiral motifs in various pharmaceutical drugs [1, 2]. For example, L-3-hydroxyadamantylglycine (L-Hag) and L-*tert*-leucine (L-Tle), which are unnatural amino acids carrying branched-chain aliphatic groups, are

essential components of a type-2 diabetes drug (i.e. saxagliptin) [3] and a HIV-protease inhibitor [4], respectively. In addition, L-norvaline carrying a linear aliphatic side chain is a key intermediate of Perindopril (i.e. an ACE inhibitor) [5]. Growing industrial demands for the aliphatic amino acids have spurred massive research efforts to develop preparative methods via biocatalysis owing to the lack of the fermentative methods yet to access the unnatural amino acids. Several biocatalytic strategies have been developed by kinetic resolution of racemic amino acid derivatives using acylase, lipase and protease [6-8] or asymmetric amination of keto acids using transaminase and dehydrogenase [9-11]. Because of a two-fold higher maximum yield, the asymmetric synthesis is often favored over the kinetic resolution [12, 13].

Branched-chain amino acid transaminase (BCTA) is a major metabolic producer of the natural branched-chain amino acids such as L-valine, L-leucine and L-isoleucine [14]. BCTA displays a perfect match in the substrate specificity for a broad range of aliphatic α-keto acids, which enables production of the unnatural amino acids such as L-Tle and L-Hag [11]. Moreover, compared to the amino acid dehydrogenases [9, 10], no requirement of an expensive external cofactor such as NADH renders BCTA promising for industrial applications in the asymmetric synthesis of the aliphatic amino acids. However, a crucial setback in the scale-up of the BCTA reaction is a neutral equilibrium position of the most BCTA reactions (i.e. equilibrium constant ≈ 1) [14]. To overcome the equilibrium problem, it was demonstrated that removal of a keto acid coproduct by spontaneous decarboxylation or enzymatic conversion using dehydrogenases could facilitate an equilibrium shift [11].

In the previous report, we demonstrated that the thermodynamically neutral BCTA reactions can be driven to completion by coupling a ω-transaminase (ω-TA) reaction as an equilibrium shifter [15]. Note that ω-TA is capable of employing primary amines as an amino donor and thereby the resulting ω-TA reaction between the primary amine and the keto acid is

thermodynamically favorable [16]. To couple the BCTA and ω-TA reactions, L-homoalanine was used as a shuttling substrate. Isopropylamine was used as an amino donor for the ω-TA from *Ochrobactrum anthropi* because isopropylamine was regarded as an ideal amino donor due to easy removal of the volatile deamination product (i.e. acetone) [17]. However, this strategy cannot be generalized to other ω-TAs because isopropylamine is rather a poor substrate for most ω-TAs. For example, two typical ω-TAs, *i.e.* an (*S*)-selective ω-TA from *Vibrio fluvialis* JS17 and an (*R*)-selective ω-TA form *Arthrobacter* sp., are known to show negligible activities for isopropylamine [18, 19]. Note that the ω-TA from *O. anthropi* used in the previous study shows exceptionally high activity for isopropylamine (i.e. 43 % activity relative to that for (*S*)-α-methylbenzylamine ((*S*)-α-MBA)) [20]. In this study, we seek to develop a generally applicable strategy in which an amine substrate of choice is reactive toward most ω-TAs (i.e. benzylamine and α-MBA) and thereby any ω-TA displaying desirable enzymatic properties for a manufacturing setting, including high operational stability and low product inhibition, can be used for a process scale-up.

2. Materials and methods

2.1. Chemicals

Pyridoxal 5′-phosphate (PLP) was purchased from Sigma Aldrich Co. (St. Louis, USA). Pyruvic acid was obtained from Kanto Chemical Co. (Tokyo, Japan). Trimethylpyruvate was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). 2-(3-Hydroxy-1-adamantyl)-2-oxoethanoic acid was purchased from Hi-Tech Chemical Co. (Chongqing, China). *n*-Hexane was purchased from Duksan Chemical Co. (Ansan, South Korea). Materials used for culture media including yeast extract, tryptone and agar were purchased from Difco (Spark, USA). All other chemicals were purchased from Sigma Aldrich Co. (St. Louis, USA) and of the highest

grade available.

