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Preparation of D-threonine by biocatalytic kinetic resolution

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ABSTRACT

D-Threonine is one of the important unnatural amino acids used as chiral building blocks in pharmaceutical drugs. Owing to the presence of two chiral centers, a synthetic protocol, either through chemocatalysis or biocatalysis, has not yet been available for one-step preparation of stereochemically pure D-threonine in terms of enantiomeric and diastereomeric excesses (i.e., both >99%). Here we demonstrate that facile production of D-threonine can be implemented using threonine deaminase (TD) via kinetic resolution of DL-threonine that can be readily prepared by conventional organic synthesis. TD catalyzes the dehydration/deamination of L-threonine, leading to generation of 2-oxobutyrate and ammonia. In contrast to mild substrate inhibition of the TD activity by L-threonine (i.e., apparent inhibition constant (K_I^{app}) = 950 mM), D-threonine turned out to be a strong inhibitor (i.e., K_I^{app} = 41 mM). In addition to the enzyme inhibitions by both enantiomers of threonine, cell lysis observed during small-scale kinetic resolutions of ≥1 M DL-threonine led us to carry out a preparative-scale reaction at 500 mM racemic substrate. The preparative-scale kinetic resolution in a 50 mL reaction mixture charged with 3 g DL-threonine and 3400 U whole cells was completed at 5 h with >99% *ee* of D-threonine. Product isolation by a cation-exchange chromatography led to white solid of D-threonine (1.36 g, 90.7% isolation yield). To explore whether our strategy could afford coproduction of another valuable unnatural amino acid, the pass-through solution from the cation-exchange column was further processed by a ω-transaminase (ω-TA) reaction where 2-oxobutyrate was converted to enantiopure homoalanine using isopropylamine as an amino donor. Addition of *S*- and *R*-selective ω-TA to the pass-through solution led to 93.2 and 90.9% reaction yield within 12 h with both >99% *ee* of the produced L- and D-homoalanine, respectively.

1. Introduction

Unnatural amino acids have gained a growing attention in chemical industry owing to their versatile utilities as chiral intermediates for a wide range of pharmaceutical drugs [1–4]. D-Threonine is one of the important unnatural amino acids and is used as a chiral building block of peptidomimetic drugs for treatment of peripheral opioids side effects [5] and for analgesic applications [6]. Threonine bears two chiral centers, leading to four stereochemical isomers among which (2*S*, 3*R*) and (2*R*, 3*S*) configurations correspond to L- and D-enantiomers of threonine, respectively. In contrast to L-threonine which is mass-produced by microbial fermentation for animal feed applications [7,8], such a fermentative production is not yet available for D-threonine.

The stereochemical complexity of threonine renders stereoselective organic synthesis of D-threonine impracticable for an industrial scale-up due to low conversion and/or coproduction of stereochemical impurities [9–11]. In contrast, organic synthesis of DL-threonine with a negligible level of diastereomeric impurities has been well established [12–14]. This has spurred development of chiral resolution of DL-threonine by preferential crystallization, which requires several successive crystallizations to achieve a high enantiopurity [15–17].

In the case of biocatalytic approaches, enzymatic kinetic resolution of DL-threonine derivatives were demonstrated using acid phosphatase [18] and lipase [19]. These methods require an additional chemical step for the derivatization of racemic threonine. Another biocatalytic approach employs asymmetric synthesis of D-threonine from acetaldehyde and glycine using D-threonine aldolase [20,21]. However, this biocatalytic synthetic method has been suffered by substantial coproduction of unwanted stereochemical impurities.

In this study, we aimed at developing an industrially feasible method for production of optically pure D-threonine by enzymatic kinetic resolution of underivatized racemic threonine (Fig. 1). To

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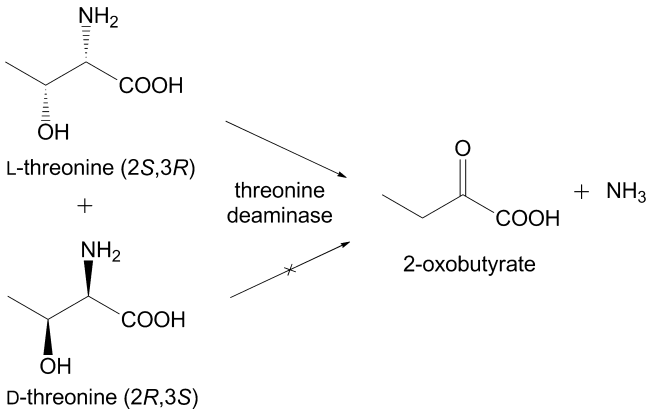

