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Novel Access to D-Valine Synthesis by Improved Variants of Bacterial Cyclohexylamine Oxidase

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Abstract: Chemo-enzymatic deracemization was applied to prepare D-valine from racemic valine ethyl ester or L-valine ethyl ester in high isolated yield (up to 95%) and excellent optical purity (ee > 99%) employing a newly evolved cyclohexylamine oxidase (CHAO) variant Y321I/M226T that exhibited 30 times higher catalytic efficiency than the wild type CHAO. Interestingly, CHAO and its variants showed opposite enantioselectivity for valine ethyl ester and phenylalanine ethyl ester.

D-Amino acids are important building blocks in pharmaceuticals, agrochemicals and food additives. [1] D-Valine in particular, exists widely in a variety of agricultural pesticides, semisynthetic veterinary antibiotics and pharmaceutical drugs, such as fluvalinate, valnemulin, penicillamine, actinomycin D, fungisporin and valinomycin. [2] It can also selectively inhibit fibroblasts proliferation in cell culture. [3] Therefore, investigation of efficient synthetic methods for D-valine is of high importance. Current state of the art approaches for the synthesis of D-valine includes chemical resolution, chemical asymmetric synthesis, and enzymatic transformation, each has its shortcomings. [2] Chemical resolution is commonly used in industry, [4] but it requires expensive chiral resolving agents and complex experimental process. Chiral auxiliaries, such as (*R*)- or (*S*)-4-phenyl-2-oxazolidinone, in chemical asymmetric synthesis are expensive. [5] Compared to chemical methods, enzymatic preparation of D-valine is an environmentally friendly process with high stereoselectivity and mild reaction conditions. To date, many enzymatic methods have been developed, for example, asymmetric degradation of DL-valine by L-amino acid oxidase; [6] stereoselective hydrolysis by D-stereospecific amidohydrolase; [7] specific hydrolysis of DL-5-isopropylhydantoin by D-hydrantoinase coupled with *N*-carbamoyl-D-amino acid amidohydrolase; [8] and asymmetric synthesis from 2-oxo-3-methylbutyric acid by D-amino acid aminotransferase [9] or D-amino acid dehydrogenase. [10]

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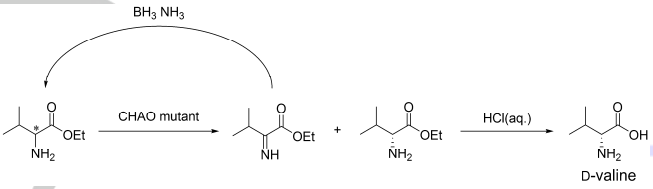
Turner *et al.* reported a deracemization strategy for the preparation of primary, [11] secondary, [12] and tertiary amines [13] as well as substituted pyrrolidines [14] employing monoamine oxidase from *Aspergillus niger* (MAO-N) mutants. Similarly, based on the substrate profile and crystal structure analysis of cyclohexylamine oxidase (CHAO) from *Brevibacterium oxydans* strain IH-35A by Lau *et al.*, [15] we have extended the substrate scope of CHAO to more bulky amines by protein engineering and examined the biocatalytic potential of these CHAO mutants. [16] Although this chemo-enzymatic deracemization strategy has been demonstrated as an effective approach for the preparation of optically pure amines, it has not been applied in deracemisation of racemic amino acid esters for the preparation of D-amino acids such as D-valine. Herein we explored the feasibility of preparing D-valine from racemic valine ethyl ester or L-valine ethyl ester employing the wild type CHAO (wt CHAO) and its genetic variants (**Scheme 1**). The improved CHAO mutants were also tested toward other amino acid derivatives to determine their activity and enantioselectivity.



Scheme 1. Deracemization of racemate ethyl valinate by employing CHAO mutant and $NH_3{\cdot}BH_3$.

Previously, the application of CHAO in biocatalysis has been focused on the deracemisation of primary and secondary amines to produce chiral amine building blocks in high yield and enantiomeric excess (*ee*). [16] In this work, we initially found that L-valine ethyl ester was a better substrate compared to L-valine, but the activity of the wt CHAO was not high (0.9 U/mg).

