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석사학위 청구논문

Rational approach for improving enantioselectivity and altering reaction mechanism of hydrolases

단백질 조작을 통한 가수분해효소의 입체선택성 향상과 반응 메카니즘의 전환

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화 학 과

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논문개요

효소는 높은 반응성과 선택성을 가지므로, 여러 합성 중간체로 사용되는 키랄 아민, 키랄 에스터, 키랄 알코올 등을 효율적으로 제조하는 데 활용되어 왔다. 많은 경우 효소는 광범위한 기질 특이성을 가지므로 다양한 종류의 반응에 응용될 수 있으며, 그 반응 조건이 온화하고, 환경 친화적인 장점을 가지고 있다. 이러한 효소의 장점에 기인하여, 청정화학이 중요시 되고 있는 최근에는 효소의 산업적 이용이 더욱 중요시 되고 있다. 그러나 효소는 경우에 따라 비천연화합물에 대한 충분한 선택성이나 반응성을 보이지 않으므로, 산업적으로 보다 효과적인 활용에 어려움이 있다. 연구자들은 이러한 문제점을 해결하기 위해 자연계에서 새로운 효소를 찾거나, 기존에 알려진 효소를 이용하여 화학적 혹은 분자생물학적 접근방법을 통해 효소의 선택성 혹은 반응성을 향상하려는 노력을 해왔다. 본 논문에서는 분자생물학적 접근방법을 통하여 효소의 기능을 변화시키는 연구를 다루었다.

본 논문에서는 첫 번째로 분자생물학적 방법을 활용하여 기존의 효소가 보이는 기질에 대한 입체선택성을 높이는 연구를 다루었다. 광학적으로 순수한 tetrahydrofuran-2-carboxylate (THFC)는 의약 산업 및 연구에 있어서 중요한 중간체이다. 본 연구에서는 단백질 조작을 통해 얻은 CAL-B 라는 *Candida antarctica*에서 추출된 lipase의 변형체들을 이용하여 THFC의 산소원자와 수소결합을 하거나 또는 반발력을 유발하도록 디자인되었다. 효소변형체들 중 일부는 wild type 보다 높은 입체선택성을 보였고, 반대되는 입체 선호도를 보였다. 유기용매에서는 I189D 변형체가 wild type보다 높은 입체선택성 (I189D, E = 3.0; wild type, E = 1.9)을 보였으며, 반대되는 입체선호도 (I189D = *R*; wild type = *S*)를 보였다. 수용액에서는 I189S 변형체가 wild type보다 높은 입체선택성 (I189S, E = 2.2; wild type, E = 1.3)을 보였다. 그

리고 대부분의 효소 변형체들은 수용액보다 유기용매에서 최대 1.6배 증가된 입체 선택성을 보였다.

두 번째로, 본 논문은 분자생물학적 방법을 활용하여 효소의 선천적인 반응성과는 다른 새로운 반응성을 도입시키는 연구를 다루었다. 본 연구는 PerPA (perhydrolase from *Pseudomonas aeruginosa*) 효소 내부에 금속이온이 결합할 수 있는 효소변형체를 생산함으로써 산화반응성을 도입시키는 것을 목적으로 하였다. 이를 위하여, 먼저 분자모델링을 통하여 반응 중심에 금속이온이 결합 가능한 위치를 선정하였다. 그리고 분자생물학적인 방법을 통하여 금속이온과 결합할 수 있는 히스티딘과 글루탐산/아스파르트산을 도입하였다. 생성된 효소변형체들 중 F165H/L32E 변형체 (specific activity: 8.9×10^{-4} μmol · sec^{-1} · mg^{-1})는 wild type (specific activity: 8.4×10^{-6} μmol · sec^{-1} · mg^{-1})과 나머지 효소변형체들에 비해 100배 높은 산화반응성을 보였다.

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Chapter 1. Introduction

Biocatalysts are referred to biological molecules that can speed up a chemical reaction. They are either enzymes or whole cells, which are originated from microorganism, plant or animal origin. Although enzymes have been used for thousands of years for a wide variety of biotechnological processes, which include the production of foods such as beer, wine, and cheese without the knowledge of enzymes. During the past few decades, the biocatalysis and the biotechnological applications have been developed by a wealth of novel methods. Biocatalysis is often called 'green chemistry', 'environmentally benign production processes' or 'sustainable development'. These words imply that the biocatalysts become important tools for a wide range of industrial applications.[1)]

Compared to conventional chemical catalysts, enzymes are remarkable catalysts and they have some unique characteristics (Table 1).[2)] The most important advantage of enzymes is their high selectivity. The selectivity includes substrate selectivity, chemoselectivity, and enantioselectivity. Such high selectivity enables enzymatic reactions not to produce many by-products. And using enzymes can avoid requiring multiple protection and deprotection steps commonly found in typical organic syntheses. In addition enzymes are environmentally benign reagents because they are completely degradable. Enzymatic reactions usually proceed in mild conditions. Enzyme acts in a range of about pH 5-8, typically around

pH 7. And the favorable temperature is around 30 ℃.

Table 1. Advantages and disadvantages of enzymes

Advantages	Disadvantages
Enzymes are very efficient catalysts	Enzymes are prone to substrate- or product-inhibitor
Enzymes can catalyze a wide range of reactions	Enzymes often need to their natural cofactors
Enzymes show high selectivities	Enzymes display their highest catalytic activity in water
Enzymes are environmentally acceptable	Enzymes may cause allergies
Enzymes are under mild conditions	The extreme process condition leads to deactivation of enzyme
Enzymes are compatible with each other	

1.1. Hydrolases

Hydrolases are a group of enzymes catalyzing hydrolysis. They are classified as EC 3 in the EC number and further classified into several subclasses, based on their acting on bonds (Table 2).[3)]

Table 2. The definitions of hydrolase class and subclasses[a]

Class	Enzyme	Type of reaction	Subclasses	
3	Hydrolases	Hydrolysis reactions	3.1	Acting on ester bonds
			3.2	Glycosylases
			3.3	Acting on ether bonds
			3.4	Acting on peptide bonds (peptide hydrolases)
			3.5	Acting on carbon-nitrogen bonds, other than peptide bonds
			3.6	Acting on acid anhydrides
			3.7	Acting on carbon-carbon bonds
			3.8	Acting on halide bonds
			3.9	Acting on phophorus-nitrogen bonds
			3.10	Acting on sulfur-nitrogen bonds
			3.11	Acting on sulfur-sulfur bonds
			3.12	Acting on carbon-sulfur bonds
			3.13	Acting on carbon-sulfur bonds

[a] http://www.expasy.org/enzyme/

The representative hydrolases are lipases (EC 3.1.1.3) and esterases (EC 3.1.1.1). These enzymes are useful in many industrial applications and academic studies because of their interesting characteristics. First, hydrolases show broad substrate specificity and can catalyze formations of various synthetic intermediates. Second, hydrolases are relatively cheap, do not require co-factors, and have high stability. Third, hydrolases catalyze not only hydrolysis but also various related reactions - esterification, transesterification (alcoholysis), acidolysis, interesterification, and aminolysis (Figure 1).[4)]

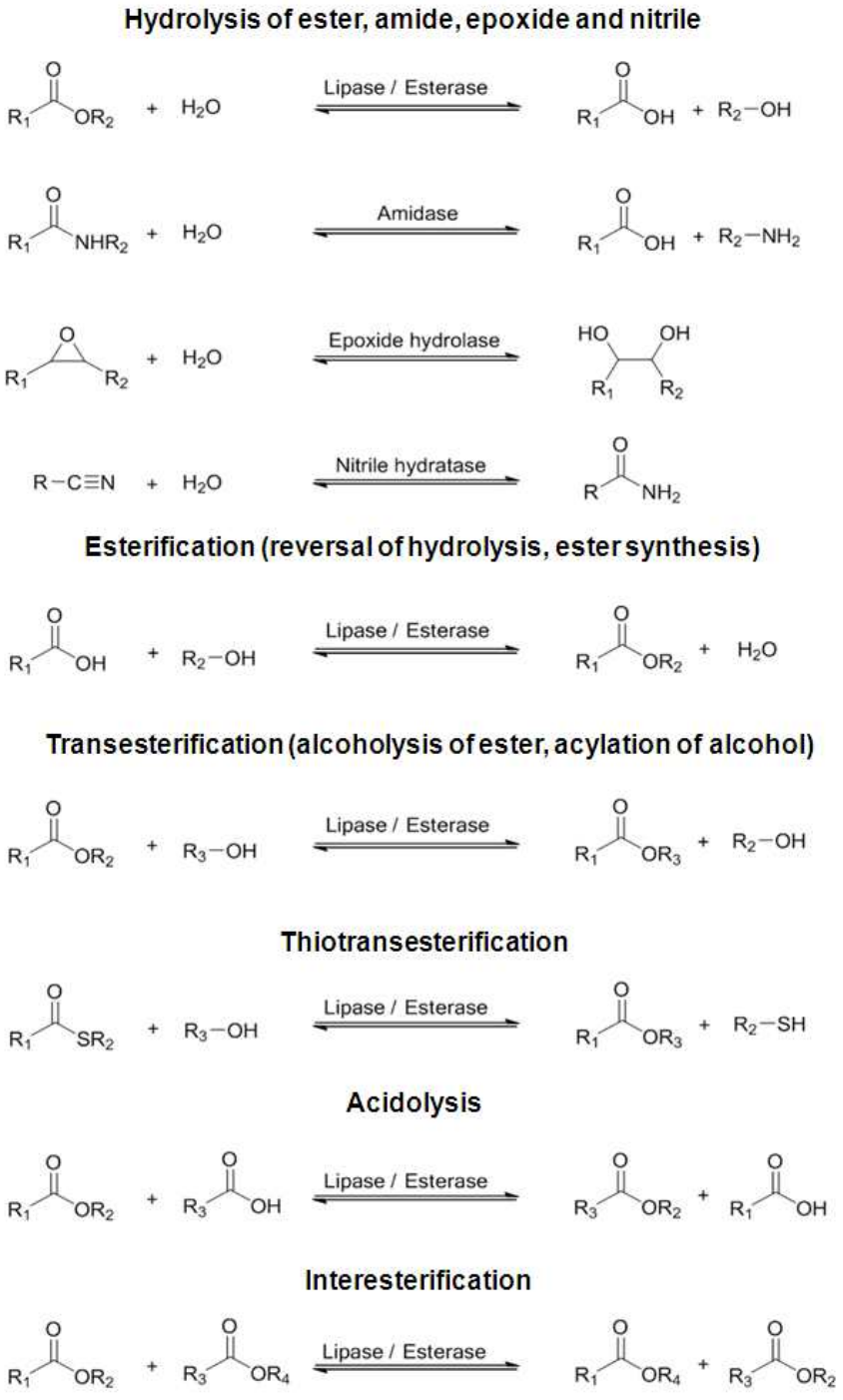
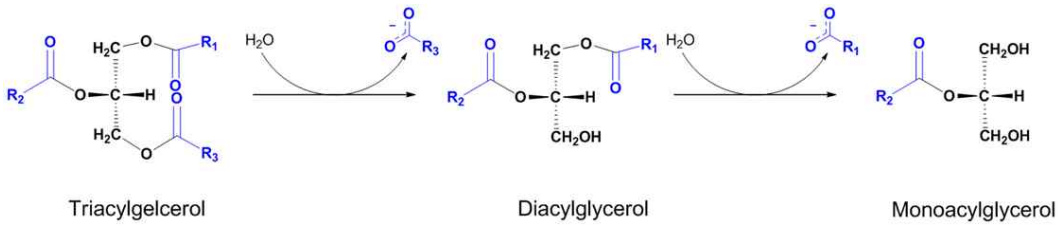


Figure 1. Reactions catalyzed by hydrolases

1.1.1. Lipase

Lipases (EC 3.1.1.3) are ubiquitous enzymes and are found in most organisms. They belong to the family of serine hydrolases and α/β-fold family. They contain the catalytic triad, Asp/Glu-His-Ser, which is similar to that of serine proteases.[5] Although both lipases and esterases catalyze hydrolysis of esters, lipases preferentially catalyze the hydrolysis of water-insoluble esters such as triglycerides to form fatty acids, diacyglycerol, monoacylglycerol, and glycerol (Scheme 1).[6] In addition, lipases not only catalyze the hydrolysis of natural esters but also unnatural esters with high enantio- or regioselectivity. These characteristics of lipases make them suitable for organic synthesis and in preparation of enantiomerically-pure pharmaceuticals and synthetic intermediates.[7]



Scheme 1. Natural action of lipases. Lipases convert triglycerols into fatty acids and monoacylglycerol.

A distinct property of lipases is their interfacial activation that is not observed for esterases (Figure 2). This phenomenon is that their activity is much higher when substrates are present at a water-oil interface compared to the water phase. This implies that lipases do not require water-soluble substrates. The process is caused by their lid/flap (α helical segment). Most but not all lipases (an exceptional example: lipase B from *Candida antarctica,* which have only a small or no lid) have a lid/flap, which can control access of substrates to the active site. When the substrate and the water-oil phase exist, the lid opens and then lipase becomes active, whereas the lid closed and the enzyme remains inactive without substrate.[8)]

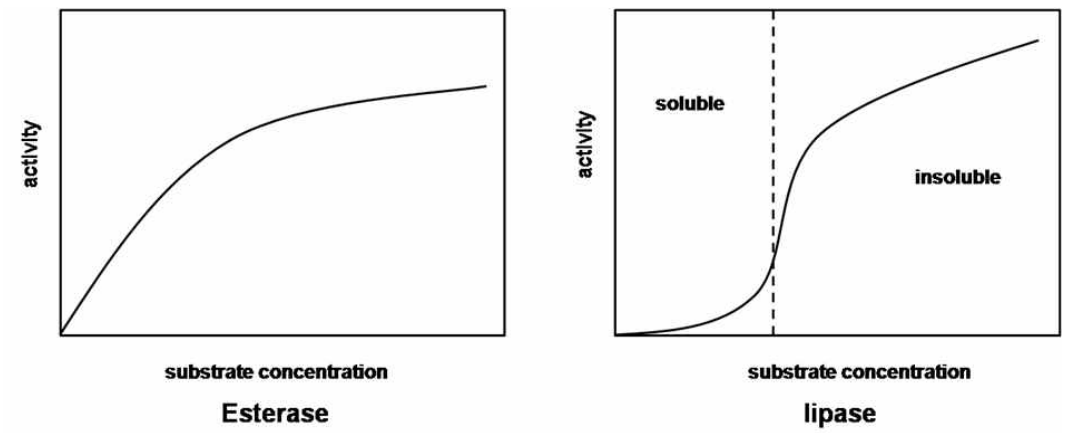


Figure 2. Kinetic behaviors of esterases and lipases. Esterases follow Michaelis-Menten kinetics and lipase kinetics shows interfacial activation.