Fig. 1. Schematics for the kinetic resolution of DL-threonine using threonine deaminase. D-Threonine is obtained from the reaction mixture when L-threonine is completely converted to 2-oxobutyrate and ammonia.

this end, we choose threonine deaminase (TD, EC 4.2.1.16) which is a pyridoxal phosphate (PLP)-dependent enzyme and performs stereoselective conversion of L-threonine into 2-oxobutyrate and ammonia [22,23]. Preparative-scale kinetic resolution and product isolation were performed to demonstrate scalable production of D-threonine. In addition, we demonstrated that 2-oxobutyrate generated from the kinetic resolution could be converted to another valuable unnatural amino acids using ω-transaminase (ω-TA).

2. Materials and methods

2.1. Chemicals and bacterial strains

D-Threonine was purchased from Alfa Aesar (Ward Hill, USA). L-Threonine and isopropylamine were purchased from Junsei Chemical Co. (Tokyo, Japan). Materials used for preparation of 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. Bacterial strains used for this study were *Escherichia coli* BL21(DE3) cells transformed with the pET21a(+) expression vector harboring a TD gene cloned from *E. coli* DH5α and the pET28a(+) vectors harboring ω-TA genes from *Ochrobactrum anthropi* and *Arthrobacter* sp., that were constructed previously [24–26].

2.2. Preparation of cell suspension, cell-free extract and purified enzyme

Overexpression of the enzymes was carried out as described previously with minor modifications [27]. *E. coli* BL21(DE3) cells carrying the expression vectors were cultivated in a LB medium (typically 1 L) containing 50 μg/mL ampicillin or kanamycin at 37 °C. Protein expression was induced by 0.1 mM IPTG at 0.4 OD_{600} and the cells were allowed to grow for 10 h. Cells were harvested by centrifugation, washed twice with potassium phosphate buffer (50 mM, pH 7) and then resuspended in the same buffer (typically 10 mL). This cell suspension was used for the whole cell reactions.

To prepare cell-free extracts, the harvested cell pellet was resuspended in a 15 mL cell disruption buffer (50 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.1 mM PMSF, 0.02% sodium azide and 0.5 mM PLP in 50 mM Tris–HCl at pH 7). Cells were disrupted by ultrasonication and then cell debris were removed by centrifugation (10,000 × *g*, 60 min, 4 °C). The resulting supernatant was used as the cell-free extract for enzyme reactions.

In the case of ω-TAs, purified proteins were used for enzyme reactions. Purification of ω-TAs was performed on ÄKTAprime plus (GE Healthcare, Piscataway, USA) as described previously [27]. The cell-free extract was loaded on a HisTrap HP column (GE Healthcare) and eluted by an elution buffer (20 mM sodium phosphate, 0.5 M sodium chloride, 0.5 mM PLP, pH 7.4) with a linear gradient of imidazole (0.05–0.5 M). A HiTrap desalting column (GE Healthcare) was used for removing imidazole using an exchange buffer (50 mM sodium phosphate, 0.15 M sodium chloride and 0.1 mM PLP, pH 7).

2.3. Enzyme assay

Unless otherwise specified, enzyme assays were carried out at 37 °C in 50 mM phosphate buffer (pH 7). One unit of TD is defined as the enzyme amount catalyzing formation of 1 μmole 2-oxobutyrate in 1 min at 50 mM L-threonine. One unit of ω-TA is defined as the enzyme amount catalyzing formation of 1 μmole acetophenone in 1 min at 10 mM pyruvate and 10 mM (*S*) or (*R*)-α-methylbenzylamine, depending on the stereoselectivity of the ω-TA. After 10 min reaction, the enzyme reaction (typically 100 μL reaction volume) was stopped by adding 600 μL acetonitrile. For the initial rate measurements (i.e., conversions <10%), 2-oxobutyrate and acetophenone for TD and ω-TA, respectively, were analyzed by HPLC.

2.4. Substrate inhibition of TD

To examine substrate inhibition of TD by L-threonine, initial reaction rates (v_i) were measured at varying concentrations of L-threonine (i.e., 10–400 mM). To determine the apparent inhibition constant (i.e., K_I^{app}), curve fitting to a hyperbolic function (Eq. (1)) was performed using the initial rate data where the activity decrease by the substrate inhibition was observed (i.e., above 100 mM L-threonine for cell-free extract).

$$v_i = \frac{v_0}{1 + ([L] - [L]_0)/K_I^{app}} \quad (1)$$

$[L]_0$ is the concentration of L-threonine at which the activity decrease begins and v_0 represents the initial rate at $[L]_0$. The K_I^{app} value was calculated from the linear regression of $1/v_i$ against $[L] - [L]_0$.

