Asymmetric Synthesis of Unnatural L-Amino Acids Using Thermophilic Aromatic L-Amino Acid Transaminase

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Abstract Aromatic L-amino acid transaminase is an enzyme that is able to transfer the amino group from L-glutamate to unnatural aromatic α-keto acids to generate α-ketoglutarate and unnatural aromatic L-amino acids, respectively. Enrichment culture was used to isolate thermophilic *Bacillus* sp. T30 expressing this enzyme for use in the synthesis of unnatural L-amino acids. The asymmetric syntheses of L-homophenylalanine and L-phenylglycine resulted in conversion yields of >95% and >93% from 150 mM 2-oxo-4-phenylbutyrate and phenylglyoxylate, respectively, using L-glutamate as an amino donor at 60°C. Synthesized L-homophenylalanine and L-phenylglycine were optically pure (>99% enantiomeric excess) and continuously pre-cipitated in the reaction solution due to their low solubility at the given reaction pH. While the solubility of the α-keto acid substrates is dependent on temperature, the solubility of the unnatural L-amino acid products is dependent on the reaction pH. As the solubility difference between substrate and product at the given reaction pH is therefore larger at higher temperature, the thermophilic transaminase was successfully used to shift the reaction equilibrium toward rapid product formation.

Keywords: thermophilic enzyme, transaminase, unnatural amino acids, asymmetric synthesis, equilibrium-shift, enrichment culture

INTRODUCTION

Unnatural L-amino acids are commonly occurring moieties in chiral drugs such as anti-cancer compounds and viral inhibitors [1]. For example, L-homophenylalanine is an intermediate in the synthesis of the angiotensin-converting enzyme (ACE) inhibitors, Benazepril, Enalapril, and Lisinopril [2]. 3-(2-Naphthyl)-L-alanine (Nal) is a significant intermediate of neurokinin NK_1 antagonists [3]. Other unnatural L-amino acids required for the synthesis of chiral compounds are L-*tert*-leucine, L-2-aminobutyrate, L-phosphinothricin, and L-thienylalanine [4-7]. The synthesis of such unnatural amino acids is reported to involve both chemical and enzymatic routes, relying on processes such as reductive amination, transamination, asymmetric hydrogenation, and kinetic resolution [1,8-10]. The asymmetric synthesis catalyzed by transaminases is a simple and efficient way to produce enantiomerically pure unnatural amino acids for several reasons (Fig. 1A): (1) the keto acid substrates can be chemically synthesized easily, (2) the external cofactor regeneration step is not required, and (3) transaminases have highly selective enantioselectivity and broad substrate specificities [11-15].

In general, asymmetric synthesis of unnatural L-amino acids using aromatic transaminase (AroAT) has been hampered by low equilibrium constants (*i.e.*, typically close to one), and by inhibition caused by both the substrate and the product [12]. There have been several attempts to overcome those limitations using coupled enzyme reactions with acetolactate synthase [1], phosphoenolpyruvate carboxykinase [16], or ω-transaminase [5] (Fig. 1B). In the coupled reaction with acetolactate synthase, acetolactate synthase was used to convert two molecules of pyruvate into acetolactate, which is spontaneously decarboxylated to acetoin, to accelerate the reaction shift toward product formation [1,4]. Multiple couplings of deaminase and acetolactate synthase with transaminase also have been attempted in the preparation of 2-aminobutyric acid using L-threonine as an amino donor [17]. In addition, α/ω-Transaminase coupling reactions have been used in a two-liquid phase system to overcome the product inhibition of ω-transaminase and the low equilibrium constant of AroAT [18]. In this reaction, the repeated addition of solid-state substrate was

