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석사학위 청구논문

Rational design for improving amidase activity of *Candida antarctica* lipase B

아미드 결합 분해 활성을 갖는 리파제 변형체 생성에 대한 연구

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이 논문을 석사학위 논문으로 제출함

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인 준 서

김 주 현의 석 사 학 위 논 문 으 로 인 준 함 .

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성 신 여 자 대 학 교 대 학 원

논문개요

여러 가지 효소들 중 리파아제는 높은 반응성 및 선택성과 유기용매에 대한 우수한 안정성으로 인해 산업적으로나 학문적으로 사용되고 있고, 에스터의 가수분해나 그 생성 등에도 유용하게 사용되고 있다. 리파아제는 상대적으로 저렴하고, 구매가 쉽고, 여러 반응 조건에서 안정하고, 다양한 화합물에 대해 높은 반응 활성과 입체선택성을 보이기 때문에 산업적으로 유용하다. 대부분의 리파아제는 세린계 단백질 가수분해 효소와 비슷한 활성 자리(Ser-His-Asp)를 가지고 있다. 하지만 세린계 단백질 가수분해 효소는 아미드 결합을 가수분해 할 수 있는 반면에, 리파아제는 매우 낮은 반응성을 가진다. 이러한 이유는 분명하지 않으며, 산업적으로 리파제가 아미드 화합물의 제조에 활용될 수 있는 높은 반응성을 갖지 못한다. 따라서 의약품 생산의 중요한 중간체인 키랄 아민이나 아미드 결합 화합물에 높은 활성을 보이는 리파아제 변형체에 대한 연구는 산업적 측면에서 중요할 것이며, 인위적인 효소 기능의 변형이라는 학문적 측면에서도 중요할 것이다.

본 논문은 리파제의 변형체를 생성하여 아미드 화합물의 가수분해에 대한 반응성을 향상시키는 연구를 다루었다. 이를 바탕으로 세린계 단백질 가수분해 효소와 리파제의 반응성 차이에 대한 이해를 시도하였다. 선행연구의 가정을 바탕으로, 아미드 결합과 수소결합을 형성할 수 있는 아미노산들인 Met, Asp, Glu, Gln을 활성자리 주변에 도입하였다. 이들 중 I189Q가 WT (opt2CAL-B_5D)보다 *p*-nitroacetanilide (*p*-NAA)에 대한 가수분해 활성이 11배 정도 증가하였다.

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Chapter 1. Introduction

Enzymes catalyze chemical reactions by improving the reaction rate. Enzyme-catalyzed reactions made the basis of the metabolism of all living organisms. Enzyme technology has showed the application of free enzymes as well as whole cell biocatalysts in the production of goods and services. Also it is an interdisciplinary field, as an important component of continuing industrial development. Table 1 shows the broad and growing impact of enzyme applications in different industry sectors. According to rapid developments in genetic engineering, high-throughput screening, and other established or emerging technologies (Table 2).[1)]

Table 1. Impact of enzyme technology in industry [a]

Industry	Key works
Agriculture	Feed additives
	Heterologous enzyme production
Chemicals	Biocatalysis
	Bulk organic compound
Cleaning energy	New detergent enzymes
	Fuel alcohol from biomass
Food	Nutraceuticals
Pharma	Chiral compounds
	Glycoprotein engineering
	Enzymes as pharma targets
Material	Paper, textile, leather treatment

[a] The table is adapted from van Beilen, J. B.; Li, Zhi. *Curr Opin Biotechnol*. **2002**, *13*, 338-344

Table 2. Impact of emerging and established technologies on enzyme technology [a]

Technology	Key developments
Functional genomics	Enzyme discovery
	Genome link with enzyme activities
	Genomics for enzyme-based drug discovery
Protein structure determination	Structural genomics inactive
High-throughput methods	Enzyme discovery and improvement
	Sol-gel immobilized enzyme arrays
	High-throughput screening methods
Enzyme engineering	Directed evolution to improve enantioselectivity
	New enzyme activities by chemical modification
	Artificial enzymes
Combinatorial biocatalysis	Combinatorial biocatalysis review
	Enzymatic polymerization
Bioelectro-catalysis	Biosensors, bioreactors and biofuel cells
Metabolic engineering	Metabolites as industrial chemicals
Bioprocess engineering	Dynamic kinetic resolution
	Industrial biocatalysis
Regulatory aspects	Single isomer pharmaceuticals
	Pollution and waste reduction

[a] The table is adapted from van Beilen, J. B.; Li, Zhi. *Curr Opin Biotechnol*. **2002**, *13*, 338-344)

1.1. Hydrolases

EC number means the enzyme commission number. This is numerical classification scheme for enzyme based on the chemical reactions which they catalyze. Hydrolases are classified as EC 3 (Table 3).

Usage of hydrolases in organic synthesis have several advantages. First, hydrolases often accept various synthetic intermediates as substrates. Second, hydrolases frequently show high stereoselectivity toward unnatural substrates. Third, hydrolases also carry out several related reactions such as condensations (reversal of hydrolysis) and alcoholysis (a cleavage using an alcohol in place of water).[2)]

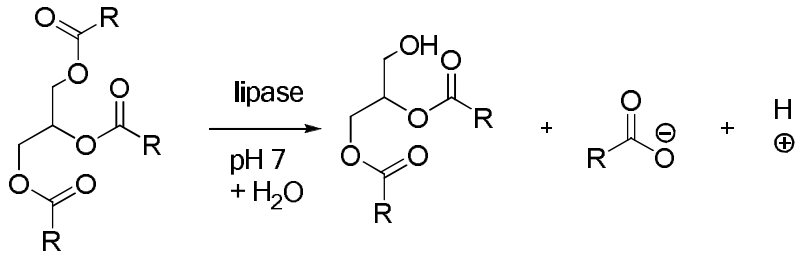
Table 3. The definitions of hydrolase class and subclasses[a]

Class	Enzyme	Type of reaction	Subclasses
3	Hydrolase	Hydrolysis reactions	3.1 Ester bonds 3.2 Sugars 3.3 Ether bonds 3.4 Peptide bonds 3.5 Carbon-nitrogen bonds 3.6 Acid anhydrides 3.7 Carbon-carbon bonds 3.8 Halide bonds 3.9 Phosphorus-nitrogen bonds 3.10 Sulfur-nitrogen bonds 3.11 Carbon-phosphorus bonds 3.12 Sulfur-sulfur bonds 3.13 Carbon-sulfur bonds

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

1.1.1. Lipase

Esterases and lipases belong to the family of serine hydrolase and α/β-fold family. They have the catalytic triad, Asp/Glu-His-Ser, which is similar to that of serine proteases. The serine residue is activated by histidine and aspartate or glutamate residue. Lipases and esterases catalyze the hydrolysis of esters by the same double displacement mechanism via an acyl enzyme intermediate.[3] Although both esterases and lipases have the hydrolysis activity of ester, lipases preferentially catalyze the hydrolysis water-insoluble esters.[4] Lipases have been isolated from a variety of sources such as archaea, microbial and mammalian sources. They hydrolyze lipids (triglycerides) to glycerol and fatty acids in nature (Scheme 1).[5] Lipases have extraordinarily broad substrate specificity. Also, they can catalyze transesterification toward a wide range of structurally diverse esters, alcohols and carboxylic acid.[6]



Scheme 1. Lipase catalyzes the hydrolysis of triglycerides (e.g. triolein) to fatty acids and glycerol.