In the case of the enzyme inhibition by D-threonine, initial rates were measured at varying concentrations of D-threonine (i.e., 0–250 mM) with the concentration of L-threonine fixed at 50 mM. To determine K_I^{app} for D-threonine, the hyperbolic decay in the enzyme activity was fitted to Eq. (2).

$$v_i = \frac{v_0}{1 + [D]/K_I^{app}} \quad (2)$$

[D] is the concentration of D-threonine and v_0 represents a reference reaction rate where the enzyme inhibition by D-threonine is absent (i.e., [D] = 0). The K_I^{app} value was calculated from the linear regression of $1/v_i$ against [D].

To examine substrate inhibition by racemic threonine, initial rates were measured with cell suspension at varying concentrations of DL-threonine (i.e., 10–500 mM). 2-Oxobutyrate produced was analyzed by HPLC for the initial rate measurements.

2.5. Small-scale kinetic resolution of DL-threonine

Unless otherwise specified, small-scale kinetic resolutions were carried out in 50 mM potassium phosphate buffer (pH 7) at 37 °C under magnetic stirring. Racemic threonine was prepared by mixing equal amount of each enantiomer. To examine the effect of substrate feeding methods, kinetic resolution reactions were carried out by adding cell suspension (final concentration = 13.5 U/mL) to the reaction mixture (5 mL reaction volume). The batch reaction starting with 300 mg DL-threonine (final concentration = 0.5 M)

was compared with a fed-batch reaction which started with 90 mg DL-threonine (final concentration = 0.15 M) and was supplemented with DL-threonine twice during the reaction (90 and then 120 mg) when the *ee* value exceeded 90%.

To compare reaction progresses of kinetic resolutions at different concentrations of DL-threonine, the reactions were started by adding cell suspension (final concentration = 68 U/mL) to the reaction mixture (1 mL reaction volume) containing 60, 120 and 180 mg DL-threonine (final concentration = 0.5, 1 and 1.5 M, respectively). Because the solubility of threonine is 745 mM at room temperature [28], solid threonine was added to the reaction mixtures instead of using a concentrated stock solution. 50 μL aliquots of the reaction mixture were taken at predetermined reaction times and were mixed with 10 μL of 5 N HCl. To determine *ee* and reaction yield, quantitative chiral analysis of DL-threonine was performed by HPLC after derivatization with a Marfey's reagent [29].

2.6. Preparative-scale kinetic resolution

Preparative-scale kinetic resolution was carried out in a 50 mL reaction mixture (50 mM phosphate buffer, pH 7) charged with 3 g DL-threonine (500 mM) and 3400 U cell suspension (430 mg dcw) at 37 °C under magnetic stirring. When the *ee* of D-threonine exceeded 99%, the reaction mixture was processed for product isolation. Cells were removed by centrifugation (10,000 × g, 20 min, 4 °C). The pH of the supernatant was adjusted to 1.0 by adding 5 N HCl and then the resulting protein precipitate was filtered off using a glass-fritted filter funnel. The filtrate solution was loaded on a glass column packed with Dowex 50WX8 cation-exchange resin (40 g), followed by washing with 0.1 N HCl (50 mL) and water (50 mL). Elution was done by 10% ammonia solution (150 mL). The elution fraction was evaporated at 50 °C and 0.1 bar, resulting in white solid of D-threonine.

2.7. Asymmetric synthesis of homoalanine

The pass-through solution from the cation-exchange column was used as a stock solution of 2-oxobutyrate after pH adjustment to 7.0 by adding 5 N NaOH. Asymmetric syntheses of L- and D-homoalanine were carried out in 1 mL reaction mixture (50 mM phosphate buffer, pH 7) containing 50 mM 2-oxobutyrate, 100 mM isopropylamine and the purified ω-TA (5.9 and 11.2 U/mL of ω-TA from *O. anthropi* and *Arthrobacter* sp., respectively) at 37 °C under magnetic stirring. Reaction yield and *ee* were monitored by quantitative chiral analysis of homoalanine after derivatization with a Marfey's reagent [29].