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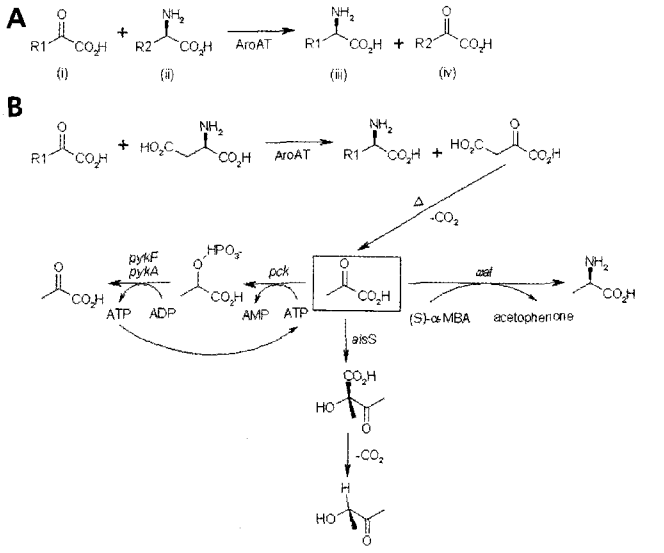

Fig. 1. Asymmetric synthesis of aromatic L-amino acids using AroAT (A) and equilibrium shift methods (B). Notations: (i) amino acceptor substrate (R1 = C_6H_5-, phenylglyoxylate; R1 = $C_6H_5CH_2$-, phenylpyruvate; R1 = $C_6H_5CH_2CH_2$-, 2-oxo-4-phenylbutyrate), (ii) amino donor substrate (R2 = CO_2HCH_2-, L-aspartate; R2 = $CO_2HCH_2CH_2$-, L-glutamate), (iii) L-amino acid product (R1 = C_6H_5-, L-phenylglycine; R1 = $C_6H_5CH_2$-, L-phenylalanine; R1 = $C_6H_5CH_2CH_2$-, L-homophenylalanine), (iv) α-keto acid product (R2 = CO_2HCH_2-, oxaloacetate; R2 = $CO_2HCH_2CH_2$-, α-ketoglutarate). Abbreviations: AroAT, aromatic L-amino acid transaminase; *pykF*, pyruvate kinase I; *pykA*, pyruvate kinase II; *pck*, phosphoenolpyruvate carboxykinase; *alsS*, acetolactate synthase; *wat*, ω-transaminase; (*S*)-α-MBA, (*S*)-α-methylbenzylamine.

used to avoid substrate inhibition by the α-keto acid toward AroAT. Due to the low solubility of L-homophenylalanine at neutral pH, its precipitation in the reaction buffer efficiently drove the reaction equilibrium toward rapid product formation [12]. Recently, an aspartate transaminase was engineered to change the substrate specificity toward L-lysine [19]. In this engineered reaction, the keto acid product from L-lysine, 2-keto-6-aminocaproate, is spontaneously converted into Δ^1-piperideine 2-carboxylic acid, which is not involved in the reaction [19].

The use of a thermophilic transaminase offers another interesting alternative for overcoming low equilibrium constants, as the nonenzymatic decarboxylation rate of oxaloacetate is faster at higher temperature. Moreover, as we report here, the solubility difference between substrate and product is preferential at the higher reaction temperature, allowing for a faster reaction equilibrium shift toward product formation. Here, we report the use of a thermophilic transaminase for the asymmetric synthesis of unnatural amino acids such as L-homophenylalanine and L-phenylglycine. Since the solubility of unnatural α-keto acid depends on the reaction temperature, higher concentrations of α-keto acid can be used with a thermophilic transaminase to shift the reaction equilibrium in the asymmetric synthesis.

