Enhancing enantioselectivity toward but-3-yn-2-ol and altering the reaction specificity of *Candida antarctica* lipase B

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ABSTRACT

Enhancing enantioselectivity toward but-3-yn-2-ol and altering the reaction specificity of *Candida antarctica* lipase B

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With the increase of environmental concerns, enzyme-catalyzed chemical reactions has been continuously tried and developed because enzymes are generally used in relatively mild conditions, and do not produce byproducts. Moreover, enzymes exhibit excellent regio- and stereoselectivity. *Candida antarctica* lipase B (CAL-B) is one of the most widely used enzymes because it possesses high thermo- and chemo-stability. In this study, protein engineering was employed to enhance the enantioselectivity of CAL-B towards chiral *sec*-alcohols bearing small substituents (*i.e.* butan-2-ol or but-3-yn-2-ol) and to introduce an epoxidase activity to CAL-B.

First, the enantioselectivity of CAL-B was improved based on an information from homologous search. Wild-type CAL-B has been known

to have low enantioselectivity towards *sec*-alcohols with smaller substituents than a propyl group. Previously, CAL-B homologous enzyme (*Pseudozyma brasiliensis* GHG 001, PBL) was found to possesses high enantioselectivity towards *sec*-alcohols bearing small substituents. Hence, it has been hypothesized that the enantioselectivity of CAL-B can be improved by substituting the amino acids with the corresponding amino acids of PBL in the medium binding pocket. The four variants of CAL-B were prepared introducing the sequence of PBL. Three mutant enzymes, which contain S47N, exhibited high enantioselectivity, especially toward but-3-yn-2-ol (E > 200). It was confirmed that the improvement of the enantioselectivity was caused by decreasing the reaction rate for the slow enantiomer rather than increase of the reaction rate for the fast one.

Second, altering the activity of CAL-B was conducted. It has been known that CAL-B possesses perhydrolytic activity, which catalyzes to produce a peracid from carboxylic acid and hydrogen peroxide instead of water molecule. Then, the produced peracids can be used to oxidize olefins to epoxides. However, the epoxidation process occurs outside of CAL-B, and thus the product should be racemic. If one can keep the peracid inside of CAL-B, it would be expected to produce chiral epoxides because the reaction environment inside of an enzyme is asymmetric. To achieve this goal, a CAL-B mutant with cysteine (T138C/A281T) was generated, and the mutant enzyme was conjugated with a series of maleimides. It was confirmed that the conjugated system catalyzes the epoxidation of α, β-unsaturated ketones. The conversion exhibits up to

twice higher than wild-type and 1.6 times higher than the background reaction when the mutant enzyme conjugated with 3-maleimidopropionic acid, and 3-penten-2-one was used as a substrate. However, the reaction was not proceeded in an asymmetric fashion.

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Chapter 1. Introduction

1. 1. Enzyme

Enzymes are powerful biological catalysts. They have several advantages as a catalyst.[1] They are catalytically efficient and environmentally acceptable. Besides, they can work under mild reaction conditions including low pressure, neutral pH, and ambient temperature range (20-40 ℃), and they exhibit a broad reaction spectrum, generally referred to as catalytic promiscuity. However, they also have a few drawbacks. For instance, they have been evolved to be suitable for natural substrates. They generally work under narrow operating conditions and often loose their catalytic activity in organic medium. Besides, they often need a cooperation with expensive natural cofactors. To overcome these shortages, researchers have tried to improve their stability and reactivity through protein engineering. The engineered enzymes can have novel specificity and improved enantioselectivity,[2] and can also catalyze one-step reaction instead of complicated multi-step organic synthesis.[3]

1. 1. 1. Lipase

Lipase (EC 3.1.1.3) is the most widely utilized enzyme that catalyzes

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[1] Faber, K. Biotransformations in organic chemistry: a textbook, 7th ed.; *Springer Berlin Heidelberg: New York, NY*, 2017;.

[2] Chen-Goodspeed, M.; Sogorb, M. A.; Wu, F.; Raushel, F. M. *Biochemistry* 2001, *40*, 1332-1339.

[3] Mahajabeen, P.; Chadha, A. *Tetrahedron: Asymmetry* 2015, *26*, 1167-1173.

the hydrolysis of carboxylic acid ester or the reverse reaction with a high chemo-, regio-, and enantioselectivity.[4] The source of lipases is many species of animals, plants and microorganisms. They possess the catalytic triad of Ser-Asp/Glu-His, and belong to an α/β-hydrolase fold like esterase.[5]

The active site serine is buried under a helical lid, and it means that active site is inaccessible by solvents.[6] At the lipid-water interface, the lipases show markedly high activity, which is called interfacial activation. This phenomenon is caused by a conformational change of lipases. During approach of a hydrophobic substrate to the surface of lipase, a helical lid is moved, and the active site is opened to the external solvents in the lipid interface. Then, the hydrophobic surface is exposed for substrate binding.[7]

1. 1. 1. 1. *Candida antarctica* lipase B (CAL-B)

CAL-B is the most widely used lipase in the resolution of chiral *sec*-alcohols and amines.[8] CAL-B consists of 317 amino acids, and the molecular mass is about 33 kDa. It has the catalytic triad of Ser105, His224, and Asp187 specially arranged in order (Figure 1). In addition,

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[4] Reetz, M. T. *Curr. Opin. Chem. Biol.* **2002**, *6*, 145-150.

[5] Ollis, D. L.; Cheah, E.; Cygler, M.; Dijkstra, B.; Frolow, F.; Franken, S. M.; Harel, M.; Remington, S. J.; Silman, I.; Schrag, J.; Sussman, J. L.; Verschueren, K. H. G.; Goldman, A. *Protein Eng. Des. Sel.* **1992**, *5*, 197-211.

[6] Uppenberg, J.; Hansen, M. T.; Patkar, S.; Jones, T. A. *Structure* **1994**, *2*, 293-308.

[7] Brzozowski, A. M.; Derewenda, U.; Derewenda, Z. S.; Dodson, G. G.; Lawson, D. M.; Turkenburg, J. P.; Bjorkling, F.; Huge-Jensen, B.; Patkar, S. A.; Thim, L. *Nature* **1991**, *351*, 491-494.

[8] Gotor-Fernández, V.; Busto, E.; Gotor, V. *Adv. Synth. Catal.* **2006**, *348*, 797-812.

Thr40 and Gln106 make up the oxyanion hole. Unlikely most lipases, CAL-B has no or a little lid to cover the catalytic triad involved in the conformational change, and thus CAL-B has no interfacial activation.[9] CAL-B has high enantioselectivity towards chiral *sec*-alcohols due to its conformational properties.[10] The acyl and alcohol moieties of an ester substrate occupy in one of the two channels at the CAL-B active site. Again two substituents of a chiral *sec*-alcohol binds into two different binding pockets, called the large and medium binding pockets. The large binding pocket is the entrance, and the medium binding pocket locates deep inside CAL-B. The two binding pockets can distinguish the difference of the substituents in size, and this discrimination provides the enantioselectivity of CAL-B.

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[9] Martinelle, M.; Holmquist, M.; Hult, K. *Biochim. Biophys. Acta, Lipids Lipid Metab.* **1995**, *1258*, 272-276.

[10] Uppenberg, J.; Ohrner, N.; Norin, M.; Hult, K.; Kleywegt, G. J.; Patkar, S.; Waagen, V.; Anthonsen, T.; Jones, T. A. *Biochemistry* **1995**, *34*, 16838-16851.

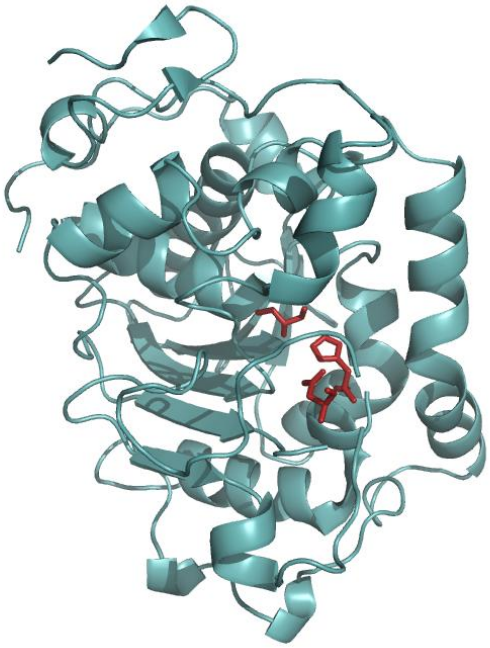

Figure 1. A crystal structure of *Candida antarctica* lipase B (PBD code: 1TCA). The catalytic triad, Ser105, Asp187, and His224, is shown as stick in brick red.

The acyl-transfer catalytic pathway of CAL-B follows ping-pong mechanism (Figure 2). It consists of two steps, the acylation and deacylation step. In the first step, the acyl portion of an ester substrate is attacked by the catalytic Ser residue activated by the catalytic His. The oxyanion hole stabilizes the tetrahedral intermediate by hydrogen bonding.[11] The acyl-enzyme is formed and the corresponding alcohol is released. Next, a water molecule enters in the acyl-enzyme, then the second tetrahedral intermediate is formed. Ultimately, in the deacylation

[11] Derewenda, Z. S.; Sharp, A. M. *Trends Biochem. Sci.* **1993**, *18*, 20-25.

step, the carboxylic acid product is released and the enzyme is returned to the free form.

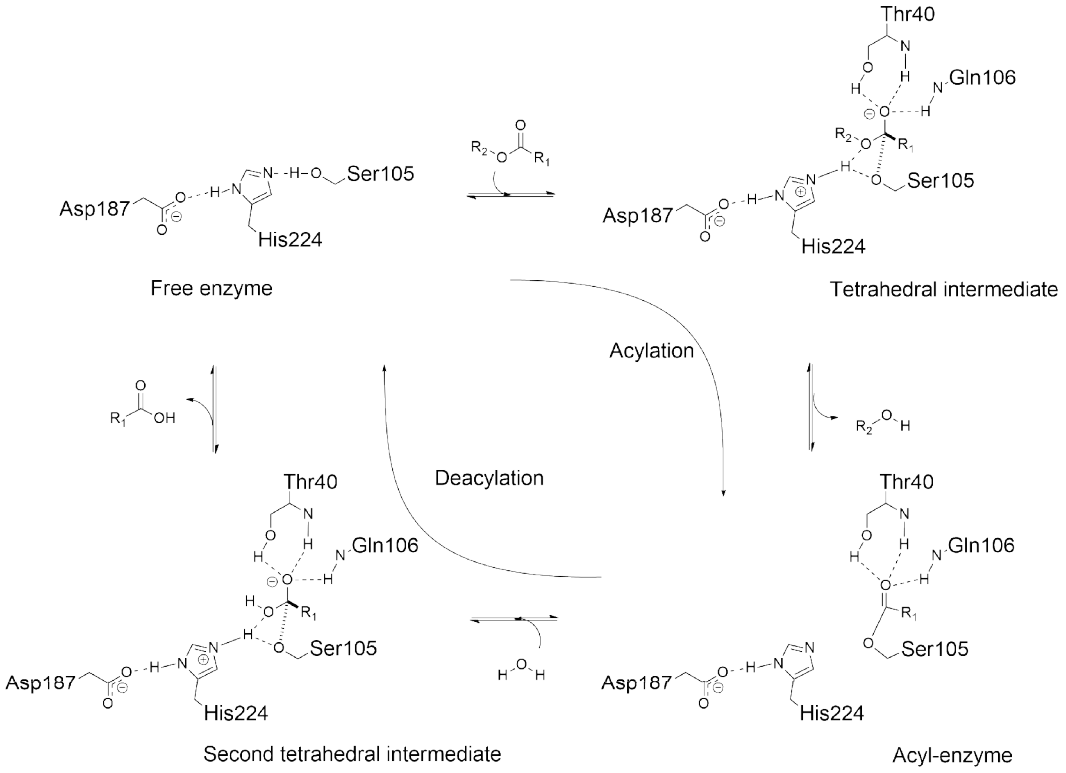

Figure 2. The catalytic mechanism of *Candida antarctica* lipase B

1. 2. Kinetic resolution

Kinetic resolution is one of the methods to separate each enantiomer from a racemic mixture. When an enzyme has excellent selectivity, the enantiopure substrate and the enantiopure product remain after the reaction finished. A successful kinetic resolution can be estimated by measuring enantioselectivity.

Enantioselectivity is represented by using the enantiomeric ratio, E,

meaning the ability of the enzyme to sort two enantiomers.[12] This value is defined as the ratio of the specificity constant of the enzyme (k_{cat}/K_M) for both enantiomers.

$$E = \frac{(\frac{k_{cat}}{K_M})_A}{(\frac{k_{cat}}{K_M})_B}$$

E = enantiomeric ratio, k_{cat} = the turnover number, K_M = the Michaelis-Menten constant, A and B = substrate enanatiomer

The E value can also be described by the difference in the Gibbs free energy of the transition state of each enantiomer binding with enzyme (Figure 3).[13][14]

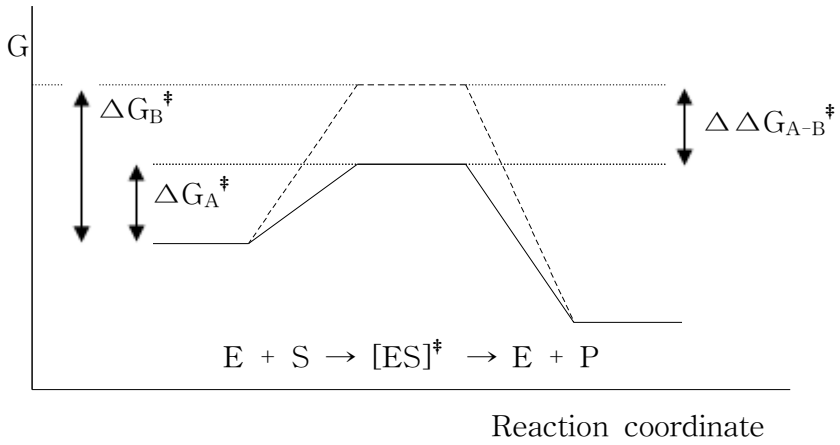

Figure 3. Reaction coordinate of enantioselective enzymatic reactions

[12] Raza, S.; Fransson, L.; Hult, K. *Protein Sci.* **2001**, *10*, 329-338.
[13] Straathof, A. J. J.; Jongejan, J. A. *Enzyme. Microb. Tech.* **1997**, *21*, 559-571.
[14] Keith, J.; Larrow, J.; Jacobsen, E. *Adv. Synth. Catal.* **2001**, *343*, 5-26.

$$\triangle\triangle G^{\ddagger} = -RT\ln\frac{v_A}{v_B} = -RT\ln E$$

$\triangle\triangle G^{\ddagger}_{A-B} = \triangle G^{\ddagger}_{B} - \triangle G^{\ddagger}_{A}$ = the difference in Gibbs free energy for activation between two enantiomers, R = gas constant (8.31 $J \cdot mol^{-1} \cdot K^{-1}$), T = temperature

1. 3. Protein engineering

Since enzymes have been evolved in living organisms, they are optimized to work in mild conditions. Therefore, enzymes have to be engineered for use at extreme temperature and pH, and in organic media for industrial applications. Protein engineering often produces enzyme variants possessing higher chemo- and thermo-stability. The modified ones are suitable to be applied to pharmaceuticals, green chemistry, and bio-fuels. Hence, protein engineering have received attention as a strong tool to improve or alter enzyme functionalities.[15]

Protein engineering can be employed to change various enzyme properties, such as stability, activity, and surface property. Protein engineering can be classified in two methods, rational design and directed evolution.[16] Rational design uses structural knowledge and is the widely used protein engineering method. Site-directed mutagenesis is the main technique in the rational design approach. Directed evolution consists of genetic recombination and screening step. Due to the need for relatively less information about enzymes, this method is getting

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[15] Kazlauskas, R. J.; Bornscheuer, U. T. *Nat. Chem. Biol.* **2009**, *5*, 526-529.
[16] Rubingh, D. N. *Curr. Opin. Biotech.* **1997**, *8*, 417-422.

more attention in industry. Recently, a combined approach, semi-rational approaches, has been reported. The approach is developed to combine the advantages in rational design as well as directed evolution.[17]

1. 3. 1. Rational design

Rational design is one of the protein engineering approaches based on the prediction of improvement of enzyme activity, selectivity or stability by molecular modeling. In order to engineer proteins by a rational design method, it is necessary to know the enzyme structure, related sequences, and catalytic mechanism.