2.8. Analytical methods

All the HPLC analysis were performed with a Waters HPLC system (Milford, USA). Analysis of 2-oxobutyrate was carried out using a Aminex HPX-87H column (Bio-Rad, Hercules, USA) with isocratic elution of water (pH adjusted to 2.0 by sulfuric acid) at 0.5 mL/min. UV detection was done at 210 nm and the column oven temperature was maintained at 40 °C. Analysis of acetophenone was performed using a Sunfire C18 column (Waters Co.) with isocratic elution of 60% methanol/40% water/0.1% trifluoroacetic acid at 1 mL/min. UV detection was done at 254 nm.

Quantitative chiral analysis of threonine and homoalanine were carried out using the Sunfire C18 column (Waters Co.) with isocratic elution of aqueous MeOH solution, 35 and 45% v/v for analysis of threonine and homoalanine, respectively, containing 0.1% trifluoroacetic acid at 1 mL/min after derivatization with a Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) which is widely used for chiral analysis of amino acids [29]. In a typical derivatization procedure, 10 μL of Marfey's reagent stock (14 mM dissolved in acetonitrile), 18 μL of sodium bicarbonate solution (1 M) and 50 μL of DMSO were added to 22 μL reaction samples (the molar ratio of Marfey's reagent to amino acid was >1.4). The reaction mixture was incubated at 40 °C for 8 h and then cooled at room temperature. 20 μL of 1 N HCl solution was added to the mixture to quench the derivatization reaction. UV detector was tuned at 340 nm.

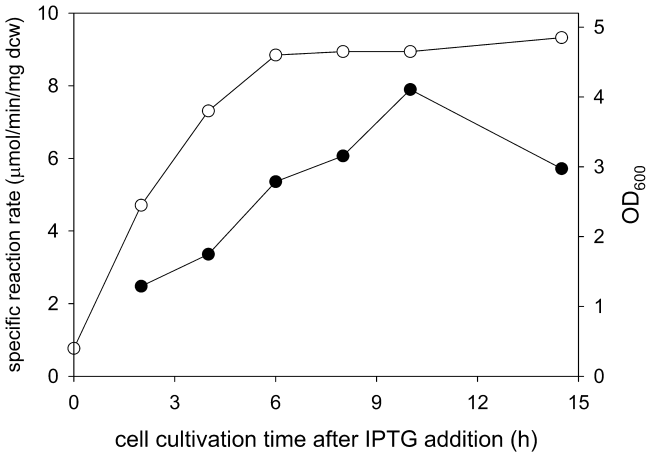

Fig. 2. Effect of the cultivation time on the specific cellular activity of TD. Specific reaction rate (closed circle) and optical density of the cell culture (open circle) were monitored for 15 h after IPTG addition to the culture broth. Specific reaction rate was measured at 50 mM L-threonine in phosphate buffer (50 mM, pH 7).

3. Results and discussion

3.1. Determination of optimal cultivation time for cell suspension preparation

Whole cells expressing intended enzymes are usually preferred over purified enzymes for a cost-effective biocatalyst formulation. Therefore, we undertook determination of an optimal cell cultivation time to achieve the highest enzyme activity required for efficient whole cell reactions. Protein expression was induced by 0.1 mM IPTG at 0.4 OD_{600} and then the cellular activity of TD normalized by a dry cell weight as well as the cell optical density was monitored for 15 h (Fig. 2). Cell growth reached a stationary phase at 6 h after the IPTG addition. However, specific cellular activity of TD kept increasing until 10 h (i.e., 7.9 U/mg dcw) and then the prolonged cell cultivation led to a decrease in the specific activity. Therefore, all the experiments later were carried out using the TD-expressing cells harvested at 10 h after the IPTG induction. Based on the specific activity and OD_{600} data, total cellular activity of TD obtainable from 1 L cell culture at 10 h was estimated to be 6300 U.

3.2. Substrate inhibition of TD

TD is involved in the metabolic pathway for synthesis of isoleucine and is one of the typical allosteric enzymes. This enzyme is known to undergo feedback inhibition by isoleucine (i.e., the end product of the metabolic pathway) as well as feedback activation by valine (i.e., the end product of the parallel pathway) [22,23]. In addition, it is known that the active site of TD shows high binding affinity for several amino acids, including 2-aminobutyric acid, alanine and D-threonine [22,23,30]. During the kinetic resolution reaction, TD should be exposed to both enantiomers of threonine much above a physiological concentration range. Therefore, we examined how the TD activity responded to high concentrations of L- and D-threonine. To this end, we measured TD activities of