MATERIALS AND METHODS

Chemicals

2-Oxo-4-phenylbutyrate (2-OPBA) was kindly obtained from CKD Research Institute (Cheonan, Korea). All other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

Analytical Methods

The quantitative analyses of L-homophenylalanine, L-phenylalanine, L-phenylglycine, and 2-oxo-4-phenylbutyrate were performed by HPLC using a C18 Symmetry® reverse phase column (Waters, Milford, MA, USA) at 210 nm with isocratic elution of a water/methanol mixture (70:30, v/v) at a flow rate of 1 mL/min. Separation of enantiomers was achieved by isocratic elution of a perchloric acid solution (pH 2) on a Crownpak CR(+) column (Daicel, Japan) with a flow rate of 0.7 mL/min at 5°C and subsequent analysis at 210 nm. The quick qualitative chiral analyses of aromatic L-amino acids were performed on chiral TLC plates (methanol-water-acetonitrile, 50:50:200, v/v/v, Macherey-Nagel, Germany). α-Keto acids were analyzed by HPLC on an Aminex-87H column (Bio-Rad, Hercules, CA, USA) at 210 nm using 5 mM sulfuric acid as an isocratic eluent at 40°C.

Screening of Thermophilic Microorganisms Showing Transaminase Activity

Soil samples were incubated at 60°C for 2 h in 100 mL of minimal medium containing 50 mM L-homophenylalanine as the sole nitrogen source [12]. After the initial 2-h incubation, 10 mL of supernatant was transferred into 90 mL of minimal medium. After 2 days of cultivation at 60°C, 10 mL of the culture broth was transferred to 90 mL of the same minimal medium. In order to simulate enrichment culture, this process was repeated three times. After the enrichment steps, the culture broth was spread on to minimal media agar plates (3%, w/v) and cultivated at 60°C until colonies were observed. Each colony on the plate was cultivated in the same minimal media at 60°C, and the transaminase activities were measured using a two-step whole-cell reaction to remove the intracellular background synthetic activity as described previously [12].

Preparation of Partially Purified AroAT from *Bacillus* sp. T30

The cultured cells were centrifuged at 4,000 rpm for 15 min at 4°C. The cell pellets were washed twice with 25 mL of ice-cold PBS. Finally, the cells were resuspended in 5 mL of sonication buffer containing 50 mM phosphate buffer (pH 7.2), 20 μM pyridoxal 5′-phosphate, 2 mM EDTA, 1 mM PMSF, 10% (w/v) glycerol and 0.01%

(v/v) β-mercaptoethanol. The cells were then sonicated for 30 min in an ice-bath. Cell debris was removed by centrifugation at 12,000 rpm for 20 min at 4°C, and the supernatant was fractionated between 30 and 70% (w/v) ammonium sulfate saturation. The precipitate was resuspended in 2 mL of the sonication buffer, dialyzed against the same buffer, and loaded on a Sephacryl S200 (Pharmacia Biotech Inc., Piscataway, NJ, USA) column (1.6 × 60 cm) equilibrated with 50 mM phosphate buffer (pH 7.0) containing 150 mM NaCl. Fractions containing AroAT activity were pooled, concentrated, desalted by ultrafiltration using an Amicon YM30 (Millipore, Billerica, MA, USA), and stored at −70°C prior to use.

Asymmetric Syntheses of L-Homophenylalanine and L-Phenylglycine

To carry out the asymmetric syntheses of L-homophenyl-alanine and L-phenylglycine, aliquots of the partially purified cell extract (70 U) of *Bacillus* sp. T30 were added to 5 mL of 500 mM Tris-HCl (pH 7.5) containing 150 mM of the corresponding α-keto acid (*i.e.*, 2-oxo-4-phenyl-butyrate or phenylglyoxylate) and 300 mM L-glutamate. To terminate the reaction, 1 N hydrochloric acid was added to the reaction samples for rapid quenching and to adjust the pH to 2. Both L-homophenylalanine and L-phenylglycine completely dissolved under these conditions. After removing the aggregates by centrifugation, the samples were analyzed by HPLC and TLC.