Craik *et al.* demonstrated that the relationship between the protein structure and function can be explained by designing site-specific mutations from the an expected structure of rat trypsin.[18] The mutant enzymes were generated by replacing Gly216 and Gly226 with alanine in the substrate binding sites. Through modeling study, the mutant enzymes were predicted to interfere with the binding of the enzyme and the substrate. As a result, although the catalytic rate was reduced, the substrate selectivity was improved, and it implied that protein structure and function are related.

1. 3. 1. 1. Site-directed mutagenesis

Site-directed mutagenesis is the method for specific change of the

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[17] Chica, R. A.; Doucet, N.; Pelletier, J. N. *Curr. Opin. Biotech.* **2005**, *16*, 378-384.

[18] Craik, C. S.; Largman, C.; Fletcher, T.; Roczniak, S.; Barr, P. J.; Fletterick, R.; Rutter, W. J. *Science* **1985**, *228*, 291-297.

DNA sequence of a target gene. The process is related to finding a suitable location for replacement, removal or addition of a residue by polymerase chain reaction (PCR).[19] In site-directed mutagenesis, replacing the sequence of the desired residues with what is already known is called sequential alteration.

1. 3. 2. Directed evolution

Directed evolution is another protein engineering approach that mimics the natural selection process to obtain desired properties of an enzyme. The process of gene diversification generates randomly mutated genes of interest. The process of selecting genes therefrom are repeated to obtain mutants with desired characteristics. In contrast of rational design, this approach enables to manipulate an enzyme and to expand a variant library without in depth information about the relationship between protein structure and function. However, a high-throughput screening method is necessary to obtain useful results from vast amounts of variants.

1. 4. Epoxidation

Epoxidation is a type of reactions that formation of epoxide (also called oxirane). Epoxide is a three-membered cyclic ether. Since this ring compound is close to an equilateral triangle, it is highly reactive and useful in many applications.

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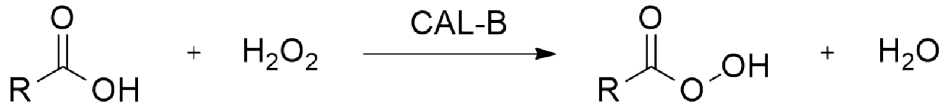
[19] Ho, S. N.; Hunt, H. D.; Horton, R. M.; Pullen, J. K.; Pease, L. R. *Gene* **1989**, *77*, 51-59.

Several serine hydrolases containing the Ser-His-Asp catalytic triad can catalyze perhydrolysis to form peracids from carboxylic acids and hydrogen peroxide.[20][21] CAL-B is known as a lipase capable of catalyzing perhydrolysis (Scheme 1).[22] Using perhydrolase activity of CAL-B can induce epoxidation reactions.



Scheme 1. CAL-B-catalyzed perhydrolysis of carboxylic acid with hydrogen peroxide

1. 5. Outline of this thesis

This thesis deals with two studies: one is related to increase of the enantioselectivity of CAL-B and the other introducing of an epoxidase activity to CAL-B. In chapter 2, the binding pocket of CAL-B was adjusted to improve the enantiomeric ratio towards but-3-yn-2-ol. In chapter 3, it was attempted to alter the reactivity of CAL-B to an epoxidase by introduction of an artificial cofactor.

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[20] Bernhardt, P.; Hult, K.; Kazlauskas, R. J. *Angew. Chem. Int. Ed Engl.* **2005**, *44*, 2742-2746.

[21] Yin, D. L.; Bernhardt, P.; Morley, K. L.; Jiang, Y.; Cheeseman, J. D.; Purpero, V.; Schrag, J. D.; Kazlauskas, R. J. *Biochemistry* **2010**, *49*, 1931-1942.

[22] Patkar, S.; Vind, J.; Kelstrup, E.; Christensen, M. W.; Svendsen, A.; Borch, K.; Kirk, O. *Chem. Phys. Lipids* **1998**, *93*, 95-101.

Chapter 2. Enhancing enantioselectivity of *Candida antarctica* lipase B toward but-3-yn-2-ol

Abstract

Candida antarctica lipase B (CAL-B) is one of the most useful enzymes in kinetic resolution of chiral *sec*-alcohols. However, CAL-B exhibits low enantioselectivity towards chiral *sec*-alcohols that have smaller substituents than a propyl group (*i.e.* butan-2-ol and but-3-yn-2-ol). To improve the enantioselectivity of CAL-B towards such *sec*-alcohols bearing small substituents, we altered the binding pocket of CAL-B like as PBL by site directed mutagenesis. The medium-binding pocket of CAL-B was chosen as the mutation site based on the information from our previous study. We produced four mutants of CAL-B, T42V, S47N, 42-47, and T42V/S47N. Enantioselectivity of the CAL-B wild-type and mutant enzymes for transesterification towards butan-2-ol and but-3-yn-2-ol was determined. Vinyl acetate and vinyl butyrate were used as an acyl donor. Enantioselectivity of the mutant enzymes (S47N, 42-47, and T42V/S47N) was improved up to 50 times compared to that of the wild-type CAL-B, especially toward but-3-yn-2-ol.

2. 1. Introduction

Preparation of enantiopure chiral *sec*-alcohol has become more important in fine chemical and pharmaceutical industry.[23] Enantiopure chiral compounds preparation is generally achieved by asymmetric catalysts. Lipases are also a kind of a natural asymmetric catalyst and are becoming more commonly used in preparation of chiral *sec*-alcohols. Among lipases, *Candida antarctica* lipase B (CAL-B) is one of the most widely used biocatalysts because of its high enantioselectivity, a broad range of substrates, and tolerance to organic solvents.[24] Molecular basis of its enantioselectivity towards chiral *sec*-alcohols has been well documented based on its structural feature.[25] The active site of CAL-B consists of two pockets, one for the acyl moiety and the other for the alcohol moiety of ester substrates. Again, the alcohol binding pocket of CAL-B divided into two pockets, the large and medium binding pockets. The larger substituent of a chiral *sec*-alcohol binds into the large binding pocket while the smaller substituent, smaller than a propyl group, binds into the medium binding pocket.[26] This implies that CAL-B cannot discriminates the difference between a methyl and ethyl group because the medium binding pocket can accept both. In fact,

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[23] Csajági, C.; Szatzker, G.; Rita Tőke, E.; Ürge, L.; Darvas, F.; Poppe, L. *Tetrahedron: Asymmetry* **2008**, *19*, 237-246.

[24] Engström, K.; Vallin, M.; Syrén, P.; Hult, K.; Bäckvall, *Org. Biomol. Chem.*, **2011**, *9*, 81-82

[25] Kazlauskas, R. J.; Weissfloch, A. N. E.; Rappaport, A. T.; Cuccia, L. A. J. *Org. Chem.* **1991**, *56*, 2656-2665.

[26] Rotticci, D.; Hæffner, F.; Orrenius, C.; Norin, T.; Hult, K. *J. Mol. Catal. B: Enzym.* **1998**, *5*, 267-272.

CAL-B exhibits low enantioselectivity toward butan-2-ol (E = 6-7).

Previously our research group has surveyed enantioselectivity of two CAL-B homologs, lipase from *Sporisorium reilianum* SRZ2 (SRL) and lipase from *Pseudozyma brasiliensis* GHG 001 (PBL), towards a series of chiral *sec*-alcohols.[27] Interestingly, PBL exhibited higher enantioselectivity towards butan-2-ol and but-3-yn-2-ol compared to those of CAL-B and SRL. The medium binding pocket of PBL is probably smaller than CAL-B and SRL. Hence, it can be hypothesized that modification of the medium binding pocket of CAL-B to that of PBL may increase the enantioselectivity of CAL-B towards butan-2-ol and but-3-yn-2-ol by decrease of the space of the medium binding pocket. In this study, we investigated the effect of alteration of the medium binding pocket on enantioselectivity of CAL-B towards butan-2-ol and but-3-yn-2-ol.

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[27] Kim, Y.; Park, S. *Bull. Korean Chem. Soc.* **2017**, *38*, 1358-1361.

2. 2. Results and Discussion

Selection of the mutation sites

In the previous study, it was reported that lipase from *Pseudozyma brasiliensis* GHG 001 (PBL), a homologous lipase of CAL-B, possesses distinct enantioselectivity compared to that of CAL-B.[28] The enantioselectivity of PBL toward but-3-yn-2-ol is much higher (E > 200) than that of CAL-B. The different size of the medium binding pocket between PBL and CAL-B may account for the distinct enantioselectivity, although their sequences are quite similar. Therefore, it can be hypothesized that enantioselectivity of CAL-B can be improved by altering the size of the medium binding pocket by substitution with the corresponding sequence of PBL. The medium binding pocket of CAL-B is composed of the residues from 39 to 48, and the sequence from 42 to 47 residues is different between PBL and CAL-B. Hence, the sequence from 42 to 47 residues of CAL-B was substituted with the corresponding sequence of PBL and four mutants including T42V, S47N, 42-47 (T42V/T43D/P45R/S47N), and T42V/S47N were created. (Table 1, The replaced residues are marked in red.) The 42-47 mutant enzyme has the medium binding pocket with the same sequence as PBL.

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[28] Kim Y. H. **2017**, 1358-1361.

Table 1. The mutation sites of CAL-B

Enzyme		medium binding pocket residues	
wt[a]	39	GTGTTGPQSF	48
T42V		GTGVTGPQSF	
S47N		GTGTTGPQNF	
42-47		GTGVDGRQNF	
T42V/S47N		GTGVTGPQNF	

[a]wt indicates the CAL-B wild-type enzyme.

Hydrolytic activity towards *p*-nitrophenyl acetate (*p*NPAc) and *p*-nitrophenyl butyrate (*p*NPBu)

The hydrolytic activity of the wild-type and mutant enzymes was measured with *p*NPAc and *p*NPBu (Table 2). The higher hydrolytic activity was obtained in order of the wild-type, T42V, S47N, 42-47, and T42V/S47N enzymes toward *p*NPAc and T42V, the wild-type, S47N, 42-47, and T42V/S47N enzymes toward *p*NPBu. Overall, the activity of the T42V mutant enzyme was comparable to that of the wild-type enzyme, and the other mutants showed less than half activity compared to that of the wild-type enzyme.

Table 2. Hydrolytic activity of the wild-type and mutant enzymes

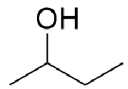
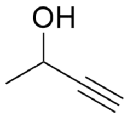
Enzyme	Activity (μmol/min/mg)	
	*p*NPAc	*p*NPBu
wt	1.44	2.31
T42V	1.10	2.58
S47N	0.609	0.558
42-47	0.461	0.537
T42V/S47N	0.392	0.444

Measurement of the enantioselectivity (*E*) for transesterification reaction towards butan-2-ol and but-3-yn-2-ol

Enantioselectivity (*E*, enantiomeric ratio[29]) for transesterification reaction towards butan-2-ol and but-3-yn-2-ol was measured (Table 3). All four mutant enzymes slowly catalyzed the reaction than the wild-type enzyme. But the *E* values of the mutant enzymes were more than twice higher except for T42V. Especially, the *E* values of the S47N, 42-47, and T42V/S47N mutant enzymes toward but-3-yn-2-ol are noticeably high ($E > 200$).

[29] Chen, C. S.; Fujimoto, Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294-7299.

Table 3. Enantioselective transesterification of *sec*-alcohol catalyzed by CAL-B wild-type and mutants[a]

Entry	Substrate	Acyl donor	wild-type			T42V			S47N			42-47			42V/47N		
			Time (h)	Conv. (%)	*E*	Time (h)	Conv. (%)	*E*	Time (h)	Conv. (%)	*E*	Time (h)	Conv. (%)	*E*	Time (h)	Conv. (%)	*E*
1	 butan-2-ol	Vinyl acetate	8	41.9	5.7	44	41.8	6.7	26	33.9	14.0	45	38.7	12.9	53	30.5	13.2
2		Vinyl butyrate	4	44.2	7.2	20	46.3	8.0	20	43.9	30.7	35	31.5	25.7	48	40.1	24.9
3	 but-3-yn-2-ol	Vinyl acetate	16	52.2	5.4	48	37.0	4.4	23	40.4	> 200	48	37.6	> 200	60	32.1	> 200
4		Vinyl butyrate	4	39.0	4.0	20	40.0	3.2	17	45.1	> 200	25	40.6	> 200	43	39.4	> 200

[a]Substrate, 0.2 mmol; acyl donor, 0.6 mmol; amount of enzymes: 200 μg of wt, T42V, and S47N, and 400 μg of 42-47 and 42/47; *tert*-butyl methyl ether (2 mL). The values are measured at least twice and averaged.

Specific activity of the wild-type and mutant enzymes in the reaction with *rac*-but-3-yn-2-ol

Specific activity was calculated by measuring the initial rate in the reaction with *rac*-but-3-yn-2-ol and vinyl acetate or vinyl butyrate (Table 4 and table 5). For all enzymes, the specific activity for the reaction with vinyl butyrate were higher than that for the reaction with vinyl acetate. Besides, all mutant enzymes exhibited lower specific activity than the wild-type enzyme, while the S47N mutant enzyme exhibited higher activity than the other mutant enzymes.