1.1.2. *Candida antractica* lipase B (CAL-B)

The yeast *Candida antarctica* was originally isolated in Antarctica and was found to produce two lipase variants (CAL-A and CAL-B). They show different characteristics.[9] CAL-A depends on calcium ion and more thermostable than CAL-B. CAL-B is metal-independent lipase and active on a broad range of non-natural esters with high selectivity. CAL-B has been used more often in preparative applications because selectivity is major concern in organic synthesis. CAL-A shows the interfacial activation while CAL-B is no interfacial activation because CAL-B contains small or no lid/flap region that covers the active site. Uppenberg et al. solved the structure of CAL-B in 1994 (Figure 3).[10]

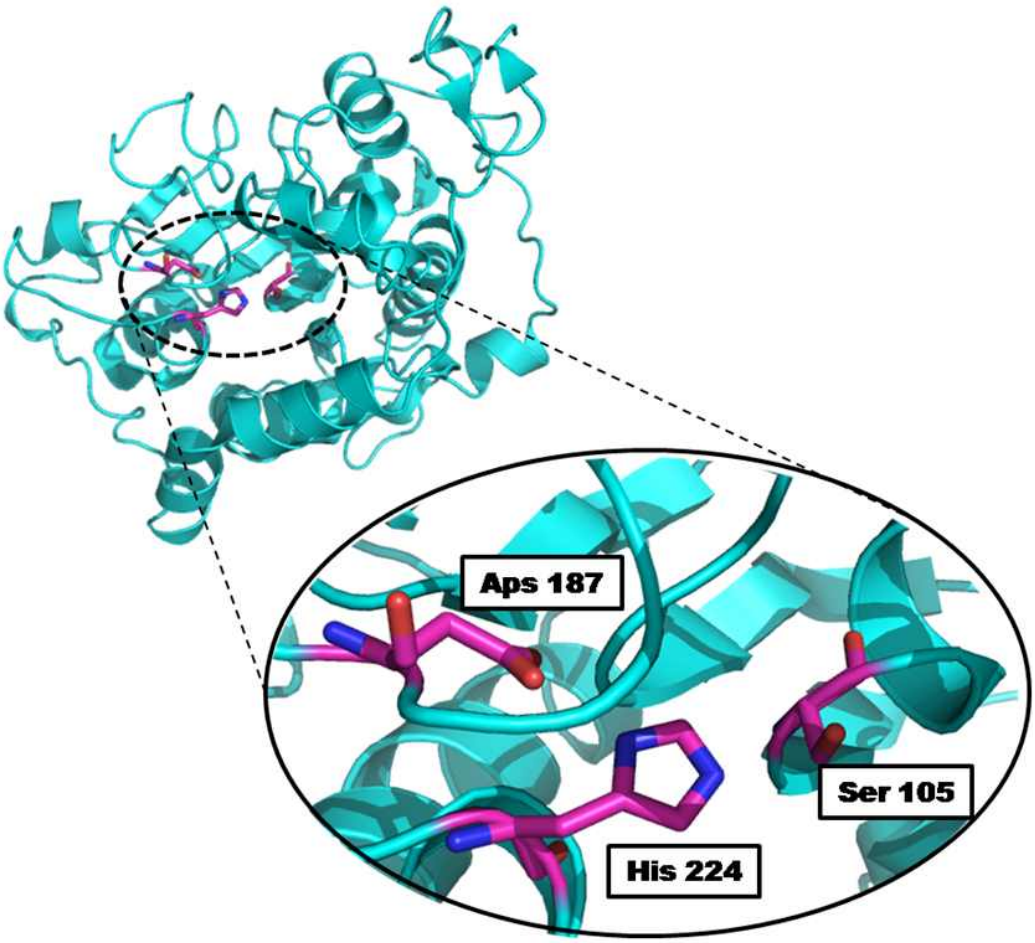


Figure 3. The structure of *Candida antarctica* lipase B. CAL-B belongs to the α/β hydrolase fold family. The catalytic triad consists of Ser105, Asp187 and His224. They are represented in stick.

CAL-B is composed of 317 amino acids and has a molecular weight of 33 kDa. CAL-B contains the catalytic triad, Ser105-His224-Asp197, common to serine hydrolases. CAL-B catalyzes acyl-transfer reaction through the bi-bi mechanism (Figure 4).[5)]

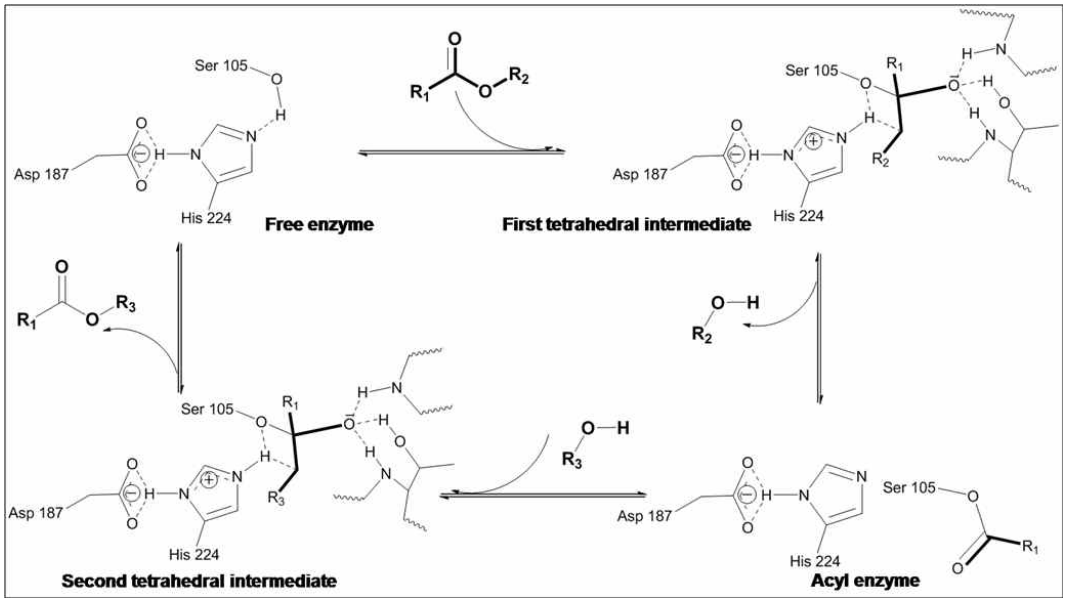


Figure 4. CAL-B-catalyzed reaction following a ping-pong mechanism. The catalytic residues, Ser105, Asp187, and His224, form the basis of this mechanism. The first substrate enters the active site and the first tetrahedral intermediate. An acyl enzyme intermediate forms by the release of the alcohol and then is attacked by the second substrate to form the second tetrahedral intermediate. Release of the second product regenerates the free enzyme.

1.2. Oxidases

Oxidation plays an important role in organic chemistry for introduction of a functional group into the starting material or intermediates. Conventional oxidation catalysts have several drawbacks (Table 3). Oxidases can solve the unsolved problems of oxidation reactions in conventional chemical catalyst and provide feasible alternatives to chemical synthetic routes with their high selectivities.[11)]

Table 3. The drawbacks of conventional catalysts

Entry	Drawbacks
1	Many oxidants are based on toxic metal ions which are harmful to the environment
2	Undesired side reactions are formed easily
3	Molecular oxygen is the most inexpensive and unharmful oxidant, but cannot be used efficiency.
4	It is difficult to perform oxidations with a regio- and stereoselectivity.

Oxidoreductases are a large class of enzymes catalyzing biological oxidation/reduction. They are classified as EC 1 in the EC number and further classified into several subclasses, based on substrates (Table 4).[3)]

Table 4. The definitions of oxidase class and subclasses[b]

Class	Enzyme	Type of reaction	Subclasses	
1	Oxidoreductase	Oxidation/ reduction	1.1	Acting on the CH-OH group of donors
			1.2	Acting on the aldehyde or oxo group of donors.
			1.3	Acting on the CH-CH group of donors.
			1.4	Acting on the CH-NH(2) group of donors
			1.5	Acting on the CH-NH group of donors.
			1.6	Acting on NADH or NADPH
			1.7	Acting on other nitrogenous compounds as donors.
			1.8	Acting on a sulfur group of donors
			1.9	Acting on a heme group of donors.
			1.10	Acting on diphenols and related substances as donors.
			1.11	Acting on a peroxide as acceptor.
			1.12	Acting on hydrogen as donor.
			1.13	Acting on single donors with incorporation of molecular oxygen.
			1.14	Acting on paired donors, with incorporation or reduction of molecular oxygen.
			1.15	Acting on superoxide as acceptor
			1.16	Oxidizing metal ions
			1.17	Acting on CH or CH(2) groups.
			1.18	Acting on iron-sulfur proteins as donors.
			1.19	Acting on reduced flavodoxin as donor.
			1.20	Acting on phosphorus or arsenic in donors.
			1.21	Acting on x-H and y-H to form an x-y bond.
			1.97	Other oxidoreductases.

[b] http://www.expasy.org/enzyme/

Oxidases can be grouped according to their requirement for the oxidant (Figure 5).[12] Dehydrogenases depend on a nicotinamide cofactor [NAD(P)H] and catalyze hydrogen removal but do not involve in active oxygen intermediates. Oxygenases and oxidases react with molecular oxygen. Oxygenases introduce one or two oxygen atoms into their substrates. Monoxygenases catalyze the introduction of one atom of oxygen into a substrate molecule, utilizing cofactor [NAD(P)H] to supply reducing potential for the supply of electrons to the substrate. This system can be metal-, heme-, or flavin-dependent. For example, cytochrome P450 is mediated reaction by cofactors containing a transition metal (Fe) and heme group. In dioxygenases-reactions, two oxygen atoms are simultaneously transferred onto the substrate. Oxidases mainly catalyze the electron-transfer onto molecular oxygen. They include flavoprotein oxidases, metallo-flavin oxidases, and heme-protein oxidases.

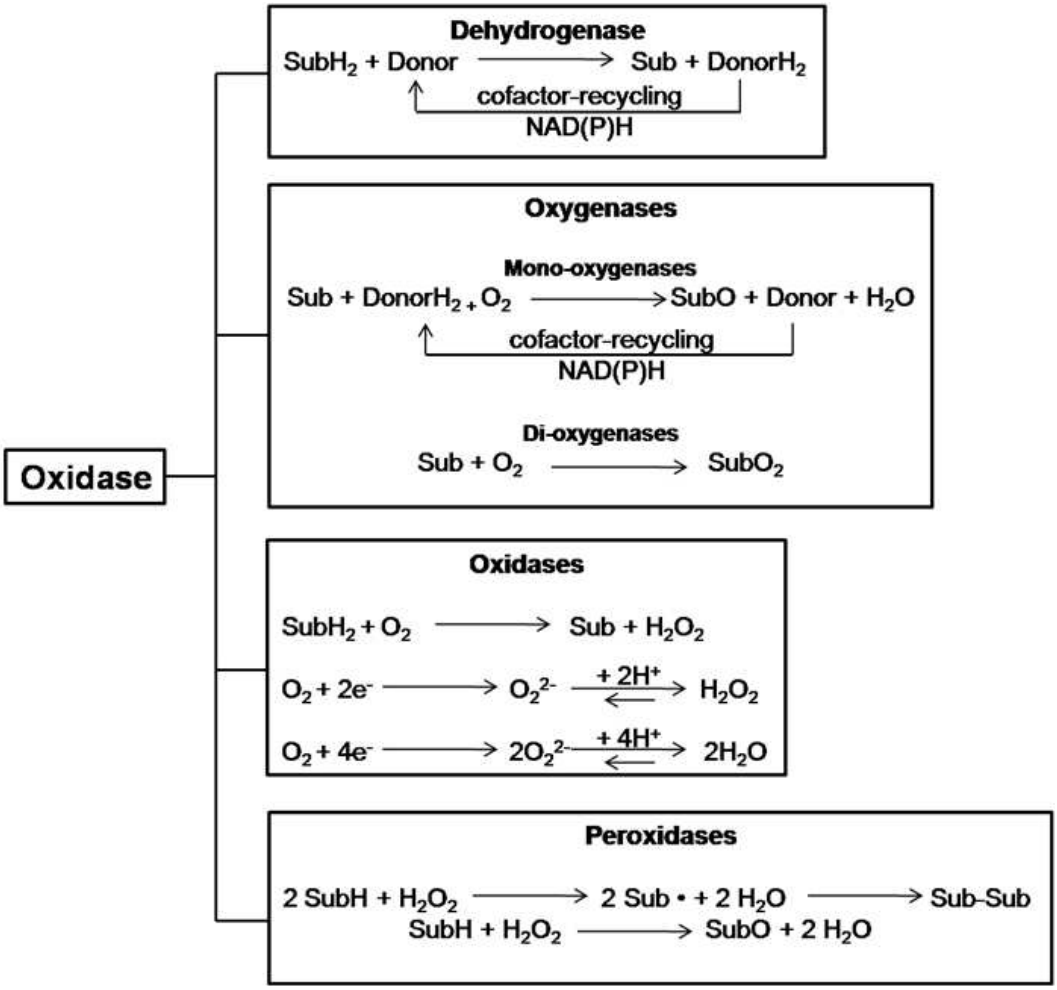


Figure 5. Grouping of oxidases according to their requirements for the oxidant.

1.2.1. Perhydrolase (Metal-free haloperoxidase)

Peroxidases can react with oxygen, generally producing reactive oxygen intermediates and then react further with reducing substrate.[12] The importance of peroxidases for organic synthesis is that they can use chemical oxidants, such as hydrogen peroxide. Hydrogen peroxide is an ideal oxidant as it is cheap, highly active, and environmentally friendly (producing water as the reduction product). Therefore the oxidation reaction by peroxidases with hydrogen peroxide is attractive and effective.[11]

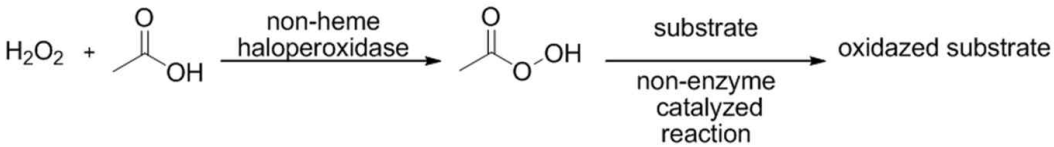
Haloperoxidases (E.C. 1.11.1) are a type of peroxidase that is able to catalyze oxidation of halides with peroxides such as hydrogen peroxide. They have been found from various sources, such as plants, animals, and microorganisms. And these enzymes are classified according to their cofactor dependence (Table 5).[13]

Table 5. The classifications of peroxidase

Entry	Enzyme
1	Heam-cotaining haloperoxidase
2	Vanadium-containing haloperoxidase
3	Metal-free haloperoxidase (perhydrolase)

Among the haloperoxidases, perhydrolases (metal-free haloperoxidase) have advantages compared to heme-containging haloperoxidase or vanadium-containing haloperoxidase.[14] Perhydrolases contain neither a heme group nor any metal ions. Therefore, they can't be inactive through oxidative destruction of the heme porphyrin ring by the oxidant. And they do not need to generate cofactor system, which prohibits industrial applications of haloperoxidases.[15),16)]Therefore, perhydrolases are suitable for industrial applications.

