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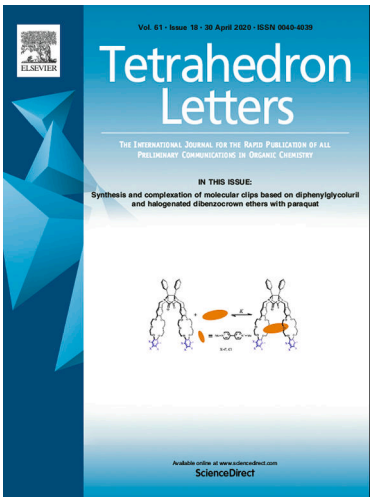

Enhancing Enantioselectivity of *Candida antarctica* Lipase B towards Chiral *sec*-Alcohols Bearing Small Substituents Through Hijacking Sequence of A Homolog

Seonghyeon Yi, Seongsoon Park

PII: S0040-4039(21)00403-2
DOI: https://doi.org/10.1016/j.tetlet.2021.153186
Reference: TETL 153186

To appear in: *Tetrahedron Letters*

Received Date: 25 March 2021
Revised Date: 15 May 2021
Accepted Date: 17 May 2021

Please cite this article as: Yi, S., Park, S., Enhancing Enantioselectivity of *Candida antarctica* Lipase B towards Chiral *sec*-Alcohols Bearing Small Substituents Through Hijacking Sequence of A Homolog, *Tetrahedron Letters* (2021), doi: https://doi.org/10.1016/j.tetlet.2021.153186

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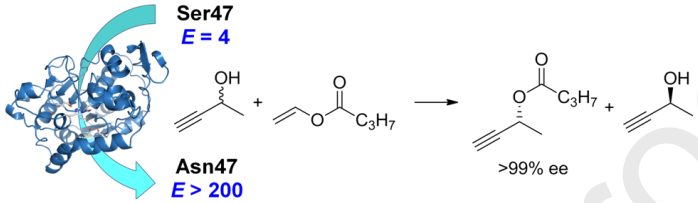

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Enhancing Enantioselectivity of *Candida antarctica* Lipase B towards Chiral *sec*-Alcohols Bearing Small Substituents Through Hijacking Sequence of A Homolog

Seonghyeon Yi and Seongsoon Park*

Department of Chemistry, Center for NanoBio Applied Technology, Sungshin Women's University, Seoul 01133, Republic of Korea

ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
Candida antarctica lipase B
homologous enzyme
kinetic resolution
lipase
protein engineering

ABSTRACT

Candida antarctica lipase B (CAL-B) exhibits extraordinary enantioselectivity towards most chiral *sec*-alcohols but not towards *sec*-alcohols bearing substituents smaller than a propyl group (*i.e.*, (±)-but-3-yn-2-ol (E = 4) and (±)-butan-2-ol (E = 7)). Previously, we reported a homologous enzyme (lipase from *Pseudozyma brasiliensis* GHG001, PBL) of CAL-B, which exhibited high enantioselectivity of CAL-B towards (±)-but-3-yn-2-ol (E > 200). Based on the result, we hypothesized that the comparison of their local sequence or structure would provide a clue for enhancing the enantioselectivity of CAL-B. In this paper, we report enhancing enantioselectivity of CAL-B towards (±)-but-3-yn-2-ol and (±)-butan-2-ol through the substitution of the local sequence of CAL-B with that of PBL. The sequence-substituted mutant of CAL-B exhibited much higher enantioselectivity towards (±)-but-3-yn-2-ol (E > 200) and (±)-butan-2-ol (E > 25).

Candida antarctica lipase B (CAL-B; also referred to as *Pseudozyma antarctica* lipase B) is one of the most extensively used biocatalysts in the preparation of enantiopure *sec*-alcohols because of its extraordinary enantioselectivity, high chemo-tolerance, and thermo-stability.[1] It has been known that CAL-B is able to efficiently distinguish the difference in the size between a methyl group and a substituent larger than an ethyl group, and thus exhibits high enantioselectivity towards a broad range of *sec*-alcohols (mostly E > 200; E, enantiomeric ratio defined by Shi *et al*.[2]).[3] However, CAL-B does not obviously exhibit high enantioselectivity towards all *sec*-alcohols. Especially, CAL-B exhibits poor enantioselectivity towards *sec*-alcohol bearing substituents smaller than a propyl group. For instance, the enantiomeric ratios of CAL-B are 7 and 4 towards (±)-butan-2-ol and (±)-but-3-yn-2-ol, respectively, while exhibiting E > 200 toward (±)-pentan-2-ol. To date, it has not been reported to improve enantioselectivity of CAL-B towards *sec*-alcohols bearing small substituents, and thus improving its enantioselectivity towards such *sec*-alcohols has still remained a challenging task.