Table 4. Specific activity with vinyl acetate[a]

	Specific activity (μmol/min/mg)					
	1	2	3	Average	STDEV[b]	S.E.[c]
wt	1.20×10^{0}	1.18×10^{0}	1.53×10^{0}	1.30×10^{0}	1.71×10^{-2}	9.90×10^{-3}
T42V	2.16×10^{-1}	1.33×10^{-1}	2.06×10^{-1}	1.85×10^{-1}	4.54×10^{-2}	2.62×10^{-2}
S47N	6.75×10^{-1}	4.99×10^{-1}	2.95×10^{-1}	**4.89×10^{-1}**	1.90×10^{-1}	1.10×10^{-1}
42-47	1.39×10^{-1}	1.43×10^{-1}	1.43×10^{-1}	1.42×10^{-1}	2.55×10^{-3}	1.48×10^{-3}
42V/47N	8.47×10^{-2}	1.22×10^{-1}	1.19×10^{-1}	1.09×10^{-1}	2.07×10^{-2}	1.20×10^{-2}

[a]*rac*-but-3-yn-2-ol 0.4 mmol; vinyl acetate 1.2 mmol; amount of enzymes: 80 μg of wt, 300 or 1000 μg of T42V, 100 or 300 μg of S47N, 800 or 1000 μg of 42-47, and 1000 or 2000 μg of T42V/S47N; *tert*-butyl methyl ether (8 mL for wt and 4 mL for mutant enzymes) is used as solvent. but-3-yn-2-ol 0.2 mmol and vinyl acetate 0.6 mmol in 4 mL of MTBE were used with wt enzyme at first. [b]STDEV is standard deviation. [c]S.E. is standard error.

Table 5. Specific activity with vinyl butyrate[a]

	Specific activity (μmol/min/mg)					
	1	2	3	Average	STDEV	S.E.
wt	1.10×10^{0}	9.30×10^{-1}	2.24×10^{0}	1.42×10^{0}	7.14×10^{-1}	4.12×10^{-1}
T42V	3.34×10^{-1}	2.89×10^{-1}	6.86×10^{-1}	4.36×10^{-1}	2.17×10^{-1}	1.25×10^{-1}
S47N	7.00×10^{-1}	4.17×10^{-1}	1.21×10^{0}	**7.76×10^{-1}**	4.03×10^{-1}	2.33×10^{-1}
42-47	1.89×10^{-1}	1.59×10^{-1}	3.18×10^{-1}	2.22×10^{-1}	8.48×10^{-2}	4.90×10^{-2}
42V/47N	9.56×10^{-2}	1.46×10^{-1}	3.05×10^{-1}	1.82×10^{-1}	1.09×10^{-1}	6.30×10^{-2}

[a]*rac*-but-3-yn-2-ol 0.2 mmol; vinyl acetate 0.6 mmol; amount of enzymes: 80 μg of wt, 300 or 450 μg of T42V, 80 or 240 μg of S47N, 400 or 600 μg of 42-47, and 800 or 1200 μg of T42V/S47N; *tert*-butyl methyl ether (8 mL for wt and 4 mL for mutant enzymes) is used as solvent.

Specific activity of the wild-type and S47N mutant enzymes in the reaction with the fast enantiomer, (*R*)-(+)-but-3-yn-2-ol, and vinyl butyrate

The specific activity of the S47N mutant enzyme was compared with that of the wild-type enzyme for the fast enantiomer ((*R*)-(+)-but-3-yn-2-ol) (Table 6). The specific activity of the wild-type enzyme toward (*R*)-(+)-but-3-yn-2-ol showed 0.6-fold compared to racemic substrate (Table 5), and the S47N mutant enzyme exhibited 0.8-fold. It is presumed that the enantioselectivity of the mutant enzyme was improved by reducing the activity of the slow enantiomer of substrate.

Table 6. Specific activity toward (*R*)-(+)-but-3-yn-2-ol

	Specific activity (μmol/min/mg)					
	1	2	3	Average	STDEV	S.E.
wt[a]	$1.01x10^{0}$	$7.99x10^{-1}$	$7.53x10^{-1}$	$8.54x10^{-1}$	$1.37x10^{-1}$	$7.93x10^{-2}$
S47N[b]	$6.32x10^{-1}$	$5.95x10^{-1}$		$6.13x10^{-1}$	$2.66x10^{-2}$	$1.88x10^{-2}$

[a](*R*)-(+)-but-3-yn-2-ol 0.1 mmol; vinyl butyrate 0.3 mmol; amount of enzymes 80 μg; 8 mL of *tert*-butyl methyl ether as solvent for wt, [b](*R*)-(+)-but-3-yn-2-ol 0.2 mmol; vinyl butyrate 0.6 mmol; amount of enzymes 160 μg; 4 mL of *tert*-butyl methyl ether as solvent for S47N mutant enzyme

2. 3. Experimental Section

General Methods

Chemicals were purchased from TCI or Thermo Fisher Scientific. The vector (pBADgⅢa) was purchased from Invitrogen Korea (Seoul, Korea). Poly-Prep chromatography columns and the protein assay solution were purchased from Bio-Rad. The Ni-NTA agarose resin was purchased from QIAGEN. CIRCLEGROW® Broth was purchased from MP Biomedicals. DNA sequencing was carried out by Solgent Co. (Daejeon, Korea). Gas Chromatography was analyzed by Agilent 6890N with a chiral capillary column (Cyclosil-B 30 m × 0.25 mm).

Site directed mutagenesis

Opt2_5D_CAL-B[30] was used as the template to obtain the mutant genes by an overlap-extension PCR with the mutagenesis primers (Table 7). The mutant genes were digested by *Nco* I and *Sal* I and ligated with the pBADgⅢa vector digested by the same restriction enzymes. Then the plasmids were transformed in the *E.* coli strain (Top 10).

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[30] Jung, S.; Park, S. *Biotechnol. Lett.* **2008**, *30*, 717-722.

Table 7. Primer for mutagenesis

F1_5DCALB_T42V	5'-GGCACCGGTGTGACTGGCCCGCAGTCTTTC-3'
R1_5DCALB_T42V	5'-GAAAGACTGCGGGCCAGTCACACCGGTGCC-3'
F1_5DCALB_S47N	5'-ACCACTGGCCCGCAGAACTTCGACAGCAACTGGATTCCACTG-3'
R1_5DCALB_S47N	5'-CCAGTTGCTGTCGAAGTTCTGCGGGCCAGTGGTACC-3'
F1_5DCALB_T42V/S47N	5'-GTGACTGGCCCGCAGAACTTCGACAGCAACTGGATTCCACTG-3'
R1_5DCALB_T42V/S47N	5'-GTTCTGCGGGCCAGTCACACCGGTGCCCGGTACCA-3'
F1_5DCALB_42-47	5'-GTGGATGGCCGTCAGAACTTCGACAGCAACTGGATTCCACTG-3'
R1_5DCALB_42-47	5'-GTTCTGACGGCCATCCACACCGGTGCCCGGTACCA-3'

Expression and purification of the wild-type and mutant enzymes

An overnight culture was prepared by using 15 mL of CIRCLEGROW® medium with ampicillin (15 μL, 100mg/mL) at 37 ℃ and 180 rpm. The overnight culture (5 mL) was inoculated in 500 mL of CIRCLEGROW® medium with 500 μL of ampicillin (100 mg/mL), and the diluted culture was grown at 37 ℃ and 180 rpm until OD_{600} = 0.5. Protein expression was initiated by the addition of a solution of L-(+)-arabinose (500 μL, 2% w/v) and the culture was incubated at 20 ℃ for 20 h. Cells were harvested by centrifugation at 10,000 rpm and 4 ℃ for 20 min.

Cell pellets were resuspended in a BES buffer (5 mL per 1 g of cell, 5

mM, pH 7.2). After sonication, cells were frozen and thawed. Then, cells were broken up by syringe pumping, and soluble and insoluble fractions were separated by centrifugation at 10,000 rpm and 4 ℃ for 10 min. Soluble fraction, the crude extract, was shaken with Ni-NTA resin for 1h and loaded onto a Poly-Prep chromatography column for purification. The column was washed three times with a lysis buffer (5 mL, 50 mM NaH_2PO_4; 300 mM NaCl; 10 mM imidazole, pH 8.0) and a wash buffer (5 mL, 50 mM NaH_2PO_4; 300 mM NaCl; 20 mM imidazole, pH 8.0) in order. Elution was performed with an elution buffer (5 mL, 50 mM NaH_2PO_4; 300 mM NaCl; 250 mM imidazole, pH 8.0). The buffer of elution fraction was exchanged with a BES buffer (three times with 20 mL, 5 mM, pH 7.2) by a centrifugal concentrator. The fractions were analysed by SDS-PAGE.

Determination of the concentration of lipase

The concentration of the purified enzyme solutions was measured by the Bradford assay.[31] A standard solution (1 mg/mL) of bovine serum albumin (BSA) was prepared and diluted in range of 0.05-0.5 mg/mL. Each BSA solution (10 μL) or appropriately diluted enzyme solution (10 μL) were mixed with assay solution (200 μL). After 5 minutes, the absorbance was measured at 595 nm, and the enzyme concentration was calculated using the BSA calibration curve.

Measuring hydrolytic activity of the wild-type and mutant

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[31] Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248-254.

enzymes with *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate

The enzyme solution (5 μL) was mixed with 100 μL of an assay solution (20 μL of 200 mM *p*-nitrophenyl acetate or *p*-nitrophenyl butyrate in acetonitrile; 870 μL of acetonitrile; 11,110 μL of 5 mM BES buffer). Then, the absorbance changes at 404 nm were measured within 5 or 7 second intervals.

Immobilization of lipase on Celite 545

A lipase solution (1 mL, 10 mg/mL) containing sucrose (60 mg) was mixed with Celite 545 (1 g). After the suspension was shaken for 15 min, the suspension was spread on a weighing dish, covered with perforated aluminum foil, and dried overnight under a fume hood.

Measurement of enantiomeric ratio (*E*) toward transesterification reaction

Substrate (0.2 mmol, 18.3 μL of butan-2-ol or 15.7 μL of but-3-yn-2-ol) and acyl donor (0.6 mmol, 55.4 μL of vinyl acetate or 76.2 μL of vinyl butyrate) were dissolved in *t*-butyl methyl ether (MTBE, 2 mL) with an immobilized lipase (200 μg of CAL-B wild-type, T42V or S47N, or 400 μg of 42-47 or T42V/S47N). The reaction mixture was then stirred at 25 ℃ and 500 rpm. The reaction was stopped at conversion around 40%. Enantiomeric ratio was calculated by the method from Chen *et al.* using the enantiomeric excesses of both starting alcohol and product ester.[32] GC analysis conditions for confirmation of the

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[32] Chen, C. S.; Fujimoto, Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294-7299.

amount of substrates and products are shown in the table 8.

Table 8. GC analysis conditions for enantioselectivity determination

Substrate	Acyl donor	GC analysis condition
Butan-2-ol	Vinyl acetate	25 ℃/30 min - 2 ℃/min - 55 ℃/5 min
Butan-2-ol	Vinyl butyrate	30 ℃/10 min - 1 ℃/min - 85 ℃/10 min
But-3-yn-2-ol	Vinyl acetate	30 ℃/10 min - 0.5 ℃/min - 40 ℃ - 2 ℃/min - 60 ℃/10 min
But-3-yn-2-ol	Vinyl butyrate	70 ℃/10 min - 2 ℃/min - 100 ℃/10 min

Comparison of the specific activity of CAL-B wild-type and S47N

Substrate, acyl donor (three equivalent), and immobilized enzyme were mixed and stirred in MTBE at 25 ℃ and 500 rpm. Small portion of the reaction mixture was retrieved within 20 min intervals for 160 min. Since the reaction rate was different for each lipase, the reaction volume increased and the amount of lipase added was adjusted.

For measuring the initial rate of fast enantiomer, (*R*)-(+)-but-3-yn-2-ol, vinyl butyrate (three equivalent), and hexadecane (0.1 equivalent) as internal standard were mixed in MTBE and stirred at 25 ℃. GC analysis conditions are same as those for enantioselectivity measurement.

Appendix

Purification band of proteins in SDS-PAGE

SDS-PAGE analysis figures of CAL-B wild-type and mutants are followed: wild-type CAL-B, T42V, S47N, 42-47, and T42V/S47N.

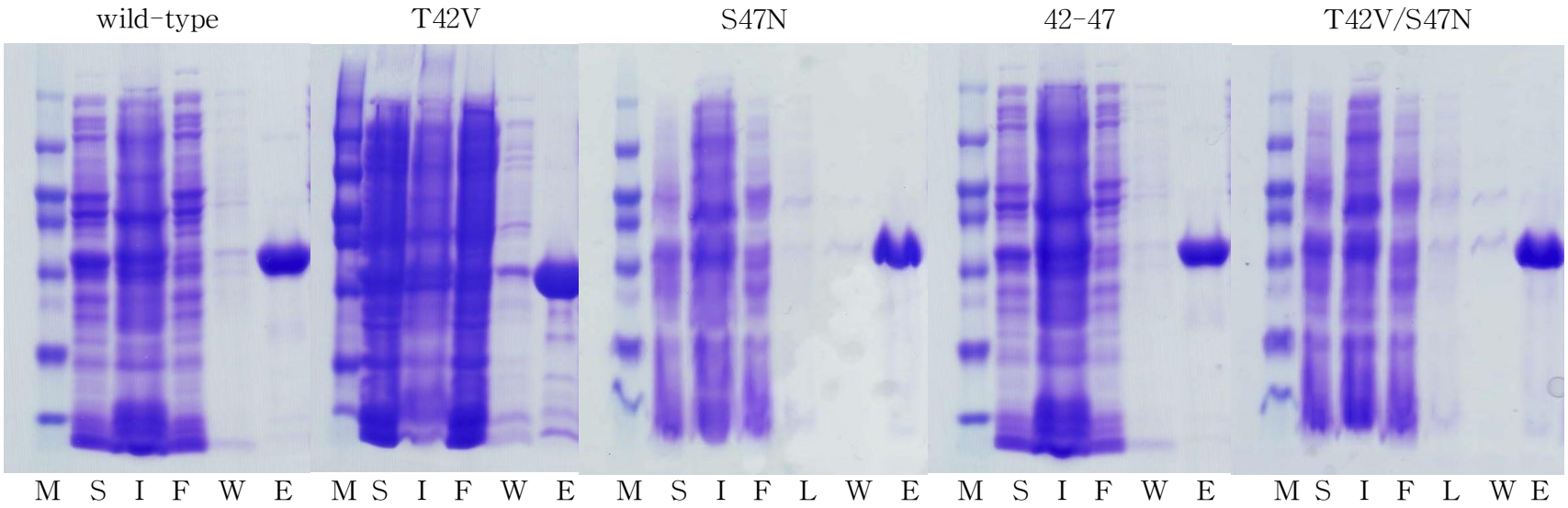

Figure A1. SDS-PAGE analyses of the wild-type and mutant enzymes. S: soluble fraction; I: insoluble fraction; F: flow through; W: washing; E: elution.