Solubility Measurements

Excess amounts of L-homophenylalanine, 2-oxo-4-phenylbutyrate, L-phenylglycine and phenylglyoxylate were added, beyond their respective saturation concentrations, to 50-mL cells equipped with pH-stat (Mettler, Switzerland) under different temperature conditions. After thoroughly mixing for 1 h at several different conditions, the pH of the mixture was adjusted with 1 N sulfuric acid or 1 N sodium hydroxide. The resulting mixture was then filtered, and the concentration of each component in the filtrate was measured by HPLC as described above.

RESULTS AND DISCUSSION

Solubility Changes according to the Temperature and the pH

As an alternative driving force to shift the transaminase reaction equilibrium toward product formation, we have paid particular attention to the solubilities of both substrates and products. While the solubility of 2-oxo-4-phenylbutyrate is dependent on temperature, the solubility of L-homophenylalanine is dependent on the pH of the reaction mixture (Figs. 2A and 2B). The solubility differences between 2-oxo-4-phenylbutyrate and L-homophenylalanine at 37°C (ΔS_{37}) and 60°C (ΔS_{60}) in the reaction solution are 84.9 and 247.5 mM, respectively. The pH-dependent solubility profile of L-homophenylalanine

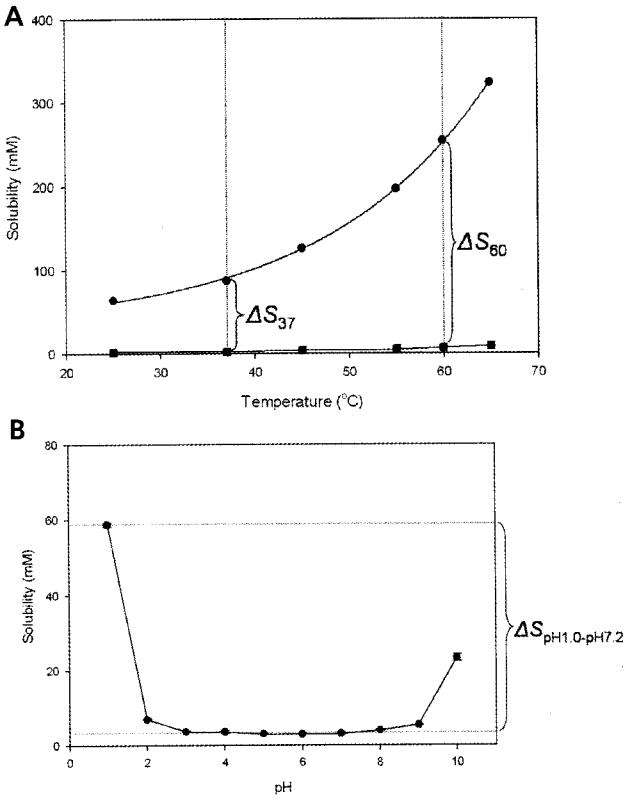

Fig. 2. Effect of temperature (A) on the solubility of 2-oxo-4-phenylbutyric acid (●) and L-homophenylalanine (■) at pH 7.5. ΔS_{37} and ΔS_{60} indicate the solubility difference between 2-oxo-4-phenylbutyric acid (●) and L-homophenylalanine (■) at 37°C and 60°C, respectively. Effect of pH (B) on the solubility of L-homophenylalanine (●) at 37°C. $\Delta S_{pH1.0\text{-}pH7.2}$ shows the solubility difference of L-homophenylalanine between pH 1.0 and 7.2.

suggested that it would precipitate in the reaction solution at either temperature, giving rise to a reaction equilibrium shift toward L-homophenylalanine formation. Provided the reaction was not hampered by severe substrate inhibition by 2-oxo-4-phenylbutrate, the effect of the solubility difference on the equilibrium shift was expected to be more prominent at higher temperatures. The solubility-difference profiles of phenylglyoxylate and L-phenylglycine showed identical trends to that of 2-oxo-4-phenylbutyrate and L-homophenylalanine (data not shown). In addition to the equilibrium shift effect, the low solubility of L-homophenylalanine (<2 mM) and L-phenylglycine (<5 mM) in neutral reaction solution was also quite helpful for alleviating the product inhibition and recovering the products. As higher reaction temperatures favor the equilibrium shift, we expected that thermophilic AroAT would give us attractive means to synthesize unnatural aromatic L-amino acids.