In addition, enantiopure but-3-yn-2-ol has been used as a useful building block to synthesize various compounds[4] including bioactive compounds, such as cytotoxic butenolide (-)-akolactone,[5] (-)-Enigmazole A, [6] Fenleuton, [7] Frondosin B,[8] HIV protease inhibitor, [9] iodoalynes (+)-pancratistatin, [10] Meayamycin,[11] and tetrahydronaphtyridine.[12] Hence, the preparation of enantiopure but-3-yn-2-ol has garnered much attention, and the effort for identifying or developing a hydrolase to resolve (±)-but-3-yn-2-ol has been continued. However, most hydrolases exhibited low to moderate enantioselectivity (E = 1.0-26) as CAL-B does.[13, 14] To improve enantioselectivity of a hydrolase toward (±)-but-3-yn-2-ol, Bornscheuer and coworkers have employed a combined approach of directed evolution and site-direct mutagenesis on an esterase from *Pseudomonas fluorescens* (PFE).[15] They have improved the enantioselectivity of PFE from E = 3 to 96 through screening over 7,000 clones, but the enantiomeric ratio has not reached to 200. In this paper, we report a successful result for enhancing enantioselectivity of CAL-B towards (±)-but-3-yn-2-ol.

Recently, we have found a homolog (lipase from *Pseudozyma brasiliensis* GHG001, PBL) of CAL-B possessing higher enantioselectivity towards (±)-butan-2-ol (E > 200) and (±)-but-3-yn-2-ol (E = 17), although its catalytic activity is much lower than that of CAL-B.[16, 17] This result was rather surprising and unexpected because PBL possesses a 72% sequence identity with CAL-B, and thus it was expected that they share a common structure and function or selectivity. Hence, we hypothesized that the comparison of local sequences between PBL and CAL-B can provide a clue to improve the enantioselectivity of CAL-B towards (±)-butan-2-ol and (±)-but-3-yn-2-ol.

The enantioselectivity of lipases generally follows the Kazlauskas rule,[18] which provides an estimation of enantioselectivity of a lipase towards chiral *sec*-alcohols (see below Figure 1). The rule is based on the structural features of lipase related to the substrate-binding pockets. CAL-B also follows the rule and exhibit (*R*)-selectivity. CAL-B possesses two

* Corresponding author. Tel.: +82-2-920-7646; e-mail: spark@sungshin.ac.kr

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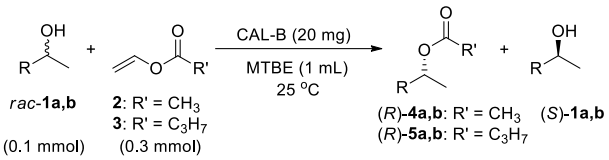


Substrate	Acyl donor	5D-CAL-B			CAL-B-42-47			CAL-B-T42V/S47N			CAL-B-T42V			CAL-B-S47N		
		Time (h)	Conv. (%)	E^b	Time (h)	Conv. (%)	E	Time (h)	Conv. (%)	E	Time (h)	Conv. (%)	E	Time (h)	Conv. (%)	E
 *rac*-**1a**	**2**	8	41.9	5.7	45	38.7	12.9	53	30.5	13.2	44	41.8	6.7	26	33.9	14.0
	3	4	44.2	7.2	35	31.5	25.7	48	40.1	24.9	20	46.3	8.0	20	43.9	30.7
 *rac*-**1b**	**2**	16	52.2	5.4	48	37.6	> 200	60	32.1	> 200	48	37.0	4.4	23	40.4	> 200
	3	4	39.0	4.0	25	40.6	> 200	43	39.4	> 200	20	40.0	3.2	17	45.1	> 200

[a]Reaction condition: enzyme, 20 mg (1%, immobilized on Celite); *rac*-**1a** or **1b**, 0.1 mmol; acyl donor (vinyl acetate **2** or vinyl butanoate **3**), 0.3 mmol, MTBE, 1 mL; 25ºC.