Comparison of enantioselectivity on GC analysis graphs

As the enantiomeric ratio increased, the difference of product peaks was noticeable in GC analysis results. The GC results of the transesterification reaction toward but-3-yn-2-ol with vinyl butyrate at the zero and 40% conversions were shown in figure A2: (a) wild-type CALB; (b) T42N; (c) S47N; (d) 42-47; and (e) T42V/S47N. The retention time of the substrates was 3.2 min for the fast enantiomer and 3.4 min for the slow enantiomer. And vinyl butyrate was detected at 3.7 min. The products were appeared at 10.3 min and 10.8 min for the fast and slow enantiomers, respectively.

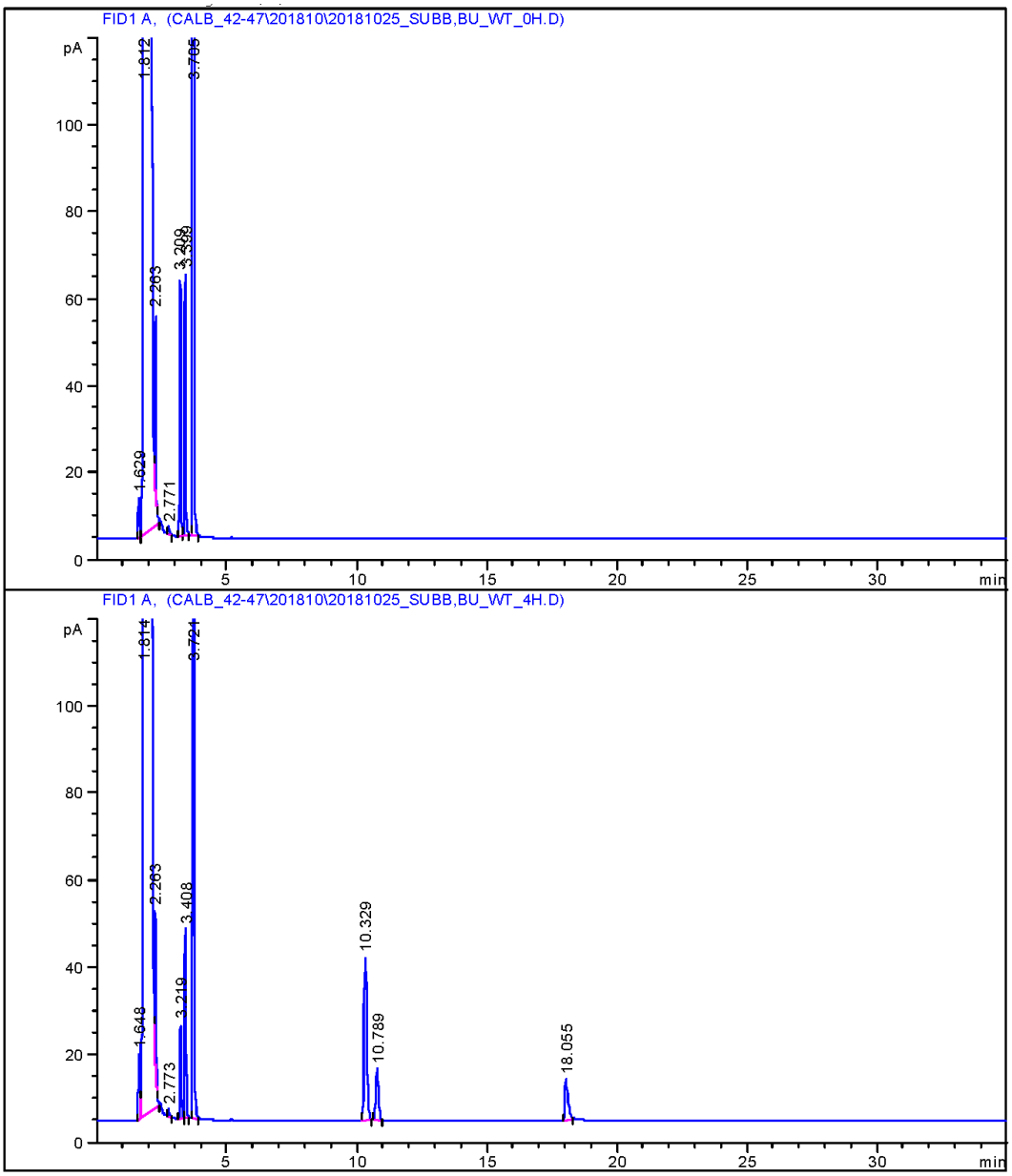

Figure A2(a). GC analysis results of wild-type CAL-B

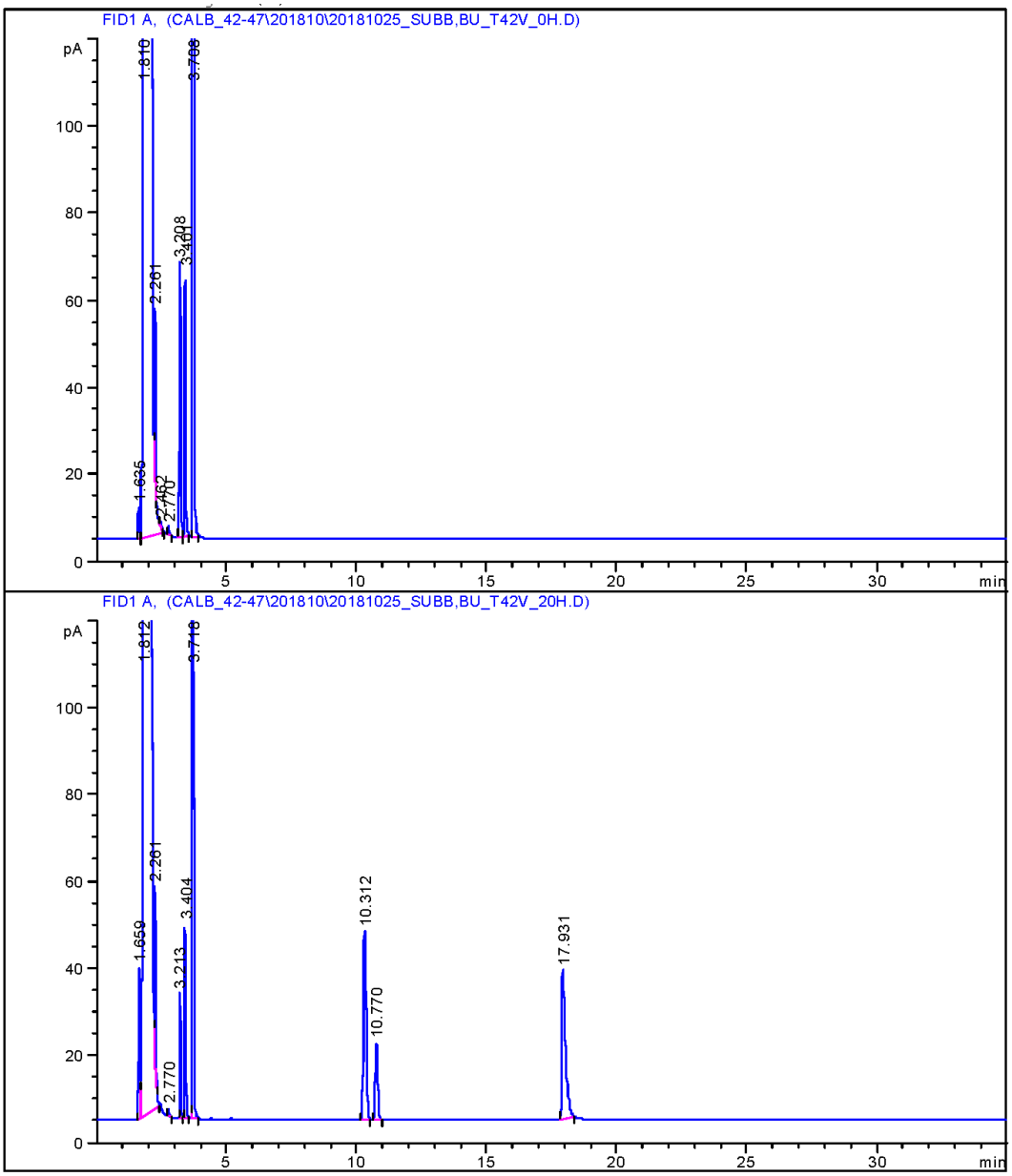

Figure A2(b). GC analysis results of T42V mutant enzyme

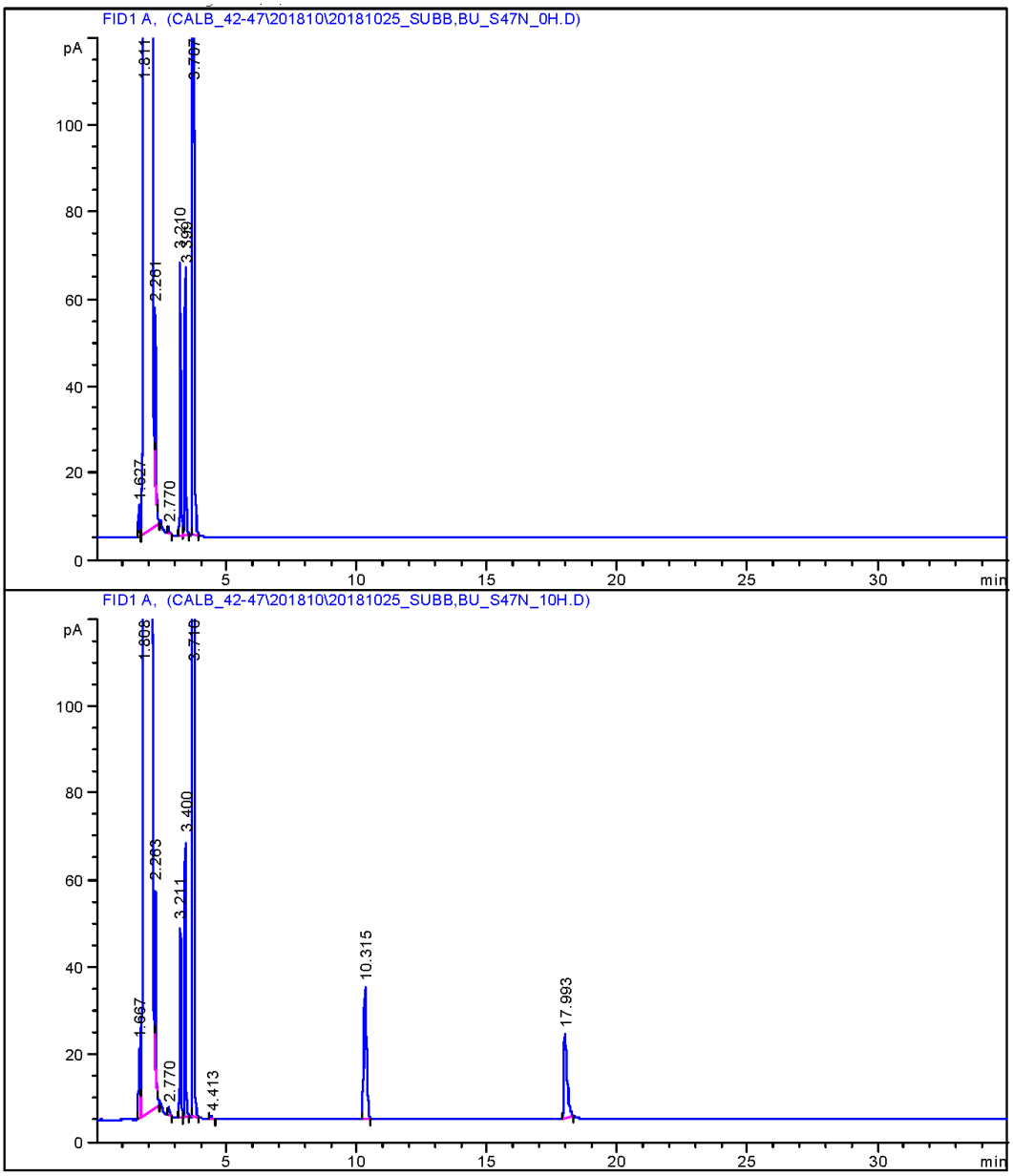

Figure A2(c). GC analysis results of S47N mutant enzyme

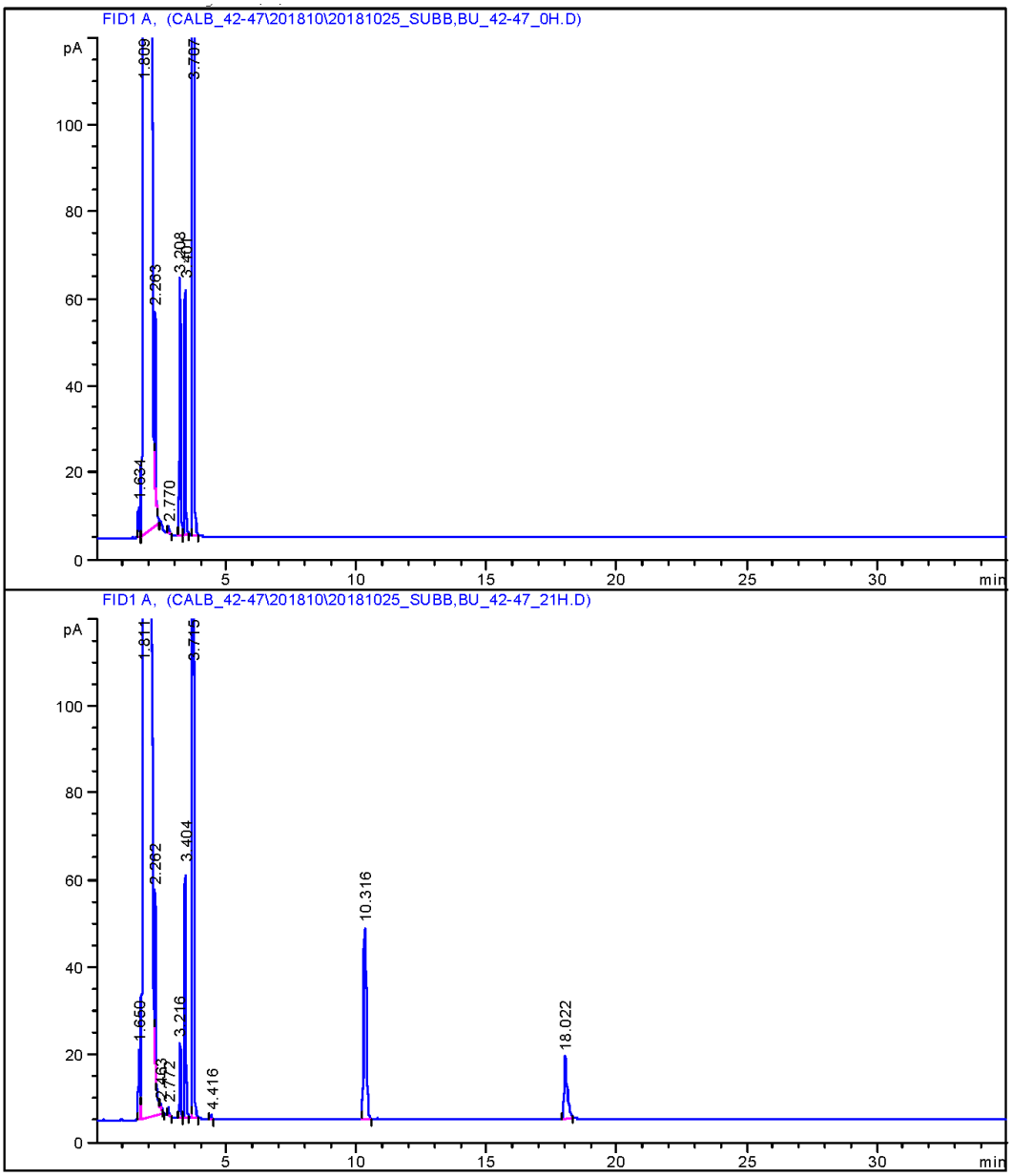

Figure A2(d). GC analysis results of 42-47 mutant enzyme

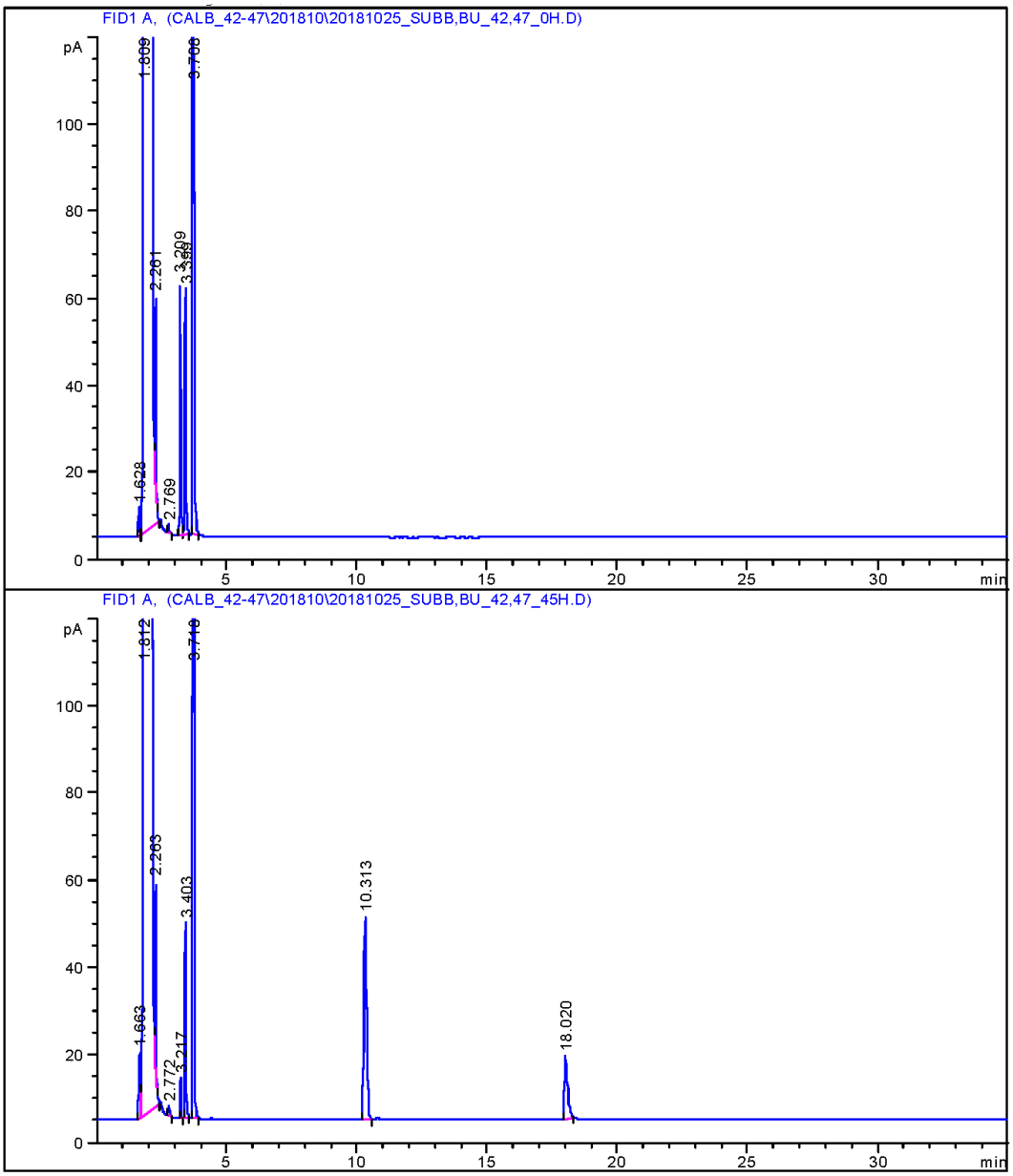

Figure A2(e). GC analysis results of T42V/S47N mutant enzyme

Chapter 3. Converting *Candida antarctica* lipase B to epoxidase by introducing an artificial cofactor

Abstract

Chemical reactions using enzymes have several advantages, such as being environmentally friendly and producing almost no by-products. Researchers are continuing their study to use enzymes more diversely. One of the most challenging approaches is to change the reactivity of enzymes being used. *Candida antarctica* lipase B (CAL-B) is one of the most widely applied lipase because it has thermo- and chemo-resistance and is stable in organic solvents. Recently, epoxidation reactions using hydrogen peroxide have attracted attention since hydrogen peroxide is a high source of oxygen and the by-product is solely water. It would be an useful finding if CAL-B can be converted to an epoxidase. Then epoxidation could be performed at milder conditions. In previous report, we have discussed that particular CAL-B mutants can perform perhydrolysis faster than the CAL-B wild-type. Herein, we report a further study on the feasibility of engineering CAL-B in an attempt to do the enantioselective epoxidation reactions.

3. 1. Introduction

Catalytic promiscuity refers to the ability of one enzyme to conduct another side reaction in addition to its own main reaction. For example, lipases or esterases, which are hydrolases, have somewhat inherent perhydrolytic activity, and their perhydrolytic activity even can be enhanced through protein engineering and reaction optimization. Since peroxycarboxylic acid and epoxide are used to prepare chemically and biologically helpful compounds, they have been interested in preparation methods. In order to develop the preparation process under mild conditions, research has been conducted on the production of the peroxycarboxylic acid and epoxidation of alkene using enzymes.[33][34] Kazlauskas *et al.* improved the perhydrolase activity of esterase from *P. fluorescens* in aqueous solution to that of a conventional perhydrolase by generating a single site mutant enzymes.[35] In addition, the molecular basis for this was presented through modeling of the reaction intermediate. The enzymatic epoxidation reaction proceeds in the order of formation of peroxycarboxylic acid through lipase-catalyzed perhydrolysis, epoxidation of alkene, and producing the corresponding epoxide (Scheme 1).[36] Using this approach, the epoxide can be produced from catalytic