The perhydrolases can catalyze the reversible formation of peroxyacids from carboxylic acids and hydrogen peroxide (Scheme 2).[17] The reaction mechanism for PerPA (perhydrolase from *Pseudomonas aeruginosa*) as an example of perhydrolase has been proposed (Figure 6).[18] The catalytic triad is composed of three residues, Ser-His-Asp, similar to esterase/lipase. They can catalyze various reactions. For example, they catalyze halogenation of various aromatics or alkenes and oxidation of many sulfides. The scheme 2 shows two-step reaction composed of enzymatic and non-enzymatic reactions. Thus, the real oxidation reaction can't show regio-, chemo-, and enantioselectivity.[19]



Scheme 2. The reaction catalyzed by perhydrolases. The first reaction is formation of peroxyacids in the presence of hydrogen peroxide in the

enzyme and the followed reaction is the non-enzymatic oxidation of substrate. The reaction shows that the perhydrolase can provide peroxyacid (generally powerful oxidizing reagents) for oxidation without regio-, chemo- or enantioselectivity.

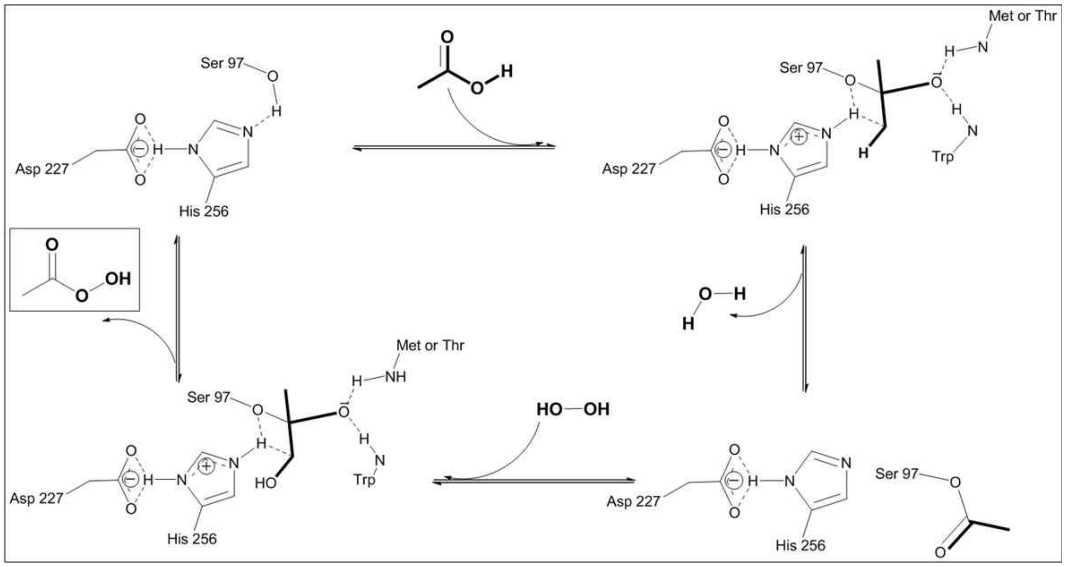


Figure 6. The mechanism for PerPA (perhydrolase from *Pseudomonas aeruginosa*). The first reaction is the nucleophilic attack on the carboxyl carbon atom of the acid by Ser97 and the formation the acyl enzyme by release of water. The second reaction is that hydrogen peroxide attacks acyl enzyme to form a peracid.

1.2.2. Perhydrolase from *Pseudomonas aeruginosa* (PerPA)

The amino-acid sequence of PerPA (perhydrolase from *Pseudomonas aeruginosa*) was identical to putative chloroperoxidase of *Pseudomonas aeruginosa* PAO1 genome sequence. PerPA has a molecular weight of 30.4 kDa.

PerPA showed halogenation activity which was the activity of already known bacterial perhyrolases. The halogenation of phenol red and monochlorodimedone (MCD) were performed by PerPA to estimate halogenation activity. The specific activity of PerPA towards phenol red and MCD were 11.9 units/mg and 8.5 units/mg.[18)]

The crystal structure has not been solved yet (Figure 7). However, a homology structure can be constructed based on the crystal structure of esterase from *Pseudomonas putida* (pdb code: 1ZOI) with 82 % sequence identity.

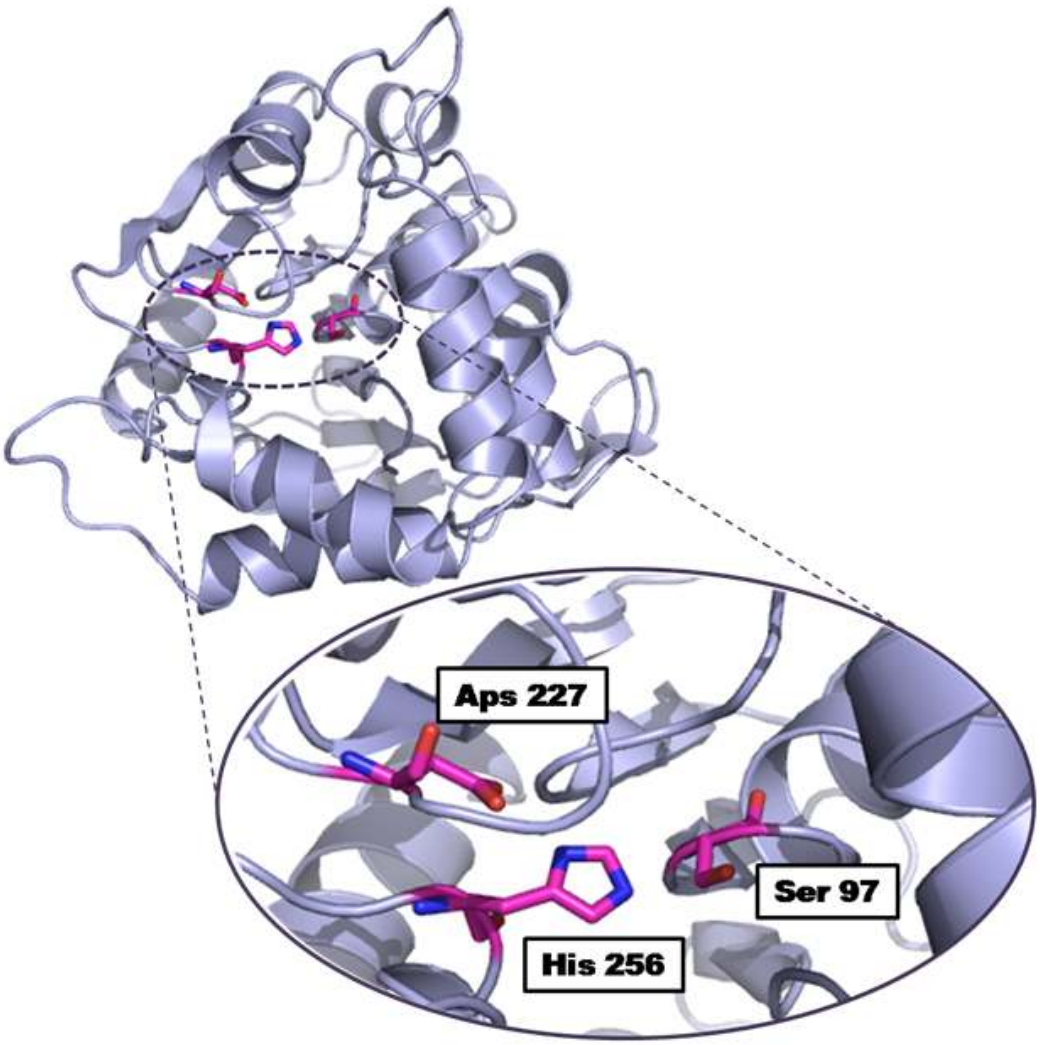


Figure 7. A homology model of PerPA. The sequces of PerPA was 100 % identical to chloroperoxidase of *pseudomonas aerogisa* PA01 sequeces. The catalytic triad consists of Ser97, Asp227, and His256. They are represented in stick.

1.3. Protein engineering

The industrial application of biocatalysts requires high reactivity, stability, and selectivity under process conditions. In many cases, the stability of the enzymes is too low in the organic solvent and at the pH or temperature required for reaction. To overcome these problems, the biocatalyst and the biocatalytic process need to be modified.[20] One can create optimized mutant enzymes to fulfill desired industrial synthesis from wild-type enzyme by protein engineering. Modification of enzyme can improve the stability of the wild-type enzyme and the performance towards non-natural reactions, or change the enzyme to catalyze a new reaction. Protein engineering is divided into two approaches - rational and random mutagenesis (Figure 8).[21]

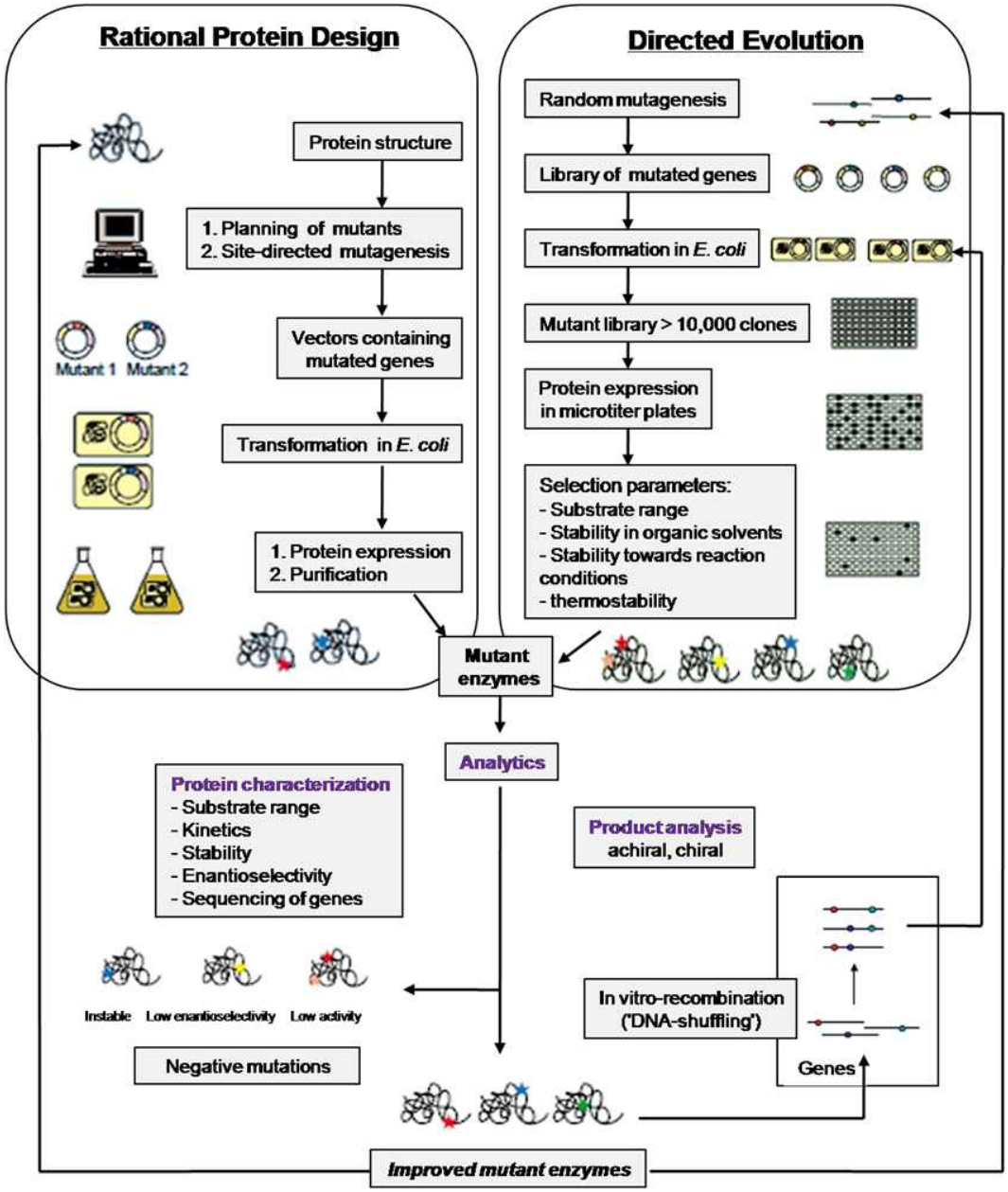


Figure 8. Comparison of rational protein design and directed evolution. Both approaches can be repeated and combined until biocatalysts with desired abilities are generated (The figure is adapted from Bornscheuer,U. T.,Pohl, M. *Curr. Opin. Chem. Biol.*, **2001**, *5*, 137-142)

Rational design requires both the structure of the enzyme (or homology model) and knowledge about the relationships between sequence and mechanism. Rational design methods of protein engineering involve the selection of specific amino acid residues for alteration to achieve the desired reactions. Generally computational chemistry techniques have been used to confirm target amino acids. And site-directed mutagenesis is utilized to change amino acids to increase the selectivity, activity, and the stability of enzymes. The rationally designed enzymes enable the identification of the important amino acids that might function modulation or change the given enzymatic reaction. Therefore, this method is useful to find optimal biocatalytic process and reaction conditions for desired reaction. In many cases, the rational design method can make a change close to the active site mutation to improve the catalytic performance. It is too difficult to predict the effect by distant mutation. This fact is sometimes disadvantage of the rational design engineering. However, many cases showed that the rational design can improve the selectivity, activity and reactivity. For example, Magnusson et al. introduced substrate-assisted catalysis in CAL-B by deletion of a hydroxyl group in the oxyanion hole using site-directed mutagenesis. The activity of the enzyme decreased dramatically, but could be restored partly if a hydroxyl group was introduced in the correct position in the substrate. Accordingly, the enantioselectivity, E, in hydrolysis of ethyl 2-hydroxypropionate increased from 1.6 to 22 for the mutant Thr40Val.[22)]