Screening of Thermophilic Microorganisms

To isolate the thermophilic microorganisms with AroAT activity, enrichment cultures were performed at 60°C us-

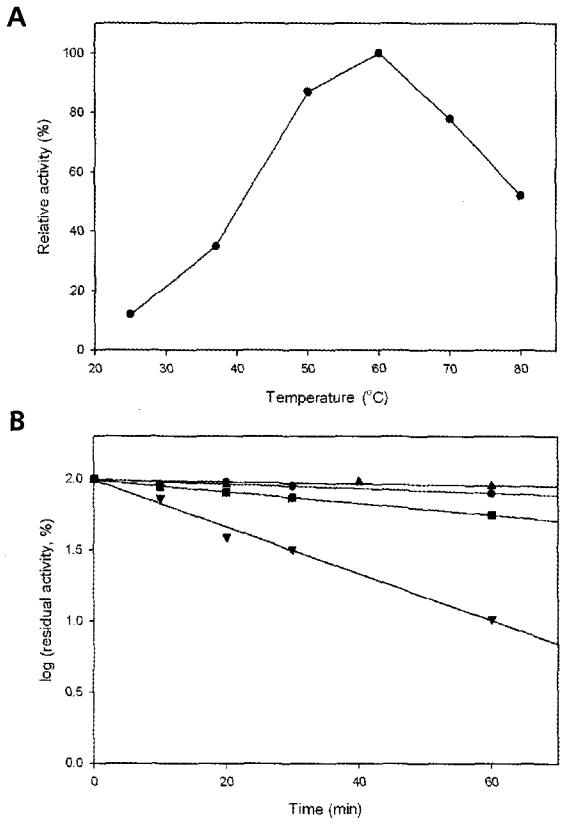

Fig. 3. Effect of temperature on the reaction rate (A) and on the stability (B) of AroAT from *Bacillus* sp. T30. Symbols: 37°C (▲), 60°C (●), 70°C (■), and 80°C (▼).

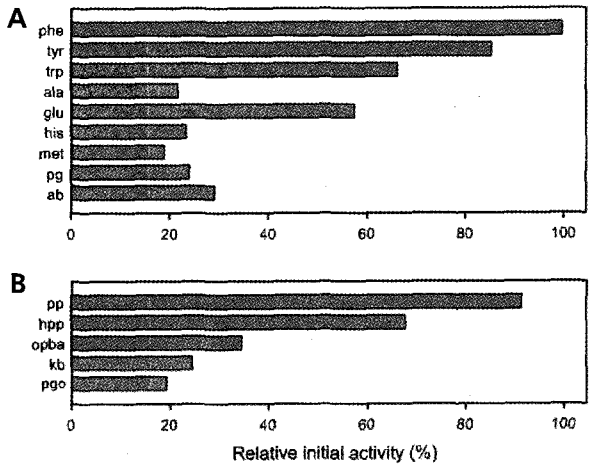

Fig. 4. Amino donor (A) and acceptor (B) specificity of AroAT from *Bacillus* sp. T30. Cell extracts containing 0.5 U of AroAT*Bs* were added to the reaction media comprised of 10 mM 2-OPBA and 50 mM Tris-HCl (pH 7.5) with each amino donor (30 mM). When measuring amino acceptor specificity, cell extracts containing 0.5 U of AroAT*Bs* were added to the reaction media comprised of 30 mM of L-glutamate and 50 mM Tris-HCl buffer (pH 7.5) with each α-keto acid (10 mM), respectively. Abbreviations are ala (L-alanine), phe (L-phenylalanine), tyr (L-tyrosine), trp (L-tryptophan), glu (L-glutamate), met (L-methionine), his (L-histidine), pg (L-phenylglycine), ab (L-aminobutyrate), opba (2-oxo-4-phenylbutyrate), pp (phenylpyruvate), hpp (hydroxyphenylpyruvate), pgo (phenylglyoxylate), and kb (2-oxo-butyrate).