[b]Enantioselectivity was represented by the enantiomeric ratio defined by Sih *et al.*[2]

binding pockets to accept two different substituents (large and small substituents) at the chiral carbon atom of *sec*-alcohol. One is referred to as the large binding pocket, which is the entrance of the substrate, and the other is referred to as the medium binding pocket. The medium binding pocket is located in the deep inside of CAL-B. The rule suggests that the medium binding pocket accepts the smaller substituent rather than the larger one. The previous experimental results demonstrated that CAL-B can accept a substituent smaller than a propyl group.[3] It means that CAL-B cannot distinguish the difference in the size between a methyl group and an ethyl or acetylene group. Hence, we hypothesized that the difference of the sequences corresponding to the medium binding pockets between PBL and CAL-B would account for their distinct enantioselectivity. The medium binding pocket of CAL-B is composed of Thr40, Thr42, Ser47, and Trp104, while the corresponding residues of PBL are Thr40, Val42, Asn47, and Trp104. Because the residue 104 is identical in both enzymes, we compared the sequences of the region between the residues 39-48. In the region between the residues 39 and 48, the residues 42, 43, 45, and 47 of PBL are not identical to those of CAL-B (Figures 1a and S1). Among these residues, the side chains of the two residues 42 and 47 are oriented to the active site (Figure 1b). As an initial approach, we decided to replace the sequence of the residues 42-47 of CAL-B with the corresponding sequence of PBL.

The corresponding gene for the residues 42-47 of PBL was synthesized and introduced to replace the corresponding gene of the template CAL-B (5D-CAL-B), which we previously prepared to improve expression levels with identical enantioselectivity and activity of the wild type CAL-B.[19] The mutant gene was transformed and functionally expressed in *E. coli* (Top10) (Figure S2). After immobilizing the mutant enzyme (CAL-B-42-47) on celite, we have conducted transesterification of (±)-butan-2-ol and (±)-but-3-yn-2-ol with vinyl acetate or vinyl butyrate to determine the enantioselectivity. Interestingly, the mutant enzyme exhibited higher enantioselectivity towards both substrates compared to 5D-CAL-B. The enantioselectivity of the mutant CAL-B-42-47 towards (±)-butan-2-ol and (±)-but-3-yn-2-ol was noticeably improved about 3-4 times (E = 13 or 26) and 40-50 times (E > 200; > 99% ee for (*R*)-**4b** and (*R*)-**5b**, respectively, compared to those of the 5D-CAL-B (E = 6-7 and

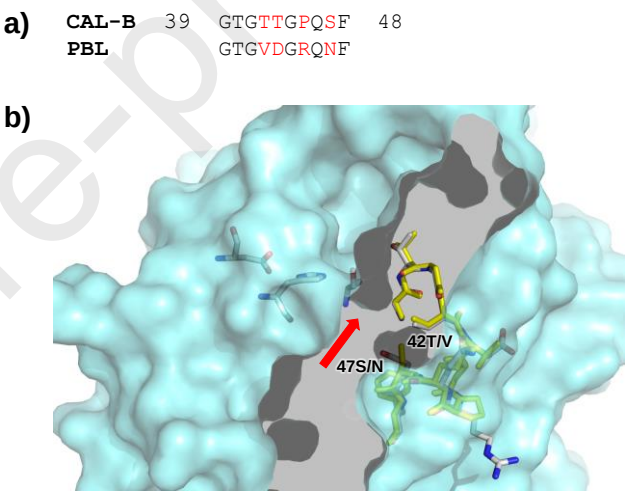
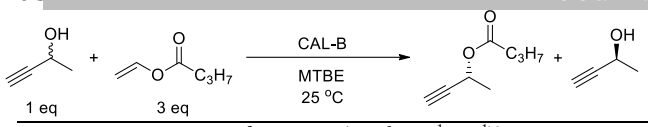


Figure 1. a) The sequence alignment of the stereospecific pocket between CAL-B and PBL. b) A section image of an overlapped structure of CAL-B and PBL. The catalytic triad (Asp187-His224-Ser105) and the residues (39-48) of the stereospecific pocket are represented by a stick model. The stereospecific pocket residues of CAL-B are colored by yellow, while the corresponding residues are colored by atomic elements. The arrow indicates the stereospecificity pocket.

4-5) (Table 1 and Figure S3). The enantioselectivity of CAL-B-42-47 is similar to that of PBL towards (±)-butan-2-ol (E = 13 or 27) and (±)-but-3-yn-2-ol (E >200).[17] The result indicates that altering the stereospecificity pocket influences the enantioselectivity of CAL-B. It is worth noting that this high enantioselectivity (E > 200) toward (±)-but-3-yn-2-ol has not been achieved to date.

