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COMMUNICATION

Structure-guided engineering of *Pseudomonas dacunhae* L-aspartate β-decarboxylase for L-homophenylalanine synthesis

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Structure-guided engineering of *Pseudomonas dacunhae* L-aspartate β-decarboxylase (AspBDC) resulted in a double mutant (R37A/T382G) with remarkable 15,400-fold improvement in specific activity reaching 216 mU/mg, towards the target substrate 3(*R*)-benzyl-L-aspartate. A novel strategy for enzymatic synthesis of L-homophenylalanine was developed by using the variant as biocatalyst affording 75% product yield within 12 h. Our results underscore the potential of engineered AspBDC for the biocatalytic synthesis of pharmaceutical relavent and value added unnatural L-amino acids.

L-Homophenylalanine is an important nonproteinogenic amino acid that can be used for the synthesis of various angiotensin converting enzyme inhibitors, including benezepril, delapril, enalapril, imidapril, linsinopril, and tempocapril.[1] It can also serve as a precursor for the proteasome inhibitor drug, carfilzomib.[2] Given the importance of L-homophenylalanine in the pharmaceutical industry, several enzymatic methods for the synthesis of L-homophenylalanine have been developed due to the mild reaction conditions and excellent selectivities (chemoselectivity, regioselectivity, and/or enantioselectivity) of biocatalysis.[3] However, the kinetic resolution method catalyzed by acylase suffers from the low theoretical yield (50%), and a racemization step of the substrate is required to achieve a 100% theoretical yield.[4] Although both transaminase[5] and amino acid dehydrogenase[6] enable the asymmetric synthesis of L-homophenylalanine, transaminase requires an excessive amount of amino donor for driving the reaction equilibrium toward the direction of product formation, which would complicate the down-stream process, whereas expensive cofactor (NADH or NADPH) is necessary for the amino acid dehydrogenase. Therefore, there is still a great need for the creation of novel L-homophenylalanine synthetic strategies.

L-Aspartate β-decarboxylase (AspBDC, EC 4.1.1.12) is a pyridoxal 5'-phosphate (PLP) dependent enzyme that catalyzes the irreversible β-decarboxylation of L-aspartate to L-alanine and CO_2.[7] Unlike most amino acid decarboxylases, which catalyze the α-decarboxylation of amino acids giving primary amines as the product,[8] chiral amino acid (L-alanine) is produced by AspBDC. Although the AspBDC from *Pseudomonas dacunhae* has been successfully employed for the production of L-alanine since 1984,[9] its substrate scope has largely been unexplored. Very recently, *threo*-β-hydroxy-DL-aspartate and *erythro*-β-hydroxy-DL-aspartate have been shown to be catalyzed by AspBDC giving L-serine, albeit with reaction rates at least 1,000-fold and 16,700-fold slower than that with L-aspartate.[7] We envisioned L-aspartate analogs with other substituents at the C_β atom might be accepted by AspBDC, thereby providing a novel access to other L-amino acids, in particular unnatural L-amino acids.

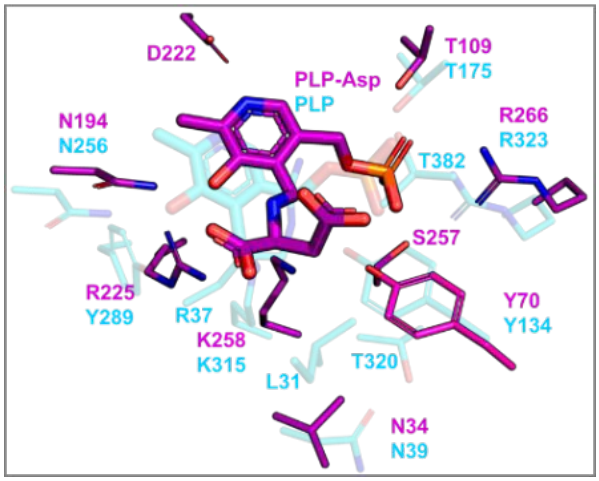

Fig. 1 Superimposed structures of AspBDC (PDB: 2ZY4, cyan) and substrate-bound AspAT Y225R/R386A (PDB: 1ARH, purple).