ing minimal culture media containing L-homophenylalanine as the sole nitrogen source. Since the local concentration of 2-oxo-4-phenylbutyrate converted from L-homophenylalanine would be higher around the fast-growing cells on the minimal agar plate than that around the slow-growing cells, colony size is an indicator of high transaminase enzymatic activity. After isolating colonies based on their colony size, the transaminase activity of the isolated cells was measured at 60°C. Following three rounds of the culture enrichment and the two-step selection process, we isolated *Bacillus* sp. T30, which displayed an excellent L-specific enantioselectivity for the asymmetric synthesis of unnatural aromatic L-amino acids. To confirm its AroAT activity, a PLP-dependent enzyme inhibitor cocktail, comprised of 1 mM each of hydroxylamine, (aminooxy) acetic acid, and gabaculine, was added to the AroAT reaction mixture and its transaminase activity was measured. The synthetic activity of either whole cells or cell extracts was completely inhibited in the reactions with the inhibitor cocktail, confirming that the enzymatic activity from *Bacillus* sp. T30 is the PLP-dependent transaminase activity.

Dependence of Catalytic Activity of Screened AroAT*Bs* on the Reaction Temperature

The catalytic activity of AroAT obtained from *Bacillus* sp. T30 (AroAT*Bs*) was determined as a function of temperature (Fig. 3A). The catalytic activity reached a maximum at about 60°C, then leveled off and began to decline. This trend suggested that the optimal reaction temperature of AroAT*Bs* was 60°C. We also measured the rate of irreversible thermal inactivation of the AroAT*Bs* as a function of temperature. The measured half-lives of the AroAT*Bs* at 37, 60, 70, and 80°C were approximately 28, 12, 4.2, and 1.1 h, respectively (Fig. 3B), and deactivation energy was measured as 653.41 kJ/mol using Arrhenius equation [20,21]. Together, these measurements indicated that AroAT*Bs* can be used as a biocatalyst for the synthesis of unnatural L-amino acids by taking advantage of the large solubility difference between substrate and product at higher temperatures to accelerate an equilibrium shift favoring product formation.

Substrate Specificities of Thermophilic AroAT*Bs*

The substrate specificities of AroAT*Bs* toward amino donors were measured at 60°C, using selected L-amino acids as an amino donor and 2-oxo-4-phenylbutyrate as an amino acceptor. AroAT*Bs* showed the highest activity toward aromatic amino acids such as L-phenylalanine, L-tyrosine, and L-tryptophan, confirming that its amino donor specificity is similar to that of AroAT (Fig. 4A). However, unlike other AroATs, AroAT*Bs* exhibited a very different nature toward L-phenylglycine as an amino do-

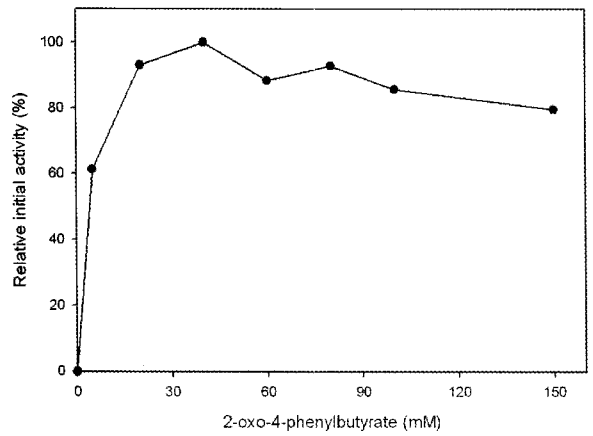

Fig. 5. Effect of 2-oxo-4-phenylbutyrate (2-OPBA) concentration on AroAT*Bs* activity. Each reaction began with the addition of 0.5 U of AroAT*Bs* to the reaction media at 60°C. Reaction media contained various concentrations of 2-OPBA, 300 mM L-glutamate, and 500 mM Tris-HCl buffer (pH 7.5).