Besides, we decided to identify which residue is essential for the improved enantioselectivity. We selected two residues (T42 and S47) of CAL-B as a candidate because the residues are different from those of PBL (V and N, respectively). Moreover, the side chains of the residues are oriented to the active site and contributed to forming the stereospecificity pocket. Hence, we hypothesized that the substitution of these two residues presumably influences the binding of the substrate. We have prepared a double mutant (CAL-B-T42V/S47N) and measured

Tab



	specific activity (μmol·min^{-1}·mg^{-1})[a]	
Enzyme	(*R*)-but-3-yn-2-ol	(*S*)-but-3-yn-2-ol
5D-CAL-B[b]	0.51 ± 0.12[d]	0.25 ± 0.08
CAL-B-S47N[c]	0.21 ± 0.02	0.0018 ± 0.0005

[a]The specific activity was calculated based on the amount of protein.

[b]Reaction condition for 5D-CAL-B: 5D-CAL-B, 10 mg (1%, immobilized on Celite); (*R*)- or (*S*)-but-3-yn-2-ol, 0.1 mmol; vinyl butanoate, 0.3 mmol, MTBE, 4 mL.

[c]Reaction condition for CAL-B-S47N: CAL-B-S47N, 40 mg (1%, immobilized on Celite); (*R*)- or (*S*)-but-3-yn-2-ol, 0.1 mmol; vinyl butanoate, 0.3 mmol, MTBE, 4 mL.

[d]All reactions were conducted at least three times and averaged. The errors were represented by the standard deviation.

the enantioselectivity towards (±)-butan-2-ol as well as (±)-but-3-yn-2-ol. As expected, the double mutant (CAL-B-T42V/S47N) exhibited as high enantioselectivity as CAL-B-42-47 (Table 1). This indicates that either both residues or one of the residues serves the key role for the improved enantioselectivity. Hence, we have prepared two single mutants (CAL-B-T42V and CAL-B-S47N). The two mutant enzymes were successfully expressed, and their enantioselectivity was measured. The two mutants exhibited distinct enantioselectivity. The enantioselectivity of CAL-B-T42V towards (±)-butan-2-ol and (±)-but-3-yn-2-ol was *E* = 7-8 and 3-4, while the enantioselectivity of CAL-B-S47N was *E* = 14-30 and >200, respectively. The result clearly indicates which residue significantly influences the enantioselectivity of CAL-B. Introducing asparagine at the residue 47 noticeably improved the enantioselectivity of CAL-B, especially toward (±)-but-3-yn-2-ol. The significantly improved enantioselectivity toward (±)-but-3-yn-2-ol by introducing asparagine at the residue 47 may be elucidated by one of the following two rationales. One of the clarifications is that the reaction rate for the fast enantiomer by the mutant enzyme increased more than that by the template enzyme. On the other hand, the reaction with the slow enantiomer by the mutant enzyme becomes much slower than that by the template enzyme. This can be clarified by measuring kinetic parameters for both enantiomers. However, obtaining the kinetic parameters for the slow enantiomer is difficult because the reaction rate of the slow enantiomer is too low to be detected at the low concentration of the substrate. Hence, we have compared the specific activity towards the fast and slow enantiomers (Table 2). The specific activities of the template 5D-CAL-B and CAL-B-S47N mutant enzymes have been measured by the reactions towards (*R*)-but-3-yn-2-ol and (*S*)-but-3-yn-2-ol with vinyl butanoate. Since the CAL-B-S47N enzyme is less active than the 5D-CAL-B enzyme (see Table 1), a larger amount of the CAL-B-S47N enzyme was used to obtain reliable reaction rates. Reaction was carried out at 25°C in methyl *tert*-butylether (MTBE). The reaction mixtures were retrieved within a particular interval of time, and the reaction progress was analyzed by gas chromatography (GC). The specific activities were calculated based on the amount of protein used instead of the total amount of immobilized enzymes.

