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Short communication

Chemoenzymatic synthesis of L-3,4-dimethoxyphenyl-alanine and its analogues using aspartate aminotransferase as a key catalyst

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ABSTRACT

In this study, a chemoenzymatic synthesis method for the production of L-3,4-dimethoxyphenyl-alanine and its analogues from phenylpyruvate derivatives was developed. The aspartate aminotransferase from *Escherichia coli* was engineered by error prone PCR and the improved variants were identified. When 3, 4-dimethoxy phenylpyruvate was added by fed-batch on a preparative scale, L-3,4-dimethoxyphenyl-alanine was formed in 95.4% conversion and > 99% ee with the best aspartate aminotransferase variant as the catalyst. This study provided an efficient method for the production of methoxy substituted phenylalanines using the engineered aspartate aminotransferase.

1. Introduction

L-3,4-Dimethoxyphenyl-alanine (L-DM-Phe, that is L-veratrylglycine) as an unnatural amino acid, is a key building block for the synthesis of a variety of biologically significant molecules, such as anti-Parkinson drug L-Dopa [1,2], isoquinoline derivatives [3], chemosensors [4], tricycles [5], chiral catalysts [6], aminoacylated tRNAs [7], benzo[*a*]quinolizidines [8] and anti-cancer agents [9]. Because of the wide application of L-DM-Phe, an increasing attention has been devoted to the synthesis of L-DM-Phe and its derivatives in recent years, which are mainly synthesized chemically [10–12].

Chemical approaches to L-DM-Phe are mostly based on the catalysis employing chiral metal catalysts, including Rhodium (I) bis(norbornadiene) hexafluorophosphate [3], [(*S*)-BINAPRuCl (benzene)]Cl [10], and chiral Nickel(II) Schiff base complexes of glycine and alanine [11], generally followed by the asymmetric hydrogenation reaction. Additionally, L-DM-Phe ester was recently synthesized using cinchona alkaloid derivatives as chiral catalyst in 88% ee and 60% yield [13]. Therefore, the enzyme-catalyzed reaction is an attractive alternative to the asymmetric chemical reaction in the synthesis of L-DM-Phe.

Currently, the commonly employed enzymes in the synthesis of L-DM-Phe are acylase [1] and protease [4]. However, these two enzymes only could selectively hydrolyze the L-precursor of D, L-DM-Phe via enzymatic resolution, and consequently the yield is < 50%. Transaminases have attracted considerable attention recently because of their remarkable potency in reductive amination reactions for the synthesis of chiral amines [14–16]. As one of the members of transaminase family, aspartate aminotransferase (AspAT, E C 2.6.1.1) possesses the similar characteristics of transaminase, which is a pyridoxal 5-phosphate (PLP)–dependent enzyme that catalyzes the reversible transamination between acidic amino acids, aspartate and glutamate, and their corresponding 2-oxo acids via the ping-pong mechanism [17–20]. According to our previous study [21], AspAT from *Escherichia coli* catalyzed the conversion of 4-methylsulfonyl phenylpyruvic acid to L-4-methylsulfonyl phenylalanine using L-aspartate as the amino donor with a high conversion of 95%. Encouraged by this result, we have explored the use of *Escherichia coli* AspAT to synthesize other phenylalanine derivatives, especially for the synthesis of L-DM-Phe and its analogues.

The present work focused on the development of *Escherichia coli* AspAT variants for the production of the target compounds. A three-step chemical-biocatalytic process was designed in which the derivatives of phenylpyruvic acid were chemically synthesized by two-step process and then converted by recombinant AspAT in aqueous media (Scheme 1). When L-aspartate was used as the amino donor, pyruvate as amino acceptor spontaneously produced from the by-product oxaloacetate and was converted into L-alanine during the biotransformation [22,23]. Due to the side reaction and the chemical instability of phenylpyruvate substrates, the conversion of the wild type AspAT is not very satisfactory, and therefore AspAT was evolved