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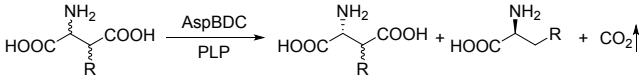
In this work, we report the structure-guided engineering of the AspBDC from *Pseudomonas dacunhae*[10] aiming to develop a novel strategy for L-homophenylalanine synthesis. The engineered enzyme showed a 15,400-fold improvement in specific activity towards the target substrate (*R*)-3-benzyl-L-aspartate over the native AspBDC. Enzymatic synthesis of L-homophenylalanine was achieved on a semi-preparative scale by using the engineered AspBDC as the biocatalyst, with high conversion (~75%). These results uncover the great potential of creating different AspBDC variants through protein engineering for efficient synthesis of structurally diverse unnatural L-amino acids.

Table 1 Specific activity of AspBDC towards different substrates[a]



R_1 = H
R_2 = OH
R_3 = CH_3
R_4 = $CH_2C_6H_5$

Substrate	Specific activity (mU/mg)		Fold
	WT	R37A/T382G	
R_1[b]	$(2.4 \pm 0.1) \times 10^4$	$(3.1 \pm 0.2) \times 10^3$	0.13
R_2	$(2.7 \pm 0.2) \times 10^1$	$(1.5 \pm 0.1) \times 10^1$	0.56
R_3	$(3.7 \pm 0.1) \times 10^0$	$(1.2 \pm 0.1) \times 10^1$	3.2
R_4	$(1.4 \pm 0.2) \times 10^{-2}$	$(2.2 \pm 0.1) \times 10^2$	1.5×10^4

[a] Activity was measured in KPB buffer (0.1 M, pH 6.0) containing purified enzyme, 0.5 mM PLP and 10 mM substrate (for R2 and R3, 20 mM substrate was used) at 30°C. The concentration of product formed was determined by HPLC and used to calculate the specific activity.
[b] L-aspartate was used.

We first examined whether AspBDC could be exploited for the other unnatural L-amino acids synthesis by using substrates with substituents at the C_β carbon of L-aspartate with different polarity and size. (Table 1). As expected, when wild-type AspBDC (2 mg/mL) was incubated at 30 °C with 5 mM 3(*R*, *S*)-hydroxy-DL-aspartate in 100 mM potassium phosphate buffer (pH 6.0), 1.7 mM L-serine was produced after 6 h reaction, which was confirmed by comparison with commercial L-serine standard (Fig. S1). These results are consistent with previous studies.[7] Interestingly, when AspBDC was incubated with 3(*R*, *S*)-methyl-DL-aspartate (20 mM), approximately 35% of the substrate was stoichiometrically converted to the key precursor of the antituberculosis drug ethambutol,[11] L-2-aminobutyrate (7.1 mM) as confirmed by HPLC and UPLC-MS (Fig. S2 & Fig. S3**)**. It should be noted that in a previous study by Novogrodsky and Meister, 3(*R*, *S*)-methyl-DL-aspartate could not be accepted as substrate by AspBDC from *Alcaligenes faecalis*.[12] Based on the configuration of the product and the conversion (> 25%), it seems that AspBDC selectively catalyzes the β-decarboxylation of L-aspartate derivatives other than its D-configured counterparts, whereas both enantiomers at the C_β atom are accepted by AspBDC, and possibly with different reaction rates. Based on the above results, 3(*R*,*S*)-benzyl-L-aspartate was synthesized chemically (see supporting information) and subjected to β-decarboxylation by AspBDC. To our delight, very low level of β-decarboxylase activity of AspBDC towards 3(*R*,*S*)-benzyl-L-aspartate was observed, and the corresponding product L-homophenylalanine was confirmed by comparison with commercial standard (Fig. S4**)** and UPLC-MS (Fig. S5**)**. The specific activity of AspBDC towards the substrates were then determined showing an order of L-aspartate (R_1) ($\times 10^4$) >> 3(*R*,*S*)-hydroxy-DL-aspartate (R_2) ($\times 10^1$) > 3(*R*,*S*)-methyl-DL-aspartate (R_3) ($\times 10^0$) >> 3(*R*,*S*)-benzyl-L-aspartate (R_4) ($\times 10^{-2}$) (Table 1). These results demonstrated that AspBDC has low level of β-decarboxylase activity towards β-substituted L-aspartate analogs and may possibly serve as a template to create highly active variants through protein engineering.