Lipases are industrially and academically important biocatalysts because they show many attractive aspects for researches. First, they usually display elaborate chemoselectivity, stereoselectivity, and regioselectivity. Second, they are available in large quantities from microbial organisms, namely fungi and bacteria. Third, the crystal structures of many lipases have been solved. Forth, they do not require cofactors. These advantages make lipases the most widely used group of biocatalysts in organic chemistry. The commercial use of lipases is comprised of a wide variety of different applications in the area of detergents, and the production of food ingredients and enantiopure pharmaceuticals.[7)]

Lipases and esterase have a distinctive property. Lipases show interfacial activation but it is not observed for esterase (Figure 1). It means that lipases become active when substrates are at a water-oil interface. This phenomenon can be explained through 3D-structure analysis. The active site of lipases was covered by a lid-like polypeptide chain constituted one or two amphipathic α-helices. This lid structure offers preventing the active site from substrate molecules. When the substrate stays at water-oil phase, the lid opens and then lipase turns active, whereas without substrate, the lid is closed and the enzyme remains inactive.[8)]

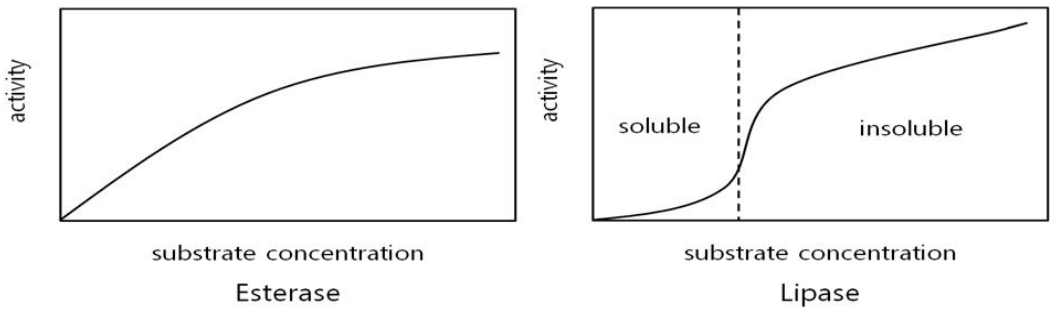


Figure 1. Kinetics of esterase and lipase. Esterases follow Michaelis-Menten kinetic and lipases show interfacial activation.

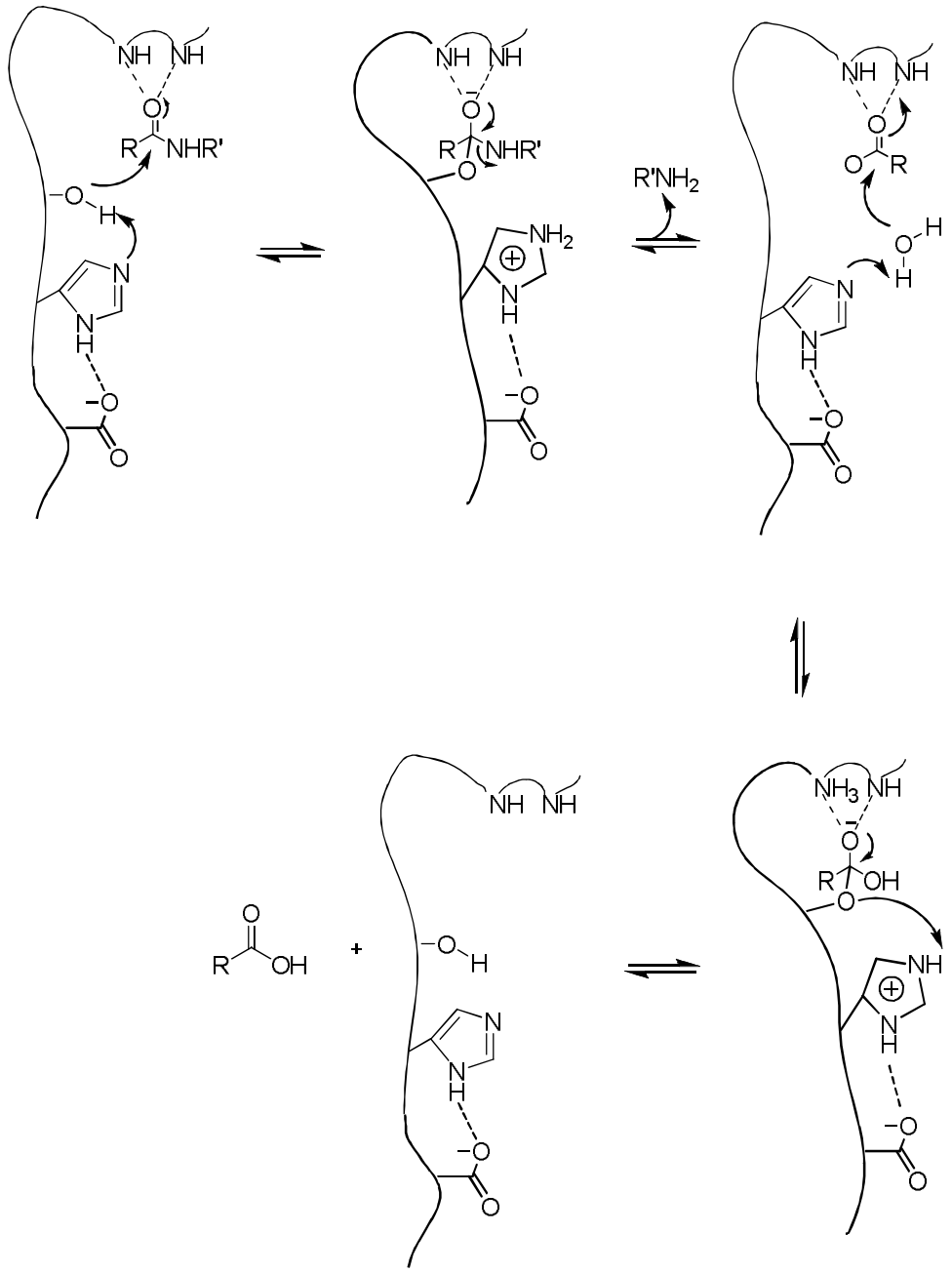
1.1.2. Serine-protease

Serine proteases contain the nucleophilic serine residue at the active site. It has the catalytic triad, Asp-His-Ser, which is similar to that of lipases.[9] Serine proteases can classify at least four different structure. These four clans are representative by chymotrypsin, subtilisin, carboxypeptidase Y, and Clp protease.[10] Besides, serine proteases with novel catalytic triads have been discovered. These proteases have been found in prokaryotes, eukaryotes, archae, and viruses.[11]

Hydrolysis of amide bond is difficult compared to cleavage of ester bond. Amide bonds are very stable due to electron donation from the amide nitrogen to the carbonyl. Also, water is not so nucleophilic and amines are poor leaving group. Thus, proteases usually activate an amide bond via the interaction of the carbonyl oxygen with a general acid, and may also distort the peptide bond to disrupt resonance stabilization. Proteases activate water through a general base, and protonate the amine prior to exclusion.