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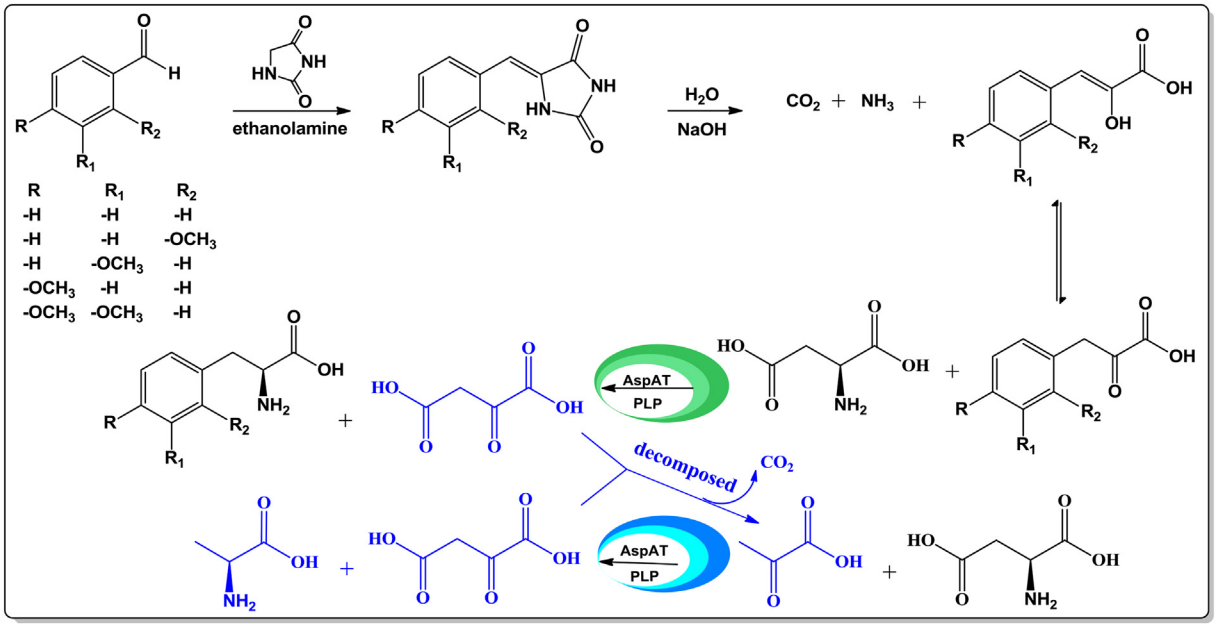

Scheme 1. Synthesis of L-DM-Phe and its analogues via the engineered or wild type AspAT. AspAT = aspartate aminotransferase from *Escherichia coli K-12*, PLP = pyridoxal phosphate.

toward higher activity by error prone PCR. We present here a promising chemoenzymatic approach for the synthesis of methoxy-substituted phenylalanine using the engineered AspAT.

2. Experimental section

2.1. Materials

Lactose and pyridoxal-5′-phosphate (PLP) were acquired from Sigma (St. Louis, MO., USA). Silica gel GF254 plate (5.0 × 10.0 cm) was purchased from Haiyang Chemical Co. Ltd. (Qingdao, China). All other chemicals and reagents used in this work were of analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Restriction enzymes and kits for genetic manipulation were purchased from Takara Bio (Dalian, China). Primers were synthesized, and their sequences were determined by Genscript Corp. (Nanjing, China). Instant Error-prone PCR Kit was purchased from Tiandz Co. Ltd. (Beijing, China).

2.2. Microorganisms and shake flask fermentation

The wild type AspAT gene was amplified by PCR using the primers, and the mutated AspAT genes were constructed by error-prone PCR using the same primers. All of the recombinant cells were constructed by cloning recombinant pET28a plasmids into *E. coli* BL21 (DE3). The engineered colonies were picked from the LB-kanamycin plates and inoculated in conical deep 96-well plates to construct the variant library. The AspAT proteins were expressed in the fermentation medium, and the fermented broth was centrifuged to collect wet cells (see Supporting information) [24–26]. When the cells for a preparative scale were needed, the wet cells were lyophilized for a minimum of two days and stored at −20 °C until used for the reactions.