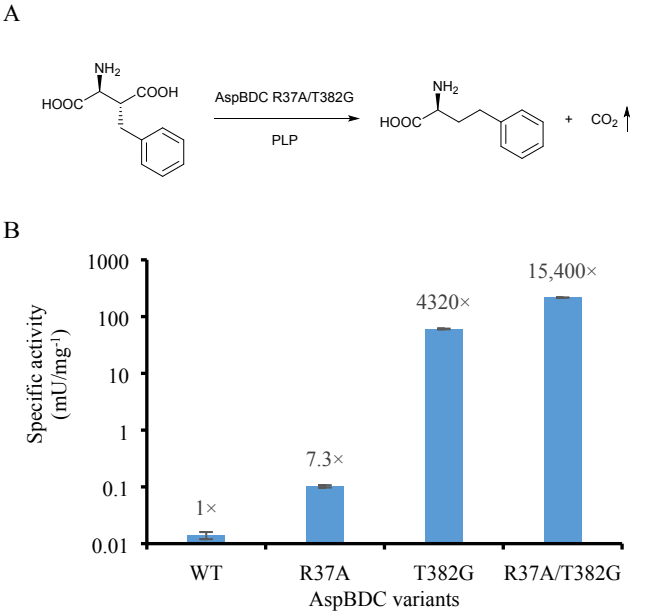


Fig. 2 Decarboxylase activity of AspBDC variants. A) Reaction scheme of AspBDC-catalyzed β-decarboxylation of 3(*R*)-benzyl-L-aspartate; B) Comparison of the decarboxylase activity of AspBDC and its variants towards 3(*R*)-benzyl-L-aspartate.

Promoted by these initial results, we then sought to enhance the catalytic efficiency of AspBDC by protein engineering, and due to the lack of high-throughput screening method for AspBDC, a structure-guided engineering strategy focusing on the active site amino acids was therefore applied for this purpose.[13] Although the crystal structure of AspBDC from *Pseudomonas dacunhae* complexed with PLP has been solved, the active site was shielded by the Schiff-base formed between Lys315 and PLP, thus preventing successful docking of L-aspartate into the active site of AspBDC. It is noteworthy that the active site of AspBDC is remarkably similar to that of aspartate aminotransferase despite their quaternary structures

are different.[14] Most importantly, the double (Y225R/Y386A) and triple (Y225R/R292K/Y386A) mutants of aspartate aminotransferase from *Escherichia coli* have been demonstrated to catalyze the β-decarboxylation of L-aspartate to L-alanine and CO_2.[15-16] Therefore, the crystal structure of AspBDC was superimposed with that of aspartate aminotransferase double mutant (Y225R/Y386A) complexed with PLP and L-aspartate (PDB ID: 1ARH) aiming to figure out the amino acids potentially interacting with the C_β atom of L-aspartate (Fig. 1). To this end, eight amino acids (Leu31, Arg37, Asn39, Tyr134, Phe204, Lys315, Thr320, and Thr382) surrounding the C_β atom of L-aspartate within 6 Å were selected for alanine scanning in an attempt to identify hot spot residues in which mutations give rise to improved β-decarboxylase activity. Improved variants were identified by measuring their total turnover numbers (TTN) in the decarboxylation of 3(*R*)-benzyl-L-aspartate for 24 h (Fig. 2A).