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[33] Kirk, O.; Christensen, M. W.; Damhus, T.; Godtfredsen, S. E. *Biocatalysis* **1994**, *11*, 65-77.

[34] Chua, S.; Xu, X.; Guo, Z. *Process Biochem.* **2012**, *47*, 1439-1451.

[35] Bernhardt, P.; Hult, K.; Kazlauskas, R. J. *Angew. Chem. Int. Ed.* **2005**, *44*, 2742-2746.

[36] Björkling, F.; Godtfredsen, S. E.; Kirk, O. *J. Chem. Soc., Chem. Commun.* **1990**, 1301-1303.

amounts of carboxylic acids. Researchers have demonstrated a more efficient method of epoxidation.[37]

Chiral epoxide is an important intermediate in the synthesis of various chiral compounds. Enzymatic stereoselective epoxide synthesis has been actively studied as an alternative to conventional chemical approaches.[38] In this study, it was hypothesized that the attachment of peracid inside of CAL-B achieves stereoselectivity in subsequent epoxidation. The cofactor compounds were introduced into CAL-B, the enzyme that is actively used in research as a stable enzyme, then improved epoxidation reactivity was observed.

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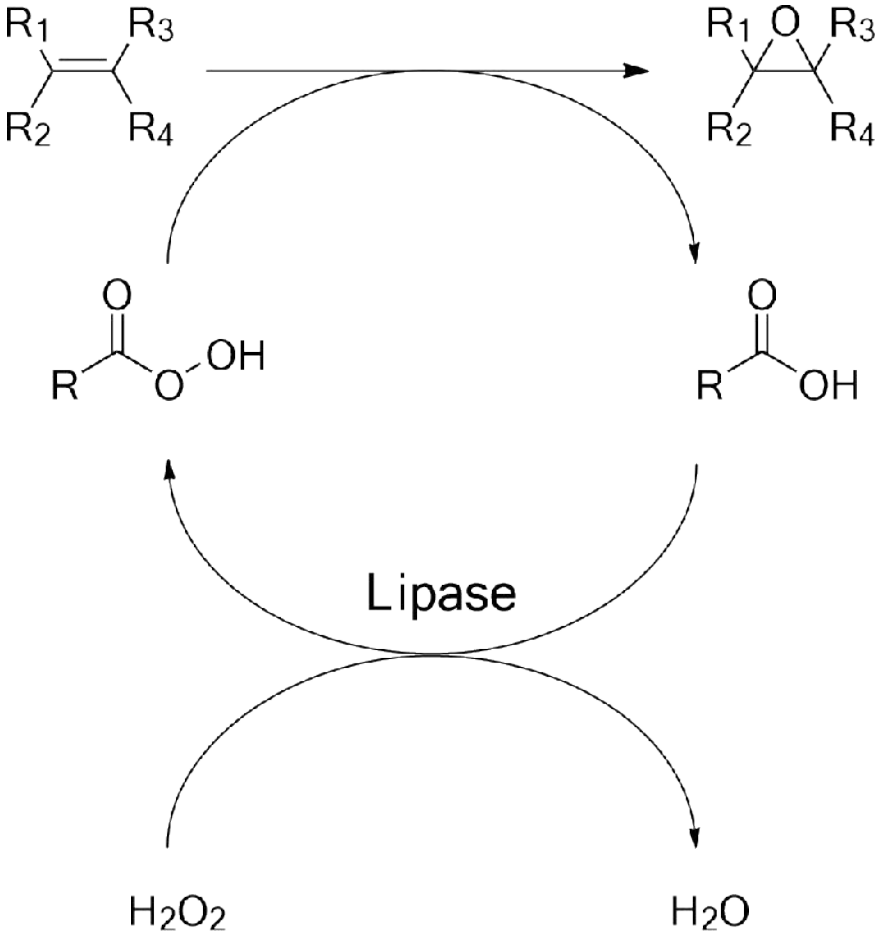
[37] Zhou, P.; Wang, X.; Yang, B.; Hollmann, F.; Wang, Y. *RSC Adv.* **2017**, *7*, 12518-12523.

[38] Hwang, S.; Choi, C. Y.; Lee, E. Y. J. *Ind. Eng. Chem.* **2010**, *16*, 1-6.



Scheme 1. Epoxidation of alkenes using peroxycarboxylic acids produced from lipase-catalyzed perhydrolysis

3. 2. Results and Discussion

Preparation of mutant enzymes

Previously in our research group, two-fold higher perhydrolytic activity of A281T mutant enzyme of CAL-B was investigated in comparison with wild-type, based on a potential water channel of CAL-B.[39] Alanine of 281 residue is considered to constitute that. In this study, eight mutant enzymes identified the possibility of epoxidase reactivity of A281T mutation of CAL-B through improved perhydrolytic activity: wild-type CAL-B; A281T mutant enzyme; three double mutant enzymes, T138C/A281T, A141K/A281T and Q157C/A281T, were prepared by site-directed mutagenesis of the other sites from the A281T mutant; and three single mutant enzymes, T138C, A141K and Q157C, were obtained from wild-type for comparison.

Measurement of perhydrolytic activity by MCD assay

Perhydrolytic activity of mutant and the wild-type enzymes were compared using monochlorodimedone (MCD) (Figure 1). A141K/A281T and A281T showed the best activity, followed by A141K, T138C/A281T and T138C in order. In contrast, mutant enzymes in which Q157 was substituted with cysteine showed little perhydrolytic activity in both the single mutant (Q157C) and the double mutant (Q157C/A281T). That of the wild-type enzyme was similar with A141K.

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[39] Wittrup Larsen , M.; Zielinska, D. F.; Martinelle, M.; Hidalgo, A.; Jensen, L. J.; Bornscheuer, U. T.; Hult, K. *ChemBioChem* **2010**, *11*, 796-801.

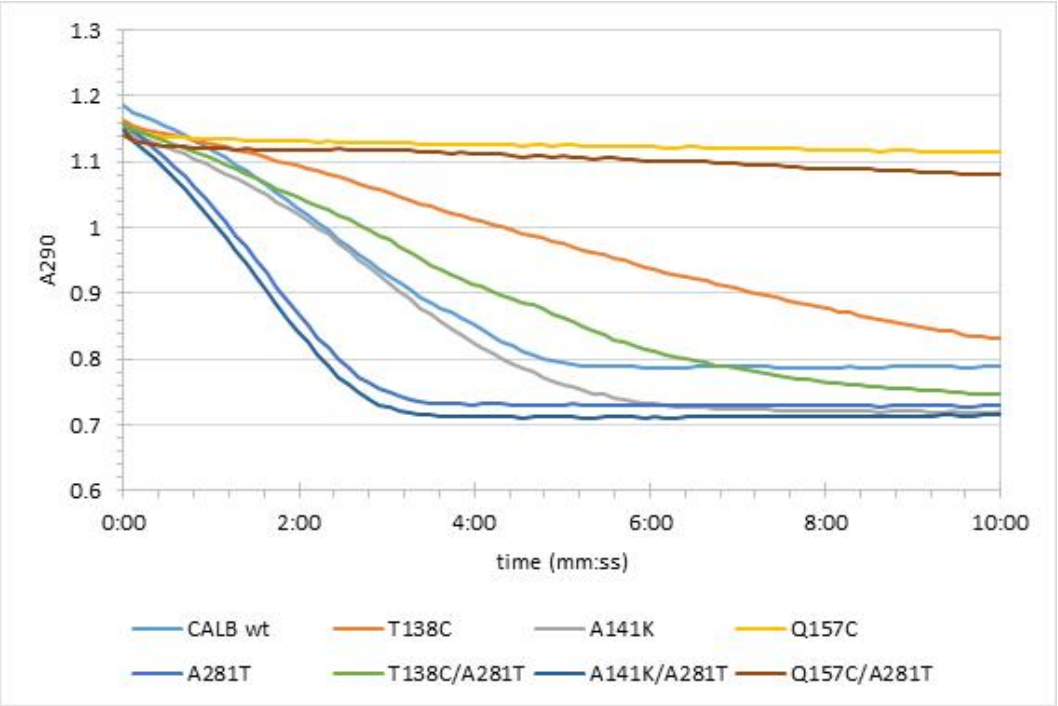


Figure 1. Comparison of perhydrolytic activity of CAL-B wild-type and mutants

Hydrolytic activity measurement through *p*NPAc and *p*NPBu

Hydrolytic activity of CAL-B mutant and wild-type enzymes was measured to ensure that it retains their own activity towards *p*-nitrophenyl acetate (*p*NPAc) and *p*-nitrophenyl butyrate (*p*NPBu). When using *p*NPAc, reactivity was greater in the order of T138C, T138C/A281T, wild-type, A141K, A141K/A281T and A281T (Figure 2). Q157C and Q157C/A281T did not exhibit the activity. When *p*NPBu was used in the measurement, the activity of A141K/A281T, A281T, A141K, and CAL-B wild-type was comparable, and T138C/A281T, T138C, Q157C, Q157C/A281T had very low activity (Figure 3).