Generally, serine proteases display the double displacement mechanism for hydrolysis of amide bond (Scheme 2). Serine residue attacks the carbonyl of the substrate, supported by histidine residue acting as a general base, to make a tetrahedral intermediate. The resulting His-H^+ is stabilized by the hydrogen bond to aspartate residue. The oxyanion of the tetrahedral intermediate is stabilized by interaction with the main chain NHs of the oxyanion hole. The tetrahedral intermediate disintegrates with emission of leaving group, assisted by histidine residue

acting as a general acid, to make the acylenzyme intermediate. Then water attacks the acylenzyme, assist histidine residue, making a second tetrahedral intermediate. This intermediate disintegrates, evicting serine residue and carboxylic acid product.[12)]



Scheme 2. The generally accepted amidase mechanism for serine proteases.

1.1.3. *Candida antarctica* lipase B

Lipase B from *Candida antarctica* (CAL-B) is one of the most widely used biocatalysis. CAL-B has a molecular weight 33 kDa. It shows the typical α/β hydrolase fold. The active site is composed of a Ser-His-Asp catalytic triad.[13] The structure of CAL-B was determined by X-ray crystallography (Figure 2).[14]

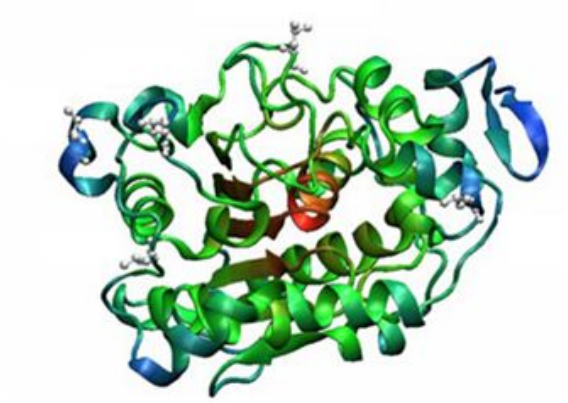
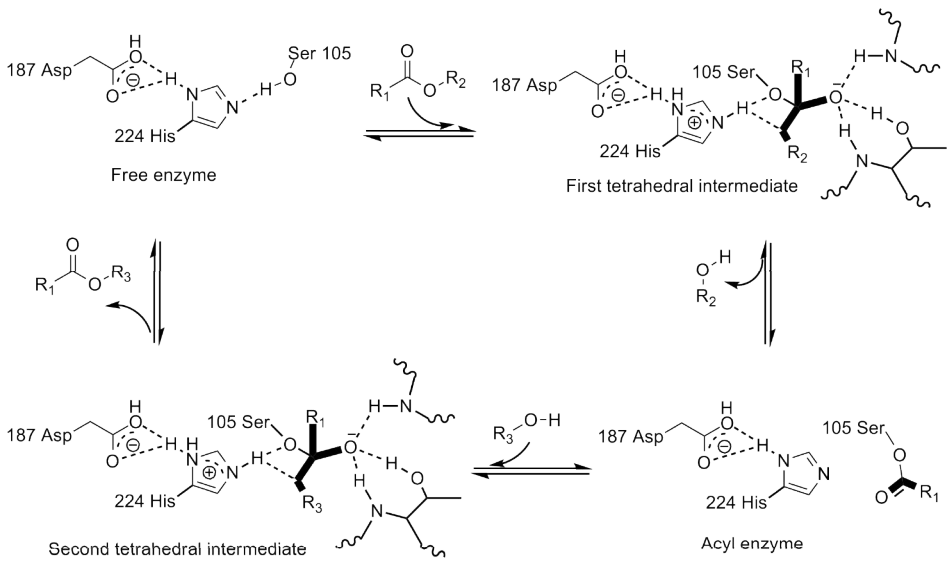


Figure 2. Structure of *Candida antarctica* lipase B (pdb code: 1LBS)

CAL-B catalyzes acyl-transfer reaction (Scheme 3). The first substrate enters the active site and makes the first tetrahedral intermediate. An acyl enzyme intermediate forms by the release to 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.[15]

The active site pocket is composed of two channels, one hosting the acyl- and the other hosting the alcohol-moiety of the substrate. These channels are responsible for the high regio- and stereo- selectivity of CAL-B towards secondary alcohols.[14]



Scheme 3. CAL-B catalyzes acyl-transfer reaction by a ping-pong mechanism

1.2. Protein engineering

Researchers need advanced enzymes for industrial application. Due to poor substrate solubility, breakdown of unstable products, or competing chemical reactions, the conditions for an enzyme reaction may be unsuitable for large-scale applications. For this reason, modification of an enzyme is required and genetical modification of an enzyme is called protein engineering.[16)]

The protein engineering combines biological, structural, chemical, and combinatorial approaches to explore protein functions and molecular interactions. Biochemist have done researches of protein engineering to explore advanced novel therapies and to maintain scientific innovation. Protein engineering through mutagenesis is generally performed using two approaches, rational design and directed evolution.[17)] Figure 3 shows the schematic diagrams for the two approaches.