Two mutations with significantly increased (> 5-fold) β-decarboxylase activity were obtained with TTN 6-fold (R37A) and 20-fold (T382A) that of the wild-type enzyme, respectively. (Table S2). Another two variants (Y134A and F204A) also resulted in 2-fold enhanced activity compared to wild-type AspBDC. No significant change in activity was observed with L31A, N39A, and T320A. However, substituting Lys315 with Ala dramatically reduced the activity, possibly because of its important role in the reaction mechanism of AspBDC.[13] Another mutation (K17A) previously shown to be more active than the wild-type enzyme for the decarboxylation of L-aspartate was also constructed,[9] and its decarboxylase activity towards 3(*R*)-benzyl-L-aspartate was measured, giving a 10-fold improvement in TTN (Table S2). The two hot spot residues (Arg37 and Thr382) were then subjected to site saturation mutagenesis (Table S1). Substitution of Arg37 with the other 18 amino acids did not lead to further improvement in activity, but gave rise to a variant (R37F) with activity comparable to that of R37A, with R37A still being the best mutant enzyme (Fig. S6). Interestingly, screening of the T382X (X represents the other 19 amino acids) resulted in the discovery of a best mutant enzyme (T382G) which displayed a considerable 123-fold enhancement in TTN for the decarboxylation of 3(*R*)-benzyl-L-aspartate (Fig. S7). Based on the above results, the best single mutants were combined aiming to further increase the catalytic efficiency of AspBDC, and their specific activities were determined (Fig. 2B & Table S3). Notably, combination of T382G with R37A resulted in a double mutant (R37A/T382G) with up to 15,400-fold enhancement in specific activity compared to the wild-type enzyme, achieving 216 mU/mg. However, the combination of T382G with R37F did not further increase the activity, but decreased the activity by 40% as compared with T382G. A 2.4-fold improvement in specific activity than that of T382G was observed when T382G was combined with K17A. Further introducing K17A into the double mutant R37A/T382G led to a triple mutant (K17A/R37A/T382G) with comparable activity to its parental double mutant, giving a specific activity of 198 mU/mg, which is 14100-fold higher than the wild-type enzyme (Table S3). This phenomenon might be explained by the fact that both Lys17 and Arg37 occupy part of the substrate entrance tunnel,[10] and substitution of either of these two residues with smaller one (*e.g.* Ala) is sufficient to allow free access of substrate to the active site. Therefore, the introduction of K17A into R37A/T382G did not lead to an additive effect.

Table 2 Kinetic parameters of AspBDC and mutant R37A/T382G towards 3(*R*)-benzyl-L-aspartate

Mutant	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}\,mM^{-1}$)
WT	$(1.3 \pm 0.2) \times 10^3$	2.0×10^{-1}	1.5×10^{-4}
R37A	$(1.0 \pm 0.3) \times 10^2$	7.2×10^{-3}	7.2×10^{-5}
T382G	$(5.1 \pm 1.7) \times 10^1$	2.0×10^{0}	3.9×10^{-2}
R37A/T382G	$(2.6 \pm 0.1) \times 10^2$	9.5×10^{1}	3.7×10^{-1}

With the improved AspBDC in hand, the specific activity of the best mutant enzyme (R37A/T382G) towards the substrates tested in this work was investigated (Table 1). The results showed that the specific activity of the variant towards 3(*R*,*S*)-methyl-DL-aspartate was also improved but with only 3.2-fold enhancement. In contrast, the specific activity towards L-aspartate and 3(*R*, *S*)-hydroxy-L-aspartate was lowered by 7.7-fold and 1.8-fold, respectively. Moreover, since 3(*R*)-benzyl-L-aspartate was used as the target substrate for the screening instead of 3(*R, S*)-benzyl-L-aspartate, the catalytic potential of the best variant towards 3(*S*)-benzyl-L-aspartate was evaluated and compared with 3(*R*)-benzyl-L-aspartate by performing reactions on an analytical scale (Fig. S8). Strikingly, in contrast to the wild-type AspBDC, which catalyzes the decarboxylation of 3(*R*)-benzyl-L-aspartate and 3(*S*)-benzyl-L-aspartate with similar reaction rates, the variant obviously preferred 3(*R*)-benzyl-L-aspartate, achieving 65% product yield after 12 h reaction, whereas the product yield of the reaction using 3(*S*)-benzyl-L-aspartate as the substrate was merely 5%. The choose of 3(*R*)-benzyl-L-aspartate as the target substrate for the screening of improved variants might be the reason for this phenomenon, consistent with the idea "you only get what you screen for".[17] The kinetic constants of the wild-type AspBDC and its variants (R37A, T382G, and R37A/T382G) towards 3(*R*)-benzyl-L-aspartate were also determined (Table 2 & Fig. S9). For R37A, both the k_{cat} and K_M were reduced by 28- and 13-fold, respectively, compared to the wild-type enzyme, suggesting that the removal of the side chain of Arg37 facilitates the access of the substrate into the active site. Similar results were also observed with the same mutation but with a different substrate (L-aspartate).[10] In the case of T382G, the decrease of K_M by 26-fold and an increase in k_{cat} by 10-fold together contributed to a two orders of magnitude improvement in activity. These results indicate that the mutant T382G might provide better accommodation of the target substrate in the active site with a more favourable conformation for the reaction to happen. As for R37A/T382G, an increase in k_{cat} from 0.2 s^{-1} for the wild-type enzyme to 95 s^{-1} for the variant and a decrease in K_M from 1300 mM for the wild-type to 260 mM for the