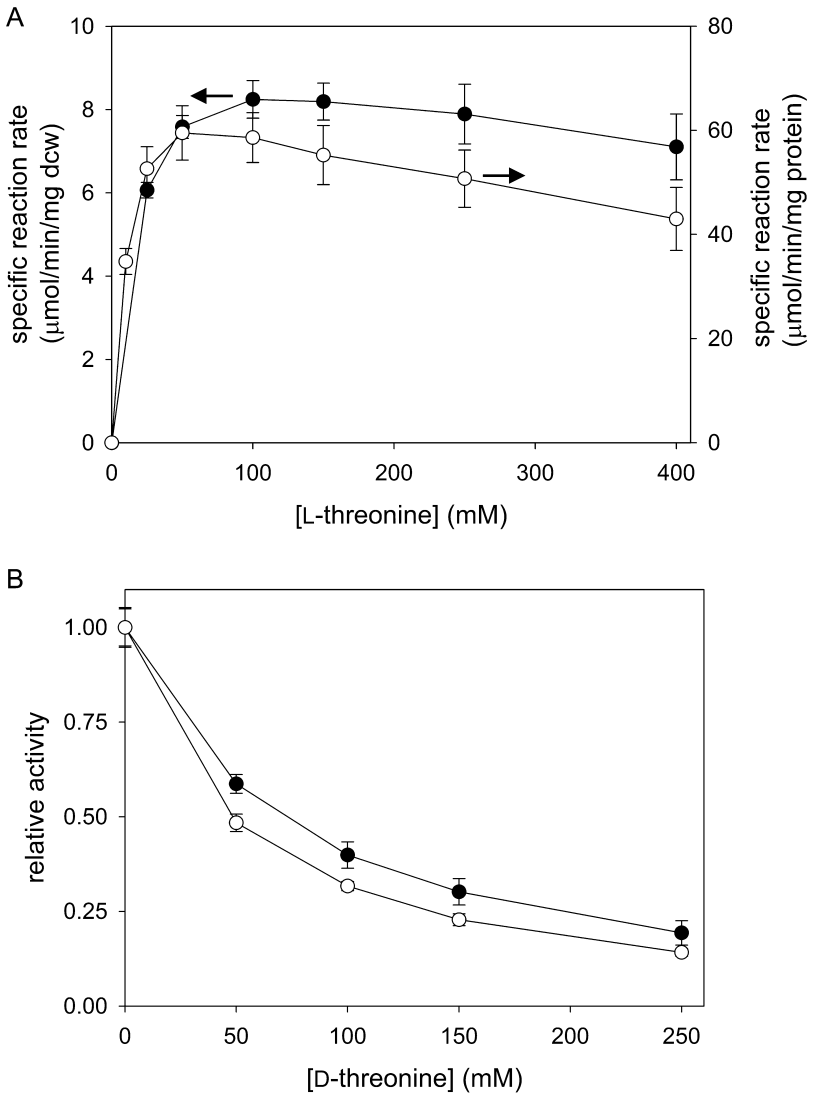

Fig. 3. Substrate inhibitions of TD by (A) L-threonine and (B) D-threonine. Specific reaction rate of whole cell (closed circle) and crude extract (open circle) represents initial rate normalized by cell or protein concentration. Relative activity was measured at varying concentration of D-threonine under a fixed L-threonine concentration (50 mM).

both whole cells and cell-free extract at varying concentrations of each threonine enantiomer.

We found that the TD activities in both enzyme formulations showed mild substrate inhibitions by L-threonine higher than 100 and 150 mM for cell-free extract and whole cells, respectively (Fig. 3A). The whole cells displayed slightly lower substrate inhibition than the cell-free extract did, presumably because cellular membrane acts as a diffusion barrier to L-threonine and the resulting intracellular concentration would be lower than the extracellular one. The inverse of the specific reaction rate measured with cell-free extract was subjected to a linear regression against the concentration of L-threonine ($r^2 = 0.98$), leading to the apparent inhibition constant (K_I^{app}) = 950 ± 210 mM. The substrate inhibition by L-threonine has not yet been reported because all the kinetic measurements reported to date were done at less than 100 mM L-threonine [22,31,32]. To the best of our knowledge, this is the first report on the substrate inhibition of TD.