2.2. Expression and purification of TAs

Overexpression of His_6-tagged TAs was carried out as described previously with minor modifications [21]. *Escherichia coli* BL21(DE3) cells carrying the expression vectors (i.e. pET28a(+) harboring the TA gene) were cultivated in LB medium (typically 1 L) containing 50 μg/mL kanamycin. Protein expression was induced by IPTG at 0.4 OD_{600} and the cells were allowed to grow for 10 h. The culture broth was centrifuged and the resulting cell suspension was subjected to ultrasonic disruption. Protein purification was carried out as described previously [21]. Molar concentrations of the purified TAs were determined by measuring UV absorbance at 280 nm.

2.3. Enzyme assay

Typical enzyme assays were carried out at 37 °C in 50 mM phosphate buffer (pH 7). One unit of BCTA is defined as the enzyme amount catalyzing formation of 1 μmole 2-oxobutyrate in 1 min at 20 mM trimethylpyruvate and 20 mM L-homoalanine. One unit of ω-TA from *Paracoccus denitrificans* (PDTA) is defined as the enzyme amount catalyzing formation of 1 μmole acetophenone in 1 min at 20 mM pyruvate and 20 mM (*S*)-α-MBA. After 10 min reaction, the enzyme reaction (100 μL reaction volume) was stopped by adding 600 μL acetonitrile. For the initial rate measurements, 2-oxobutyrate and acetophenone for BCTA and PDTA, respectively, were analyzed by HPLC.

2.4. Cascade enzyme reactions to produce aliphatic amino acids

Unless otherwise specified, the cascade reactions were performed in 50 mM phosphate buffer (pH 7) at 37 ^{o}C. In the biphasic reactions, 3 or 6 mL of hexane was carefully overlaid on

the aqueous reaction mixture (1 mL) and the reactions were carried out without agitation. Aliquots of the aqueous and organic phases (30 μL each) were taken at predetermined reaction times and mixed with 180 μL acetonitrile. The reaction progresses were monitored by HPLC analysis of the keto acid substrate or the amino acid product.

2.5. Preparative-scale synthesis and isolation of L-Hag

Preparative-scale extractive cascade reaction for asymmetric synthesis of L-Hag was carried out at 37 °C under magnetic stirring in an oven-dried beaker charged with 50 mL reaction mixture containing 2-(3-hydroxy-1-adamantyl)-2-oxoethanoic acid (2.5 mmol), L-homoalanine (0.25 mmol), *rac*-α-MBA (6 mmol), PLP (5 μmol), BCTA (0.18 μmol), PDTA (0.7 μmol) and potassium phosphate (50 mM, pH 7.0). Hexane (300 mL) was added to the aqueous reaction mixture. When the conversion exceeded 90 %, the aqueous reaction mixture was subjected to product isolation.

The aqueous reaction mixture (50 mL) was separated from the organic phase using a separation funnel and the pH was adjusted to 1.0 by adding 5 N HCl (4.5 mL). Protein precipitate was removed by filtration through a glass-fritted filter funnel. The pH of the filtrate solution was adjusted to 7.0 by adding 5 N NaOH solution (6 mL). The resulting solution was evaporated at 30 °C and 0.1 bar until the final volume reached around 2 mL. The precipitate formed during the evaporation was obtained by filtration, washed by 10 mL EtOH and then dried overnight in a drying oven (75 °C). The resulting white solid of L-Hag was subjected to mass spectrometric analysis and elemental analysis as described in the Supplementary Data.

2.6. HPLC analysis

Analysis of acetophenone was performed on a Waters HPLC system (Milford, USA) using a Symmetry C18 column (Waters Co.) with isocratic elution of 60 % methanol/40 % water,

both containing 0.1 % trifluoroacetic acid, at 1 mL/min. Detection was done with a UV detector tuned at 254 nm. Retention time of acetophenone was 3.8 min.