Random mutagenesis methods, such as error-prone PCR and DNA shuffling, can create thousands of enzyme variants. After screening or selection, the best variants can be selected. This process is often used

several times over and is called 'directed evolution' referring to Darwin's evolution theory. For example, directed evolution of an esterase from *Pseudomonas fluorescens* yields a mutant with excellent enantioselectivity and activity for the kinetic resolution of a chiral building block, the secondary alcohol *rac*-but-3-yn-2-ol. A triple mutant showed high enantioselectivity (E = 89) in a kinetic resolution to yield the building block (*S*)-but-3-yn-2-ol. And a double mutant showed excellent selectivity (E = 96) and activity (20 min for 50 % conversion, which corresponds to 1.25 U per mg of protein).[23)]

1.4. Definition of the enantiomeric ratio

In the enzymatic kinetic resolution, the selectivity of a resolution is called the enantiomeric ratio, E. This value implies the ability of an enzyme to distinguish enantiomers. This is defined as the ratio of specificity constants (k_{cat}/K_M) for both enantiomers.[24)]

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

E: enzyme

A and B: enantiomeric substrates

v: reaction velocities of A and B

k_{cat}: the rate constant for [ES]→ E + P or the turnover number

K_M: the Michaelis-Menten constant

E is related to the difference in Gibbs free energy of activation between the two enantiomers, $\Delta\Delta G_{A-B}^{\ddagger}$ (figure 9).[25]

$$\triangle\triangle G^{\ddagger} = -RTln\frac{v_A}{v_B} = -RTlnE$$

$\Delta\Delta G_{R-S}^{\ddagger}$: the difference in Gibbs free energy of activation between the two enantiomers

R: gas constant (8.31 $Jmol^{-1}K^{-1}$)

T: temperature

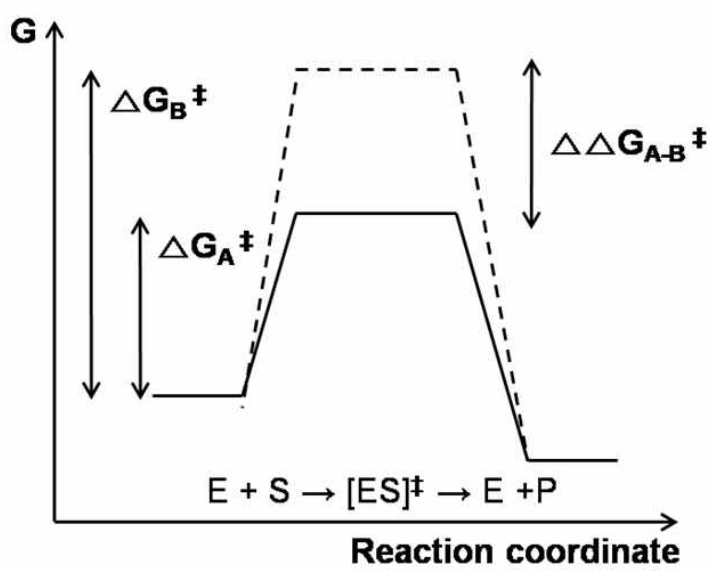


Figure 9. Energy profiles for an enzymatic reaction with a chiral substrate

1.5. Altering selectivity and reaction mechanism of hydrolases - outline of this thesis

Enzymes are remarkable catalysts because of their high selectivity and activity toward natural substrates under mild conditions. However, enzymes sometimes do not have enough selectivity and activity toward unnatural substrates. Enhancing their selectivity and activity can be achieved by molecular biological modification. This thesis deals with modification of hydrolases to alter selectivity or functionality by protein engineering.

In chapter 2, this chapter is described to increase enantioselectivity of CAL-B (lipase from *Candida Antarctica*) by molecular biological approach. Rationally designed protein engineering of CAL-B was applied to increase enantioselectivity for kinetic resolution of butyl tetrahydrofuran-2-carboxylate.

Chapter 3 deals with introduction of peroxidase activity to perPA (perhydrolase from *pseudomonase aeruginosa*) by protein engineering. We have designed an artificial metalloenzyme by imitation of natural one. In order to accept a metal ion, we introduced His and Glu/Asp into the enzyme, like natural metalloenzyme.

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Chapter 2. Improving the enantioselectivity of *Candida antarctica* lipase B for kinetic resolution of butyl tetrahydrofuran-2-carboxylate

Abstract: Enantiopure tetrahydrofuran-2-carboxylate (THFC) is an important intermediate in the pharmaceutical industry and research. However, there has not been established an efficient way of yielding of optically pure THFC by conventional synthesis. We present increasing the enantioselectivity of CAL-B in kinetic resolution of butyl tetrahydrofuran-2-carboxylate by rationally designed protein engineering. Some mutants showed not only higher enantioselectivity but also reverse enantio-preference compared to wild type. In organic solvent, the I189D mutant enzyme showed higher enantioselectivity with reversed enantio-preference (E = 3.0, R-selective) than the wild-type enzyme (E = 1.9, S-selective). And in organic solvent, E values of most enzymes showed higher enantioselectivity than those in the aqueous media up to 1.6 times.

Introduction

Preparation of enantiomerically pure compounds is one of the most active fields in synthetic chemistry because chirality is a key factor in the efficiency of therapeutic agents. The enzymatic kinetic resolution is an advanced tool for preparation of enantiopure compounds using remarkable chemo-, regio-, and stereoselectivity of enzyme.[1)]

Enantiopure tetrahydrofuran-2-carboxylate (THFC) is an important intermediate as well as a building block in the pharmaceutical industry and research. The *(R)*-tetrahydrofuran-2-carboxylic acid was first incorporated into a penem skeleton to give furopenem, which is a clinically efficient non-natural β-lactam antibiotic (Figure 1).[2)] However, there has not been established the efficient way of yielding optically pure THFC by conventional synthesis.

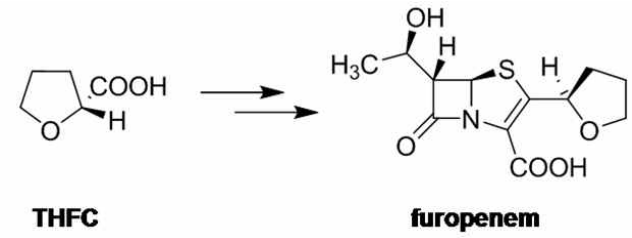


Figure 1. Structure of THFC and furopenem

Enzymatic kinetic resolution could be one of the methods to produce the enantiopure THFC. Hydrolases can be used to resolve THFC. However, most biocatalysts including CAL-B have low enantioselectivity toward butyl tetrahydrofuran-2-carboxylate. This study deals with present increasing the enantioselectivity of CAL-B in a kinetic resolution of butyl tetrahydrofuran-2-carboxylate by rationally designed protein engineering.

Experimental section

Chemicals and buffers were purchased from Sigma-Aldrich. LB-broth was purchased from Merck. Pfu DNA polymerase and restriction enzymes (*Nde* I and *BamH* I) were purchased from Enzynomics (Daejeon, Korea). DNA oligomers were obtained from Sigma-proligo (Singapore) and Bionics (Korea). DNA sequencing was performed by Solgent Co. (Daejeon, Korea). The Ni-NTA agarose resin was purchased from QIAGEN. Polystyrene-coated magnetic beads were donated from our lab.

Mutagenesis of CAL-B genes

Opt2CAL-B, lipase from *Candida antarctica*, was optimized expression in *E.coli*.[3] The opt2CAL-B was subcloned into pBAD/gⅢa vector containing restriction sites for *Nco*I and *Sal*I (Figure 2). The mutant genes were created by Quikchange mutagenesis with mutagenesis primers. The sequences of the opt2CAL-B and mutagenesis primers are listed in Table 1. After the PCR reactions, the plasmids of the mutants were transformed into *E.coli* (TOP10).

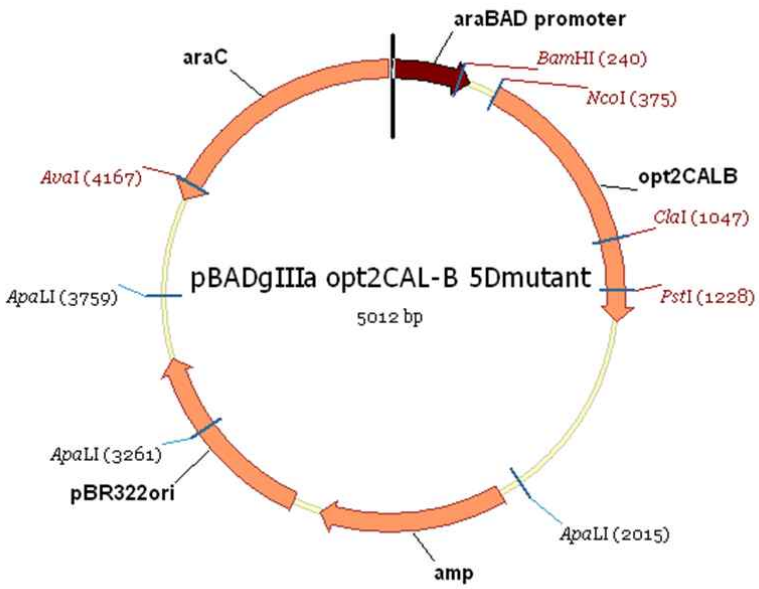


Figure 2. The vector map of opt2CAL-B as a template for mutagenesis.

Table 1. Codon-optimized sequences of CAL-B and mutagenesis primers

Sequence (opt2CAL-B)
CTG CCG TCT GGT TCC GAT CCG GCT TTC TCC CAG CCG
AAA TCC GTG CTG GAC GCG GGT CTG ACC TGT CAG GGT
GCT TCT CCA AGC AGC GTG TCT AAA CCG ATC CTG CTG
GTA CCG GGC ACC GGT ACC ACT GGC CCG CAG TCT TTC
GAC AGC AAC TGG ATT CCA CTG TCC ACC CAA CTC GGT
TAT ACT CCT TGC TGG ATC TCT CCG CCG CCG TTT ATG
CTG AAC GAT ACT CAG GTA AAC ACT GAA TAC ATG GTA
AAC GCT ATC ACC GCT CTG TAC GCA GGT TCT GGT AAC
AAC AAA CTG CCA GTG CTG ACC TGG TCC CAG GGT GGT
CTG GTT GCA CAA TGG GGC CTG ACT TTC TTC CCG TCT
ATC CGT TCT AAA GTG GAC CGT CTG ATG GCA TTC GCT
CCG GAC TAC AAA GGT ACT GTG CTG GCT GGC CCG CTG
GAT GCA CTG GCT GTA TCT GCG CCA TCC GTG TGG CAG
CAG ACC ACT GGT TCT GCG CTG ACC ACT GCA CTG CGT
AAC GCT GGT GGT CTG ACC CAG ATC GTT CCG ACT ACT
AAC CTG TAC AGC GCA ACC GAT GAG ATC GTT CAG CCG
CAG GTA TCT AAC TCC CCG CTG GAT TCT TCT TAC CTG
TTC AAC GGT AAG AAC GTT CAG GCT CAG GCT GTT TGT
GGC CCG CTG TTC GTT ATC GAT CAC GCA GGT TCC CTG
ACC TCC CAG TTC AGC TAT GTG GTT GGC CGC TCT GCT
CTG CGC TCC ACC ACT GGT CAA GCG CGC TCT GCT GAC
TAC GGC ATC ACC GAC TGC AAC CCG CTG CCG GCG AAC
GAC TTA ACC CCG GAA CAG AAG GTT GCA GCT GCG GCT
CTG CTG GCA CCG GCT GCA GCT GCA ATT GTT GCG GGC
CCG AAA CAG AAC TGC GAA CCG GAC CTG ATG CCG TAC
GCT CGT CCG TTC GCG GTT GGT AAA CGC ACT TGT TCT
GGC ATC GTA ACT CCG

Mutagenesis primers
CAL-B D134K_F 5′-CGTCTCATGGCATTTGCCCCTAAATATAAAGGCACAGTGCTCGCG-3′ CAL-B D134K_R 5′-CGCGAGCACTGTGCCTTTATATTTAGGGGCAAATGCCATGAGACG-3′
CAL-B D134N_F 5′-CGTCTGATGGCATTCGCTCCGAACTACAAAGGTACTGTGCTGGCT-3′ CAL-B D134N_R 5′-AGCCAGCACAGTACCTTTGTAGTTCGGAGCGAATGCCATCAGACG-3′
CAL-B D134Q_F 5′-CGTCTGATGGCATTCGCTCCGCAGTACAAAGGTACTGTGCTGGCT-3′ CAL-B D134G_R 5-′AGCCAGCACAGTACCTTTGTACTGCGGAGCGAATGCCATCAGACG-3′
CAL-B V190G_F 5′-GCCACCGACGAAATTGGCCAGCCGCAGGTCTCC-3′ CAL-B V190G_R 5′-GGAGACCTGCGGCTGGCCAATTTCGTCGGTGGC-3′
CAL-B Q157K_F 5′-TCTGCGCCATCCGTGTGGCAGAAAACCACTGGTTCTGCGCTGACC-3′ CAL-B Q157K_R 5′-GGTCAGCGCAGAACCAGTGGTTTTCTGCCACACGGATGGCGCAGA-3′
CAL-B I189D_F 5′-TACAGCGCAACCGATGAGGATGTTCAGCCGCAGGTATCT-3′ CAL-B I189D_R 5′-AGATACCTGCGGCTGAACATCCTCATCGGTTGCGCTGTA-3′
CAL-B I189S_F 5′-CTGTACAGCGCAACCGATGAGTCTGTTCAGCCGCAGGTATCTAAC-3′ CAL-B I189S_R 5′-GTTAGATACCTGCGGCTGAACAGACTCATCGGTTGCGCTGTACAG-3′

Protein expression and purification

The *E.coli* (TOP10) containing the mutant gene was inoculated to LB medium (15 ml) containing ampicillin (15 μl, 100 mg/ml) and incubated at 37°C for 16 h. And the overnight culture (1 ml) was added to LB medium (100 ml) containing ampicillin (100 μl, 100 μg/ml) and incubated at 37 °C and 200 rpm to an $O.D_{600}$ of 0.5-0.6. After adding an arabinose solution (1 ml, 2% w/v) to induce the protein expression, the culture was incubated at 37 °C and 200 rpm for 6 h. Then the culture was centrifuged for 15 min at 4,000 rpm and the pellet was resuspended in a lysis buffer (5 ml/g wet wt; NaH_2PO_4, 50 mM; NaCl, 300mM; imidazole, 10 mM pH 8.0 adjusted with NaOH) and distrupted by sonication. The soluble fraction was separated from cell debris by centrifugation. The cell debris was dissolved in a 8 M urea solution (4 ml containing 1 mM dithiothreitol) for SDS-PAGE analysis. Ni-NTAagarose resin (1 ml, 50% w/v slurry) was added to the supernatant (4 ml) and the mixture was shaken at 4 °C for 1 h. The lysate-Ni-NTA mixture was loaded on a Poly-Prep column (Bio-Rad), and eluted twice with the lysis buffer (2 ml) and then washed three times with the wash buffer (4 ml; NaH_2PO_4, 50 mM; NaCl, 300 mM; imidazole, 20 mM; pH 8.0 adjusted with NaOH). The His_6-CAL-B enzyme was eluted from the column with four volumes of the elution buffer (0.5 ml; NaH_2PO_4, 50 mM; NaCl, 300 mM; imidazole, 250 mM; pH 8.0 adjusted with NaOH). Eluate (2 ml) from the Ni-NTA column containing the purified CAL-B was exchanged from the elution buffer to BES (5 mM, pH 7.2) using a centrifugal device (Amicon Ultra-15, Millipore). And the wild-type enzyme was expressed and purified as above.