2.3. Library screening

Take into consideration the importance of L-DM-Phe, we selected the clones by comparing the conversion of DMPPA. The positive clones were collected and confirmed by sequencing. The master plates containing variant cells were stored at −80 °C for further analysis or cultivation.

The screening of the random variant library of AspAT was performed by two-step screening procedure. Firstly, the variant whole cells were directly used to convert the substrates, and the reaction results were roughly screened by TLC [27]. Secondly, the enzymes in predominant colonies were purified by Kits, and then their conversion solutions were further analyzed by HPLC. To identify the effective clones, we used the wild type AspAT as the control group to compare it with all of the screening clones (see Supporting information).

2.4. Analytical methods and enzyme assays

All of phenylpyruvate derivatives and their corresponding products were directly analyzed by HPLC, and the enantioselectivity of chemoenzymatic synthesis was determined by derivation of amino acids with the Marfey's reagent (see Supporting information) [28]. Apparent kinetic parameters of the purified wild-type and 170a mutant AspAT were obtained using nonlinear regression fitting to the Michaelis-Menten equation. The apparent *K*m and k_{cat} values were determined by varying the concentrations of phenylpyruvic acid derivatives from 0.2 to 20 mM (see Supporting information 1.5).

2.5. Scaling up the fermentation, bioconversion, and isolation of methoxy substituted phenylalanine

According to our protocols [24–27], the fermentation was operated in a 7 L scale. At the end of the fermentation process, fermentation broth was centrifuged to obtain wet cells. The wet cells were lyophilized and collected after fermentation with a weight of 58.8 ± 1.4 g, which were then mixed with substrates under the optimum reaction conditions for bioconversion in a 20 L reactor.

The bioconversion of methoxy substituted phenylalanine was generally operated as follows: L-Asparate (1317.7 g, 9.9 mol) and phenylpyruvate substrates powder (3 mol) were dissolved in water to make 15 L solution. The pH of the resulting solution was adjusted with 2 M sodium hydroxide to 8, and then 0.001215 mol PLP, 300 mL Triton X-100 (10%, v/v), and the engineered 170a AspAT cells (75 g) were added successively. The remaining phenylpyruvate substrates were separately added (3 mol, 1.5 mol and 1.5 mol) at intervals of 1 h. The

reaction mixture was stirred under N_2 at 220 rpm for 12 h after the complete addition.

L-DM-Phe precipitated with progressing bioconversion reaction, and the post-treatment of L-DM-Phe was similar to that of L-tyrosine [24,25]. The reaction mixture was centrifuged, and the collected L-DM-Phe was purified according to the difference of solubility at the different pH (see supporting information). The spectral characterization of L-DM-Phe was shown as follows: $[\alpha]_D^{25} = -4.3^o$ ($lit^{11} = -4.4^o$, $c = 1$, 4 M hydrochloric acid), ^{1}H NMR (D_2O) (Bruker DRX600, Germany) δ: 6.88 (d, 1H, $J = 8.2$ Hz), 6.81 (s, 1H), 6.75 (dd, 1H, $J = 1.2$ Hz, 8.2 Hz), 3.76 (s, 3H), 3.74 (s, 3H), 3.38 (dd, 1H, $J = 5.9$ Hz, 6.8 Hz), 2.84 (dd, 1H, $J = 5.5$ Hz, 13.6 Hz), 2.70 (dd, 1H, $J = 7.2$ Hz, 13.6 Hz). ^{13}C NMR (D_2O, Bruker DRX600, Germany) δ: 182.4, 147.8, 146.7, 131.3, 122.0, 112.9, 111.8, 57.3, 55.7, 55.6, 40.3 (Fig. S1–2). Based on the similar operation, the other methoxy substituted phenylalanines were obtained, and their structures were confirmed by ^{1}H NMR and ^{13}C NMR as shown in Supporting information (Fig. S3–10).