α-Keto acids were analyzed using an Aminex HPX-87H column (Bio-Rad, Hercules, USA) with isocratic elution of 5 mM H_2SO_4 solution at 0.5 mL/min. UV detection was carried out at 210 nm. Column oven temperature was set to 40 °C. Retention times of the keto acids were 12.0 (2-oxobutyrate), 14.2 (2-oxopentanoic acid), 11.9 (3-methyl-2-oxobutyrate), 17.7 (2-oxohexanoic acid), 15.3 (4-methyl-2-oxopentanoic acid), 9.7 (trimethylpyruvate) and 11.7 min (2-(3-hydroxy-1-adamantyl)-2-oxoethanoic acid).

For determination of amino acid concentration and enantiomeric excess, 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) was used for amino acid derivatization [22]. The GITC-derivatives of the amino acids were resolved on the Symmetry column at a flow rate of 1 mL/min with UV detection tuned at 254 nm. Retention time of L/D-enantiomers of homoalanine was 26.9/28.7 min at isocratic elution of 40 % methanol (A)/60 % water (B), both containing 0.1 % trifluoroacetic acid,. Retention time of L/D-enantiomers of Hag was 21.1/34.4 min at isocratic elution of 45 % A/55 % B. Retention time of L/D-enantiomers of norvaline, valine, leucine, Tle were 10.1/14.9, 8.8/13.3, 15.2/23.4 and 11.6/19.0 min, respectively, at isocratic elution of 50 % A/50 % B. Retention time of L/D-enantiomers of norleucine was 6.0/7.9 min at isocratic elution of 60 % A/40 % B.

3. Results and discussion

3.1. Extractive cascade reaction

To construct the extractive cascade reaction setup (Figure 1), we chose a BCTA cloned from *Escherichia coli* [15] and a (*S*)-selective ω-TA from *Paracoccus denitrificans* (PDTA) [23]. L-Homoalanine (i.e. a shuttling substrate) was used as an amino donor for the BCTA

reaction and its deamination product (i.e. 2-oxobutyrate) was converted back to L-homoalanine by the ω-TA reaction in which the amino group to be transferred was supplied from a primary amine, leading to recycling of the shuttling substrate and a consequent thermodynamic shift of the BCTA reaction driven by the highly exergonic ω-TA reaction. Owing to the recycling of the shuttling substrate, the cascade reaction requires L-homoalanine in a much lower amount than the keto acid substrate. Benzylamine and α-MBA, i.e. typical reactive amine substrates for all known ω-TAs, were employed as the amino donor for PDTA in the cascade reactions. For example, PDTA showed a 14-fold higher activity for (*S*)-α-MBA relative to that for isopropylamine [20]. This renders (*S*)-α-MBA much more efficient as an amino donor for PDTA like other ω-TAs. The ketone product from the ω-TA reaction was known to exert a severe inhibition on the ω-TA activity [16]. For example, the product inhibition constant of PDTA for acetophenone was measured to 2.4 mM in the reaction between (*S*)-α-MBA and pyruvate [24]. Therefore, an organic solvent was overlaid on the aqueous phase where the cascade reaction was occurring, so the inhibitory ketone could be selectively extracted to reduce the product inhibition [16]. Note that all the substrates and products, except the ketone, are not extractable because of their ionic characters. Hexane was used as the extractant of benzaldehyde and acetophenone of which the partition coefficients were measured to be 11.1 [25] and 16.4 [26], respectively, in the hexane/water biphasic system.

3.2. Effect of ketone removal

To examine whether the extractive ketone removal improves the cascade reaction rate, we compared reaction progresses of the synthesis of L-Tle in a monophasic and a biphasic system using benzylamine as the amine substrate (Figure 2). Note that PDTA possesses a high activity

for benzylamine (i.e. 96 % activity relative to (*S*)-α-MBA) [23]. Owing to a cheap price, racemic homoalanine rather than an enantiopure L-form was used as the shuttling substrate (0.1 molar equivalent of kinetically effective L-homoalanine to trimethylpyruvate). As expected, the biphasic reaction led to a much higher conversion yield than that of the monophasic reaction devoid of extraction of the inhibitory benzaldehyde. In both reactions, enantiomeric excesses of the produced L-Tle were higher than 99.9 % owing to the stringent stereoselectivity of BCTA.