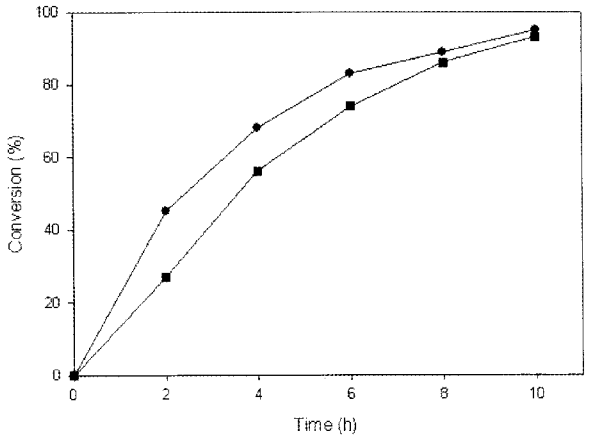

Fig. 6. Asymmetric syntheses of L-homophenylalanine (●) and L-phenylglycine (■) using AroAT*Bs*. Each reaction began with the addition of 50 U of AroAT*Bs* to the reaction media at 60°C. Reaction media contained 150 mM of the respective amino acceptor, 300 mM L-glutamate, and 500 mM Tris-HCl buffer (pH 7.5).

nor. The amino acceptor specificity of AroAT*Bs* was measured in a reaction solution comprised of 30 mM L-glutamate and 50 mM Tris-HCl buffer (pH 7.5) with 10 mM of each amino acceptor at 60°C (Fig. 4B). Like other AroATs, AroAT*Bs* showed the highest activity toward phenylpyruvate and hydroxyphenylpyruvate. However, while most of AroATs are inactive toward phenylglyoxylate, AroAT*Bs* was able to use phenylglyoxylate as an amino acceptor with relatively high reactivity (*i.e.*, 21.3% of reactivity toward L-phenylpyruvate).

Both the amino donor and amino acceptor specificities suggest that the active site of AroAT*Bs* is comprised of two substrate-binding pockets (*i.e.*, the large binding pocket and the small binding pocket) around a PLP-Lys Schiff base, similar to other AroATs [22]. However, as most AroATs do not show substrate specificity toward L-phenylglycine and phenylglyoxylate, the structure of the substrate-binding region of AroAT*Bs* is predicted to be quite different from those of other AroATs. Furthermore, as most AroATs show relatively high catalytic activity toward L-aspartate, which cannot be utilized by AroAT*Bs*, we have concluded that the active site structure of AroAT*Bs* is quite different from other AroATs, making AroAT*Bs* an even more preferable biocatalyst for the asymmetric synthesis of unnatural aromatic L-amino acids.