Conjugation of CAL-B to the polystyrene coated magnetic beads

The polystyrene coated magnetic beads (500 mg) were suspended in 5 ml of MES buffer (0.1 M, pH 5.0). After addition 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 500 mg), the suspension was shaken for 20 min at room temperature. The beads were separated and washed three times with 5 ml of BES buffer (5 mM, pH 7.2). The magnetic beads were resuspended in 1 ml of BES buffer (5 mM, pH 7.2) and then 1 ml of enzyme (1.0 mg/ml) was added. The mixture was incubated overnight at 4 ℃ and the enzyme-conjugated magnetic beads were washed three times with 1 ml of BES buffer (5 mM, pH 7.2). The amount of bound enzymes was determined to be about 1 mg per g of beads by the Bradford dye protein assay method.

Synthesis of (±)-ethyl tetrahydrofuran-2-carboxylate

Tetrahydro-2-furoic acid (10 ml, 0.104 mol) was added to ethanol (40 ml, 0.1713 mol) and ten drops of sulfuric acid added. The reaction mixture was refuluxed for 24 h. Ethanol was evaporated by a rotavap and the concentrated was diluted with diethyl ether (10 ml). The ether solution was washed twice with a saturated sodium carbonate aqueous solution (40 ml). The ether layer was concentrated by rotovap and dried over anhydrous sodium sulfate.

^{1}H NMR (500 MHz, $CDCl_3$): δ 4.59-4.56 (1H, t); 4.36-4.30 (2H, m);

4.17-4.02 (2H, m); 2.39-2.11 (2H, m); 2.10-2.03 (2H, m); 1.42-1.40(3H, t).

^{13}C NMR (125 MHz, $CDCl_3$, δ): 173.67, 77.52-77.01, 69.60, 61.16, 30.42, 25.48, 14.46.

Synthesis of (±)-butyl tetrahydrofuran-2-carboxylate

Tetrahydro-2-furoic acid (10 ml, 0.104 mol) was added to butanol (40 ml, 0.438 mol) and ten drops of sulfuric acid added. The reaction mixture was refuluxed for 24 h. Ethanol was evaporated by a rotavap and the concentrated was dilluted with diethyl ether (10 ml). The ether solution was washed twice with a saturated sodium carbonate aqueous solution (40 ml). The ether layer was concentrated by rotovap and dried over anhydrous sodium sulfate.

^{1}H NMR (500 MHz, $CDCl_3$): δ 4.50-4.48 (1H, dd); 4.21-4.16 (2H, t); 4.08-3.94 (2H, q); 2.31-2.27 (2H. m); 2.07-1.94 (2H, m); 1.71-1.58 (2H, m); 1.46-1.39 (2H, m); 1.00-0.96 (3H, t)

^{13}C NMR (125 MHz, $CDCl_3$, δ): 173.78, 77.53-77.10, 69.58, 65.00, 31.86, 30.47, 25.48, 19.31, 13.94.

Transesterification of (±)-ethyl tetrahydrofuran-2-carboxylate

Butanol (365 μl, 4 mmol), (±)-ethyl tetrahydrofuran-2-carboxylate (0.1442 g, 1 mmol) and molecular seives (60 mg, 4A powder) were added to a suspension of the enzyme-conjugated polystyrene coated magnetic beads (100 mg) in methyl tert-butyl ether (6 ml) and the reaction mixture was shaken at 25 °C. A small volume of the reaction mixture (100 μL) was

retrieved with intervals (0, 1, 3, 24, 48, 72h) diluted in and diethyl ether (1.4 ml). The reaction progress was analyzed by a GC (Agilent 6890N) with a chiral capillary column (Cyclosil-B 30 m × 0.25 mm): initial column temperature 50 °C for 5 min, ramp up to 120 °C at a rate of 1.0 °C/min and then held at 120 °C for 10 min.

Determination of enantioselectivity for hydrolysis of (±)-butyl tetrahydrofuran-2-carboxylate

The hydrolysis reaction was monitored using a pH-stat. The reaction was started by an addition of the enzyme solution (0.5 mg/ml, 50 μL in 5 mM BES buffer, pH 7.2) to a solution of (±)-butyl tetrahydrofuran-2-carboxylate (150 ul of 1 M in acetonitrile) and acetonitrile (650 uL) in 10 mM BES buffer (10,150 μl, pH 7.2). The reaction was maintained at pH 7.2 by a controlled addition of 0.1 N sodium hydroxide. Reaction was stopped by extraction with diethyl ether (5 mL) at around 40% conversion. The organic layer was dried by anhydrous sodium sulfate and analyzed by GC with a chiral capillary column: initial column temperature 50 °C for 5 min, ramp up to 120 °C at a rate of 1.0 °C/min and then held at 120 °C for 10 min. Enantioselectivity was calculated using the method of Chen et al. from enantiomeric excesses of both starting ester and alcohol product.[4)]

Retention time: (*S*)-ethyl tetrahydrofuran-2-carboxylate: 41 min
(*R*)-ethyl tetrahydrofuran-2-carboxylate: 44 min
(*S*)-butyl tetrahydrofuran-2-carboxylate: 66 min
(*R*)-butyl tetrahydrofuran-2-carboxylate: 67 min

Results and Discussion

Selection of mutation sites

To improve the enantioselectivity of CAL-B for kinetic resolution of tetrahydrofuran-2-carboxylate (THFC), we selected three residues D134, Q157, and I189 based on the crystal structure (Figure 3). We introduced residues to construct hydrogen bond or repulsive interaction with the oxygen atom of THFC.

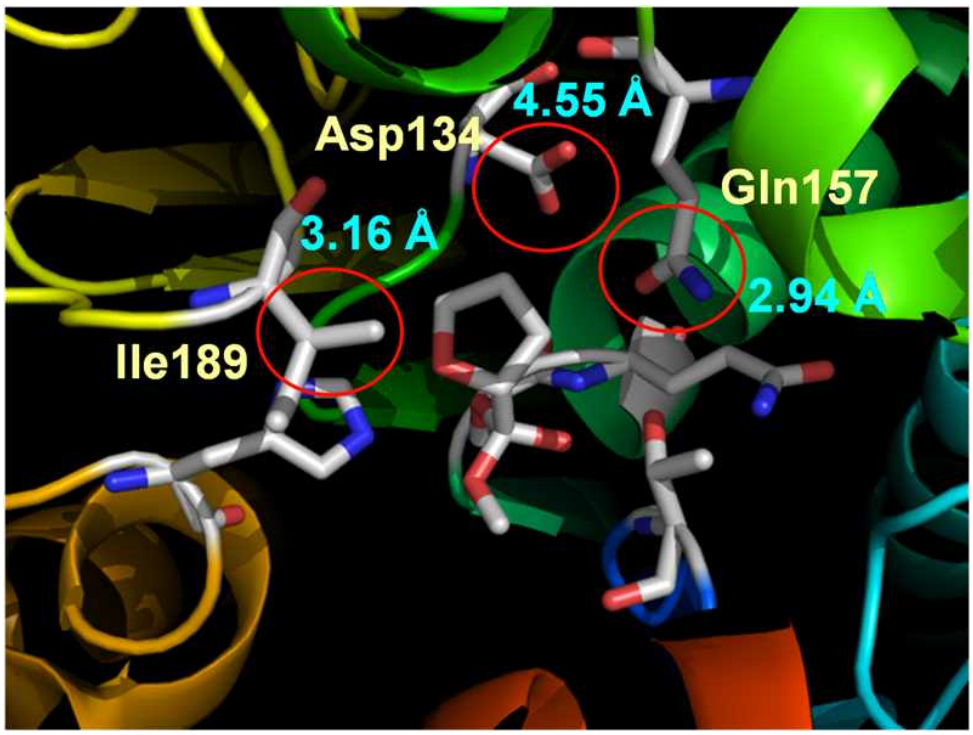


Figure 3. A docking structure of THFC in CAL-B. selected three residues D134, Q157, and I189, which are located near the active site.

The construction of hydrogen bond can be provided by the residues, such as lysine, asparagine, glutamine, and serine. The side chain of these amino acids may be interact with the oxygen atom, which exists at the chiral center of the substrate. And the hydrogen bond may provide a favorable interaction between the side chain and one of the enantiomers. Alternatively repulsive interaction may resolve the counter-enantiomer to wild-type enzyme. We have chosen two amino acids such as aspartate and glutamate for making a repulsive interaction (Figure 4).

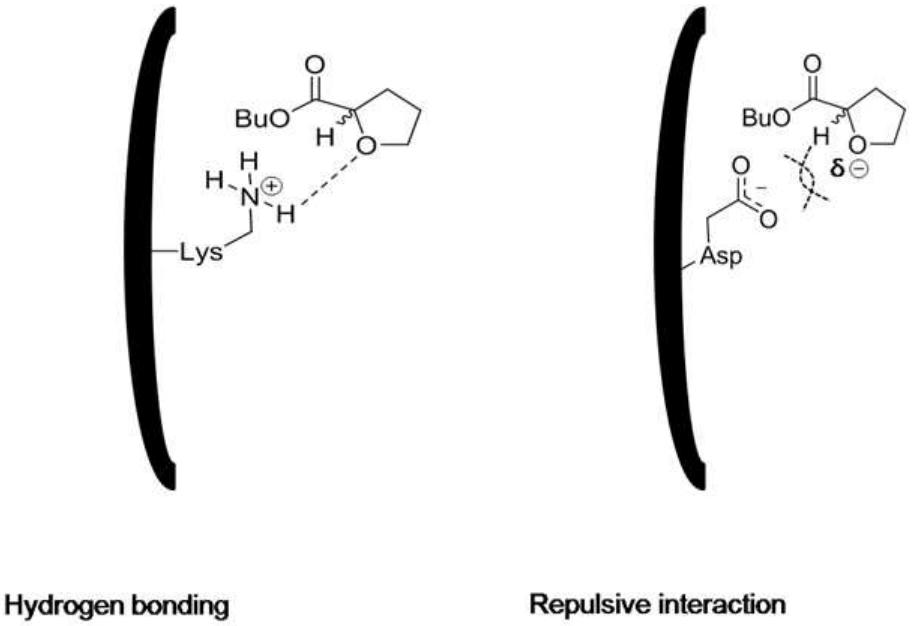


Figure 4. The effects of construction of hydrogen bond and repulsive interaction in kinetic resolution of THFC.

The D134 residue was mutated to lysine, asparagine, and glutamine and also the V190 residue was changed to glycine. The substituted position V190 have to be controlled to create enough space for accepting the bulky amino acids at the D134. Thus, we have replaced the V190 residue with a small amino acid, glycine. The substitution at position 157 and 189 was expected to introduce the repulsive interaction or hydrogen bond with the substrate. The mutants are listed in Table 2.

Table 2. The list of variants

Entry	Enzyme
1	D134K V190G
2	D134N V190G
3	D134Q V190G
4	Q157K
5	I189D
6	I189S

Protein expression and purification

Most mutant enzymes were expressed as good as the wild-type CAL-B. The SDS-PAGE analysis showed that the purification of the enzymes were above 95 % (Figure 5).

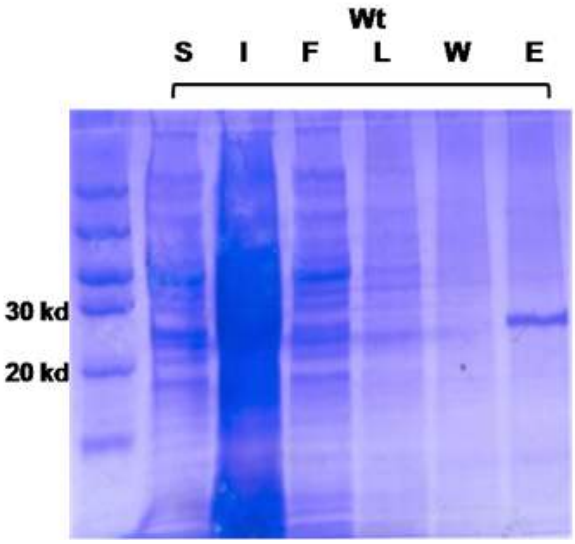


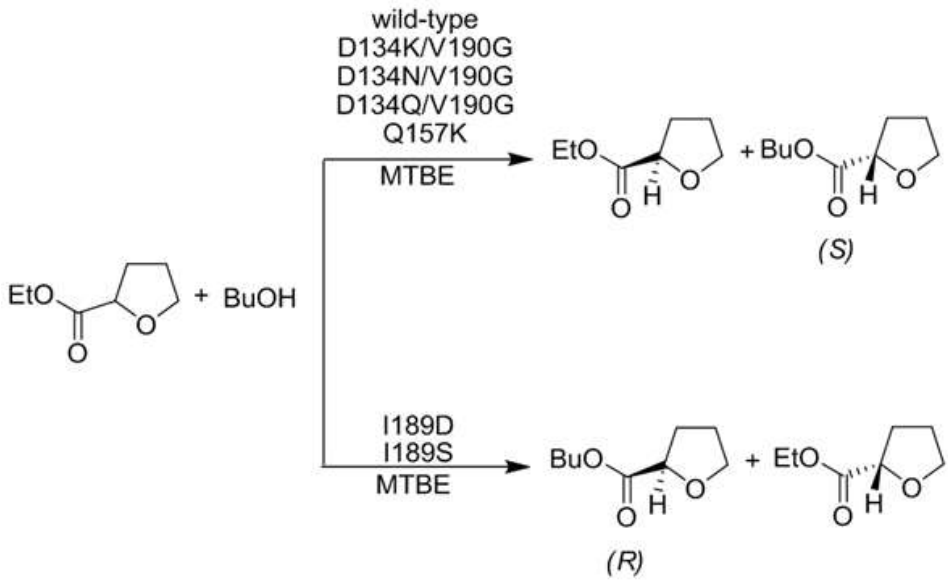
Figure 5. SDS-PAGE analysis of CAL-B wild-type enzyme. SDS-PAGE was performed on a 12% polyacrylamide gel and stained using the Coomassie brilliant blue. M, molecular weight marker; S, soluble fraction; I, insoluble fraction; F, flow-through fraction; L, lysis buffer fraction; W, wash buffer fraction; E, elution buffer fraction.