We thus sought for CHAO variants that may have an improved activity by modelling L-valine ethyl ester into the active site of CHAO. [15a] Effectively, 11 amino acid residues (F88, T198 L199, M226, Q233, Y321, F351, L353, F368, P422, Y459) lining the binding pocket of CHAO were selected and mutated to six typical amino acids (Ala, Ile, Phe, Trp, Thr, Tyr) by site-specific mutagenesis (**Figure S1** in the Supporting Information). The resultant mutants were assayed against L-valine ethyl ester. [17] As a result, four substitutions (T198I, L199F, M226T and Y321I) were found to possess a significantly improved catalytic activity compared to the wt CHAO enzyme (**Table S1** in the Supporting Information). In order to obtain better mutants toward L-valine ethyl ester, iterative mutagenesis was performed on the best mutant Y321I. Consequently, three double mutants, Y321I/T198I, Y321I/L199F, and Y321I/M226T were created and assayed. The best mutant Y321I/M226T was selected as template for the last

round of mutagenesis resulting in Y321I/M226T/T198I, Y321I/M226T/L199F, and Y321I/M226T/T198I/L199F.

The kinetic parameters of the wt CHAO and its variants against substrate L-valine ethyl ester showed that three of the single amino acid substitutions (T198I, L199F and Y321I) displayed an improved catalytic efficiency (2-13 folds) whereas that of M226T was marginal compared to the wt CHAO (**Table 1**). According to the structure of CHAO, [15a] M226 is located at the entrance of the tunnel distal to the active site. Hence the M226T mutation may have a low influence on the activity. Interestingly, T198I, L199F, and Y321I all have high k_{cat} values but their high K_m in 5 mM range resulted in a sharp decline of catalytic efficiency. In contrast, the catalytic efficiency of the double mutant Y321I/M226T was 30 fold higher than the wt CHAO. Interestingly, iterative mutagenesis of M226T and Y321I yielded triple (Y321I/M226T/T198I) and quadruple mutants (Y321I/M226T/T198I/L199F) that possessed higher k_{cat} than other mutants, but their high K_m values resulted in lower catalytic efficiencies.

Table 1. Kinetic parameters of wt CHAO and mutants toward L-valine ethyl ester.

Enzyme[a]	K_m (mM)	k_{cat} (min^{-1})	k_{cat} / Km ($min^{-1}mM^{-1}$)
wt CHAO	7.5	38.5	5.1
T198I	5.0	65.2	13.0
L199F	5.2	55.3	10.6
M226T	5.1	33.3	6.5
Y321I	1.1	75.0	66.4
Y321I/ T198I	1.8	130.7	72.2
Y321I/ M226T	1.2	186.2	152.2
Y321I/ M226T/T198I	2.7	212.6	77.5
Y321I/M226T/T198I/F199F	5.8	221.4	38.2

[a] Y321I/L199F and Y321I/ M226T/L199F failed to be expressed

Then we applied the Turner-deracemization technique [11, 13b, 13d, 18] to racemic valine ethyl ester and L-valine ethyl ester by using *E. coli* whole cells of CHAO and its double mutant Y321I/M226T in combination with the nonselective chemical reducing agent ($NH_3 \cdot BH_3$) at pH 6.5 and 30 °C. The time course study (**Figure 1**) showed that the reaction was fast and linear in the first two hours, resulting in up to 99% and 77% *ee* for mutant Y321I/M226T. The deracemization process of L-valine ethyl ester plateaued in the next one hour, with a modest increase in *ee* to 99%. As a result, D-valine ethyl ester was isolated after 4 h, and hydrolyzed to D-valine by 2 mol/L of HCl solution. After removal of the solvent, D-valine hydrochloride was obtained in 95% and 91% yield. Although the reaction was also fast and linear in the first two hours for CHAO, resulting in 58% and 19% ee, respectively, the *ee* leveled out at a lower value (62% and 64% ee).

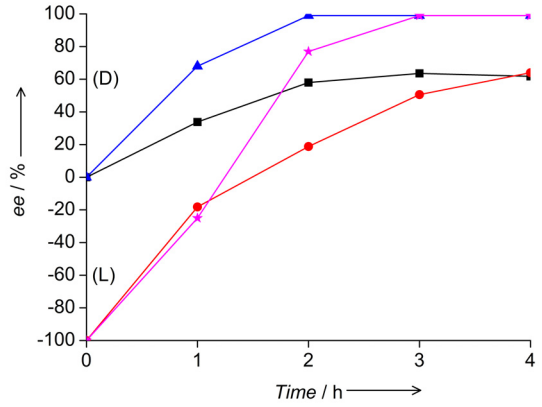


Figure 1. Time course of deracemization of D/L-valine ethyl ester and L-valine ethyl ester by employing CHAO or its mutant Y321I/M226T and borane–ammonia complex. D/L-valine ethyl ester with CHAO (■) or its mutant Y321I/M226T(▲), L-valine ethyl ester with CHAO (●) or its mutant Y321I/M226T(★).