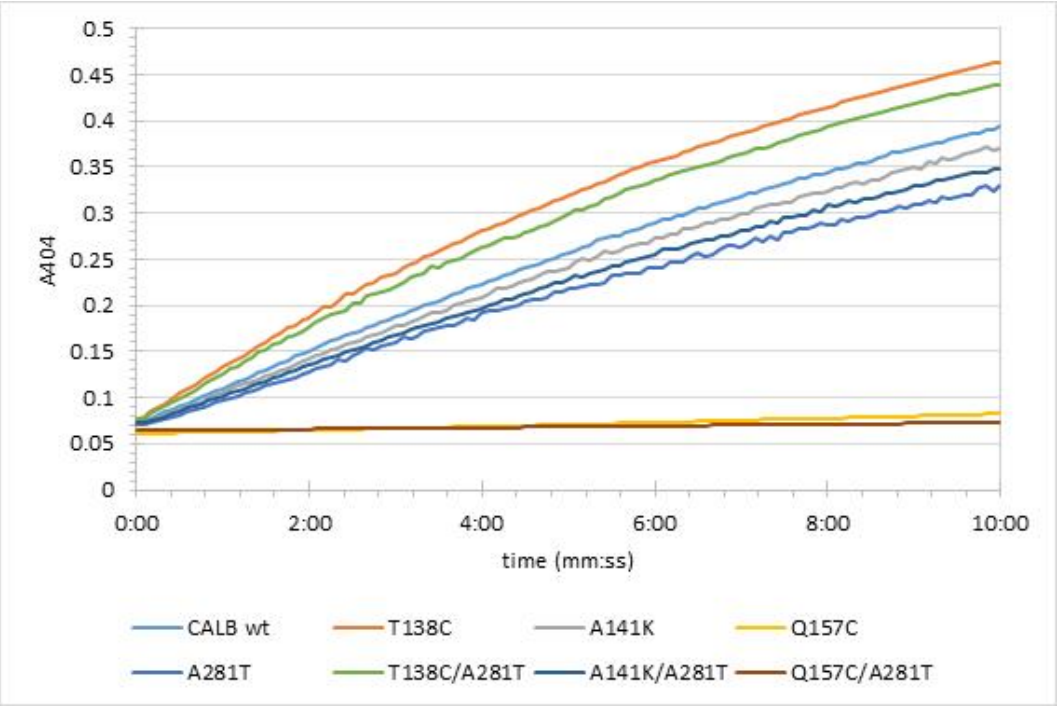


Figure 2. Hydrolytic activity of CAL-B wild-type and mutants with *p*NPAc

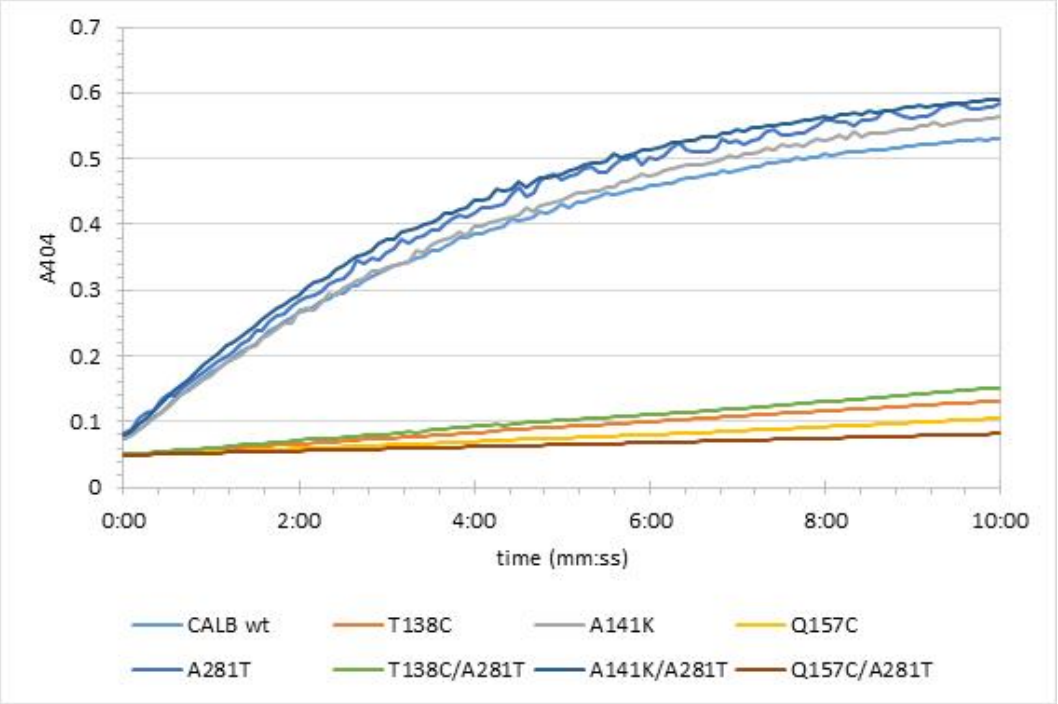


Figure 3. Hydrolytic activity of CAL-B wild-type and mutants with *p*NPBu

Maleimides conjugation on Cysteine

3-Maleimidopropionic acid (3ma), 4-maleimidobutyric acid (4ma), and 6-maleimidohexanoic acid (6ma) were bound to the cysteine of the mutant enzymes. Then the decrease in hydrolytic activity was measured since the hydrolytic activity of the enzymes could be disturbed by the conjugation chemical. To find the conditions of conjugation, the mutant enzymes containing T138C were mixed with a series of concentrations of maleimide variants in PBS buffer (pH 6.5) at 4 ℃. It is thought that the coupling at room temperature can induce enzyme denaturation, thus the coupling temperature was set at 4 ℃ and the change of activity was

confirmed at 1 h or more time interval. Determined conjugation condition for 3ma was 3ma of 0.01 M and 24 h or 0.02 M and 8 h (Figure 4(a)). For 4ma, it was 0.01 M and 40 h or more or 0.02 M and 24 h or more (Figure 4(b)). And for 6ma, 0.02 M and more than 40 h was appropriate (Figure 4(c)).

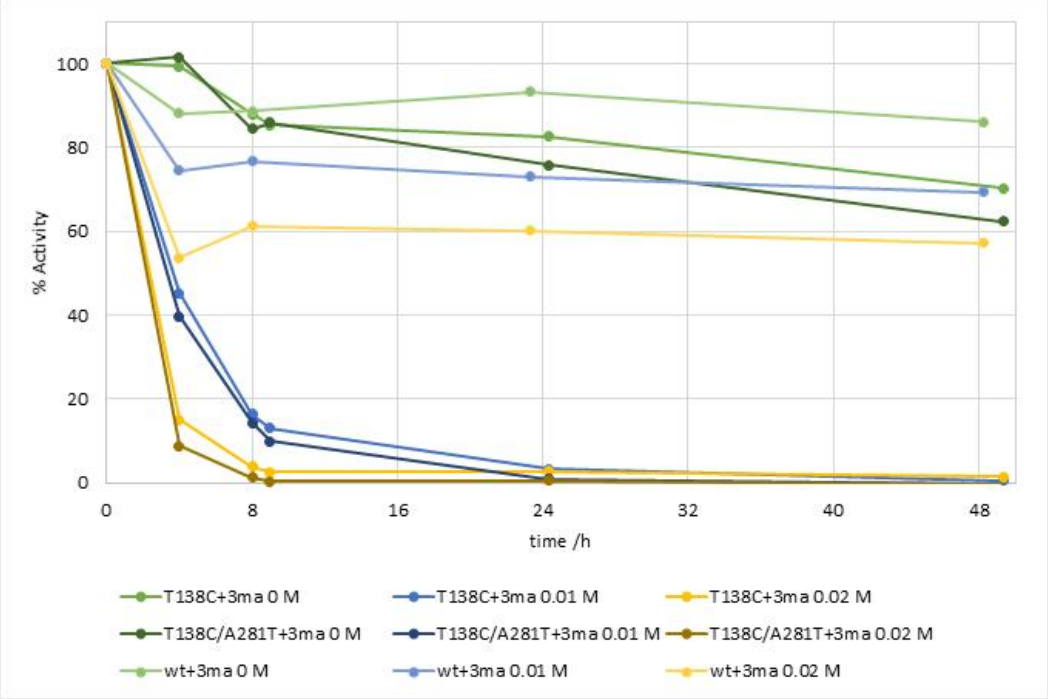


Figure 4(a). Determination of hydrolytic activity using *p*NPAc for identify the 3-maleimidopropionic acid coupling progress

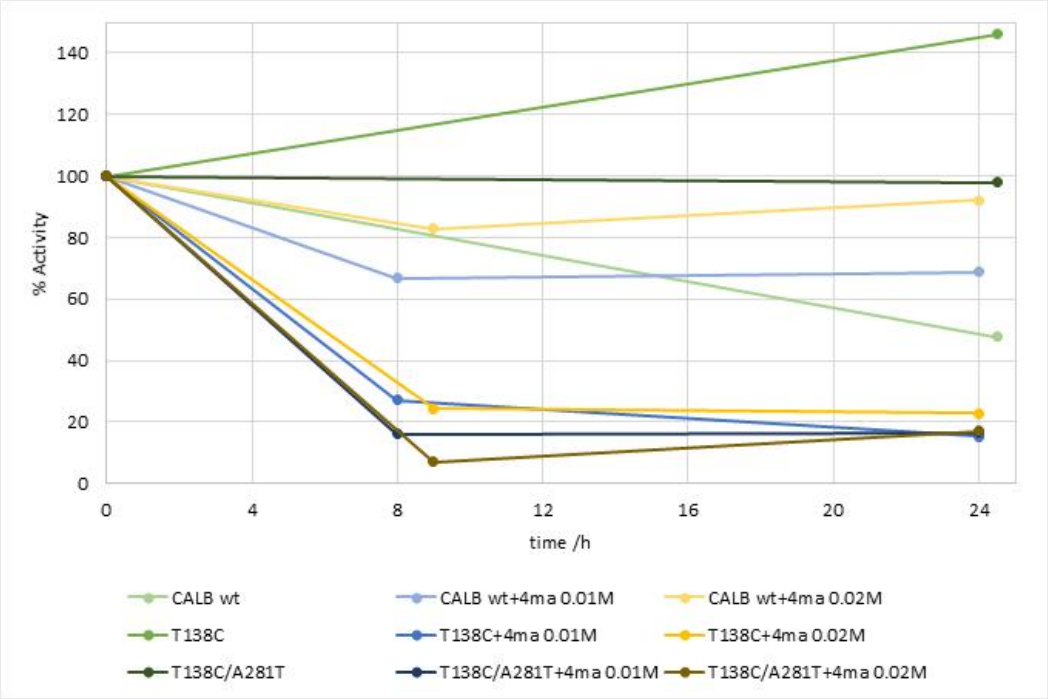


Figure 4(b). Determination of hydrolytic activity using *p*NPAc for identify the 4-maleimidobutyric acid coupling progress

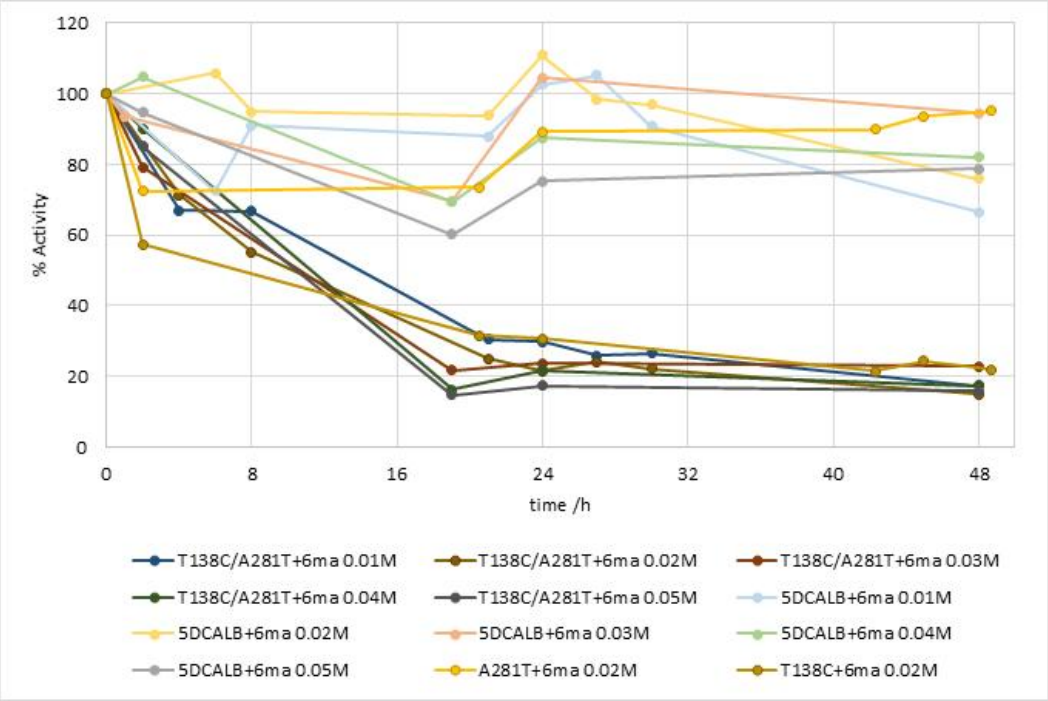


Figure 4(c). Determination of hydrolytic activity using *p*NPAc for identify the 6-maleimidohexanoic acid coupling progress

The maleimidocarboxylic acids conjugation on the mutant enzyme was identified by mass analysis. The mass of conjugated enzymes were compared with the T138C/A281T mutant enzyme to confirm whether the maleimidocarboxylic acids were stuck substantially. It is verified by the result (Table 1). The observed mass of mutant enzyme was slightly different from the theoretical mass since the mass of enzyme can have an observational error by the solvent molecules around the enzyme.

