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To be cited as: *ChemBioChem* 10.1002/cbic.202000488

Link to VoR: https://doi.org/10.1002/cbic.202000488

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A single-transaminase-catalyzed biocatalytic cascade for efficient asymmetric synthesis of L-phosphinothricin

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Abstract: A single-transaminase-catalyzed biocatalytic cascade was developed by employing a desired biocatalyst ATA-117-Rd11 that showed high activity toward 2-oxo-4-[(hydroxy)(methyl)phosphinoyl] butyric acid (PPO) and α-ketoglutarate, and low activity against pyruvate. The cascade successfully promotes highly asymmetric amination reaction for the synthesis of L-phosphinothricin (L-PPT) with high conversion (>95%) and > 99% *e.e.*. In the scale-up experiment, using 10 kg pre-frozen *E. coli* cells harboring ATA-117-Rd11 as catalyst, 80 kg PPO was converted to ≈70 kg L-PPT after 24 hours with a high *e.e.* value (>99%).

Phosphinothricin (2-amino-4-[(hydroxy)(methyl)phosphonoyl] butanoic acid, PPT, also known as glufosinate) is spectral, low toxicity, non-selective herbicide that is widely used for genetically modified crops.[1] However, the currently commercial PPT is a racemic mixture containing about 50% D-PPT that is no herbicidal activity and harmful to the environment. Therefore, it is necessary to develop a feasible route to synthesize optically pure L-PPT. Non-enzymatic synthesis of L-PPT by asymmetric hydrogenation,[2] Strecker synthesis,[3] and Michael addition,[4] has been intensively studied. But, it often raised the problems of complex synthesis process, high cost of catalyst, and environmental pollution.[5] Recently, the biological method of synthesizing L-PPT has attracted more and more attention due to its advantages of low impact on the environmental, high atom utilization rate, and high product yield, in which amidase,[6] nitrilase,[7] deacetylase,[8] amino acid dehydrogenase[9] and transaminase[10] were involved.

Transaminase (TA, EC 2.6.1.X) catalyzes a transamination reaction between an amino acid and an α-keto acid, which is important in the synthesis of natural and unnatural amino acids.[11] Thus, the application of transaminase to the synthesis of L-PPT (**1b**) from 2-oxo-4-[(hydroxy)(methyl)phosphinoyl] butyric acid (PPO, **1a**) is a promising route (Figure 1A). However, organic synthesis by TAs usually suffer from the unfavorable reaction equilibrium so that net conversion of **1a** to L-PPT is thermodynamically limited and very difficult to reach >85%.[12] In order to shift the reaction balance to L-PPT and increase the conversion of transamination, one way is to use a large number of amino donors or smart amino donors.[12] For instance, in the TA-catalyzed reaction, Schulz et al utilized a quadruple equivalent of an amino donor (toward **1a**) for synthesis of **1b**, which yielded a conversion of 90% with an *e.e.* >99%.[13] However, there are still two major drawbacks of this strategy. The highly loaded amino donor may cause a substrate inhibition, and a large amount of unconverted amino donor will increase the difficulty of product post-processing.

To break these bottlenecks, a biocatalytic cascade was developed by coupling of two transaminases that are an aspartate transaminase from *Bacillus stearothermophilus* (*Asp*TA) and a glutamate: oxaloacetate transaminase (GOT) (Figure 1B). In the cascade, glutamate was used as an amino donor, and aspartate (**1d**) was used as co-substrate that was converted into α-ketoglutarate (**1c**). **1c** is easily decarboxylated to pyruvate in the presence of metal ions.[14] This biocatalytic cascade, using 1.2 equivalent **1d**, reached a high reaction conversion (85%), and a 96% *e.e.* of **1b**.[15] Since only few amino donor was remained in the reaction system, the post-processing is relatively simple. In spite of the success of *Asp*TA-GOT coupled cascade, two different transaminases were required in the cascade, resulting high cost of biocatalyst, and redundant fermentation process.[16] Another disadvantage of the *Asp*TA-GOT coupled cascade is that the pyruvate formed in the decarboxylation step can lead to a side reaction where **1f** is formed by transamination, which limited the maximum conversion and may complicate the product purification.