3. Results and discussion

3.1. AspAT mutants obtained by error-prone PCR

Three clones 10b, 170a and 261b were selected from about 4000 independent variants library, which exhibited higher catalytic efficiency than the wild-type enzyme (see Supporting information 1.3). Sequencing analysis clearly revealed that 10b was mutated at two amino acid positions (F170S, R316H), 170a was mutated at four amino acid positions (N165Y, R316H, A381Tand P399L), 261b was mutated at two amino acid positions (R316H, T366A). The conversions of methoxy substituted phenylpyruvates were analyzed under the catalysis of these variants (Fig. 1). The synthesis of phenylpyruvic acid and its derivatives were described in Supporting information and the structures were confirmed by ^{1}H NMR and ^{13}C NMR (Fig. S11–20).

As shown in Fig. 1, all the conversions of DMPPA catalyzed by the AspAT variants increased in comparison to the wild type AspAT. The wild type AspAT catalyzed the transamination reactions with all the methoxy substituted phenylpyruvates in approximately 70% conversion but phenylpyruvate in 83% conversion. The variant 170a increased the conversions of all substrates by 7% at least, in particularly, the conversions of DMPPA and PPA were 91% and 94%, respectively. The variants 10b, 261b could effectively catalyze all methoxy substituted phenylpyruvates and phenylpyruvate, except 2-methoxy substituted phenylpyruvate. The results illustrated the mutations may influence the enzymatic activities, thus providing the effective mutation sites for further improving AspAT activity.

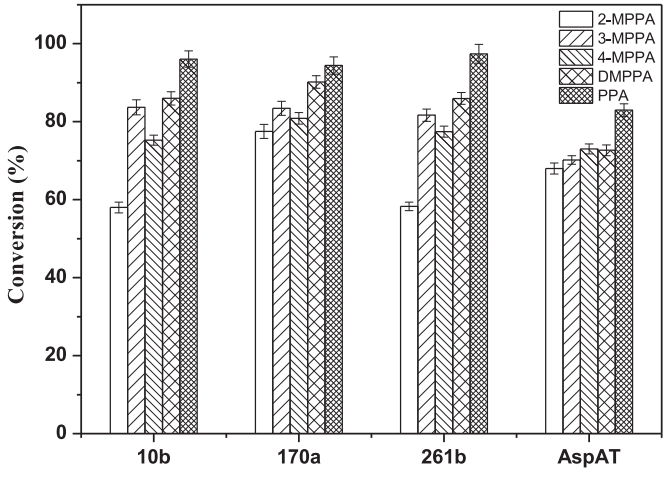

Fig. 1. The conversions of phenylpyruvate and its derivatives with the purified AspATs as catalyst. Phenylpyruvate substrates (200 mM, 125 μL, pH = 8), L-aspartate (220 mM, 125 μL, pH = 8) and 2 μL PLP (1%, m/v) were mixed in 100 mM Tris-HCl buffer (200 μL, pH = 8) and separately catalyzed by the purified AspAT solution from the variants (50 μL, 0.003 μg/μL) at 40 °C with 170 rpm shaking for 8 h.
PPA = Phenylpyruvic acid; 2-MPPA = 2-Methoxy phenylpyruvic acid; 3-MPPA = 3-Methoxy phenylpyruvic acid; 4-MPPA = 4-Methoxy phenylpyruvic acid; DMPPA = 3, 4-dimethoxy phenylpyruvic acid.