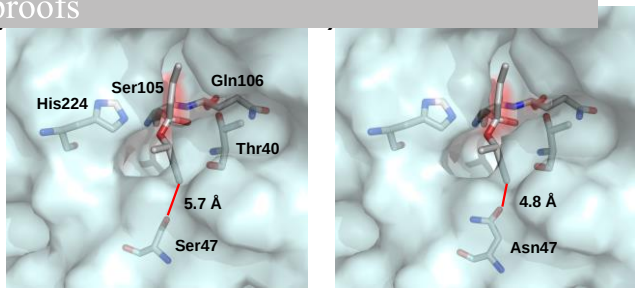


Figure 2. A proposed tetrahedral structure with (*S*)-but-3-yn-2-ol. a) 5D-CAL-B. b) The CAL-B-S47N mutant. The substrate ((*S*)-but-3-yn-2-ol), the catalytic residues (His224 and Ser105), and the oxyanion hole (Thr40 and Gln106) are represented by a stick model. The solid lines represent the distances between the Oγ atom of serine or the Oδ1 atom of asparagine and the terminal carbon atom of the acetylene group.

The template 5D-CAL-B enzyme exhibited about 50% lower specific activity for the slow enantiomer ((*S*)-but-3-yn-2-ol) compared to the fast enantiomer ((*R*)-but-3-yn-2-ol), whereas the CAL-B-S47N enzyme exhibited about 120 times lower specific activity toward the slow enantiomer compared to the fast enantiomer. These results clearly demonstrate that the introduction of asparagine at the residue 47 makes the reaction for the slower enantiomer much slower. Hence, it can be concluded that the binding of the slower enantiomer in the CAL-B-S47N mutant at the transition state is less favorable than that in the template CAL-B.

Molecular modeling was conducted to understand the influence of introducing asparagine at the residue 47 on the improved enantioselectivity of the CAL-B-S47N mutant. After the generation of the tetrahedral intermediates of the wild type CAL-B and CAL-B-S47N mutant enzymes for the slow enantiomer, the structures were energy minimized. However, molecular modeling did not provide a rationale for explaining the molecular basis of the improved enantioselectivity. Energy-minimized structures for both enzymes are not much distinct from each other except the distances between the terminal carbon atom of the acetylene group and the Oγ atom of serine or the Oδ1 atom of asparagine (Table S1 in supporting information). Although the distance between the Oδ1 atom of asparagine and the terminal carbon of the acetylene group (4.8 Å) is shorter than the distance between the Oγ atom of serine and the terminal carbon atom (5.7 Å) (Figure 2), both distances are considered as beyond optimum distance for van der Waals interaction. This implies that asparagine itself may not significantly influence the binding of the slow enantiomer because the side chain of asparagine is still far from the slow enantiomer. One reasonable hypothesis for the improved enantioselectivity is that water molecules may hydrogen bond to the side chain of asparagine and thus interrupt binding of the slow enantiomer at the transition state.[20]

In the current study, we demonstrated that the enantioselectivity of CAL-B towards *sec*-alcohols bearing small substituents can be improved by utilizing information from homologous enzymes of CAL-B. Nature has divergently evolved enzymes and so has produced various homologous enzymes. Each evolved homologous enzyme may possess specific characteristics for certain purposes. This implies that the detailed comparison of the local sequence between homologous enzymes may provide a clue to alter or improve enzyme function. We adapted the sequence information of the medium binding pocket from one of the homologous enzymes of CAL-B and replaced the sequence of CAL-B with that. The created mutant enzyme

(±)-but-3-yn-2-ol. Besides, we identified that the substitution of serine 47 with asparagine is responsible for the improved enantioselectivity through the preparation of a series of mutants. However, improving the enantioselectivity by altering the residue 47 is unlikely to be rationally expected because the residue 47 is located rather far away from the reaction center, although the residue is deep inside of CAL-B. Hence, the current result is not readily achievable by a conventional rational design approach for protein engineering. And also, the directed evolution may not be able to obtain the current results by screening a small number of clones because many results show that most residues identified by directed evolution are found on the surface of an enzyme.[21] Therefore, the current approach would be valuable in improving or altering enzyme functions.

Acknowledgments

This work was supported by the Sungshin Women's University Research Grant of 2018-1-11-045/1.

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Supplementary Material

Supplementary data (experimental details, Figures S1-S3 and Table S1) to this article can be found online.

Hig

- *Candida antarctica* lipase B (CAL-B) exhibits high enantioselectivity towards *sec*-alcohols.
- However, CAL-B shows low enantioselectivity towards *sec*-alcohols bearing small substituents.
- A recently found homologous enzyme of CAL-B exhibited high enantioselectivity.
- Substitution with the local sequence of the homolog may improve the enantioselectivity of CAL-B.
- Enantioselectivity of the created mutant CAL-B was higher than 50 times toward but-3-yn-2-ol.