Transesterification

(±)-Ethyl tetrahydrofuran-2-carboxylate was resolved by wild-type enzyme and mutant enzymes-catalyzed transesterification. We used an immobilized enzyme because the activity of enzymes suspended in organic solvents decreases significantly. We covalently immobilized the enzyme onto polymer-coated magnetic beads, which were prepared in our lab.

In organic solvent, E values of most enzymes showed more increase than aqueous reaction. Each mutant (D134, D134N/V190G, and D134Q/V190G) showed similar enantioselectivity to that of the wild type.

However, the enantiopreference of I189D and I189S differ from those of the wild-type and the other mutants. They showed *(R)*-preference and higher enantioselectivity.



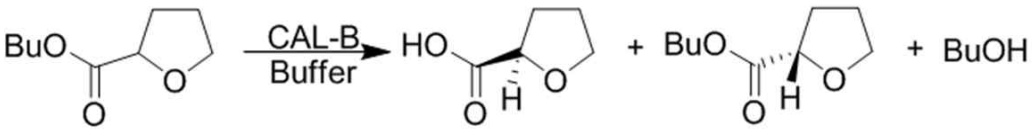
Scheme 1. Transesterification of (±)-ethyl tetrahydrofuran-2-carboxylate

Table 3. Enzymatic resolution of racemic butyl tetrahydrofuran-2-carboxylates using CAL-B in MTBE

Entry	Enzyme	E	Preference
1	Wild-type	1.9	S
2	D134K V190G	1.8	S
3	D134N V190G	1.7	S
4	D134Q V190G	1.6	S
5	Q157K	2.0	S
6	I189D	3.0	R
7	I189S	1.4	R

Hydrolysis reaction

The hydrolysis by the wild-type and mutant enzymes showed similar results to those of esterification. The Hydrolysis reaction of (±)-butyl tetrahydrofuran-2-carboxylate with wild-type enzyme yields the (*R*)-preference, while mutant D134K V190G enzyme and mutant Q157K enzyme yield the (*S*)-preference.



Scheme 2. Hydrolysis of (±)-butyl tetrahydrofuran-2-carboxylate

Table 4. Enzymatic resolution of racemic butyl tetrahydrofuran-2-carboxylate using CAL-B in buffer solution

Entry	Enzyme	E	Preference
1	Wild-type	1.3	R
2	D134K V190G	1.1	S
3	D134N V190G	1.1	R
4	D134Q V190G	1.2	R
5	Q157K	1.9	S
6	I189D	1.6	R
7	I189S	2.2	R

Conclusion

This study deals with present increasing the enantioselectivity of CAL-B in a kinetic resolution of butyl tetrahydrofuran-2-carboxylate by rationally designed protein engineering. Some mutants showed not only higher enantioselectivity but also reverse enantio-preference compared to the wild-type enzyme. In organic solvent, the I189D mutant enzyme showed higher enantioselectivity with reversed enantio-preference (E = 3.0, R-selective) than the wild-type enzyme (E = 1.9, S-selective). In aqueous reaction, the E value of the I189S enzyme (E = 2.2, R-selective) was higher than that of the wild-type enzyme (E = 1.3, R-selective). Although some mutants showed reverse preference and slightly improved the enantioselectivity, the value was not enough for synthetic applications. Therefore, a new system should be designed. Combination of site-directed mutagenesis and the random mutagenesis could be employed for improving the enantioselectivity and find fine-tuning enzyme.

2.5 Reference

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Chapter 3. Creating a peroxidase from a perhydrolase by introducing a metal ion into the active site

Abstract: PerPA (perhydrolase from *Pseudomonas aeruginosa*) is an enzyme that is able to catalyze perhydrolysis in aqueous media. PerPA contains Ser-His-Asp catalytic triad showing high similarity to esterase/lipase and do not have any cofactors and a metal ion. We have designed an artificial metalloenzyme by imitation of a natural metalloenzyme. In general, the catalytic metal ion of many metalloenzyme is coordinated by Glu/Asp and two His. We introduced such residues in PerPA to accept metal ion and to catalyze peroxidation with hydrogen peroxide. Several mutants of PerPA that were created by site-directed mutagenesis increased the binding affinity to a metal ion and some mutants showed more increase peroxidase activity than wild-type enzyme, especially mutant F165/L32E enzyme (100-fold increased).

Introduction

Oxidation plays an important role in organic chemistry and biotechnological chemistry. Oxidoreductases can provide feasible alternative to chemical synthetic routes due to their high selectivities.

Peroxidases are suitable for many applications because they use hydrogen peroxide as an oxidant. Most natural peroxidases are metalloenzyme containing a heme group. They often become inactive through oxidative destruction of heme porphyrin ring by the oxidant during reaction. Metal free haloperoxidase can be used for oxidation without enzyme destruction by the oxidation.

However, the actual oxidation by haloperoxidase occurs out of the active site of the enzyme and thus no regio-, chemo-, and enantioselectivity are expected.[1)] We have designed an artificial metalloenzyme by imitation of a natural metalloenzyme. In general, the catalytic metal ion of many metalloenzyme is coordinated by Glu/Asp and two His. We introduced such residues in PerPA (perhydrolase form *Pseudomonas aeruginosa*) to accept metal ion and to catalyze peroxidation with hydrogen peroxide.

Experimental section

Chemicals and buffers were purchased from Sigma-Aldrich. LB-broth was purchased from Merck. Pfu DNA polymerase and restriction enzymes (*Nde* I and *BamH* I) were purchased from Enzynomics (Daejeon, Korea). DNA oligomers were obtained from Sigma-proligo (Singapore) and Bionics (Korea). DNA sequencing was performed by Solgent Co. (Daejeon, Korea). The plasmid containing PerPA was donated from Dr. Song (KRICT).

Subcloning and mutagenesis of PerPA

The PerPA gene was subcloned into pJOE2972n vector using the primers containing restriction sites for *Nde* I and *BamH* I (Figure 1). The mutant genes were created by Quikchange mutagenesis with the mutagenesis primers. The sequences of the PerPA gene and mutagenesis primers are listed in Table 1. The plasmids of the mutants were transformed into *E.coli* (DH5α, TOP10, BL21, and BL21(DE3)pLysS).

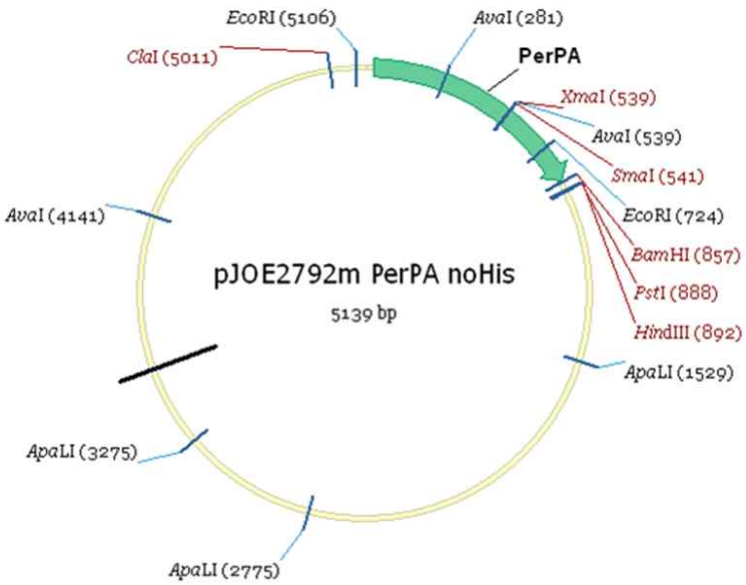


Figure 1. The map of the plasmid containing PerPA as a template for mutagenesis.

Table 1. The sequences of PerPA and mutagenesis primers

Sequence (PerPA)
ATG GGT TAC GTG ACA ACG AAG GAT GGC GTC GAA CTC
TTC TAC AAG GAC TGG GGG CCG CGC GAC GCC CAG GTG ATC
TAC TTC CAT CAT GGC TGG CCA CTG AGT TCC GAC GAC TGG
GAT GCG CAG ATG CTG TTC TTC CTC GCC GAG GGC TTT CGC
GTA GTG GCC CAC GAT CGC CGT GGC CAC GGT CGC TCC AGC
CAG GTC TGG GAC GGC CAT GAC ATG GAC CAC TAC GCC GAC
GAC GTG GCG GCG GTG GTC GAG CGC CTC GGG GTG CGC GGG
GCG ATC CAT GTC GGC CAT TCC ACC GGC GGC GGC GAG GTC
GTC CAC TAC ATC GCC CGC TAT CCC GAC GAC CCG GTG CCG
AAG GCG GCG ATC ATC AGC GCG GTA CCG CCG CTG ATG GTG
AAG ACC GAG GGC AAT CCG GGC GGC CTG CCG AAG AGC GTC
TTC GAC GAT CTC CAG GCG CAG CTC GCG GCC AAT CGC GCG
CAG TTC TAC CAG GAC ATT CCC GCC GGC CCG TTC TAT GGC
TAC AAC CGT CCC GGG GCG CAG CCC TCG GAA GGG ATC GTC
CGC AAC TGG TGG CGG CAG GGC ATG ATG GGC GGC GCC AAG
GCC CAC TAC GAT GGG ATC GTG GCC TTT TCC CAG ACC GAC
TTC AGC GAT GAC CTG AAG CGC ATC GAC ATC CCG GTG CTG
GTG ATG CAT GGC GAC GAC GAC CAG ATC GTG CCC TAC GAG
AAT TCC GGC GTA CTC TCG GCG AAG CTG CTG CGC AAC GGC
ACG CTG AAG ACC TAT CCG GGG TTC CCG CAC GGC ATG CCG
ACC ACC CAG GCC GAG GTG ATC AAC GCC GAC CTG CTG GCC
TTC ATC CGC GGC TGA

Mutagenesis primers (PerPA)
F-PerPA-F165H 5′-CATTCCCGCCGGCCCGCATTATGGCTACAACCGTC-3′ R-PerPA-F165H 5′-GACGGTTGTAGCCATAATGCGGGCCGGCGGGAATG-3′
F-PerPA-L32E 5′-TATTCCATCATGGCTGGCCAGAAAGTTCCGACGACTGGGAT GCG-3′ R-PerPA-L32E 5′-CGCATCCCAGTCGTCGGAACTTTCTGGCCAGCCATGATGGA ATA-3′
F-PerPA-H95E/S96G 5′-GGCGATCCATGTCGGCGAAGGCACCGGCGGCGGCGAGG-3′ R-PerPA-H95E/S96G 5′-CCTCGCCGCCGCCGGTGCCTTCGCCGACATGGATCGCC-3′
F-PerH-F165E 5′-ATTCCCGCCGGCCCGGAATATGGCTACAACCGT-3′ R-PerH-F165E 5′-ACGGTTGTAGCCATATTCCGGGCCGGCGGGAAT-3′
F-PerPA-H255D/P258H 5′-TATCCGGGGTTCCCGGATGGCATGCATACCACCCAGGCCGAG-3′ R-PerPA-H255D/P258H 5′-CTCGGCCTGGGTGGTATGCATGCCATCCGGGAACCCCGGATA-3′
F-PerPA-H255E/P258H 5′-ATCCGGGGTTCCCGGAAGGCATGCATACCACCCAG-3′ R-PerPA-H255E/P258H 5′-CTGGGTGGTATGCATGCCTTCCGGGAACCCCGGAT-3′

Protein expression and purification

The *E.coli* (DH5α, TOP10, BL21, and BL21(DE3)pLysS) containing the mutant gene was incubated to LB medium (15 ml) containing ampicillin (15 μl, 100 mg/ml) and incubated for 16 h at 37 °C. And the overnight culture (1 ml) was added to LB medium (100 ml) containing ampicillin (100 μl, 100 μg/ml) and incubated at 37 °C and 200 rpm to an $O.D_{600}$ of 0.5-0.6. Addition of L-rhamnose (1 ml, 20% w/v) induced the protein expression and the culture medium was incubated at 37 °C and 200 rpm for 6 h. Then the culture was centrifuged for 15 min at 4,000 rpm and the pellet was resuspended in sodium phosphate solution (5 ml) and distrupted by sonication (amp 30 %, power on: 10 sec, power off: 10 sec, running time: 90 sec). The soluble fraction was separated from cell debris by centrifugation. The cell debris was dissolved in 8 M urea solution (4 ml) for SDS-PAGE analysis. The soluble fraction was filterated by a filter device (0.22 μm syringe driven filter unit, Millipore), purified with anion exchange chromatography (Vivapure IEX maxi Q type column, Sartorius stedim biotech), and analyzed by SDS-PAGE. The purified protein solution was exchanged from the elution buffer to BES (5 mM, pH 7.2) using a centrifugal device (Amicon Ultra-15, Millipore).

Conjugation of PerPA to polymeric beads[2)]

The polymeric beads (100 mg, Lewatit CNP, Bayer chemicals) were suspended in ethanol (1 ml), and degased for 5 min and resuspended in MES buffer (1 ml, 0.1 M, pH 5.0). After addition of 100 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), the suspension was shaken for 20 min at room temperature. The beads were separated and washed three times with 5 ml of BES buffer (5 mM, pH 7.2). The activated beads were resuspended in 1 ml of BES buffer (5 mM, pH 7.2) and then 1 ml of enzyme solution (6.3 mg/ml) was added. The mixture was incubated overnight at 4 °C. And the enzyme-conjugated commercial beads were washed three times with 1 ml of BES buffer (5 mM, pH 7.2). Generally, the amount of bound enzyme was determined to be about 5.7 mg per g of beads by the Bradford dye protein assay method.

Enzyme activity assay : Peroxidase activity[3)]

The assay solution was prepared by mixing ddH_2O (105 μl), potassium phosphate buffer (16 μl, 100 mM, pH 6.0), hydrogen peroxide solution (8 μl, 0.5 wt%), and pyrogallol solution (16 μl, 5 w/v %) (Scheme 1). The enzyme (5 μl) was added to the above assay solution. The total volume was 150 μl. The reaction was monitored at 415 mm for 60 min using a microplate reader.