Table 1. Confirmation of maleimidocarboxylic acid conjugation on cysteine

Enzyme or maleimidocarboxylicacid	Molecular weight	
	Theoretical	Observed (increased)
CAL-B T138C/A281T	34303.49 Da	34397.2 Da
3-ma (3-maleimidopropionic acid)	169.13 Da	
CAL-B T138C/A281T 3-ma	34,471.62 Da	34566.3 Da (169.1)
4-ma (4-maleimidobutyric acid)	183.16 Da	
CAL-B T138C/A281T 4-ma	35485.65 Da	34581.4 Da (184.2)
6-ma (6-maleimidohexanoic acid)	211.21 Da	
CAL-B T138C/A281T 6-ma	34,499.68 Da	34608.6 Da (211.4)

5-Acetylvaleric acid conjugation on Lysine

Mutant enzymes including A141K were attempted conjugation with 5-acetylvaleric acid using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). When tested in buffer conditions, enzymes are easy to denature and the immobilized mutants in organic solvent, *tert*-butyl methyl ether (MTBE), did not represent meaningful reduction in hydrolytic activity even after a week.

α, β-Unsaturated ketone epoxidation

1) Selection of substrates

A series of α, β-unsaturated ketones used in this study was selected

with reference to Kim at al.[40] Eight selected α, β-unsaturated ketones were 2-cyclohexen-1-one (EP1), 3-penten-2-one (EP2), 3-hepten-2-one (EP3), *trans*-3-nonen-2-one (EP4), 1-hexen-3-one (EP5), 1-octen-3-one (EP6), *trans*-2-hexenal (EP7), and *trans*-2-octenal (EP8) (Figure 5).

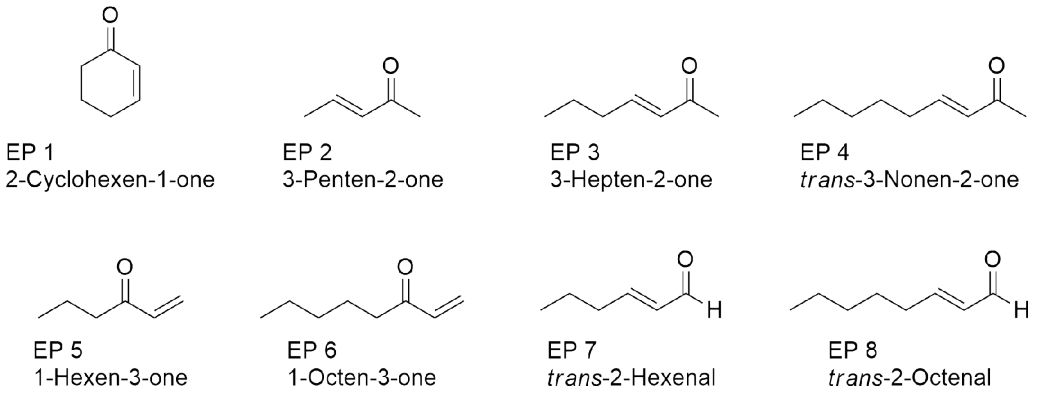


Figure 5. α, β-Unsaturated ketones for reaction substrates

2) Solvent screening and epoxidation reaction progress

Several buffers were tested for screening the reaction solvent. First, acetate buffer (1 M, pH 5.5), pentanoate buffer (0.6 M, pH 5.5), and BES buffer (5 mM, pH 7.2) were used as solvents to perform the background reaction under the enzyme-free conditions through EP1. The proper pH of the buffer was expected to be slightly acidic, since a buffer of high pH can itself cause epoxidation. As expected, BES buffer (pH 7.2) was excluded due to its high background reaction conversion. Subsequently, since CAL-B has affinity for a long substituent, EP4 was tested with various enzymes as a substrate (Figure 6). When 3ma-conjugated T138C/A281T was used, it showed a remarkably high conversion rate.

[40] Kim, J.; Jung, S.; Park, S.; Park, S. *Tetrahedron Lett.* **2011**, *52*, 2866-2868.

Therefore, EP4 was expected to be a suitable substrate for application to enantioselective epoxidation using the mutant enzyme, but the product was not detected in GC.

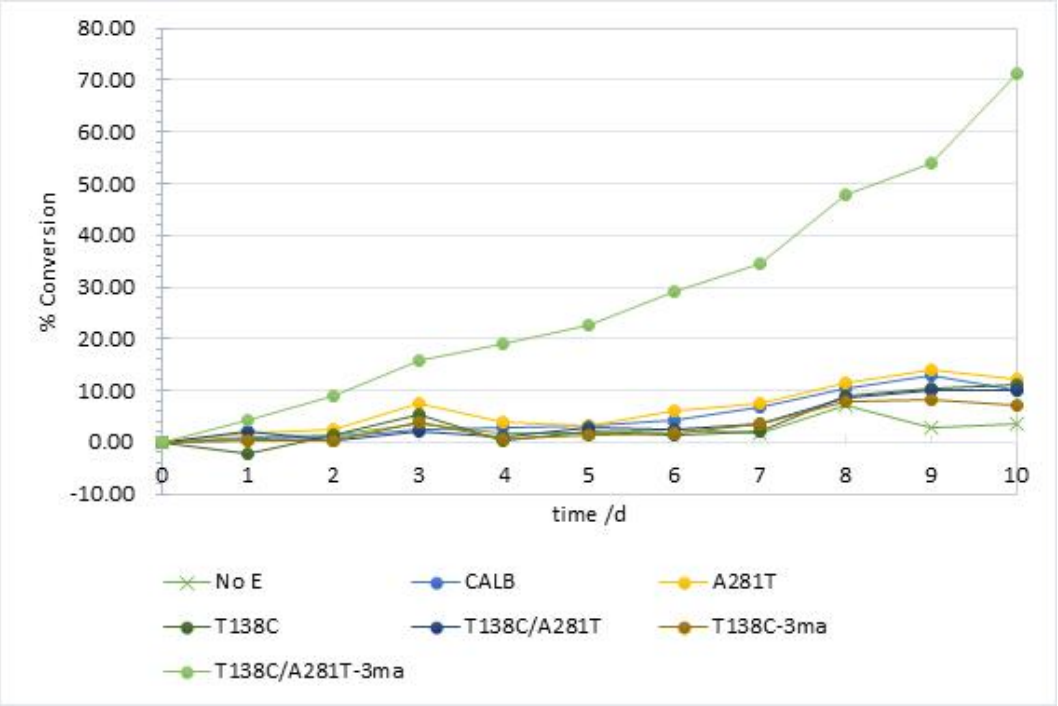


Figure 6. Conversions of *trans*-3-nonen-2-one (EP4) in pentanoate buffer (0.6 M, pH 5.5)

It was decided that the reaction was performed homogeneous by mixing the organic solvent in the buffer, considering the case where the product is not mixed in the buffer. As the organic solvent to be mixed, acetonitrile (MeCN), isopropyl alcohol (*i*PrOH), tetrahydrofuran (THF), and Tween 20 were considered. EP4, EP8 that have a long hydrophobic substituent were used as substrates, and MeCN 7% and 45% conditions were first tested in a pentanoate buffer (0.6 M, pH 5.5). Since the BG

conversion rate was high, other organic solvents were also sequentially confirmed. And since the perhydrolytic activity of CAL-B is higher in the pentanoate buffer than in the acetate buffer, the buffer for the reaction was changed. Based on the point where the mixture of 0.1 mmol of the substrates were completely mixed in 1 mL of the solvent, 30%, 50%, and 1% of *i*PrOH, THF, and Tween 20 were respectively added to acetate buffer (1 M, pH 5.5,). The conversion of the background reactions was high in the solvent mixed with Tween 20. In addition, the reactions in which *i*PrOH and THF were mixed showed a large variation, which is considered to be unstable in the reactivity of the enzyme due to the high organic solvent ratio.

The organic solvent to be mixed was kept in MeCN and the concentration of the buffer was reduced. To exclude the case where sodium contributes to the epoxidation reaction, the buffer was changed to ammonium acetate buffer (50 mM, pH 5.5). Instead of the substrate mixture, each of substrates was adjusted separately, and the MeCN ratio mixed for each substrate condition was determined: 0% acetonitrile for EP1, EP2, and EP7; 7% for EP3 and EP5; 12% for EP6; and 30% for EP4 and EP8. Although the immobilized enzyme on the bead was also considered, it was confirmed that the bead itself catalyzes the reaction. Thus the enzymes stored in PBS buffer (1 mM, pH 7.3) was used.

In addition, citrate buffer was selected and tested as another solvent candidate. Citrate presumably cannot enter the CAL-B reaction site, the buffer is not expected to be involved in the reaction and the background reaction conversion is expected to be very low. Two citrate buffers were

prepared: sodium citrate buffer (100 mM, pH 5.5) and ammonium citrate buffer (100 mM, pH 5.5). Background reaction was tested first, and sodium citrate buffer (100 mM, pH 5.5) with a lower conversion was selected. And EP7, which had the highest background conversion rate (57% on the 4 days after), was excluded from the next step. Subsequently, experiments were performed under various enzyme conditions, and results observed up to 4 days were compared (Figure 7). Except when EP4 was used as a substrate, the conjugated mutant enzymes exhibited high conversion rate than background condition. Especially the conversion toward EP2 with the T138C/A281T-3ma mutant enzyme showed 1.6 times higher than background condition and twice higher than wild-type after four days. Since the products were obtained as racemic mixture in all results, however, further studies are required to accomplish the enantioselective epoxide products.

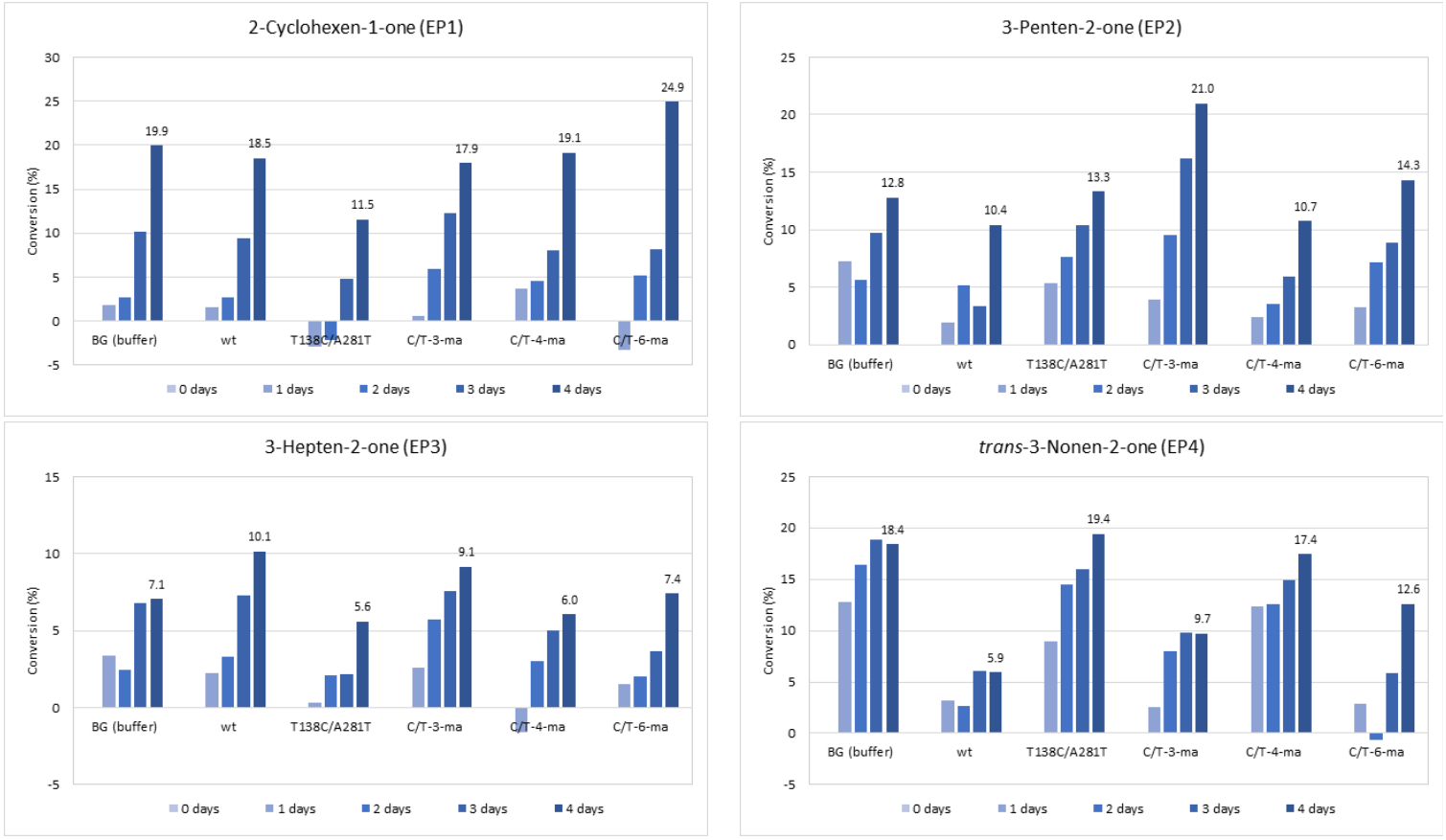
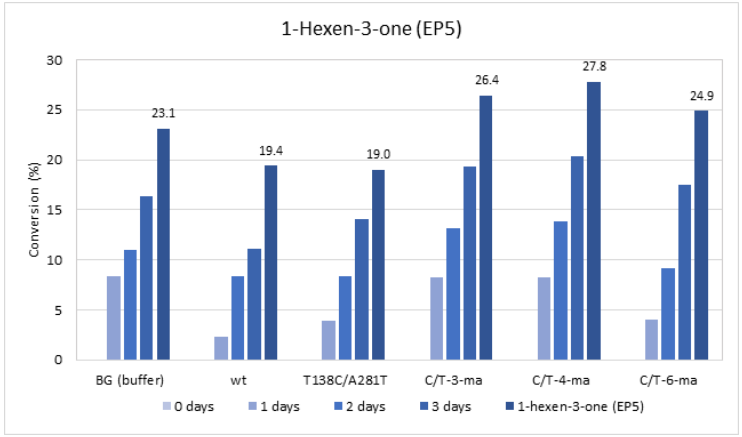
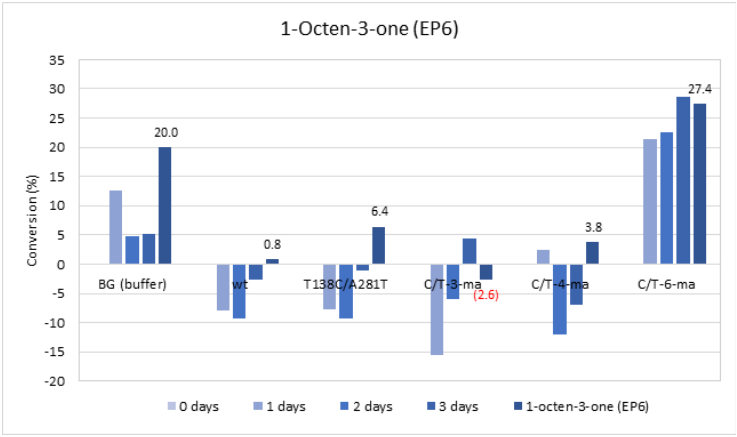


Figure 7. Conversion of the epoxidation reactions according to the substrates and the enzymes. CAL-B T138C/A281T mutant enzyme is abbreviated as C/T.