Substrate and Product Inhibition

To shift the reaction equilibrium with a higher concentration of substrate, with the shift driven by the solubility difference, there should not be severe substrate inhibition by the amino acceptor in the transamination reaction catalyzed by AroAT*Bs*. Therefore, the substrate inhibition by the amino acceptor was measured at various concentrations of 2-oxo-4-phenylbutyrate and L-glutamate at 60°C. While no substrate inhibition was observed for L-glutamate at concentrations up to 500 mM (data not shown), the catalytic activity reached a maximum at 40 mM 2-oxo-4-phenylbutyrate, and then began to decline (Fig. 5). However, the substrate inhibition profile for 2-oxo-4-phenylbutyrate showed that AroAT*Bs* maintained 80% catalytic activity even at 150 mM 2-oxo-4-phenyl-butyrate. To identify product inhibition, the initial activities were measured at different initial concentrations of α-ketoglutarate and L-homophenylalanine. No severe product inhibition was observed for either product at concentrations of up to 500 mM α-ketoglutarate and 6.5 mM L-homophenylalanine, respectively. While the relative initial AroAT activity was 80.3% at 500 mM α-ketoglutarate, no product inhibition was observed within the solubility range of L-homophenylalanine. In the case of product inhibition by L-homophenylalanine, the transaminase reaction cannot be executed with more than 6.5 mM L-homophenylalanine, due to its low solubility in the aqueous buffer solution at neutral pH. Consequently, these results indicate that product inhibition by either α-ketoglutarate or L-homophenylalanine is not a significant factor to performing the asymmetric synthesis of unnatural aromatic L-amino acids using the thermophilic AroAT. Judging from the substrate inhibition by 2-oxo-4-phenylbutyrate, the control of 2-oxo-4-phenylbutyrate concentrations in the reaction mixture will be a key factor in maintaining the transaminase activity and high total turnover number of this enzyme.

Asymmetric Syntheses of Unnatural Aromatic L-Amino Acids

To demonstrate the practical production of unnatural amino acids using AroAT*Bs*, the asymmetric syntheses of L-homophenylalanine and L-phenylglycine were performed using 2-oxo-4-phenylbutyrate and phenylglyoxylate as the amino acceptors, respectively. The solubility differences of the substrate and the product at 60°C are 198 mM for 2-oxo-4-phenylbutyrate and L-homophenyl-

alanine, and 195 mM for phenylglyoxylate and L-phenylglycine under the reaction conditions. 150 mM 2-oxo-4-phenylbutyrate and 150 mM phenylglyoxylate were converted to L-homophenylalanine and L-phenylglycine, with 95% (>99% ee^S) and 93% conversion (>99% ee^S) in 10 h by the partially purified AroAT*Bs*, respectively (Fig. 6). Due to the low solubility of L-homophenylalanine and L-phenylglycine, precipitation began immediately under the reaction conditions. During the reaction, L-homophenylalanine and L-phenylglycine were continuously precipitated in the reaction media, which efficiently shifted the reaction equilibrium toward the product formation. In addition, after completion of the reactions, L-homophenylalanine and L-phenylglycine in the reaction media were easily recovered with high purity using a simple pH change of the reaction media as described elsewhere [12].

CONCLUSION

Transaminase-catalyzed reactions have become very useful tools for the synthesis of unnatural amino acids and chiral amines via both asymmetric synthesis and kinetic resolution. While other approaches, such as the hydantoinase and dehydrogenase processes, have several limitations, including 1) low substrate solubility in aqueous reaction media and 2) requirement of cofactor regeneration, thermophilic transaminase-catalyzed process can use higher substrate concentration without the need for cofactor regeneration, resulting in a higher turnover number and a more economical bioprocess. Still, for industrial application of the transaminase reaction, several issues should be considered, such as enantioselectivity, reaction equilibrium, enzyme stability, high concentration reaction, and product separation. With this in mind, the thermophilic AroAT showed high enantioselectivity (>99% ee^S). Also, the low solubility of unnatural amino acid products under these reaction conditions makes product separation simple. In addition, thermophilic enzymes generally have higher thermal stability than mesophilic enzymes. Finally, as described above, the reaction equilibrium was successfully shifted toward the product formation by solubility differences between substrate and product under the reaction conditions.

In this article, asymmetric syntheses of L-homophenylalanine and L-phenylglycine from their corresponding α-keto acids were successfully demonstrated using an aromatic transaminase from the thermophilic *Bacillus* sp. T30, which was isolated from domestic soil samples by enrichment culture. *Bacillus* sp. T30 has intracellular AroAT activity, which is able to transfer the amino group from L-glutamate to unnatural aromatic α-keto acids to generate α-ketoglutarate and unnatural aromatic L-amino acids, respectively.

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