3.3. Asymmetric synthesis of L-Tle

The yield of L-Tle in the biphasic system reached 81 % at 53 h but did not increase upon further incubation until 107 h (Figure 2). We presumed that the leveling off of the yield resulted from high amino acceptor reactivity of benzaldehyde. PDTA displayed 91 % amino acceptor activity for benzaldehyde relative to 2-oxobutyrate [22]. Therefore, despite the extractive removal, a low concentration of benzaldehyde in the aqueous phase would strongly compete with 2-oxobutyrate for the amination step catalyzed by PDTA. This led us to change the amine substrate from benzylamine to (*S*)-α-MBA because the amino acceptor activity of PDTA for arylalkyl ketones such as propiophenone was negligible [23] and thereby the competition with 2-oxobutyrate for PDTA would be minimal. Indeed, substitution of benzylamine with α-MBA significantly improved the asymmetric synthesis of L-Tle (i.e. 94 % yield and > 99.9 % *ee* of L-Tle at 61 h) (Figure 3A). By the same reason with homoalanine, *rac*-α-MBA rather than (*S*)-α-MBA was used as the amine substrate for PDTA (i.e. 1.25 molar equivalent of kinetically effective (*S*)-α-MBA to trimethylpyruvate). This led to enantioenrichment of the racemic amine substrate during the cascade reaction (i.e. 89 % *ee* of (*R*)-α-MBA at 61 h). This could benefit the process economics when the partially enriched (*R*)-α-MBA was to be isolated and further

processed to enantiopure (*R*)-α-MBA.

To examine how well the shuttling substrate was recycled during the cascade reaction, we monitored concentrations of L-homoalanine and 2-oxobutyrate (Figure 3B). The level of 2-oxobutyrate was kept below 0.12 mM throughout the reaction, indicative of the efficient regeneration of L-homoalanine driven by the PDTA reaction. Only 0.03 mM 2-oxobutyrate was detectable at the end of the reaction, supporting that the thermodynamically favorable PDTA reaction served well as an equilibrium shifter. The strong thermodynamic coupling of the two TA reactions was attributed to the efficient removal of an inhibitory ketone and the ensuing maintenance of the high PDTA activity. We monitored acetophenone levels in both phases, showing that acetophenone was initially built up in the aqueous phase and then the aqueous acetophenone was kept constant around 0.4 mM after 21 h (Figure 3C). The concentration ratio of acetophenone in the aqueous to organic phase was 10.4 at the end of the extractive reaction (i.e. below the partition coefficient), indicating that the hexane layer was capable of further extraction of acetophenone.

3.4. Asymmetric synthesis of various amino acids

The cascade reaction performed with BCTA and PDTA employed L-homoalanine, rather than L-alanine, as the shuttling substrate. This choice was based on a much lower activity of BCTA for L-alanine than that for L-homoalanine because the active site of BCTA has been optimized for branched-chain amino acids [15]. However, PDTA shows much higher activity for pyruvate than 2-oxobutyrate, i.e. a 19-fold higher specificity constant for pyruvate than that for 2-oxobutyrate [27]. Moreover, L-alanine is cheaper than L-homoalanine. To examine whether L-homoalanine is a shuttling substrate of choice better than L-alanine, we compared reaction progresses of the cascade reaction to synthesize L-Tle in a monophasic reaction system (Figure 4). The cascade reaction performed with L-alanine led to much lower conversion than

that with L-homoalanine did, indicating that the BCTA reaction, rather than the PDTA reaction, is rate-determining under the given reaction conditions. Therefore, we kept using L-homoalanine for further reactions.