In addition to the substrate inhibition by L-threonine, TD displayed strong inhibition by D-threonine although the D-enantiomer is a catalytically inert substrate analog (Fig. 3B). D-Threonine is known to bind tightly to the active site of TD in a competitive manner [30]. Indeed, the nonproductive binding was strong enough to inhibit deamination of L-threonine (e.g., 58 ± 2 and 48 ± 2% residual activities at 50 mM D-threonine in the reactions with whole cells and cell-free extract, respectively). As observed with the substrate inhibition by L-threonine, the whole cells showed slightly lower inhibition by D-threonine than the cell-free extract did. To determine K_I^{app} of TD for D-threonine, we performed linear regression of the inverse of the relative activity of the cell-free extract against the concentration of D-threonine ($r^2 = 0.99$). The K_I^{app} value was calculated to be 41 ± 4 mM, which is in a reasonable agreement with the average dissociation constant (=19.8 mM) reported elsewhere [30].

Because of the enzyme inhibitions by both enantiomers of threonine, use of high concentrations of DL-threonine would not be desirable for an efficient kinetic resolution process. We examined how the mixed enzyme inhibitions affected the reaction rate by measuring the TD activity of whole cells at varying concentration of racemic threonine (Fig. 4). As expected, whole cells exhibited strong substrate inhibition by DL-threonine. The decrease in the reaction rate begun even at 50 mM DL-threonine and the residual activity at 500 mM was 58% relative to the maximal reaction rate.

3.3. Small-scale kinetic resolution

Based on the results in Fig. 4, initial concentration of DL-threonine around 50 mM would be optimal to exploit high reaction

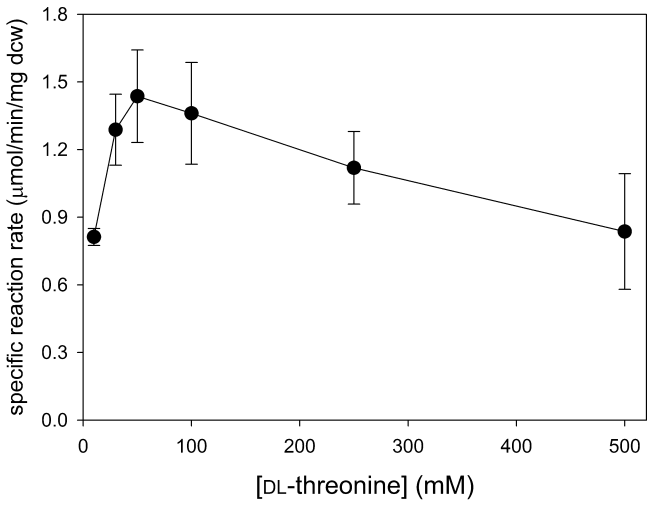

Fig. 4. Substrate inhibition of TD by racemic threonine. Specific reaction rate represents initial rate normalized by cell concentration.

rates. However, this concentration level is far below an industrial requirement when the volumetric productivity is taken into account. As trial runs before a preparative-scale reaction, we compared reaction progresses of small-scale kinetic resolutions (reaction volume = 5 mL) using different substrate feeding methods, i.e., batch vs. fed-batch reactions (Fig. 5A). Total amount of substrate doses was set to be identical (i.e., 300 mg DL-threonine) in both reactions where the initial substrate concentrations were 0.5 and 0.15 M for the batch and fed-batch reactions, respectively. The fed-batch reaction vial was additionally supplemented with DL-threonine twice during the reaction (i.e., 0.15 and then 0.2 M) when the *ee* value exceeded 90%. The rate of increase in the *ee* value of DL-threonine was slowed down as the substrate doses were added to the fed-batch reaction vial. This result is ascribable to the strong enzyme inhibition by D-threonine, as concentration of the inhibitory D-threonine overwhelmed that of the reactive L-enantiomer along with the supplementation of the racemic substrate. As expected, the batch reaction led to a lower *ee* value at the end of the reaction compared to the fed-batch reaction.