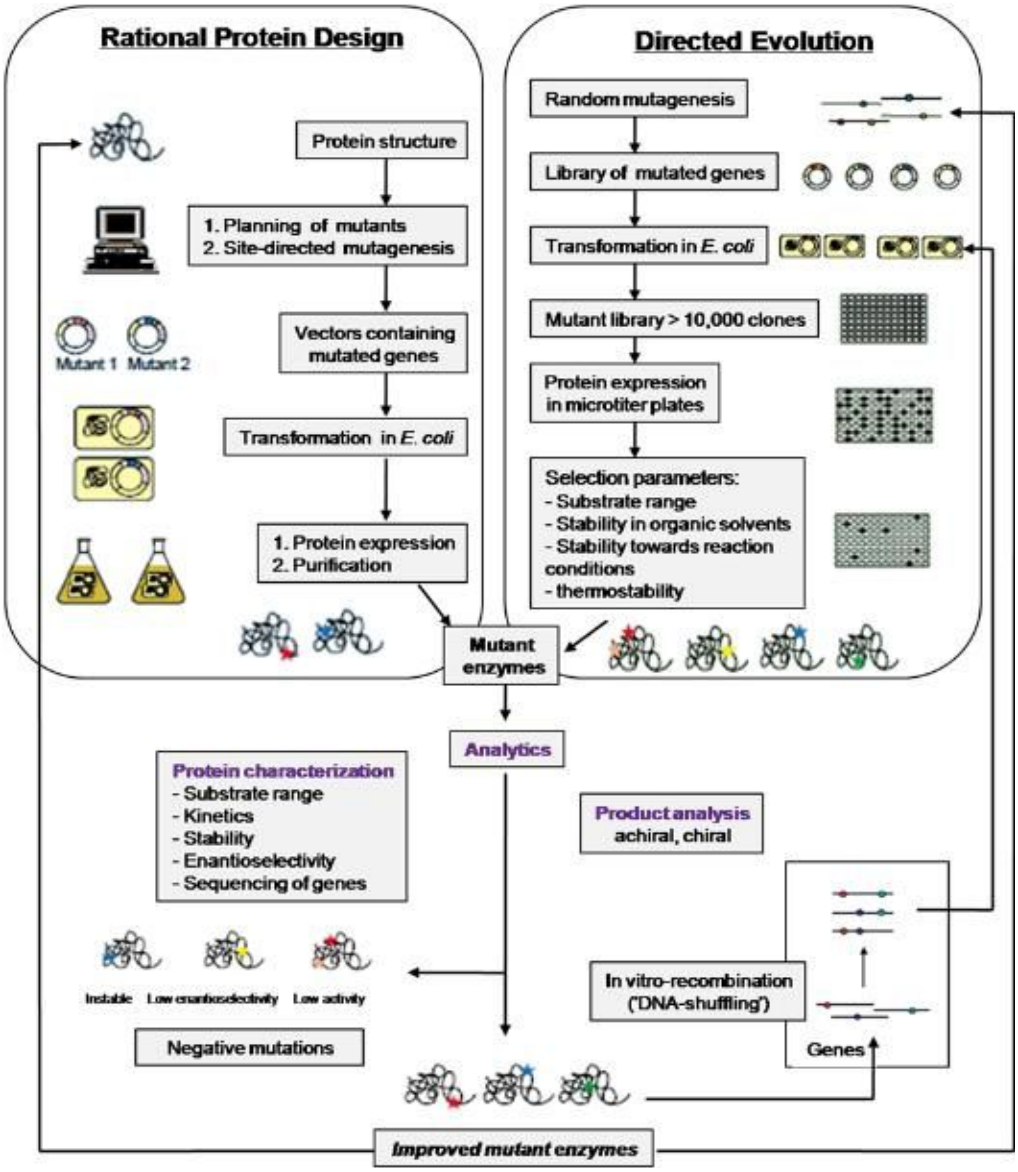


Figure 3. Comparison of rational protein design and directed evolution. These will surely help to increase our understanding of structure/function relationships and will see more combinations of both protein engineering tools (The figure is adapted from Bornscheuer, U. T.; Pohl, M. *Curr. Opin. Chem. Biol*. **2001**, *5*, 137-142).

The goal of protein engineering is as follows. First, protein engineering improves existing proteins and enzymes to make them more effective – with higher affinity, longer lasting effects and/or greater selectivity. Second, protein engineering determines three-dimensional structures of proteins and protein complexes for finding new molecules with biological activity and exploring their potential as therapeutic agents or drug targets. Third, protein engineering invents new protein/peptide technologies for applying chemical approaches to tasks in drug discovery and biology.[18)]

1.2.1. Rational design

Rational design requires detailed knowledge of enzyme structure, function, and catalytic mechanism. Rational design is based on prediction to increase the selectivity, activity and stability of enzymes by using molecular modeling.[19)]

Rational design methods of protein engineering involve the choice of specific amino acid residues for alteration to accomplish the desired reaction using site-directed mutagenesis. Depending on the purpose of the mutagenesis, amino acid substitutions are often selected by sequence comparison with homologous sequences. Sometimes, the results are ineffective because minor sequence changes by a single point-mutation may cause signigicant structural disturbance. Thus, distinction of the three-dimensional structures of wild-type enzymes and mutant is necessary to ensure that a single mutation is really site-directed.[15)]

van den Heuvel *et al.* take one rational approach to invert the stereospecificity of a vanillyl-alcohol oxidase. By site-directed mutagenesis, the putative active site base has been relocated to the opposite face of the active site cavity. The single mutants, T457E, D170A, and D170S, preferentially converted 4-ethylphenol to the (*R*)-enantiomer of 1-(4′-hydroxyphenyl)ethanol. The double mutants D170A/T457E and D170S/T457E exhibited an inverted stereospecificity of (*S*)-selective with 4-ethylphenol.[20)]

Rotticci et al. used molecular modeling to study the different binding modes for alcohol enantiomers in the active site of *Candida*

antarctica lipase B and proposed a model for its enantioselectivity. Site-directed mutagenesis was used to alter the active site residues causing unfavorable interactions between the substrate and the enzyme. A single mutation, Ser47Ala, resulted in improvement of the lipase-catalyzed resolution of 1-chloro-2-octanol from E = 14 to E = 28. (E is the enantiomeric ratio)[21)]

1.2.2. Directed evolution

Rational design requires enormous inputs of structural, mechanistic, and dynamic information.[16] In contrast, directed evolution does not need detailed structural and mechanistic information about enzymes. Directed evolution employs a random process of mutagenesis by error-prone PCR or DNA shuffling to create a library of mutagenized genes.[22)23]

Figure 4 shows the general scheme of directed evolution. Genetic diversity is first introduced into a target gene through random mutagenesis and/or recombination. Random mutagenesis methods create a library of variants containing point mutations from a single parental gene, whereas gene recombination methods create a library of chimeric variants via blockwise exchange of sequence information among the parental genes.

The library of mutant genes is then transformed into host cells in which the mutant genes are converted into their corresponding proteins. Functionally improved mutant proteins are identified through an appropriate selection or screening strategy. The same process will be repeated until the goal is achieved or no further improvement is possible.[24]

Directed evolution can be employed for engineering enantioselective biocatalysts. The Reetz group used error-prone PCR coupled with a 96-well plate based colorimetric screening method to increase the enantioselectivity of a *Pseudomonas aeruginosa* lipase toward 2-methyldecanoate.[25] After several rounds of directed evolution, the enantioselectivity of the lipase increased from E = 1.04 (2%ee) to E = 25 (90 - 93%ee). Using a similar approach, Arnold group even inverted the

enantioselectivity of hydantoinase from D-selectivity (40%ee) to moderate L-preference (20%ee at 30% conversion).[26]

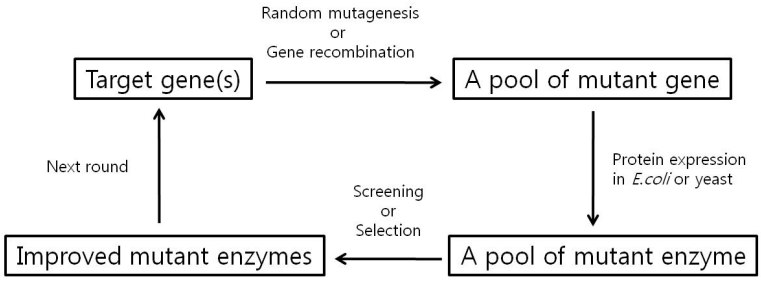


Figure 4. The general scheme of directed evolution (The figure is adapted from Chen, Z.; Zhao, H. *Encyclopedia of Chemical Processing*. **2006**, *10*, 2467-2477).