Sulfoxidation[4)]

Thioanisole (0.06 mmol) and enzyme-conjugated beads (30 mg, 1.035 x 10^{-6} mmol) were sonified in 6 ml of citrate buffer (100 mM, pH 5.0) for 5 min. Hydrogen peroxide (0.03 mmol) was added in 1 h in 4 aliquots at 10 min intervals and the reaction was carried out for 20 h. Reaction (200 ul) was stopped by extraction with diethyl ether (5 mL). The organic layer was dried by anhydrous sodium sulfate and analyzed by GC with a chiral capillary column: initial column temperature 120 °C for 5 min, ramp up to 180 °C at a rate of 2.5 °C/min and then held at 180 °C for 5 min.

Results and Discussion

Selection of mutagenesis sites.

In nature, many enzymes contain a metal ion for catalytic activity. Thermolysin is one of the metalloenzymes and has a catalytic zinc atom which is coordinated to three amino acid residues. The crystal structure revealed that two histidines (His142 and His156) and one glutamate (Glu166) coordinate to the zinc ion.[5)]

We designed to introduce such amino acids into PerPA to coordinate a metal ion like a natural metalloenzymes. We selected several locations of residues like the system of thermolysin. The locations of His95, His255, and Pro258 are corresponded to those the amino acids of thermolysin, which coordinate to the zinc ion. These amino acids were replaced to aspartate and histidine to be able to bind a metal ion. The F165H mutant enzyme was designed as a role of the distal histidine in the reaction of peroxidases. The distal histidine can increase activation and stabilization of hydrogen peroxide in the heme-containing system.[6)] As a result, the F165H mutant enzyme may cause to increase in the activation of hydrogen peroxide and can stabilize the system. Other locations of residues were chosen in consideration that the replace residues keep proper distances each other. The designed mutant enzymes are listed in Table 2.

Table 2. The list of PerPA variants.

Entry	Enzyme
1	F165H
2	F165H L32E
3	F165H H95E S96G
4	F165E S96G
5	H255D P258H
6	H255D P258H F165H
7	H255E P258H
8	H255E P258H F165H

Optimization of protein exprssion level and purification

We subcloned the PerPA gene from the pHCE19T(II) vector to pJOE2792n vector. The expression was analyzed by SDS-PAGE (Figure 2). Each mutant enzyme showed different protein expression level.

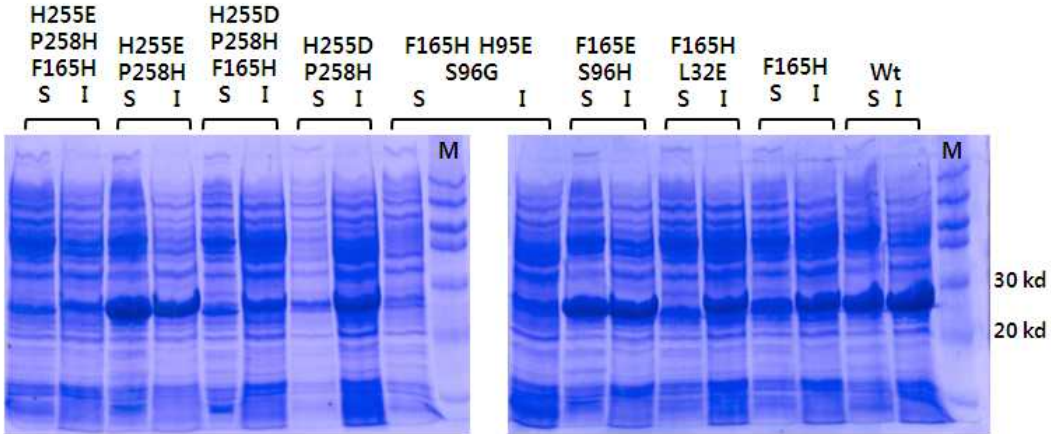


Figure 2. SDS-PAGE analysis of PerPA wild-type enzyme and mutant enzymes. PerPA has a molecular weight of 30.4 kDa. SDS-PAGE was performed on a 12% polyacrylamide gel and stained using the Coomassie brilliant blue. M, molecular weight marker; I, insoluble fraction; S.

Mutant enzymes yielded small amounts in soluble fractions. To improve the expression level, several *E.coli* strains (DH5α, TOP10, BL21, and BL21(DE3)pLysS) were tested as an expression host. However, in the case of the F165H/L32E mutant, most proteins were found as inclusion bodies (Figure 3). We attempted to improve the expression level of the mutants by control of the expression conditions such as the concentration of inducer, and the time and the temperature of expression. However these controls didn't affect much the expression level. After the protein-expression step, crude soluble fractions were partially purified with anion exchange chromatography.

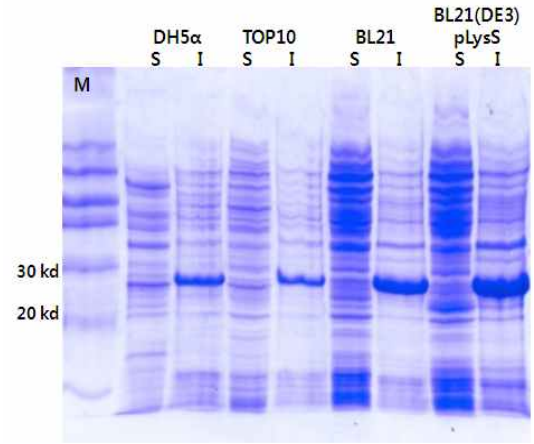
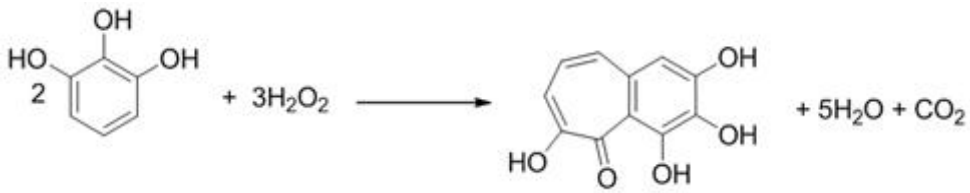


Figure 3. SDS-PAGE analysis of PerPA mutant F165H/L32E enzyme. PerPA has a molecular weight of 30.4 kDa. SDS-PAGE was performed on a 12% polyacrylamide gel and stained using the Coomassie brilliant blue. M, molecular weight marker; I, insoluble fraction; S.

Measurement of peroxidase activity

We have monitored the oxidation of pyrogallol as a model reaction of peroxidation. The formation of the product, purpurogallin, can be observed at 415 nm (Scheme 1).[3)]



Scheme 1. A model reaction for peroxidase activity. Peroxidases oxidize pyrogallol to purpurogallin using hydrogen peroxide

First we have tested peroxidase activity of crude cell-free enzymes without purification. Unfortunately, cell-free solution containing pJOE2792n showed the peroxidase activity. This result indicated that the crude cell-free system had intrinsic peroxidase activity and thus the enzyme must be purified to reduce the background reaction.

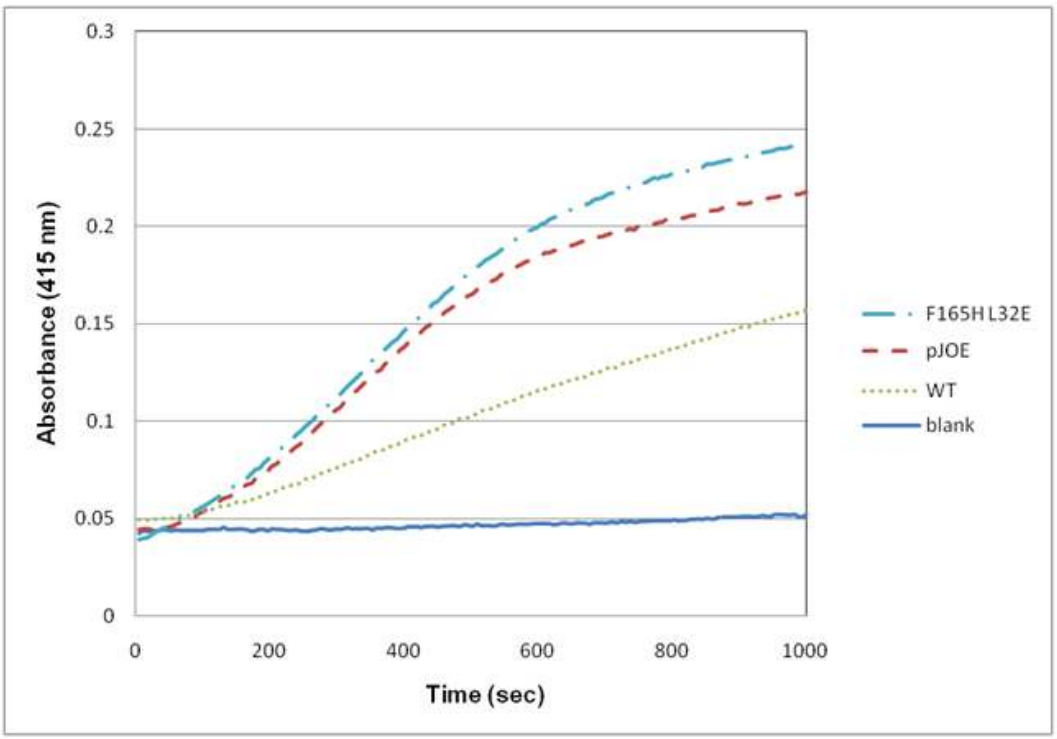


Figure 4. Peroxidase activity of pJOE vector, wild-type enzyme and among mutant enzymes, F165H L32E mutant enzyme

Purification of enzymes

Purification was performed by an anion exchange column and each elution fraction was analyzed by SDS-PAGE (Figure 5). The wild-type enzyme and most mutant enzyme yielded a large amount of proteins in the soluble fractions. But F165H/L32E enzyme was not found in the soluble fraction.

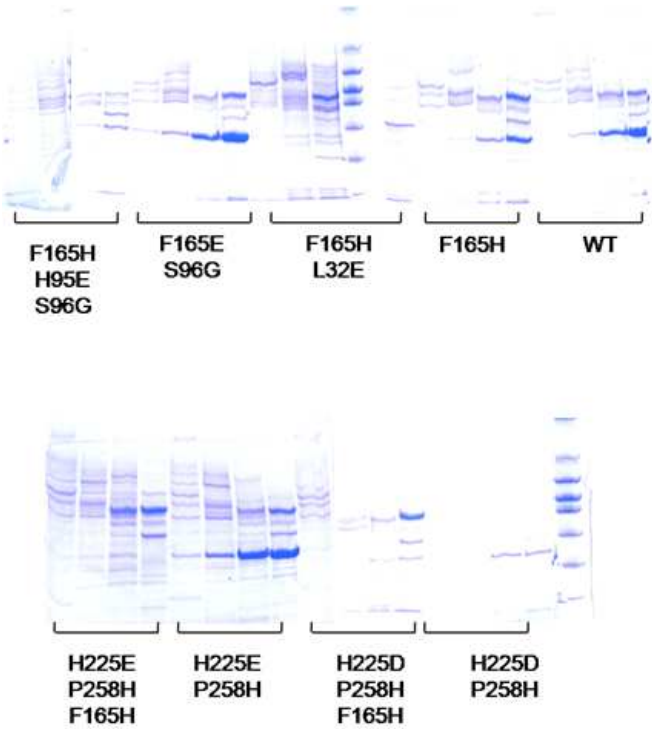


Figure 5. SDS-PAGE analysis of wild-type enzyme and mutant enzymes. Wild-type enzyme and the F165E/S96G mutant enzyme were as high as 90 % purity but F165H mutant enzyme were not obtained as above 90% purity. SDS-PAGE was performed on a 12% polyacrylamide gel and stained using the Coomassie brilliant blue.

Confirmation of peroxidase activity of the mutant producced.

To demonstrate the metal effect in the enzyme, we first treated the enzyme with 2,6-pyridine dicarboxylic acid (2,6-PDCA)[7] and ethylenediaminetetraacetic acid (EDTA)[8] to remove a potential metal ion in the enzyme. Because mutant enzyme may capture unspecific metal ions from the medium during protein-expression. 2,6-PDCA is a tridentate ligand and can form a complex with a metal ion and EDTA is a hexadentate ligand (Figure 6). After enzyme was treated with 2,6-PDCA, the peroxidase activity of enzyme was decreased. Each mutant enzyme showed different amount of decrease of peroxidase activity according to their ability of acceptable potential metal ions. While the wild-type enzyme did not decrease the peroxidase activity treatment of the chelating agents, the mutant enzymes (H255D/P258H and H255E/P258H) lost their activity (Table 3).

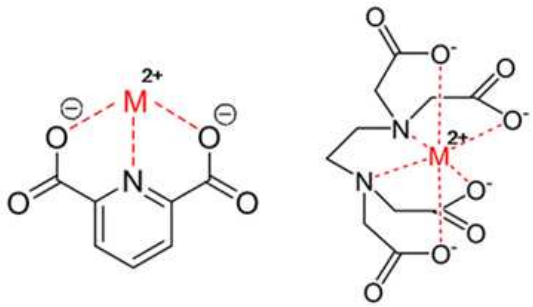


Figure 6. Metal-2,6 PDCA complex and metal-EDTA complex

Table 3. Peroxidase activity of removal of potential metal ion in wild-type enzyme and several mutant enzymes

	specific activity (μmol · sec^{-1} · mg^{-1})	EDTA (μmol · sec^{-1} · mg^{-1})	2,6-PDCA (μmol · sec^{-1} · mg^{-1})
wt	2.3×10^{-5}	4.3×10^{-5}	2.6×10^{-5}
F165H	2.5×10^{-5}	6.0×10^{-5}	6.3×10^{-5}
H255D P258H	5.9×10^{-4}	3.8×10^{-4}	2.9×10^{-4}
H255E P258H	5.1×10^{-4}	2.4×10^{-4}	2.0×10^{-4}

$$(\mu mol \cdot \sec^{-1} \cdot ml^{-1}) = \frac{\triangle A_{415nm}/time\,(\sec) \times reaction\,volume\,(ml)}{molar\,extinction\,coefficient\,(12) \times volume\,of\,enzyme\,solution\,(ml)}$$

Mutant F165H L32E enzyme showed the highest peroxidase activity, compared to other mutant enzymes and the wild-type enzyme. The mutant enzyme showed 100-fold higher relative peroxidase activity than wild-type enzyme (Table 4). This mutant may capture unspecific metal ions during expression. After the enzyme was treated with 2,6-PDCA, its peroxidase activity was maintained. Therefore, this mutant enzyme was expected for showing a high level of metal ion effect.