To examine how the substrate concentrations affected the reaction progress in a batch reaction mode, we performed small-scale kinetic resolutions (reaction volume = 1 mL) at 0.5, 1 and 1.5 M DL-threonine (Fig. 5B). Because the batch reaction shown in Fig. 5A ended up with only 79% *ee*, we used a 5-fold higher cell concentration (8.5 mg dcw/mL). Because the solubility of threonine is 0.74 M [28], solid DL-threonine was present at the beginning of the reaction in the reaction mixture for 1 and 1.5 M substrate concentrations. The *ee* value of D-threonine in the reaction starting with 0.5 M DL-threonine was 99.3% at 3 h and reached >99.9% at 6 h with 50% conversion. However, the reactions starting with 1 and 1.5 M DL-threonine showed 95.1 and 61.7% *ee* values at 11 h with 48.6 and 36.9% conversions, respectively. Prolonged incubation of the reaction mixtures until 24 h ended up with modest increases in the *ee* values (i.e., 96.2 and 63.0% *ee* values for the reactions using 1 and 1.5 M DL-threonine, respectively). Besides the stronger inhibition at the higher substrate concentration, the disappointingly slow reaction progresses after 11 h seemed to result from progressive cell aggregation and lysis that started to be observed at 6 h. In contrast, no cell aggregates were observed throughout the reaction using 0.5 M DL-threonine. It is presumable that the apparent cell lysis at ≥1 M DL-threonine is ascribed to a substantial damage of cellular membrane by solid threonine under the mixing conditions and/or too high ionic strength detrimental to cellular integrity.

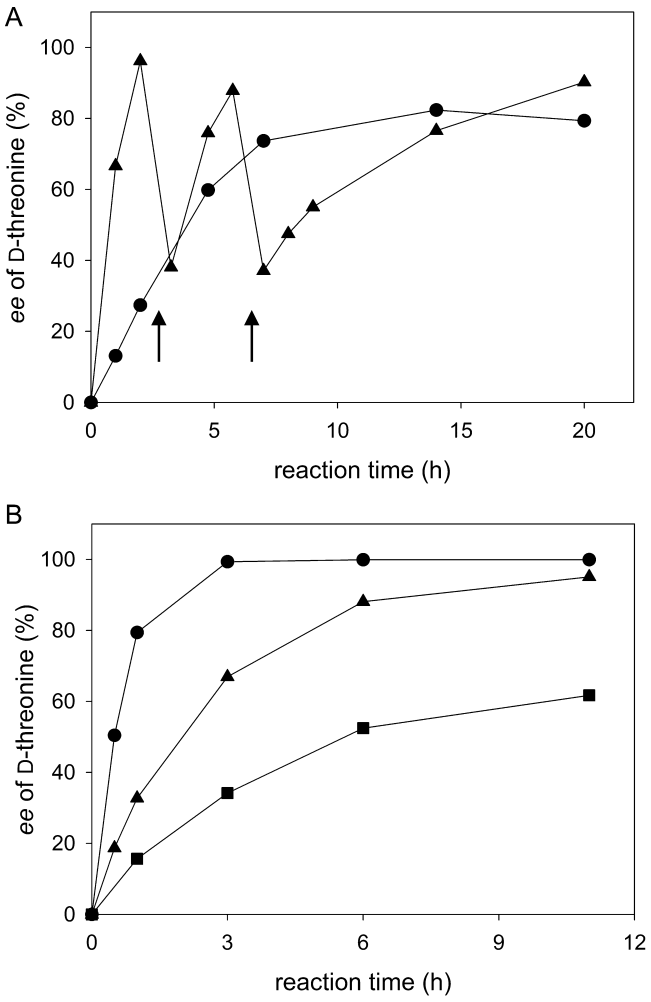

Fig. 5. Small-scale kinetic resolution of DL-threonine. (A) Effect of substrate feeding methods on the reaction progress. The batch reaction (closed circle) and the fed-batch reaction (closed triangle) were started with 0.5 and 0.15 M DL-threonine, respectively. Additional substrate feeding was carried out during the fed-batch reaction, which was indicated by arrows. Reaction conditions: TD (1.7 mg dcw/mL), total DL-threonine added (0.5 M) and phosphate buffer (50 mM, pH 7) in 5 mL reaction mixture. (B) Kinetic resolutions of different concentrations of DL-threonine. Reaction conditions: TD (8.5 mg dcw/mL), DL-threonine (0.5, 1 and 1.5 M represented by circle, triangle and square symbols, respectively) and phosphate buffer (50 mM, pH 7) in 1 mL reaction mixture.

3.4. Preparative-scale kinetic resolution

To demonstrate practical applicability of the kinetic resolution, we performed a preparative-scale reaction in a batch reaction mode. The apparent cell lysis at ≥1 M DL-threonine led us to carry out a preparative-scale kinetic resolution starting with 0.5 M DL-threonine. The reaction mixture (total 50 mL) was charged with 3 g DL-threonine, 3400 U cell suspension (430 mg dcw) and phosphate buffer (final concentration = 50 mM, pH 7), and then incubated at 37 °C under magnetic stirring. The reaction was completed within 5 h, leading to >99% *ee* of D-threonine at 50% conversion.