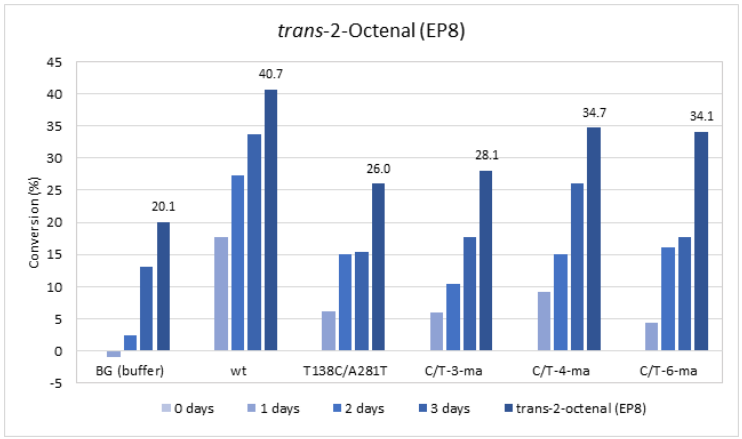


Figure 7. Conversion of the epoxidation reactions according to the substrates and the enzymes (*Continued*)

3. 3. Experimental Section

General Methods

Chemicals were purchased from TCI or Thermo Fisher Scientific. The vector (pBADgⅢa) was purchased from Invitrogen Korea (Seoul, Korea). Poly-Prep chromatography column and protein assay solution were purchased from Bio-Rad. The Ni-NTA agarose resin was purchased from QIAGEN. CIRCLEGROW® Broth was purchased from MP Biomedicals. DNA sequencing was carried out by Solgent Co. (Daejeon, Korea). Mass analysis was tested at the Life Science Laboratories Co. (Seoul, Korea). Gas Chromatography was analyzed by Agilent 6890N with a chiral capillary column (Cyclosil-B 30 m × 0.25 mm).

Site directed mutagenesis of CAL-B

Opt2_5D CAL-B[41] was used as the template of an overlap-extension PCR for performing the mutagenesis. The mutant genes were digested by Nco I and Sal I and inserted into the pBADgⅢa vector digested by the same restriction enzymes. Then the plasmids were transformed in the E. coli strain (Top 10).

Protein expression and purification

The protein expression and purification methods are referred by Chapter 2.

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[41] Jung, S. **2008**, 717-722.

Determination of the concentration of Protein

Protein concentration was calculated by measuring the absorbance at 280 nm using a Nano-drop. The calculation followed Beer-Lambert law ($A = \varepsilon bc$, A = absorbance; ε = molar coefficient of the protein; b = optical path length; c = concentration of the protein). The molar coefficients of the protein were obtained by protein parameter tool of ExPASy (https://web.expasy.org/protparam/).

Comparison of perhydrolytic activity of wild-type with mutant enzymes

The perhydrolytic activity was measured by absorbance at 290 nm, decreasing as monochlorodimedone (MCD) disappears. MCD assay solution contained 90 μL of 10 mM MCD, 43 μL of 30% wt H_2O_2, and 450 μL of 1 M NaBr in 4,167 μL of acetate buffer (1 M, pH 5.5) or pentanoate buffer (0.6 M, pH 5.5). The enzyme solution (10 μL) was mixed with assay solution (200 μL) and the absorbance changes were measured within 7 second intervals. The molar coefficient (ε) of MCD is 19,900 $M^{-1} \cdot cm^{-1}$.

Measuring of hydrolytic activity

The enzyme solution (5 μL) and assay solution (100 μL, 20 μL of 200 mM *p*-nitrophenyl acetate or *p*-nitrophenyl butyrate in acetonitrile, 870 μL of acetonitrile, 11,110 μL of 5 mM BES buffer) were mixed. And the absorbance changes at 404 nm were measured within 7 second intervals.

Maleimidocarboxylic acid conjugation on Cysteine

The composition for a series of concentrations of mutant enzyme and maleimidocarboxylic acids is followed (Table 2(a), (b), and (c)).

Conc. of 3ma (M)	0.00	0.01	0.02	0.05
Enzyme (1 mg/mL, μL)	50	50	50	50
1 M 3ma in DMSO (μL)	0	5	10	25
PBS buffer (pH 6.5, μL)	450	445	440	425
Total volume (μL)	500	500	500	500

Table 2(a). The composition for a series of concentrations of mutant enzyme and 3-maleimidopropionic acid

Conc. of 4ma (M)	0.00	0.01	0.02
Enzyme (1 mg/mL, μL)	100	100	100
500 mM 4ma in DMSO (μL)	0	20	40
PBS buffer (pH 6.5, μL)	900	880	860
Total volume (μL)	1000	1000	1000

Table 2(b). The composition for a series of concentrations of mutant enzyme and 4-maleimidobutyric acid

Conc. of 6ma (M)	0.00	0.01	0.02	0.03	0.04	0.05
Enzyme (2 mg/mL, μL)	50	50	50	50	50	50
500 mM 6ma in DMSO (μL)	0	20	40	60	80	100
PBS buffer (pH 6.5, μL)	950	930	910	890	870	850
Total volume (μL)	1000	1000	1000	1000	1000	1000

Table 2(c). The composition for a series of concentrations of mutant enzyme and 6-maleimidohexanoic acid

Epoxidation condition catalyzed by CAL-B mutants

Substrates (EP1-8, 0.05 mmol), 30% wt hydrogen peroxide (0.05 mmol), *t*-amyl alcohol as internal standard, and enzymes (100 μL, 2 mg/mL) were mixed and stirred with solvent (1 mL) in a 4 mL vial at 25 ℃ and 500 rpm. 30% wt hydrogen peroxide was added with 24h intervals. The 100 μL of reaction mixture was taken and mixed with *t*-butyl methyl ether (MTBE, 1.2 mL) containing sodium sulfite and sodium sulfate. After centrifugation, the supernatant was collected in 2 mL GC vial. GC analysis condition: 30 ℃/10 min - 3 ℃/min - 180 ℃/5 min. The substrate EP1-8 were detected at 30.1 min, 14.4 min, 27.8 min, 38.3 min, 15.8 min, 28.8 min, 23.2 min, and 34.9 min in order. The products for each substrate were detected at 36.1 and 36.6 min, 19.8 and 21.1 min, 31.3 and 32.1 min, 41.2 and 41.7 min, 28.2 and 29.6 min, 38.6 and 39.1 min, 26.4 and 26.9 min (two additional peaks were detected at 28.3 and 28.5 min in following order for EP7), and 37.2 and 37.5 min.

Conclusion and Summary

Candida antarctica lipase B (CAL-B) is one of the useful lipases in the preparation of chiral *sec*-alcohols because CAL-B is stable in organic media, and has excellent enantioselectivity towards various chiral *sec*-alcohols. However, CAL-B does not exhibit reliable enantioselectivity towards a few *sec*-alcohols bearing smaller substituents than a propyl group (*i.e.* butan-2-ol or but-3-yn-2-ol) because the medium binding pocket cannot distinguish the size difference between a ethyl and methyl group. Therefore, it can be hypothesized that alteration of the space of the medium binding pocket may influence the enantioselectivity of CAL-B towards such *sec*-alcohols. Recently, our research group found a homologous enzyme of CAL-B, which possesses high enantioselectivity toward but-3-yn-2-ol. After sequence comparison, it was revealed that a few amino acids in the medium binding pocket of CAL-B are different from those of the homologous enzyme. Based on the information, a few mutant enzymes were created by site-directed mutagenesis of the medium binding pocket of CAL-B as the corresponding amino-acids sequence of the homologous enzyme. The S47N mutant enzyme exhibited much higher enantioselectivity (E > 200) than the wild-type enzyme (E = 4-5) in transesterification reaction towards but-3-yn-2-ol. This result indicates that the information from homologous search can be utilized in a method for improving the selectivity of the enzyme.

Second, converting CAL-B to an epoxidase was studied. Although CAL-B can indirectly perform epoxidation through perhydrolysis, the

reaction occurs at outside of CAL-B without controlling stereochemistry. Hence, if an acid substrate is fixed in the active site, the expoxidation may occur in the active site, in which can provide an asymmetric environment. The threonine at the 138 residue was converted to cysteine to introduce maleimidocarboxylic acids near the active site of CAL-B because cysteine can specifically bind with the double bond of the maleimide. The conjugation of maleimidocarboxylic acid and the CAL-B mutant enzyme was confirmed by Mass spectrometry analysis. The conjugated system exhibited higher reaction rate towards the epoxidation of α, β-unsaturated ketone, although racemic products were obtained.

논문개요

환경친화적인 합성방법에 대한 관심이 높아지면서 효소를 이용한 화학 반응 연구가 지속적으로 이루어지고 있다. 효소는 일반적으로 온화한 조건에서 사용되며 반응 부산물이 거의 없는 등의 장점이 있다. 동시에 뛰어난 위치 및 입체 선택적 화학 반응을 촉진하는 유용한 생체 촉매이다. 이 중 *Candida antarctica* lipase B (CAL-B)는 열적 안정성과 내화학성이 우수한 효소로 알려져 있으며, 이러한 특징 때문에 효소 활용 화학 반응에서 연구자들이 널리 이용하는 효소이다. 본 연구에서는 이러한 CAL-B를 활용하여 작은 치환기를 갖는 이차 알콜에 대한 입체 선택성이 향상된 효소 변이체 생성을 수행하였고, 에폭시화 반응을 수행할 수 있는 효소 변이체 개발 연구를 수행하였다.

먼저, CAL-B의 입체 선택성 향상을 위해, 기존 연구에서 확인된 높은 입체 선택성을 지닌 CAL-B의 상동체 효소인 *Pseudozyma brasiliensis* GHG001(PBL)과 CAL-B 사이의 서열 비교분석으로부터 기질 치환체의 중간크기 결합 자리(medium binding pocket)의 아미노산 서열의 차이를 확인하였다. 그리고 CAL-B의 중간크기 결합 자리(medium binding pocket) 서열 대신 PBL의 서열을 도입하거나 단일자리 혹은 이중자리 치환을 수행하여 변이체들을 생성하였다. 이를 통해 본 연구에서는 CAL-B의 S47N 변이체에서 but-3-yn-2-ol에 대한 CAL-B의 입체 선택성을 50배까지 높였다(E > 200). 이러한 반응성 변화는 효소의 비활성도(specific activity)를 측정함으로써 기질의 느린 거울상 이성질체에 대한 반응성이 감소하면서 나타난 것임을 확인하였다.

두 번째로, 본 연구에서는 기존 효소의 반응성을 변화시켜 새로운 반응성

을 갖도록 시도하였다. 즉, 가수분해반응을 수행하는 CAL-B에 과산화수소수를 활용한 에폭시화 반응성을 도입하는 연구를 수행하였다. CAL-B는 물 대신 과산화수소수를 기질로 활용하여 과가수분해반응(perhydrolysis)을 수행할 수 있음이 알려져 있다. 그렇지만 CAL-B에 의한 과산화산(peracid) 생성 후에 이어지는 에폭시화 반응 등의 산화 반응은 효소 밖에서 진행되므로, 최종 산물의 입체 선택성은 기대할 수 없다. 따라서 만약 CAL-B에 의해 생성되는 과산화산을 효소 내부에 고정한다면, 연속적으로 일어나는 산화 반응의 입체 선택적 진행을 기대할 수 있을 것으로 고려된다. 이러한 가정하에 먼저 CAL-B 촉매 삼원소(catalytic triad) 주변에 cysteine을 도입한 변이체(T138C/A281T)를 생성하였으며, 여기에 인공보조인자 역할을 기대할 수 있는 maleimide류의 화합물을 도입하였다. 이를 통해 얻은 시스템은 효과적으로 α, β-불포화 케톤의 에폭시화 반응을 촉진할 수 있음이 확인되었다. 특히 3-penten-2-one를 기질로 한 반응에서 3-maleimidopropionic acid를 결합한 변이체는 바탕 반응에 대해 1.6배, 야생형 CAL-B에 대해 2배의 전환율을 나타냈다. 그러나 인공보조인자를 도입한 효소 변이체를 통한 입체 선택적 생성물은 발견하지 못해 이에 관한 추가 연구가 필요하다.

감사의 글

인준서에 사인까지 받고 나니 졸업한다는 게 이제야 실감 납니다. 그동안 주변에서 지켜봐 주신 감사한 분들에게 인사드리고자 짧게 적어봅니다.

먼저 지도교수님이신 박성순 교수님, 끝까지 이끌어주셔서 감사합니다. 중간에 포기하려 할 때도 한 발짝 앞에서 기다려 주셔서 따라가다 보니 과정을 마칠 수 있었습니다. 함께 실험실에서 지내며 하루의 절반 가까이를 함께 보낸 아름 언니, 윤재 언니. 같이 가는 사람들이 있어서 졸업까지 올 수 있었습니다. 기분도 컨디션도 오르락내리락 하는 일이 잦았는데 옆에 있어줘서 고마워요. 그리고 입학 때부터 학교생활에 많은 도움 주시고 얘기도 많이 들어준 경미 언니, 주희 언니 감사합니다.

항상 그 자리에 있어 준 가족들과 친구들에게도 고맙다는 말을 전합니다. 기억이 시작될 때부터 곁을 지켜준 엄마, 아빠, 수호, 수현, 내 가장 오랜 친구들, 정예, 소희, 유경, 해민, 세령, 꾸준히 안부 전해주는 규리, 미지, 영주, 예린, 다희, 아로, 주연, 민아, 윤정, 여진, 복길, 전공을 함께 배우면서 더 돈독해진 세은 언니, 정원, 지혜. 가끔 만나도 어제 본 것처럼 편한 사람들이 있다는 사실이 큰 도움이 됐습니다. 꾸준히 연락하고 고민도 함께 해주고 같이 추억할 거리를 만들어줘서 고맙다고 하고 싶었어요. 그리고 소중한 마이데이 친구들, 혜영 언니, 유경이, 성애 언니. 덕분에 지금까지 중에 가장 힘든 시기를 지나면서도 가장 즐겁고 행복한 시간을 보냈어요. 데이식스로 만났지만 그게 아니어도 오래 봐요, 우리.

마지막으로 대학원 생활에 대해 아낌없이 팁을 전해주시고 응원해주신 선배님들, 정수현 박사님, 민정 언니, 지원 언니, 소영 언니, 현아 언니, 예지 언니 모두 감사합니다. 덕분에 무사히 졸업까지 올 수 있었습니다. 이외에도 주변에서 저를 응원해주신 모든 분께 감사함을 전합니다.

아직 밖으로 나가기엔 부족하다고 생각하지만, 많은 분이 곁에서 도와주신 만큼 더 멋진 모습으로 성장하는 사람이 되겠습니다. 모두 행복하고 건강하시길 바랍니다. 감사합니다.