To apply the extractive cascade reaction to the synthesis of various aliphatic amino acids, we performed asymmetric amination of five additional keto acids (Table 1). All the reactions led to > 95 % yields of the amino acid products (i.e. three unnatural and two natural amino acids) within 76 h. Owing to the high stereoselectivity of BCTA, enantiopurities of all the amino acids produced were > 99.9 % *ee*. As observed during the asymmetric synthesis of L-Tle in Fig. 3, all the reactions in Table 1 led to partial enantioenrichment of α-MBA substrate (i.e. 63 - 72 % *ee* of the resulting (*R*)-α-MBA).

To demonstrate industrial applicability of the extractive cascade reaction, we performed preparative-scale synthesis of L-Hag under magnetic stirring in a 50 mL aqueous reaction mixture charged with 0.56 g 2-(3-hydroxy-1-adamantyl)-2-oxoethanoic acid (50 mM), 0.026 g L-homoalanine (0.1 molar eq. to the keto acid substrate), 0.773 mL *rac*-α-MBA (2.4 molar eq.), 34 mg BCTA (2 U/mL) and 74 mg PDTA (16 U/mL). For *in situ* removal of acetophenone, 300 mL hexane (i.e. 6 vol. to the aqueous phase) was overlaid over the aqueous reaction mixture. After 70 h, conversion yield of L-Hag reached 92 % with > 99.9 % *ee*. The resulting aqueous phase was processed to product isolation, leading to recovery of 0.32 g white solid of L-Hag (62 % isolation yield, > 99.9 % *ee*). The isolated product was subjected to mass spectrometric analysis and elemental analysis, which corresponded to pure L-Hag (see the Supplementary Data).

4. Conclusions

In this study, we demonstrate that the extractive cascade reaction composed of BCTA and

ω-TA can afford asymmetric synthesis of various aliphatic amino acids by shifting the equilibrium limit of the BCTA reaction and removing the product inhibition of the ω-TA reaction. Because the amine substrate used in this study (i.e. α-MBA) is one of the most reactive amino donors for ω-TAs, the present strategy is expected to be applicable to all the ω-TAs. To the best of our knowledge, this is the first example of the TA-based asymmetric synthesis of amino acids using extractive cascade reactions. However, feasibility of scaling up this approach should be addressed by minimizing interfacial enzyme inactivation that might result from a direct contact of the enzyme to an organic solvent (e.g. use of an enzyme membrane reactor combined with an extraction module [28]).

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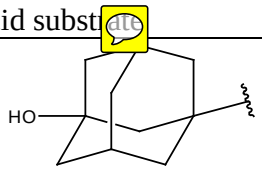
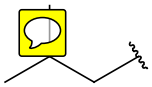
Table 1. Asymmetric synthesis of aliphatic amino acids in the extractive cascade reaction.[a]

Side chain of the keto acid substrate	Reaction time (h)	Product	Yield [b] (%)	*ee* [b] (%)
	61	L-Hag	95.6	> 99.9
	76	L-norvaline	> 99	> 99.9
	40	L-norleucine	97.8	> 99.9
	40	L-valine	> 99	> 99.9
	40	L-leucine	95.2	> 99.9

[a] Reaction conditions were 6 mL hexane combined with 1 mL aqueous reaction mixture containing keto acid (20 mM), *rac*-α-MBA (50 mM), *rac*-homoalanine (4 mM), BCTA (0.6 U/mL), PDTA (4.8 U/mL) and PLP (0.1 mM) in phosphate buffer (50 mM, pH 7). [b] Yield and *ee* of the amino acid products were measured by performing quantitative chiral HPLC analysis.

Figure legends

Fig. 1 Schematics of the extractive cascade reaction. Chemicals enclosed by ovals represent substrates that are initially fed in the reaction mixture. The desired amino acid product is shown in a box.

Fig. 2 Comparison of the cascade reactions in a monophasic and a biphasic system for asymmetric synthesis of L-Tle. Aqueous reaction mixture (1 mL) contained trimethylpyruvate (20 mM), benzylamine (30 mM), *rac*-homoalanine (4 mM), BCTA (0.6 U/mL), PDTA (4.8 U/mL) and PLP (0.1 mM) in phosphate buffer (50 mM, pH 7).

To construct the biphasic system, hexane (3 mL) was overlaid on the aqueous reaction mixture.