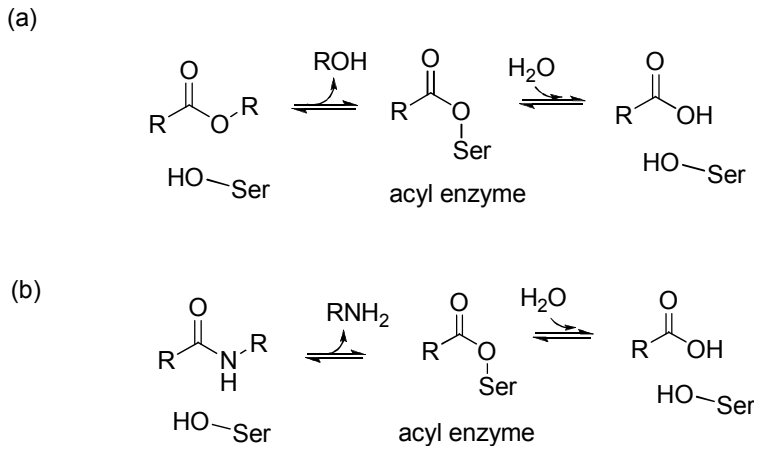
1.2.3. Combined approaches

Enzyme properties can be improved by rational design or directed evolution. Although either rational design or directed evolution is effective, a combination of both strategies will probably represents the most successful route to improving the properties and function of an enzyme. Particularly, a directed evolution may ignore the unexpected structural change from rational design. Also, it could improve an additional property of enzymes.[27)]

Reetz *et al.* showed the improvement of the enantioselectivity of a lipase. Using such a cyclical random/targeted approach, they greatly improved the enantioselectivity (E) of *Pseudomonas aeruginosa* lipase toward a *p*-nitrophenyl ester in favour of the (*2S*) enantiomer. Initial improvement from E=1.1 to E=51 was achieved by rational design and directed evolution approaches.[28)]

1.3. Outline of this thesis

Lipase and serine protease share the same catalytic triad. The lipase-catalyzed reactions proceed through corporative roles of the catalytic triad in their active sites (Scheme 4).[29]



Scheme 4. Hydrolysis mechanism of lipase and serine-protease.
(a) lipase (b) serine-protease

Therefore, it would be concluded that lipase and serine-protease show the similar catalytic activities. However, most lipases cannot cleave amide bonds although a few lipase can slowly catalyze hydrolysis of amides,[30] whereas proteases can hydrolyze esters and amides.[31] This phenomenon is not yet fully understood and comprehending its molecular basis remains a challenge for researchers.

Enhancing amide-hydrolysis activity of a lipase was reported by Nakagawa and coworkers. They improved the amidase activity of lipase from *Pseudomonas aeruginosa* toward a long-chain-fatty-acid amide by a factor of twenty eight through two-stage directed evolution. However, the detailed mechanism for the improvement was not revealed.

Recently, Cammenberg *et al.* proposed the molecular basis for the enhanced *Burkholderia cepacia* lipase (BCL)-catalyzed *N*-acylation of 1-phenylethanamine by methoxyacetate.[32] The enhancement of BCL catalyzed acylation of an amine by using ethyl methoxyacetate was reported by Balkenhohl *et al.* without the detailed mechanistic study.[33] I suggested that the hydrogen atom connected to the nitrogen atom of the amine substrate may interrupt formation of the key hydrogen bond between the catalytic histidine and the nitrogen atom of the substrate at the transition state. The disruption could be avoided by using methoxyacetate as an acyl donor. The oxygen atom of the methoxy group of the acyl donor can hydrogen bond to the nitrogen atom and thus the proton can not interrupt the key hydrogen bond. This results in stabilization of the transition state and thus accelerates the aminolysis.

Similarly, Kourist *et al.* proposed that the molecular basis of the promiscuous amidase activity of *Bacillus subtilis* esterase. They proposed that the glutamate near the active site constructs a hydrogen-bond network with the amide nitrogen through a water molecule and help to avoid the interruption by the amide proton.

It was assumed that introduction of a residue to be able to form a hydrogen bond or to interact electronically with the amide nitrogen can enhance the amidase activity of lipase as the role of methoxy group of

methoxyacetate. In this thesis, this hypothesis was exploited to increase amidase activity of *Candida antarctica* lipase B (CAL-B), which possesses low intrinsic amidase activity.

Chapter 2. Experimetal Section

Materials and Method.

Chemicals and buffers were purchased from Sigma-Aldrich. *Pfu* DNA polymerase and restriction enzyme (*Dpn* I) were purchased from Enzynomics (Daejeon, Korea) and New England Biolabs, respectively. DNA oligomers were obtained from Sigma-Proligo (Singapore). DNA sequencing was performed by Solgent Co. (Daejeon, Korea). The Ni-NTA agarose resin was purchased from QIAGEN.

Site directed mutagenesis

Opt2CAL-B_5D, a mutant of lipase B from *Candida antarctica*, was designed for higher expression in *E.coli*. The opt2CAL-B_5D was subcloned into *p*BAD/gⅢa vector containing restriction sites for *Nco* I and *Sal* I (Figure 5). The mutant genes were created by Quikchange mutagenesis with mutagenesis primers. The sequences of the opt2CAL-B_5D (Table 4) and mutagenesis primers are listed in Table 5. After the PCR reactions, the plasmids of the mutants were transformed into *E.coli* (TOP10).

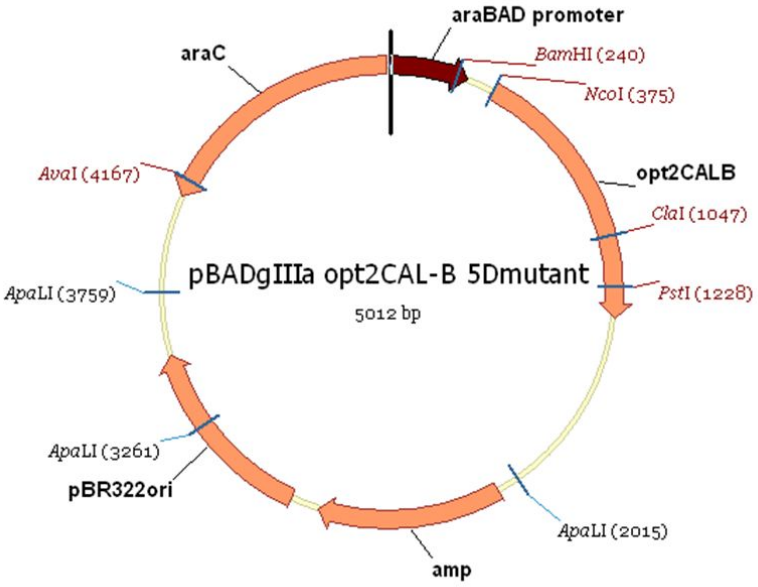


Figure 5. The vector map of opt2CAL-B_5D as a template for mutagenesis.