variant together contributed to a 2500-fold enhancement in k_{cat}/K_M. To verify the superiority of the variant over the wild-type enzyme for the β-decarboxylation of 3(*R*)-benzyl-L-aspartate in the synthesis of L-homophenylalanine, reactions were carried out on an analytical scale using wild-type AspBDC (19 mg/mL) and the variant (0.07 mg/mL) as the biocatalyst, respectively (Fig. 3). Although the biocatalyst load of the variant was merely 0.3% that of the wild-type enzyme, the reaction catalysed by the variant still significantly outperformed that of the wild-type AspBDC, and delivered an analytic product yield of 58% within 6 h, and the product yield observed with the wild-type enzyme was less than 1%. We next evaluated the synthetic potential of the variant for the enzymatic synthesis of L-homophenylalanine on preparative scale. The reaction (15 mL) was performed with 10 mM 3(*R*)-benzyl-L-aspartate as the substrate, good product yield (50%) was obtained after 5 h, and by supplementing equal amount of biocatalyst at 5 h, the product yield was further increased to 75% after 12 h biotransformation (Fig. S10).

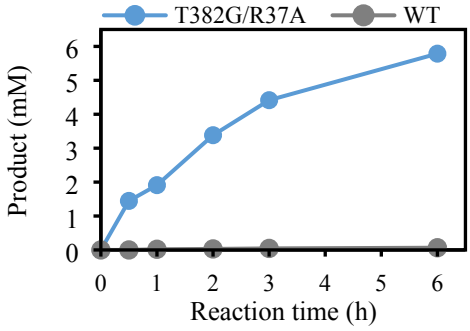


Fig. 3 Reaction progress curves for the decarboxylation of 3(*R*)-benzyl-L-aspartate catalyzed by wild-type AspBDC and mutant R37A/T382G.

In conclusion, the specific activity of AspBDC towards 3(*R*)-benzyl-L-aspartate was significantly improved by four orders of magnitude through structure-guided engineering, enabling the novel strategy for enzymatic synthesis of L-homophenylalanine. This work has demonstrated the potential of engineering AspBDC for unnatural L-amino acids synthesis. Structural studies of AspBDC R37A/T382G aiming to probe the origin of this improvement in catalytic activity and further widen the substrate scope by rational design are currently under progress.

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Conflicts of interest

There are no conflicts to declare.

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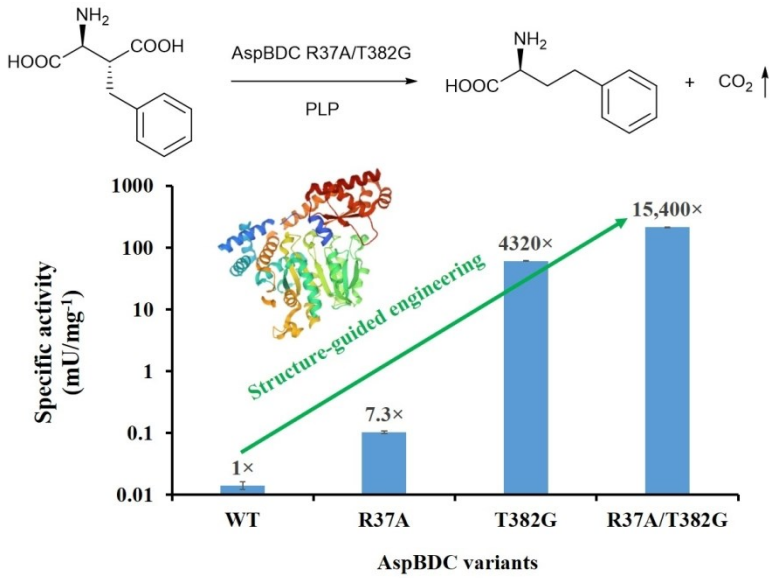

Structure-guided engineering improved the specific activity of AspBDC by 15,400-fold towards 3(*R*)-benzyl-L-aspartate, enabling the novel enzymatic synthesis of L-homophenylalanine

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