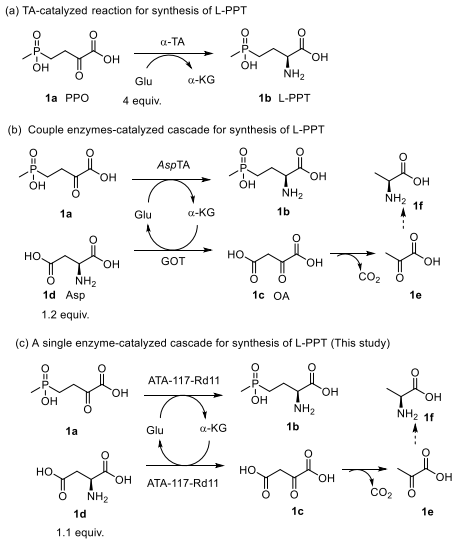

Figure 1. The scheme of biosynthesis of L-PPT. (A) a single-TA-catalyzed reaction for synthesis of L-PPT, (B) couple enzymes (TA and GOT)-catalyzed cascade for synthesis of L-PPT, (C) a single enzyme-catalyzed cascade for synthesis of L-PPT

In this work, to solve the above-mentioned problems, we developed a single-transaminase-catalyzed cascade, in which a desired transaminase displaying high activity toward PPO and α-KG and low activity toward pyruvate was obtained by virtual screening and experiments. Firstly, 50 different TAs, sharing 20%-90% similarities, were selected from NCBI database by a three-step procedure (see method section in supporting informatino). Virtual screening was performed by homology modeling and molecular docking toward PPO, α-KG and pyruvate. Table S1 summarized the docked binding energy of these 50 transaminases toward the three ligands. Only 10 transaminases showed low binding energy (<-5 kcal/mol) to both PPO and α-KG, which indicated that the desired biocatalyst is likely to be found from the 10 TAs. Among them, ATA-117-Rd11 showed the lowest binding energy, which is -5.9 and -6.7 kcal/mol to PPO and α-KG, respectively. The evolutionary conservation of the active site region of the 50 TAs were evaluated by ConSurf server (colored turquoise to maroon according to the conservation grades)[17] The conservation scores of most residues in active site were higher than 6, which indicated that they were conservative (Figure S1). Furthermore, a higher binding energy (>-4.5 kcal/mol) of pyruvate was observed from 9 out of these 10 transaminases, suggesting low possibility for pyruvate binding. Finally, the 9 transaminases were selected for the further experimental investigation.

The three ligands were used as substrate for testing TA activities. All the selected nine TAs exhibited high activities of more than 50 U/g toward **1a** (Table 1), showing good agreement with the docking results that, the binding energies between 1a and the nine TAs were all lower than -5 kcal/mol. Among them, two TAs (ATA117-Rd11 and ABAT1) displayed the highest activity of >100 U/g. Using 20 mM **1a** as substrate and glutamate as amino donor, more than 85% conversion was reached by 2 h reaction (91.7% for ATA-117-Rd11; 86.0% for ABAT1), yielding **1b** with high *e.e.* (>99%). They also showed the highest activity toward α-KG (341 U/g for ATA-117-Rd11, 199 U/g for ABAT1). Importantly, the ATA117-Rd11 showed the lowest activity toward pyruvate (only 3 U/g), which indicated that the side-reaction may not be detectable. Therefore, ATA-117-Rd11 was selected as a desired biocatalyst for development of the single-TA-catalyzed cascade. Interestingly, ATA-117-Rd11 was a highly engineered transaminase for the biomanufacture of sitaglipitin (a widely used therapeutic agent for type-2 diabetes) by 11 rounds of directed evolution, in which its substrate-binding pocket was enlarged to extend its substrate specificity.[12]

Table 1. The activities of selected enzymes toward PPO, α-KG and pyruvate

Entry	Enzyme	Genbank	Activity toward PPO (U/g)	Activity toward α-KG (U/g)	Activity toward pyruvate (U/g)	Conversion of PPO (%) [a]	*e.e.* of L-PPT (%) [b]
1	ATA-117-Rd11	3WWJ_A	115±4	341±12	3±0.2	91.7	>99
2	ATA-117	3WWI_A	83±3	145±9	6±0.5	75.9	98.4
3	*R*-selective transaminase	WP_010910285.1	75±2	143±2	20±1.1	36.2	92.3
4	AT-ωATA	EAQ46075.1	65±2	51±3	13±0.9	36.9	99.2
5	Aminotransferase	WP_011462786.1	74±3	75±4	12±1.2	21.8	98.1
6	Amine transaminase (fold IV)	WP_011349475.1	83±4	114±8	13±0.2	20.7	98.3
7	ABAT1 (4-aminobutyrate aminotransferase)	AKK18527.2	108±4	199±10	19±0.2	86.0	96.8
8	ABAT2 (4-aminobutyrate aminotransferase)	WP_001095559.1	87±5	80±6	17±0.6	46.1	94.2
9	ABAT3 (4-aminobutyrate aminotransferase)	WP_021700965.1	79±4	201±11	16±0.2	58.2	95.3

[a] $\text{Conv.}(\text{PPO}) = \frac{[\text{D-PPT}]+[\text{L-PPT}]}{[\text{PPO}]_0-[\text{PPO}]} \times 100\%$; [b] $e.e. = \frac{[\text{L-PPT}]-[\text{D-PPT}]}{[\text{L-PPT}]+[\text{D-PPT}]} \times 100\%$; [D-PPT], [L-PPT] and [PPO] are the concentrations of D-PPT, L-PPT and PPO after reaction, respectively, and $[\text{PPO}]_0$ was the initial concentration of PPO.