Table 4. Codon-optimized sequences of opt2CAL-B_5D

Sequence (opt2CAL-B_5D)
ATG GCT 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 GAC 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 GAT GAT TCT
TCT TAC CTG TTC AAC GGT AAG AAC GTT CAG GCT CAG GCT GTT TGT
GGC CCG GAC 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 GAT ACC GAC TGC AAC
CCG GAC 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 GTC GAC

Table 5. The primers used for mutagenesis

Mutagenesis primers	
F_mu_CALB_G39M	5’-CCGATCCTGGTACCGATGACCCGTACCACTGGC-3’
R_mu_CALB_G39M	5’-GCCAGTGGTACGGGTCATCGGTACCAGGATCGG-3’
F_mu_CALB_G39D	5’-AAACCGATCCTGGTACCGATGACCCGTACCACTGGC-3’
R_mu_CALB_G39D	5’-GCCAGTGGTACGGGTCATCGGTACCAGGATCGGTTT-3’
F_mu_CALB_G39E	5’-AAACCGATCCTGCTGGTACCGGAAACCGGTACCACTGGC-3’
R_mu_CALB_G39E	5’-GCCAGTGGTACCGGTTTCCGGTACCAGCAGGATCGGTTT-3'
F_mu_CALB_G281M	5’-GCGGCTCTGGCACCGATGGCAGCTGCAATTGTT-3’
R_mu_CALB_G281M	5’-AACAATTGCAGCTGCCATCGGTGCCAGAGCCGC-3’
F_mu_CALB_G281D	5’-GCGGCTCTGGCACCGGATGCAGCTGCAATTGTT-3’
R_mu_CALB_G281D	5’-AACAATTGCAGCTGCATCCGGTGCCAGAGCCGC-3’
F_mu_CALB_G281E	5’-GCGGCTCTGGCACCGGAAGCAGCTGCAATTGTT-3'
R_mu_CALB_G281E	5’-AACAATTGCAGCTGCTTCCGGTGCCAGAGCCGC-3’
F_mu_CALB_I189M	5’-AGCGCAACCGATGAGATGGTTCAGCCGCAGGTATC-3’
R_mu_CALB_I189M	5’-GATACCTGCGGCTGAACCATCTCATCGGTTGCGCT-3’
F_mu_CALB_I189D	5’-AGCGCAACCGATGAGGACGTTCAGCCGCAGGTATC-3’
R_mu_CALB_I189D	5’-GATACCTGCGGCTGAACGTCCTCATCGGTTGCGCT-3’
F_mu_CALB_I189E	5’-CAGCGCAACCGATGAGGAAGTTCAGCCGCAGGTATCTAAC-3’
R_mu_CALB_I189E	5’-GTTAGATACCTGCGGCTGAACTTCCTCATCGGTTGCGCTG-3’
F_mu_CALB_I189Q	5’-GCGCAACCGATGAGCAGGTTCAGCCGCAG-3’
R_mu_CALB_I189Q	5’-CTGCGGCTGAACCTGCTCATCGGTTGCGC-3’

Expression and purification of CAL-B

Overnight culture (1 mL of *E. coli*) was added to LB medium (100 mL; ampicillin, 100 μg/mL) and incubated at 37 ℃ and 200 rpm to an OD_{600} of 0.5. Protein expression was induced by adding arabinose (1 mL; 2% w/v) and incubated for 6 h at 25 ℃ and 200 rpm. The OD_{600} was reached to ~1.5. The cells were harvested by centrifugation (10 min, 3,800 × g, 4 ℃) and the supernatant was discarded.

The cell pellet (~0.8 g) was resuspended in the lysis buffer (5 mL/g wet weight; NaH_2PO_4, 50 mM; NaCl, 300 mM; imidazole, 10 mM; adjusted to pH 8.0 with NaOH), and lysozyme was added to 1 mg/mL. Incubation on ice for 45 min was followed by a freeze-thaw cycle at -20 ℃ and room temperature. The viscous lysate was passed several times through a sterile 20-gauge syringe needle and centrifuged (10 min, 10,000 × g, 4 ℃). The supernatant was separated from the cell debris. The cell debris was dissolved in 8M urea solution (4 mL, containing 1 mM of dithiothreitol) for SDS-PAGE analysis. Ni-NTA agarose resin (1 mL, 50% w/v slurry) was added to the supernatant (4 mL) and the mixture was stirred at 25 ℃ for 1 h. The lysate-Ni-NTA mixture was loaded on a Poly-Prep column (Bio-Rad), drained, and then washed three times with the wash buffer (4 mL; NaH_2PO_4, 50 mM; NaCl, 300 mM; imidazole, 20 mM; adjusted to pH 8.0 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; adjusted to pH 8.0 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).

Determination of the amount of the enzymes

The amount of purified enzymes was determined by absorbance at 280 nm (ε = 41,285 M^{-1} cm^{-1} calculated with tools at Swiss Prot Expasy, http://ca.expasy.org/tools/protparam.html).

Measurement of esterase activity toward hydrolysis of p-nitrophenylacetate

Esterase activity of the enzymes was measured by following the hydrolysis of *p*-nitrophenylacetate at 405 nm. The assay solution was prepared by mixing *p*-nitrophenylacetate (20 μL, 200 mM in acetonitrile), acetonitrile (870 μL), and BES buffer (5 mM, pH 7.2, 11,110 μL). The absorbance change was measured at 405 nm for 5 min after mixing the assay solution (100 μL) with the enzyme solution (5 μL). The final concentrations in the reaction solution were 0.32 mM substrate, 4.65 mM BES, 7% acetonitrile. The activity was calculated according to the method of Janes et al. where $\Delta\varepsilon$ = 17,300 M^{-1} cm^{-1}.

Measurement of amidase activity toward hydrolysis of p-nitroacetanilide

Amidase activity of the enzymes was measured from the hydrolysis of *p*-nitroacetanilide at 405 nm. The assay solution was prepared by mixing *p*-nitroacetanilide (200 μL, 100 mM in acetonitrile), acetonitrile (690 μL), and BES buffer (11,110 μL, 5 mM, pH 7.2). The assay solution (1 mL) and the enzyme solution (100 μL) were combined and incubated at 24 °C. The final concentrations in the reaction solution were 1.52 mM

substrate, 4.66 mM BES, 7% acetonitrile. The enzyme concentrations used were 1.5-2.6 μM. The absorbance change was measured at 405 nm. The activity was calculated using $\Delta\varepsilon$ = 11,100 M^{-1} cm^{-1}.

Acylation of 1-phenylethanol and 1-phenylethanamine

Acyl donor reagent (1.0 mmol) and 1-phenylethanol or 1-phenylethanamine (1.0 mmol) were added to a suspension of molecular sieves 4A (50 mg) and Novozym 435 (10 mg) in methyltetrabutylether (5.0 mL) and shaken at 20 °C. The samples (100 μL) from the reaction mixture were retrieved with intervals and analyzed by a GC with a chiral column (Cyclosil-B, 30 m × 0.25 mm): initial column temperature 80 °C for 10 min, ramp to 120 °C at a rate of 2.5 °C min^{-1}, and then held at 120 °C for 10 min.

HPLC analyses

The samples (500 μL) conducted with the hydrolysis of *p*-nitroacetanilide were diluted with acetonitrile (500 μL). The diluted samples (1 μL) were injected on a nonpolar column (3.0 × 100 mm, 1.8-μm thickness, Agilent ZORBAX Eclipse plus C18), and eluted with an aqueous methanol solution (60% v/v) containing 0.2% of acetic acid at a flow rate of 0.2 ml min^{-1} and 25 °C. The signals were detected at 320 nm. The retention times of *p*-nitroacetanilide and *p*-nitroaniline were 3.4 min and 4.1 min, respectively.