3.2. The properties of the engineered 170a AspAT

To elucidate the properties of the engineered 170a AspAT, the overall characterization including the apparent k_{cat} and K_m of the wild-type and AspAT variant for five phenylpyruvate substrates has been performed (Table 1). The SDS-PAGE analysis of the purified AspAT was shown in Fig. S21. The apparent kinetic parameters were determined as described in Supporting information (Fig. S22–23). Based on the apparent k_{cat} and K_m of the wild type and 170a variant, k_{cat}/K_m values of the examined variant increased by 3–24 fold. The 170a variant AspAT showed the highest increase in k_{cat}/K_m values for DMPPA and significantly improved its conversion from 72.5% to 91.6%. This result demonstrated that these four substituted amino acid positions were functionally important.

According to HPLC chromatogram of D, L-Phe and L-Phe (Fig. S28–30), L-Phe isomer was produced from the conversion of phenylpyruvate under the catalysis of the variant 170a AspAT. For L-DM-Phe, its L-(−) isomer characteristics was confirmed by HPLC chromatogram (Fig. S31) and the optical rotation determination [12]. Compared with HPLC results of the corresponding racemates, the unique product peaks in chromatograms for the other Phe derivatives indicated that they were all L-isomers (Fig. S32–S37). The HPLC results indicated that the ee % values of all the phenylalanine derivatives were > 99%, the products were all L-configuration and consequently the exchanged sites in 170a did not influence the chiral centers of products.

3.3. Synthesis of L-DM-Phe

So far, AspAT has never been engineered to improve the catalytic efficiency from DMPPA to L-DM-Phe. Therefore, the synthesis of L-DM-Phe was further studied among these products. After library screening, the variant 170a was selected with an approximately 91.6% conversion, which suggested DMPPA was not completely converted into the target product. DMPPA substrate in the mixture could be detected readily by HPLC, and the remaining substrate was determined to be < 0.01%. Therefore, an approximately 8% loss of the product could be attributed to the instability of DMPPA, which decomposed during the conversion process as it was observed with PPA [29]. Considering the stability of the substrate, we improved the conversion process by feeding DMPPA in batches and performing the reaction under inert gas. It turned out that the best addition method of DMPPA (9 mol) was an initial addition of DMPPA (3 mol), followed by the subsequent addition in batch, 1 h (3 mol), 2 h (1.5 mol), and 3 h (1.5 mol) on a preparative scale. As shown in Fig. 2, the conversion could reach 95.4%, which was higher than that of the one-batch addition of DMPPA (91%). The concentration of DMPPA gradually decreased during the scale-up biotransformation, and finally L-DM-Phe increased to 572 mM.

Additionally, as shown in Fig. S38 and scheme 1, the by-product, oxaloacetate produced from L-aspartate, subsequently undergoes spontaneous decarboxylation to pyruvate, and then pyruvate was converted into L-alanine by AspAT [22,23]. Nevertheless, the variant 170a produced much less L-alanine (9.4% ± 0.7%, we supposed the complete conversion of L-aspartate into L-alanine to be 100%.) than the wild type AspAT (32.3% ± 1.2%) (Fig. S38) after 8 h, which indicated the increase in DMPPA conversion for 170a AspAT was related to the decrease in alanine production.

In general, the scale-up biocatalysis might have anticipated difficulty in the separation of the target compound from the reaction

Table 1
Apparent kinetic parameters of wild type and 170a variant AspAT for phenylpyruvate substrates, pH 8, 40 °C.

Substrate	The wide type AspAT				The 170a variant AspAT			
	k_{cat} (s^{-1})	Km (mM)	k_{cat}/Km ($s^{-1}M^{-1}$)	Conversion (%)	k_{cat} (s^{-1})	Km (mM)	k_{cat}/Km ($s^{-1}M^{-1}$)	Conversion (%)
PPA	2.99 ± 0.22	4.24 ± 0.26	705.2 ± 23.3	83.4 ± 1.6	3.45 ± 0.19	0.92 ± 0.02	3750.0 ± 127.8	94.4 ± 2.2
2-MPPA	1.44 ± 0.11	12.07 ± 0.78	119.3 ± 6.1	67.8 ± 1.5	2.69 ± 0.16	5.84 ± 0.32	460.6 ± 16.4	77.5 ± 1.4
3-MPPA	1.55 ± 0.09	11.56 ± 0.72	134.1 ± 5.4	70.2 ± 1.1	2.79 ± 0.21	4.92 ± 0.38	567.1 ± 14.3	83.4 ± 1.9
4-MPPA	1.77 ± 0.13	10.14 ± 0.57	174.6 ± 9.7	73.6 ± 1.3	2.85 ± 0.24	4.12 ± 0.27	691.7 ± 17.5	80.8 ± 1.5
DMPPA	1.81 ± 0.14	10.32 ± 0.59	175.4 ± 7.2	72.5 ± 1.4	4.33 ± 0.31	1.01 ± 0.03	4287.1 ± 159.7	91.6 ± 1.6