Since the ATA-117-Rd11 exhibited higher activities toward PPO and α-KG than other selected TAs, and low activity toward pyruvate, ATA-117-Rd11 was employed as the single biocatalyst to develop the enzyme cascade. As the principle shown in **Figure 1C**, glutamate acid was used as the amino donor for supplying the amino group to PPO and the L-aspartate acid was utilized as co-substrate to regenerate the amino donor. The substrate PPO and the amino donor was initially set as 100 mM and 20 mM, respectively. The concentration of co-substrate L-aspartic acid was optimized from 100 mM to 300 mM. The highest conversion was observed when the L-aspartic acid concentration was higher than 110 mM. Furthermore, when the substrate concentration was increased to 200 mM, the optimal co-substrate concentration was 220 mM. Taken together, the optimal ratio between **1d** and **1a** was 1.1, which is substantially lower than that used in TA-catalyzed reaction (> 4 equivalent amino donor).

The effects of pH and temperature on the single-TA-catalyzed cascade were tested in range of 30 to 60 °C (temperature) and 6.0 to 11.0 (pH). The conversion was increased from 15.6% (30 °C) to 83.9% (40 °C), but decreased when the temperature is higher than 40 °C. (Figure S2A). While, when the pH was lower than 7.0 or higher than 8.5, the conversion was lower than 80%.

The optimal pH value was selected as 8.0 in Tris-HCl buffer (Figure S2B). Furthermore, the enzyme cascade was performed in a whole cell format. The cell concentration (DCW: 0.5-5 g/L) is optimized and the optimal lyophilized cell concentration is 3 g/L. (Figure S2C). Moreover, since ATA-117-Rd11 is PLP-dependent, it is necessary to optimize the PLP concentration. When the PLP concentration is higher than 0.2 mM (toward 3 g/L cells), the conversion of **1a** is higher than 80% (Figure S2D). The further increase in PLP concentration (0.2 mM) did not enhance the conversion of PPO in this cascade.

To evaluate the potential of this cascade for synthesis of L-PPT, different concentrations of PPO were used as substrate and lyophilized *E. coli* cells harboring ATA-117-Rd11 used as biocatalyst. Under optimized conditions (pH 8.0 and 40 °C), the conversion of 100 mM **1a** was almost complete (conversion > 96%, >99% *e.e.*) within 24 hours using 3 $g \cdot L^{-1}$ lyophilized *E. coli* co-expressing ATA-117-Rd11, and the side-product alanine was undetectable. To explore the performance of this cascade, the substrate concentration was gradually increased from 100 mM to 200 mM and 500 mM, and the ammonium and glucose concentrations were also increased to the same extent. As shown in Figure 2, the conversion of 200 mM and 500 mM **1a** (89.04 $g \cdot L^{-1}$) reached 95% and 92% after 24 h reaction with the same amount of biocatalyst (3 $g \cdot L^{-1}$ lyophilized cells harboring ATA-117-Rd11), affording an L-PPT with *e.e.*>99%. Compared to the reports, the highest conversion (>95%) was achieved by the ATA-117-Rd11 catalyzed cascade, in which only 3 $g \cdot L^{-1}$ biocatalyst was used with 1.1 equivalent aspartate and 0.2 equivalent glutamate (in total 1.3 equivalent amines). Due to unfavorable thermodynamic equilibrium, the higher amount amino donor (> 4 equivalent) should be employed in TA catalyzed amination reaction toward 20 g/L PPO (Table 2). Therefore, the single-TA-catalyzed cascade displays a great potential in biocatalytic synthesis of L-PPT.