Fig. 3 Extractive cascade reaction for asymmetric synthesis of L-Tle using α-MBA as the amine substrate. Reaction conditions in the 1 mL aqueous phase were trimethylpyruvate (20 mM), *rac*-α-MBA (50 mM), *rac*-homoalanine (4 mM), BCTA (0.6 U/mL), PDTA (4.8 U/mL) and PLP (0.1 mM) in phosphate buffer (50 mM, pH 7). For the ketone removal, 6 mL of hexane added to the aqueous reaction mixture. Time-course monitoring of the reaction progress was done by analyzing (A) L-Tle and trimethylpyruvate, (B) L-homoalanine and 2-oxobutyrate, and (C) acetophenone in both aqueous and organic phases.

Fig. 4 Comparison of L-homoalanine and L-alanine as a shuttling substrate. The cascade reactions were carried out under the same reaction conditions as those in Figure 3, except that 4 mM *rac*-homoalanine was replaced by 2 mM L-homoalanine or L-alanine. Organic solvent was not added over the aqueous reaction mixture.

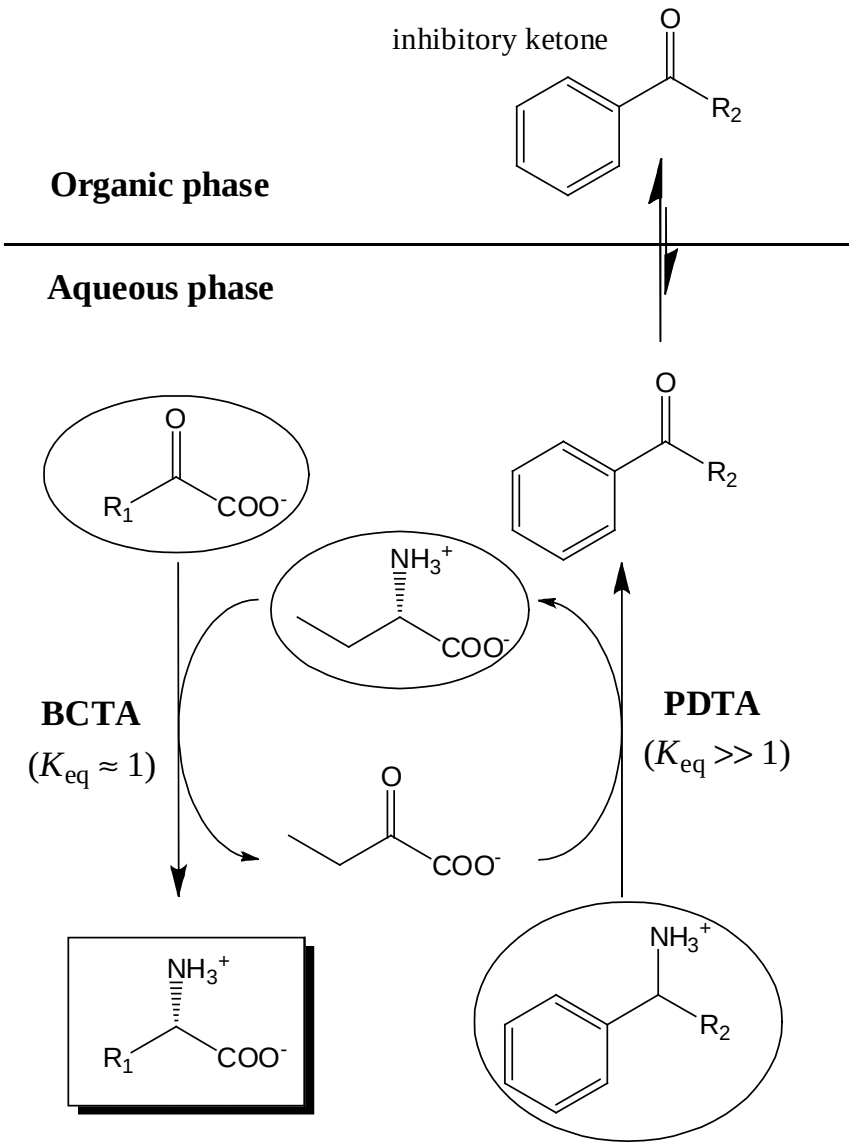


Fig. 1

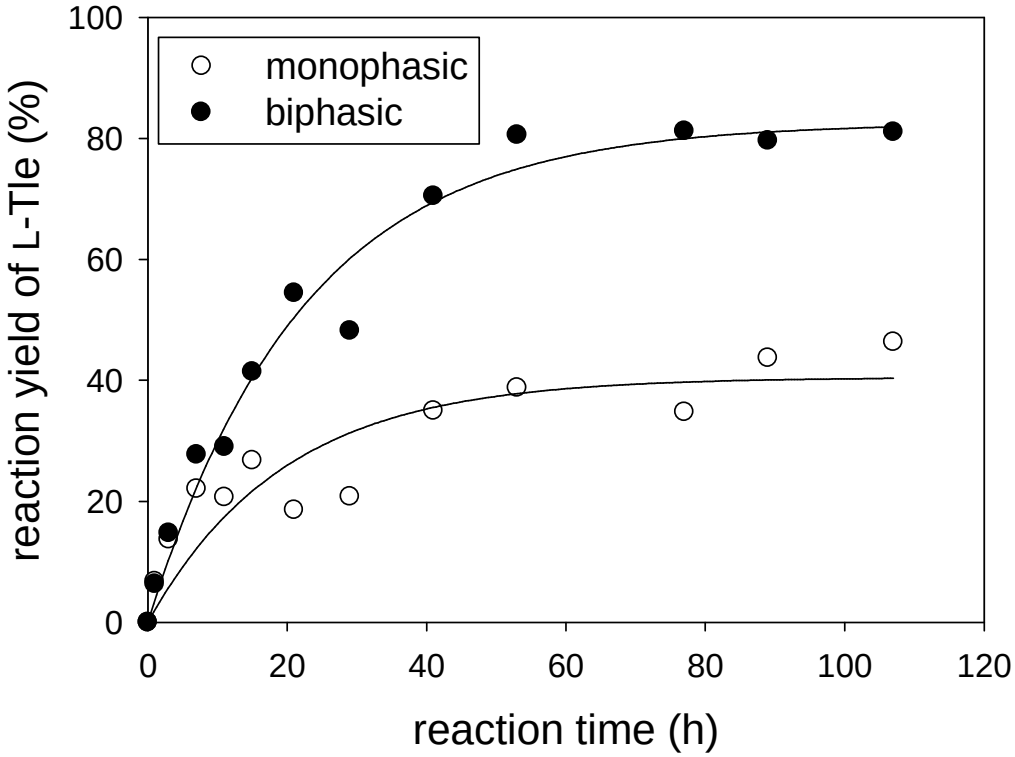


Fig. 2

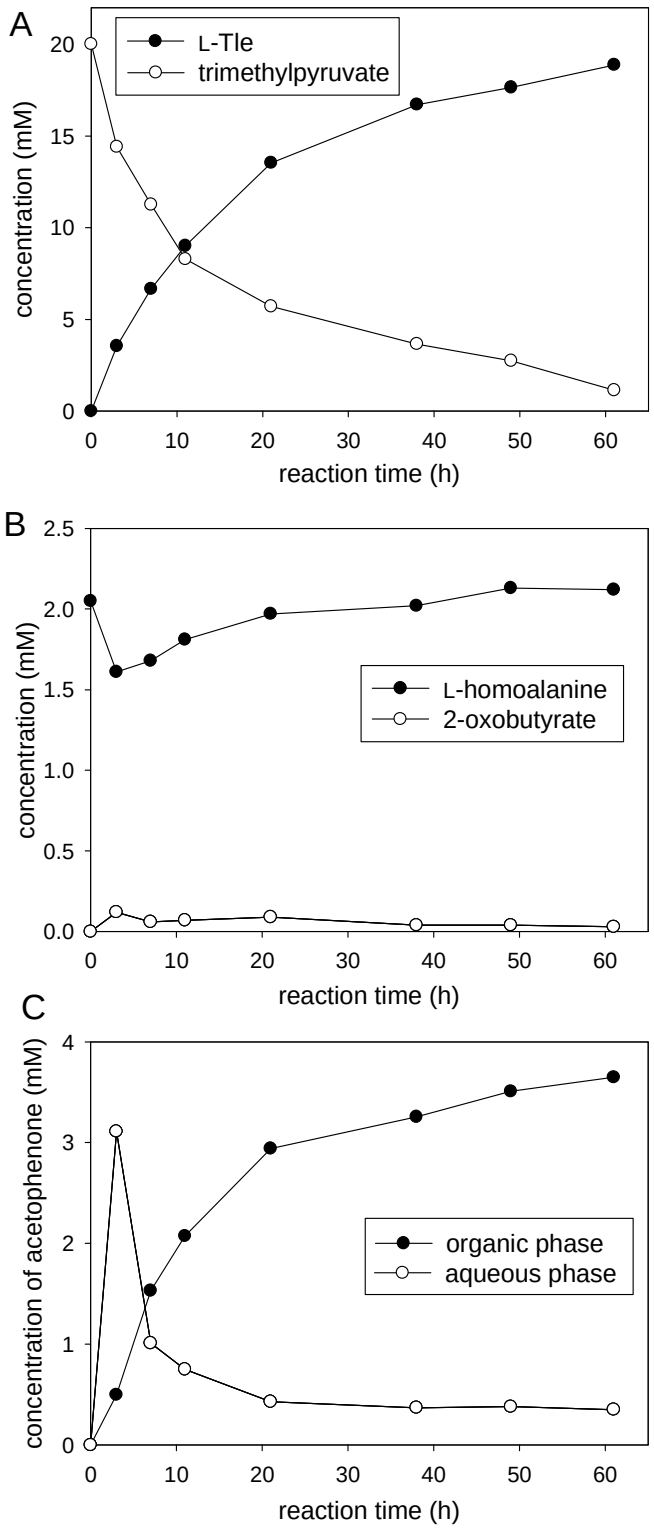


Fig. 3

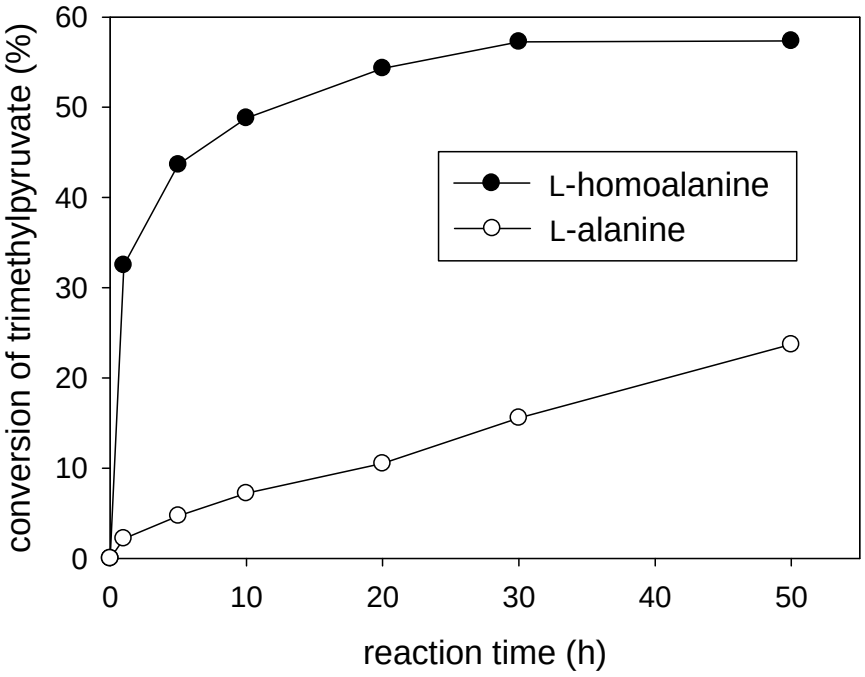


Fig. 4