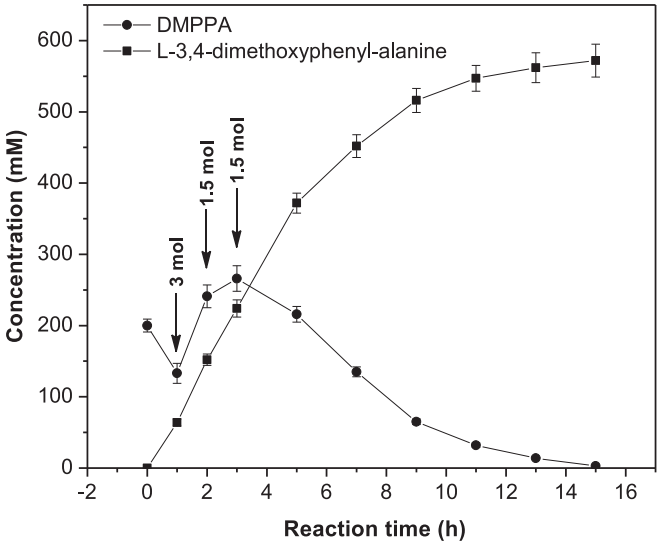

Fig. 2. Time course of enzymatic synthesis of L-DM-Phe by the variant 170a AspAT whole cell on a large 15 L scale. The reaction was initiated by adding DMPPA at the beginning (672.6 g, 3 mol) and subsequently 1 h (672.6 g, 3 mol), 2 h (336.3 g, 1.5 mol), 3 h (336.3 g, 1.5 mol) as indicated by arrows. The conversion of DMPPA to L-DM-Phe (95.4%) was the highest after 15 h incubation.

mixture. Fortunately, L-DM-Phe was readily separated since it could precipitate from the mixture as the reaction proceeded. L-DM-Phe as white crystals were collected and dried under vacuum to yield 1817 g on a preparative scale. The total yield of L-DM-Phe for this bioconversion step was 89.6%. The yield and the determined purity (> 99%) of L-DM-Phe met the demand of industrial production. Moreover, the space-time yield of the preparation of L-DM-Phe described here could be extrapolated to 193.81 kg per m^3 per day. As shown in Table S1, atom economy is usually used to evaluate the conceptual design of the synthetic strategy [1]. The atom economy of 0.497 was obtained for the present route (Table S1).

4. Conclusions

This study has provided an efficient enzymatic approach for the production of L-DM-Phe and its analogues by the engineered AspAT. The developed protocol exhibited various advantages, such as mild reaction conditions, convenient separation of product from the reaction mixture, > 99% ee and purity (99%) of the product, and the total yield (e.g. for L-DM-Phe 72.6%) of this three-step chemoenzymatic synthesis. In the present, the utilization of L-DM-Phe for the synthesis of other related pharmaceuticals is undergoing. Moreover, based on the previous protocol [30], the extensive protein engineering followed by optimization of the identified motif of aspartate transaminase to identify optimal variants for the synthesis of a wide array of potential substances is ongoing in our lab. This study has represented an example of designing chemoenzymatic processes for the synthesis of important pharmaceutical intermediates by the engineered enzyme with improved activity.

Conflicts of interest

There are no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.catcom.2018.10.033.

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