Chapter 3. Result and discussion

Selection of residues for mutagenesis

First, the amino acids were selected to be introduced for constructing a hydrogen bond with the amide nitrogen atom. Amino acids containing a side chain working as a hydrogen-bond acceptor are aspartate, asparagine, glutamate, glutamine, serine, threonine and methionine. For hydrogen bonding, the side chains of these amino acids should place near the amide nitrogen within a proper distance. In general, a typical length for hydrogen bonds is 2.7-3.2 Å. Therefore, the residues should be located within 5.1-8.0 Å from the amide nitrogen atom because the lengths of the side chains are 2.4-4.8 Å. Based on this criteria, the residues within 8.0 Å from the amide nitrogen were explored using an inhibitor bound crystal structure of CAL-B (pdb code: 1LBS) with substitution of the inhibitor by the corresponding amide and identified twelve residues in the crystal structure (Figure 6). Among these residues, we deselected Thr40, Ser105, and His224 in further consideration because these residues are involved in the catalytic machinery.

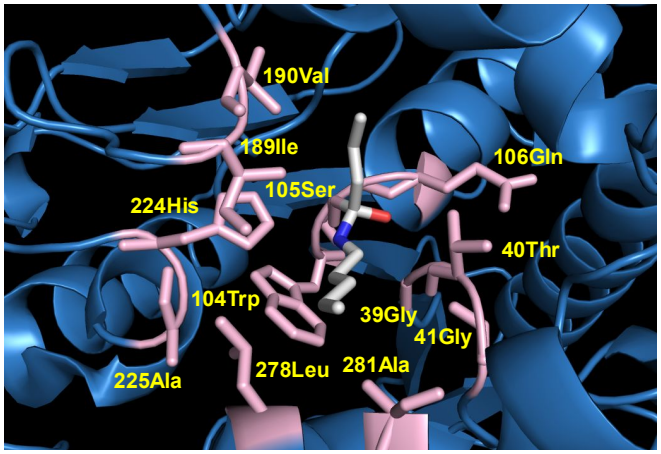


Figure 6. The residues placed within 8 Å from the amide nitrogen atom. The structure was created by substitution of the inhibitor (pdb code: 1LBS) with nitrogen atom. The stucture was generated using PyMOL 0.99 (Delano Scientific, San Carlos, CA).

In addition, the direction of the side chains is also important to construct a proper hydrogen bond. Only Gly39, Ile189, and Ala281 contain the side chains toward the amide nitrogen (Table 6). The distances between the residues and the amide nitrogen atom are 4.69-7.72 Å. To construct a hydrogen bond, an amino acid possessing a rather longer side chain is required. Thus, we chose Asp, Glu and Met to be introduced.

Table 6. The selected residues within 8.0 Å from the amide nitrogen atom to the α-carbon of a residue

Residues	Distance from the amide nitrogen atom to the α-carbon	comments
Gly39	4.69 Å	proper direction
Thr40	5.00 Å	oxianion hole
Gly41	7.52 Å	wrong direction
Trp104	7.04 Å	wrong direction
Ser105	4.46 Å	catalytic triad
Gln106	5.96 Å	wrong direction
Ile189	6.71 Å	proper direction
Val190	7.83 Å	wrong direction
His224	7.35 Å	catalytic triad
Ala225	7.97 Å	wrong direction
Leu278	7.88 Å	wrong direction
Ala281	7.72 Å	proper direction

Expression and purification of CAL-B

Mutant enzymes were expressed as much as yield of the wild-type CAL-B_5D. The purities of the enzymes were above 95% based on SDS-PAGE analyse (Figure 7).

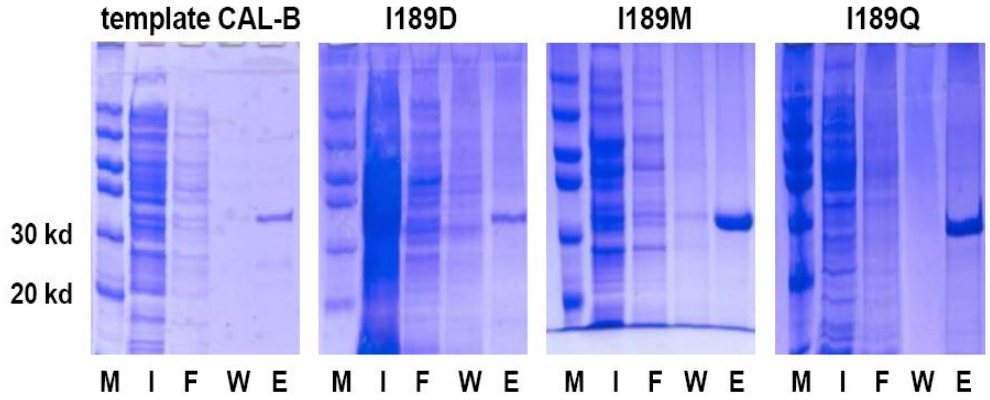
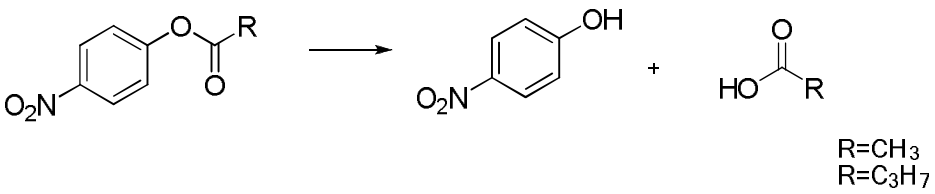


Figure 7. SDS PAGE analyses of CAL-B mutant enzymes expressed in *E. coli*. SDS-PAGE was performed on a 12% polyacrylamide gel and stained using the Coomassie brilliant blue. M, molecular weight marker; I, insoluble fraction; F, flow-through fraction; W, wash buffer fraction; E, elution buffer fraction (for details see the materials and method section).

Measurement of esterase activity toward hydrolysis of p-nitrophenylacetate

Esterase activity of mutant enzymes toward hydrolysis of *p*-nitrophenylacetate was measured (Scheme 5). The hydrolysis by the wild-type and mutant enzymes showed different esterase activity. A281E of the mutant enzymes showed the most rapid hydrolysis activity toward *p*-nitrophenyl acetate than other enzymes (Table 7).