Table 4. Peroxidase activity of wild-type enzyme and mutant F165H L32E enzyme

	specific activity (μmol · sec^{-1} · mg^{-1})
wt	8.4×10^{-6}
F165H L32E	8.9×10^{-4}

Addition of a metal ion in the wild-type and mutant enzyme

After all of the metal ions in the enzyme were removed, we attempted to introduce several metal ions, such as Fe (II), Fe (III), Mn (II), and Cu (II) to bind into the enzymes. We first try to find proper metal ions using a mimetic system of non-heme peroxidases. Manabendra Ray and coworkers preciously synthesized a ligand which has capsular cavity with the redox-active metal center and is an analogue of the catalytic triad of PerPA (Figure 7).[9] We used this ligand to find a proper metal ion. Each of metal ion was coordinated to the ligand and then peroxidase activity was monitored by oxidation of pyrogallol. Cu (II) ion itself showed high peroxidase activity. Fe (II) metal ion was suitable for peroxidase activity (Figure 8).

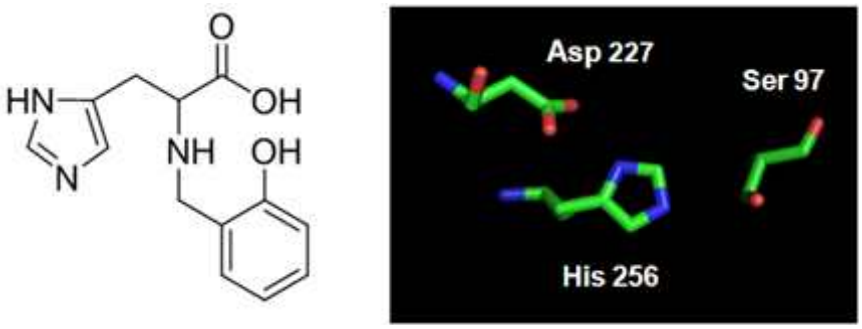


Figure 7. Molecular structure of the ligand and the catalytic triad of PerPA.

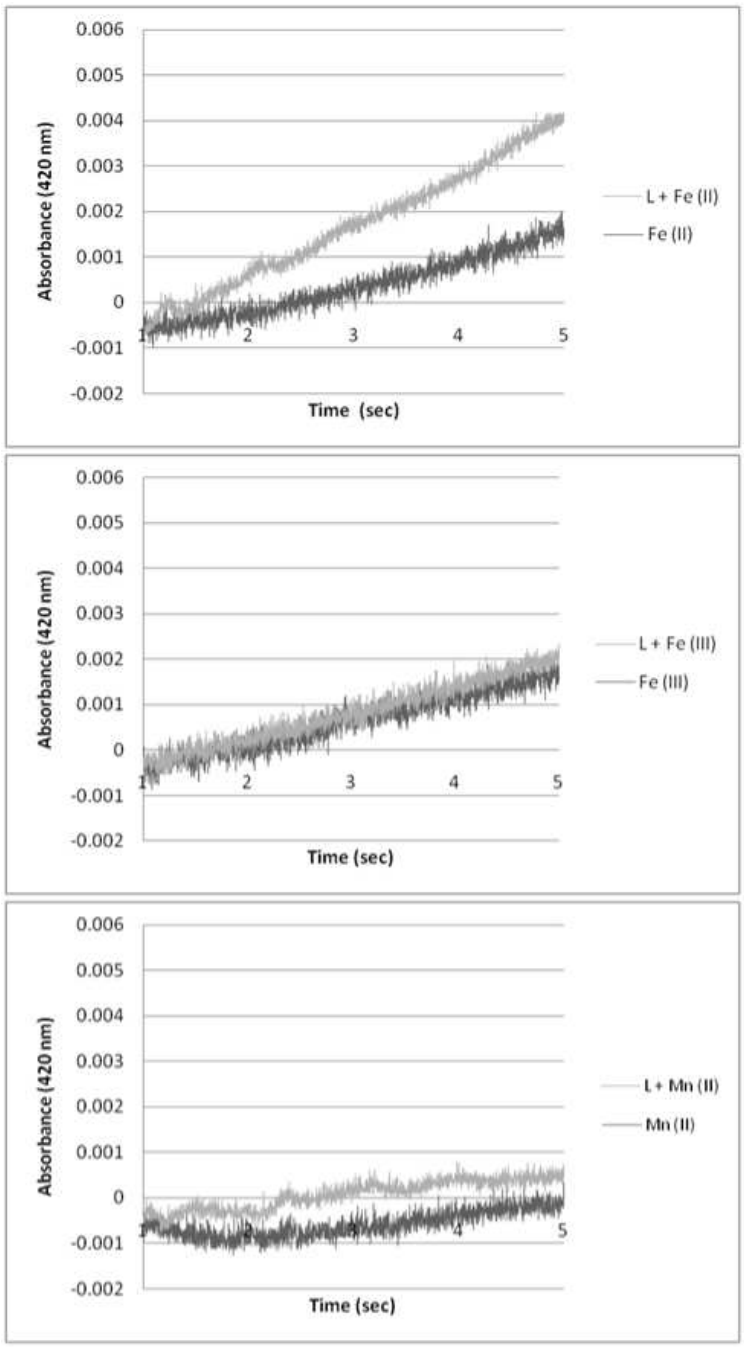


Figure 8. Peroxidase activity of several metal ions with the ligand of analogous catalytic triad

Several methods of adding a metal ion are known. Two methods were attempted. First, the dialysis method was tried but the enzyme was easily denatured. Second, a metal ion was directly added. On this occasion, the concentration of metal solution was considered. The ratio of 3 (metal) : 1 (enzyme) was the best. Too high concentration of metal ion can denature the enzyme.

Fe (II) and Fe (III) ion were introduced to mutant F165H/L32E enzyme, with the ratio of 3 : 1 and incubated at 4 °C for 16 h. And then, the enzyme solution was washed with BES buffer (5mM, pH 7.2) twice to remove excess metal ions, which potentially bound with the surface acidic residues of the enzyme. It was expected that the peroxidase activity of mutant enzyme may be more increased by the metal effect. But the result was contrary to the expectation (Table 5).

After the wild-type enzyme was treated with Fe (II) ion and Fe (III) ion, the peroxidase activity was increased. However, the mutant F165H/L32E enzyme didn't show the metal ion effect and the peroxidase activity was decreased.

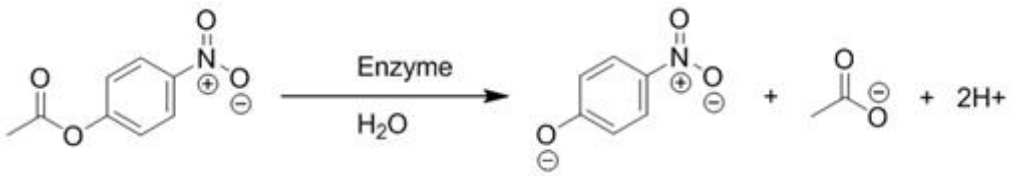
Table 5. Peroxidase activity of addition of a metal ion in the wild-type and mutant enzyme

	1st specific activity (μmol · sec^{-1} · mg^{-1})	2nd specific activity (μmol · sec^{-1} · mg^{-1})
wt	4.8×10^{-7}	4.8×10^{-7}
wt + Fe(II)	1.6×10^{-5}	7.7×10^{-6}
wt + Fe(III)	1.2×10^{-5}	4.7×10^{-6}
F165H L32E	3.6×10^{-6}	3.6×10^{-5}
F165H L33E + Fe(II)	6.5×10^{-6}	4.6×10^{-6}
F165H L34E + Fe(III)	7.4×10^{-6}	3.1×10^{-6}

The surface of PerPA was composed of acidic residues that can bind unspecific metal ion and may show the increase of the wild-type enzyme. To confirm metal ion effect clearly, another attempts were tried. If the metal ion property bind into the mutant enzyme, the enantioselective sulfoxidation or epoxidation were expected. However, enantioselective sulfoxidation was not observed (date not shown).

Attempt of refolding the F165H/L32E mutant

The simple refolding precedure is to dilute the inclusion body into a refolding buffer that allows the formation of the native structure of the protein. The native structure of PerPA has weak esterase activity. This activity can be monitored of *p*-nitrophenyl acetate. Several refolding buffer solutions and conditions were tested but the results were not clear because the purified mutant F165H/L32E enzyme showed very low hydrolysis activity, compared to wild-type enzyme (Figure 9). So the result of hydrolysis activity of this mutant enzyme was difficult to confirm the folding status.



Scheme 2. A model reaction of hydrolysis activity. The reaction was monitored at 404 mm for 5 min.

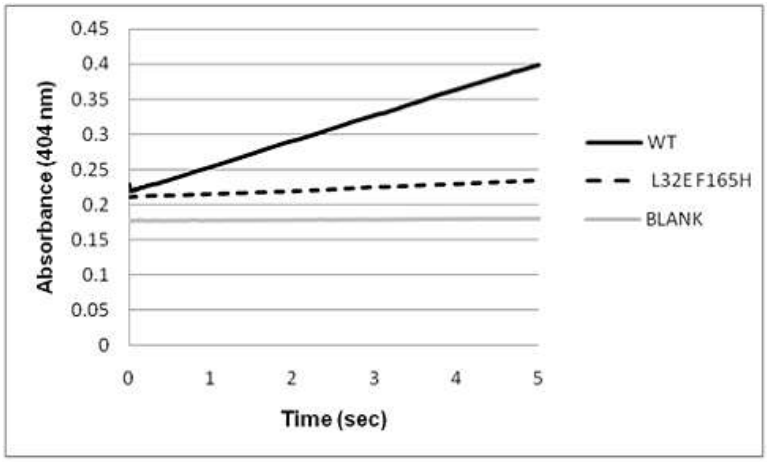


Figure 9. Hydrolysis activity of wild-type enzyme and mutant F165H L32E enzyme

Although several refolding condition has been employed, refolding was not successful and thus it was difficult to obtain the refolded protein with above 90 % purity.

Conclusion

We have attempted to a peroxidase from a perhydrolase by introducing a metal ion into the active site. Several mutants of PerPA that were created by site-directed mutagenesis increased the binding affinity to a metal ion. Some mutants showed much higher peroxidase activity than wild-type enzyme. The F165/L32E mutant enzyme (specific activity: 8.9 x 10^{-4} μmol · sec^{-1} · mg^{-1}) showed 100-fold higher peroxidase activity than wild-type enzyme (specific activity: 8.4 x 10^{-6} μmol · sec^{-1} · mg^{-1}).

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Conclusions and summary

Enzymes are remarkable catalysts because of their intrinsic high selectivity and activity toward natural substrates under mild condition. However, enzymes sometimes do not have enough selectivity or activity toward unnatural substrates. To overcome these problems, the enzyme and enzymatic process need to be modified. This thesis deals with modification of an enzyme to alter selectivity or functionality of a hydrolase by protein engineering.

In the first study, we have increased the enantioselectivity of CAL-B (lipase from *Candida antarctica*) in kinetic resolution of butyl tetrahydrofuran-2-carboxylate by rationally designed protein engineering. Some mutants showed not only higher enantioselectivity but also reverse enantio-preference compared to the wild-type enzyme. In organic solvent, the I189D mutant enzyme showed higher enantioselectivity with reversed enantio-preference (E = 3.0, R-selective) than the wild-type enzyme (E = 1.9, S-selective). In aqueous reaction, the E value of the I189S enzyme (E = 2.2, R-selective) was higher than that of the wild-type enzyme (E = 1.3, R-selective). In addition, the E values of most enzymes showed higher than those in the aqueous media. Although some mutant showed reverse preference and slightly improved the enantioselectivity, the value was not enough for synthetic applications. Therefore, a new system should be designed. Combination of site-directed mutagenesis and the random mutagenesis could be employed for further improving the enantioselectivity.

In the second study, we have introduced new catalytic mechanism to PerPA (perhydrolase from *Pseudomonas aeruginosa*) by protein engineering. We have designed an artificial metalloenzyme by imitation of a natural one. In general, the catalytic metal ion of many metalloenzyme is coordinated by Glu/Asp and two His. We introduced such residues in PerPA to accept a metal ion for peroxidation by site-directed mutagenesis. Some mutants showed much higher peroxidase activity than wild-type enzyme. The F165/L32E mutant enzyme (specific activity: 8.9 x 10^{-4} μmol · sec^{-1} · mg^{-1}) showed 100-fold higher peroxidase activity than wild-type enzyme (specific activity: 8.4 x 10^{-6} μmol · sec^{-1} · mg^{-1}).

ABSTRACT

Rational approach for improving enantioselectivity and altering reaction mechanism

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Enzymes are efficient catalysts for a wide range of industrial applications because of their high reactivity and selectivity. In addition, enzymatic reactions are environmentally benign and are performed under mild conditions. Due to these characteristics of enzymes, industrial applications of enzymes become much more important. However, many enzymes do not show enough reactivity, stability, and selectivity toward unnatural substrates under process conditions. To overcome these problems, researchers have attempted to find new enzymes from nature or modified enzymes by chemical or molecular biological approaches. This thesis deals with biological approaches to alter the functionality of hydrolases.

The first study in this thesis was performed to increase the enantioselectivity of an enzyme by molecular biological approach. Enantiopure tetrahydrofuran-2-carboxylate (THFC) is an important intermediate in the pharmaceutical industry and research. We have increased the enantioselectivity of CAL-B (lipase B from *Candida*

antarctica) towards butyl tetrahydrofuran-2-carboxylate. We introduced residues to construct a hydrogen bond or repulsive interaction with the oxygen atom of THFC. Several mutants showed not only higher enantioselectivity but also reverse enantio-preference compared to the wild-type enzyme. In organic solvent, E value of I189D was 3.0 and R-selective while the wild-type enzyme had 1.9 of the E-value and S-selectivity. And most enzyme variants showed higher enantioselectivity in organic solvents than in aqueous media.

In the second study, modification of an enzyme to introduce new enzyme function was investigated. Our goal was to introduce a peroxidase activity to PerPA (perhydrolase from *Pseudomonas aeruginosa*). For this, we first chose several residues near the active site to accept a metal ion by molecular modeling. Then we have introduced histidine and glutamate/aspartate to bind a metal ion by protein engineering. The F165/L32E mutant (specific activity: 8.9 x 10^{-4} μmol · sec^{-1} · mg^{-1}) showed 100-fold increased peroxidase activity compared to the wild-type enzyme (specific activity: 8.4 x 10^{-6} μmol · sec^{-1} · mg^{-1}).

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