To shed light on the possible molecular mechanism of the catalysis, we performed docking experiments. Docking D/L-valine ethyl ester to the wt CHAO generated several binding poses. In the D-docking position, the Cα and NH of valine ethyl ester are 4.31 Å and 4.75 Å from the N (5) of FAD. The O (4) of FAD forms a hydrogen-bonding interaction with NH of valine ethyl ester, so the NH moved and deviated from the above of N (5) of FAD as shown in **Figure 2 (a)**. In the L-docking position (**Figure 2 (b)**), the Cα and NH of valine ethyl ester are 4.22 Å and 4.44 Å from the N (5) of FAD. Y321 has a hydrogen-bonding interaction with NH of valine ethyl ester, so it places NH right above the N (5) of FAD. Therefore the L-enantiomer is favored, whereas the activity toward D-enantiomer is below detection.

Docking of D-valine ethyl ester to the mutant Y321I/M226T showed a similar result as the wt CHAO that gave negligible activity (**Figure 2 (c)**). The best docking result was obtained by L-valine ethyl ester (**Figure 2 (d)**). Here, the Cα and NH of L-valine ethyl ester are 3.75 Å and 3.13 Å from the N (5) of FAD. The hydrogen-bonding interaction formed between O (4), N (5) of FAD and NH of L-valine ethyl ester with other interaction force pull them closer and these interactions anchor the L-valine ethyl ester and promote stability. These attributes evidently led the

mutant to show higher activity and affinity to the substrate with an overall 30-fold increase in catalytic efficiency.

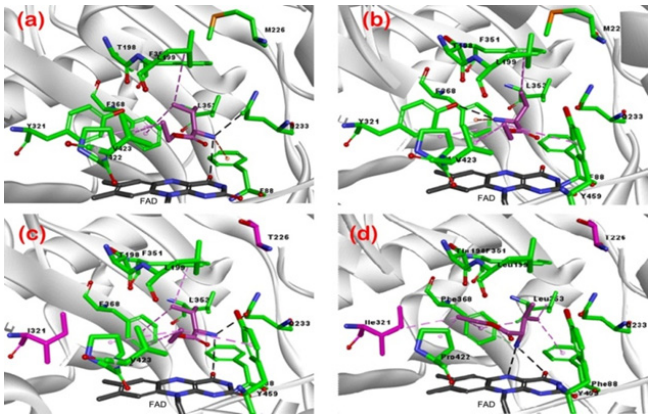


Figure 2. Docking structure of (a) D-valine ethyl ester and (b) L-valine ethyl ester in the active site of wt CHAO; Docking structure of (c) D-valine ethyl ester and (d) L-valine ethyl ester in the active site of mutant Y321I/M226T.

Table 2. Specific activity (U/mg)[a] of wt CHAO and mutants.

Substrate	CHAO	Y321I	Y321I/M226T
D-tyrosine ethyl ester	Trace[b]	0.014	0.019
L-tyrosine ethyl ester	0.029	ND[c]	0.016
D-phenylalanine ethyl ester	0.188	0.705	1.043
L-phenylalanine ethyl ester	0.018	0.013	ND[c]
D-alanine ethyl ester	0.022	0.012	ND[c]
L-alanine ethyl ester	0.117	0.106	0.141
D-prolinamide	ND[c]	ND[c]	ND[c]
L-prolinamide	ND[c]	ND[c]	ND[c]
D-valine ammonia amide	ND[c]	Trace[b]	ND[c]
L-valine ammonia amide	0.011	0.016	0.03
D-phenylglycinamide	0.023	0.021	0.034
L-phenylglycinamide	Trace[b]	Trace[b]	0.012
L-2- aminobutanamide	ND[c]	ND[c]	ND[c]
D-2-aminobutanamide	0.016	0.016	0.022

[a] One enzyme unit (U) was defined as the amount of enzyme that produced 1 μmol of hydrogen peroxide per minute. [b] Trace: activity below 0.01 U/mg. [c] ND: not detected