Table 2. Comparison of the cascade with other reported reactions for production of L-PPT

Enzyme	Substrate	Substrate loading (g/L)	Catalyst loading (g/L)	Amino donor	Time (h)	Conversion (%)	Reference
Amidase	a PPT derivative[d]	150	0.03[a]	-	6.8	49.7	[18]
α-Transaminase (single enzyme reaction)	**1a**	20	34[b]	>4 equiv. glutamate	14	91.2	[19]
*Asp*TA and GOT (Coupled enzyme cascade)	**1a**	89	0.08 for TA[a], 0.02 for GOT[a]	0.2 equiv. glutamate, 1.2 equiv. **1d**	24	Up to 85	[15]
ATA-117-Rd11 (single enzyme cascade)	**1a**	89	3[c]	0.2 equiv. glutamate, 1.1 equiv. **1d**	24	92.4	This study

[a] purified enzyme used as catalyst.
[b] The immobilized α-TA used as catalyst.
[c] The catalyst loading as dry cell weight.
[d] The rac-4-(hydroxy(methyl)phosphoryl)-2-(2-phenylacetamido) butanoic acid (a PPT derivative) used as substrate.

In a kilogram-scale experiment, a 5-ton fermenter (Figure S3) was used to produce *E. coli* cells harboring ATA-117-Rd11, which yielded approximately 150 kg *E. coli* wet cells (see supporting information for the fermenter settings in deail). In a 1000 L reaction mixture, 80 kg PPO was mixed with 10 kg pre-frozen *E. coli* cells (wet cells) harboring ATA-117-Rd11 in Tris-HCl buffer (pH = 8.0). During the 24 h reaction, the pH was automatically adjusted to 8.0 by titrating with $NH_3 \cdot H_2O$ or HCl. The samples were taken from the bioreactor every 4 h.The reaction process was monitored by the determination of residual PPO concentration and generated PPT using HPLC, as previously described.[11] The formed product was purified by an eight-step purification process shown in supporting information, [20] and the total yield was about 81% (from ≈440 mol PPO to ≈ 360 mol L-PPT). Finally, approximately 70 kg of L-PPT was obtained and characterized using HPLC (purity = 92.3% with 99% *e.e.* (Figure S4), ESI-MS (*m/z*= 180.0437, calcd. for $C_5H_{11}NO_4P^-[M]^-$: 180.0426, Figure S5) and NMR analysis (Figure S6-S7).

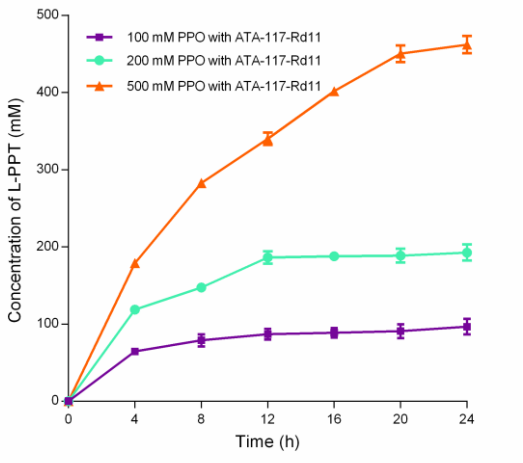

Figure 2. Biosynthesis of L-PPT using ATA-117-Rd11 catalyzed cascade toward 100, 200, and 500 mM PPO.

In summary, we demonstrated a single-transaminase catalyzed cascade that enables the high conversion of high concentration PPO. The *e.e.* of L-PPT is higher than 99% and side-product alanine is almost undetectable. In the kilogram-scale reaction, approximately 70 kg L-PPT was yielded after 24 h with a high *e.e.* value (>99%) when 80 kg PPO was loaded as substrate.

Acknowledgements

This work was financially supported by Natural Science Foundation of China (21978268, 31700693 and 31970046) and the National Defense Science and Technology Innovation Zone Foundation of China.

Conflict of interest

The authors declare no conflicts of interest.

Keywords: L-phosphinothricin • transaminase • enzyme cascade • virtual screening • biocatalysis

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10.1002/cbic.202000488
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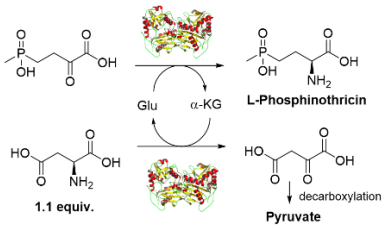

A single-transaminase-catalyzed cascade for asymmetric synthesis of L-phosphinothricin

Asymmetric synthesis of L-phosphinothricin: A single-transaminase-catalyzed biocatalytic cascade for L-phosphinothricin synthesis was developed by employing a desired biocatalyst ATA-117-Rd11. High conversion, high *e.e.* of product and low side reaction was achieved by employing only 1.3 equivalent amine donors. In a kilogram-scale experiment, ATA-117-Rd11, about 70 kg L-phosphinothricin (e.e. >99%) was yielded from 80 kg PPO within 24 hours.