To isolate the resulting enantiopure D-threonine, the reaction mixture was subjected to cell removal by centrifugation, pH adjustment to 1.0 and then removal of the protein precipitate using a glass-fritted filter funnel. The filtrate solution was loaded on a cation-exchange column packed with the Dowex 50WX8 resin. Elution of the bound D-threonine by 10% ammonia solution followed by evaporation of the elution fraction led to white solid of D-threonine (1.36 g, >99% *ee*, 90.7% isolation yield).

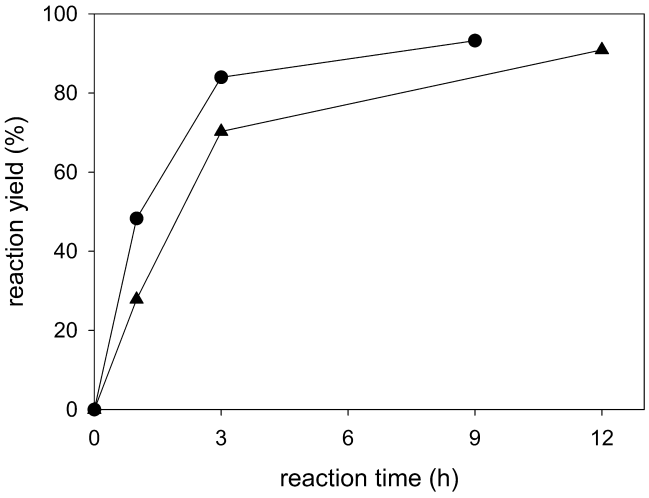

Fig. 6. Asymmetric synthesis of homoalanine from 2-oxobutyrate obtained as a side product of the TD reaction. Circle and tringle symbols represent reaction yields of the synthesis of homoalanine by OATA and ARTA, respectively. Reaction conditions: 2-oxobutyrate (50 mM), isopropylamine (100 mM), ω-TA (5.9 and 11.2 U/mL for OATA and ARTA, respectively), and phosphate buffer (50 mM, pH 7) in 1 mL reaction mixture at 37 °C.

3.5. Asymmetric synthesis of homoalanine from a side product of kinetic resolution

The pass-through solution from the cation-exchange column contained a valuable side product, i.e., 2-oxobutyrate, which can be converted to more commercially valuable unnatural amino acids, i.e., L- and D-homoalanine, by *S*- and *R*-selective ω-TA, respectively, as we demonstrated previously [33]. For example, L-homoalanine is a key intermediate for levetiracetam and brivaracetam (antiepileptic drugs) [34], and ethambutol (an antituberculosis drug) [35]. Simultaneous reactions by TD and ω-TA in one pot supplemented with DL-threonine and isopropylamine (i.e., an amino donor for the ω-TA reaction) would lead to coproduction of enantiopure homoalanine along with D-threonine. However, the one-pot reaction renders isolation of both amino acids very difficult. Therefore, we decided to go with a stepwise approach rather than the one-pot reaction.

The pass-through solution was used as a stock solution of 2-oxobutyrate after pH adjustment to 7.0 without any purification. As a proof-of-concept, we performed small-scale asymmetric conversions of 50 mM 2-oxobutyrate into L- and D-homoalanine using *S*-selective ω-TA from *O. anthropi* (OATA) and *R*-selective ω-TA from *Arthrobacter* sp. (ARTA), respectively (Fig. 6). The reaction performed with OATA showed a 93.2% reaction yield of L-homoalanine with >99% *ee* at 9 h. Likewise, the reaction with ARTA led to enantiopure D-homoalanine (i.e., >99% *ee*) with 90.9% reaction yield at 12 h.

4. Conclusions

In this study, we demonstrated preparation of optically pure D-threonine as well as both enantiomers of homoalanine by TD-catalyzed kinetic resolution of DL-threonine followed by ω-TA-catalyzed asymmetric amination of 2-oxobutyrate. Because of the lack of a cost-effective method for preparation of enantiopure D-threonine, the TD-based method developed in this study might be applicable for an industrial purpose. To this end, the cell stability problem at ≥1 M DL-threonine should be addressed. Besides, a process engineering strategy such as a fed-batch operation should be also fashioned to attenuate the enzyme inhibition at high substrate concentrations. Finally, it is notable that the process economics of the TD-catalyzed kinetic resolution can benefit by the subsequent asymmetric conversion of the unwanted side product.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2015.09.011.

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