A series of amino acid derivatives were examined as the substrates of the wt CHAO and its mutants Y321I and Y321I/M226T. Each substrate was assayed individually at 10 mM substrate concentration with the purified enzymes (**Table 2**). In the case of D-phenylalanine ethyl ester the activity of the double mutant Y321I/M226T improved significantly compared to those of the wt CHAO and mutant Y321I. Interestingly, wt CHAO and Y321I had inverse enanioselectivity toward tyrosine ethyl ester, but Y321I/M226T showed no selectivity. It is possible that residues lining the active site and entrance tunnel can influence enantioselectivity. In all cases, there was no activity towards the enantiomers of prolinamide. The enantioselectivity of both mutants toward phenylalanine ethyl ester and phenylglycinamide was D-enantiomer. In contrast, L-enantiomer was the selectivity of the mutants toward alanine ethyl ester and valine ammonia amide. These results showed that the enzymes displayed opposite enantioselectivity toward aromatic compounds.

In order to further confirm the enantioselectivity of mutant Y321I/M226T toward phenylalanine ethyl ester, deracemization of racemic phenylalanine ethyl ester was performed by employing mutant Y321I/M226T and borane-ammonia complex in 10 mL reaction. The results indicated that L-phenylalanine was obtained in up to 99% *ee* value within 8 h (**Figure S6 and S7** in the Supporting Information).

To elucidate the possible basis for the dramatic reversal of enantioselectivity, protein–ligand docking simulations using the structure of mutant Y321I/M226T indicated that D-phenylalanine ethyl ester adopted a binding mode where the benzene ring pointed out of the active site, whereas the benzene ring of L-phenylalanine ethyl ester pointed toward the active site (**Figure 3**). The latter state resulted in an increase of distance between NH of phenylalanine ethyl ester and N (5) of FAD from D-phenylalanine ethyl to L-phenylalanine ethyl. Consequently, the benzene ring was postulated to exert an important influence on the enantioselectivity of the enzyme. Valinamide and 2-aminobutanamide have the similar structure but in fact the enzyme has opposite enantioselectivity, it is an interesting result and worth further research.

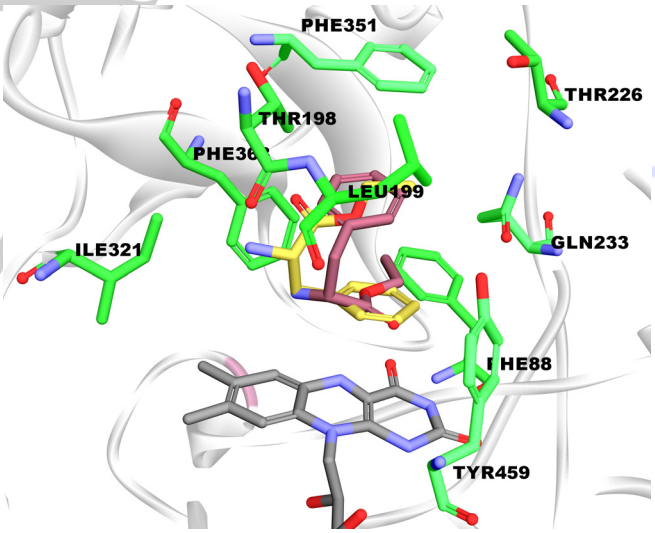


Figure 3. Docked structures of D-phenylalanine ethyl ester (dull-red) and L-phenylalanine ethyl ester (yellow).

In summary, a new enzyme variant of the flavoprotein CHAO was found useful for the synthesis of D-valine in high yield and enantiomerically pure form via the deracemization and stereoinversion of valine ethyl ester properties that are otherwise difficult to obtain by previously reported enzymatic methods. In addition, CHAO and its variants showed opposite enantioselectivity for valine ethyl ester and phenylalanine ethyl ester. Further protein engineering of CHAO is underway in our

laboratory to improve its enantioselectivity and activity towards other amino acid derivatives.

Acknowledgements

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Keywords: Deracemization • D-Valine • Monoamine Oxidase • Stereoinversion • D-Amino Acids

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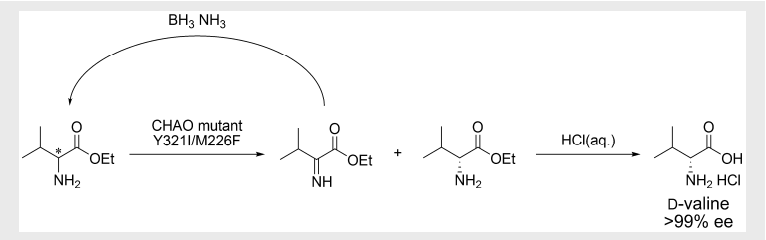

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