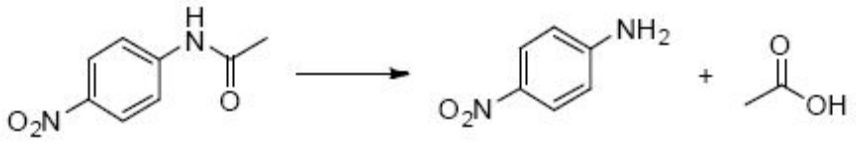
Scheme 5. The reaction to determine esterase activity

	Specific activity *p*-NPAc ($\mu molmin^{-1}mg^{-1}$)
CALB_5D_wt	4.31 ± 0.43
G39M	0.020 ± 0.0030
G39D	4.7 ± 0.25
I189M	2.3 ± 0.19
I189D	0.65 ± 0.087
I189E	0.49 ± 0.15
A281M	4.80 ± 0.12
A281D	17 ± 0.70
A281E	23 ± 1.1

Table 7. Specific activity for hydrolysis of *p*-nitrophenyl acetate(*p*-NPAc)

Measurement of amidase activity toward hydrolysis of p-nitroacetanilide

To identify amidase activity of the mutant enzymes, hydrolysis towards *p*-nitroacetanilide as a model reaction was performed (Scheme 6). The initial screening with the nine mutant enzymes was carried out at 1.5 mM substrate and 1.8 μM enzyme in a BES buffer (pH 7.2), and monitored at 405 nm. The absorbance change for 43 h clearly indicated that I189M and I189D mutant enzymes shows faster hydrolysis of *p*-nitroacetanilide than the template CAL-B (Figure 8). Aspartate and glutamate contain the same negatively charged functional group (-COO-). But the mutant enzyme substituted by aspartate solely showed higher amidase activity than the template enzyme. The 189 residue locates just above the catalytic histidine in which a positive charge develops during catalysis. Thus, the negative charge of the longer side chain of glutamate would be close enough to disrupt the charge balance of the catalytic machinery.



Scheme 6. The reaction to determine amidase activity

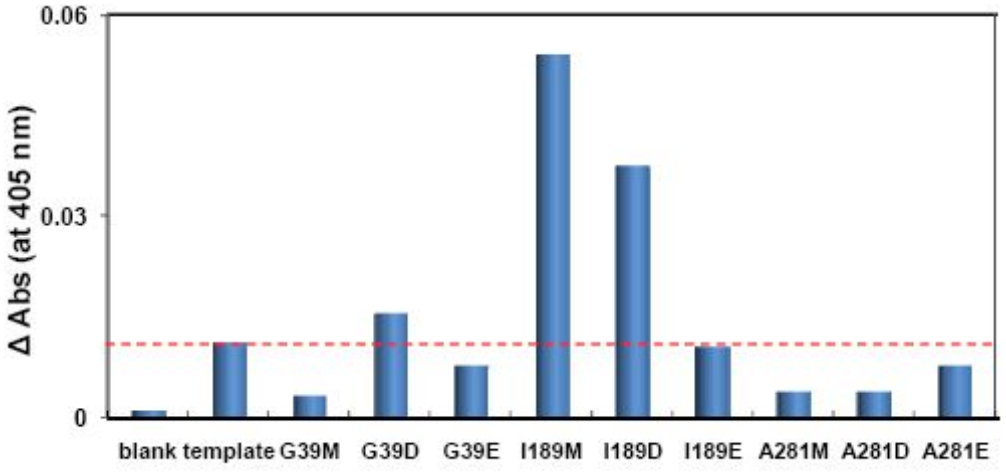


Figure 8. The absorbance change at 405 nm for 43 h

I189 mutants of hydrolysis of p-nitrophenylacetate and p-nitroacetanilide

To improve the amidase activity further, we introduced glutamine at the 189 residue, which contains an oxygen atom on the side chain and may construct stronger hydrogen bond than methionine. Dissimilarly to glutamate, glutamine does not have a negative charge on the side chain and thus would not disrupt the charge balance of the catalytic machinery at the transition state. As expected, the glutamine mutant showed faster hydrolysis of *p*-nitroacetanilide than the template enzyme as well as the methionine mutant (Figure 9).

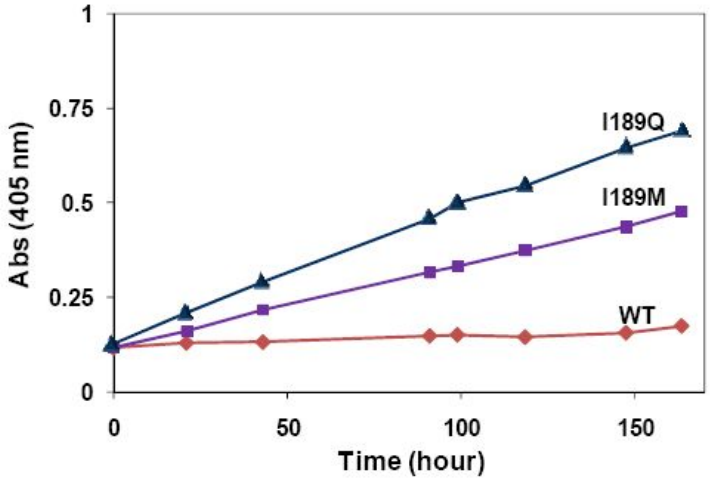


Figure 9. The absorbance change in hydrolysis of *p*-nitroacetanilide

In order to compare esterase and amidase activities of the template, I189D, I189M, and I189Q enzymes, we measured the initial rates of hydrolysis of *p*-nitrophenylacetate and *p*-nitroacetanilide, respectively (Table 8).

Mutation on the 189 residue decreased the esterase activity by 20-80%. The methionine mutant enzyme retained 80% of esterase activity of the template enzyme. I189D and I189Q mutant enzyme showed 20% and 30%, respectively, of the esterase activity compared to that of the template enzyme. These decreases of esterase activity are presumably due to influence on the charge balance by introduction of a polar residue on the 189 residue. However, as already confirmed, the three mutant enzymes show higher amidase activity than the template enzyme.

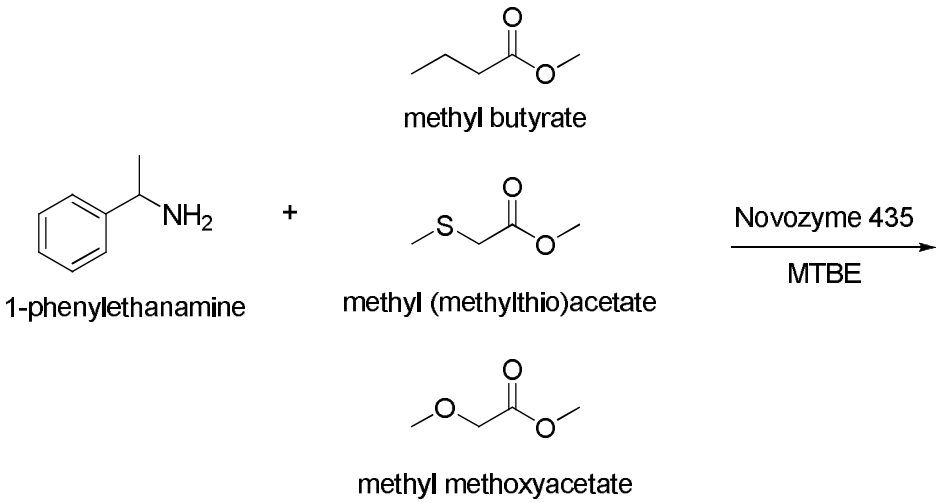
The highest amidase activity was obtained by the glutamine mutant enzyme and the initial rate increased by a factor of 11. These results clearly indicate that introduction of residue acting as a hydrogen-bond acceptor is a key to enhancing amidase activity of CAL-B. The template enzyme or the wild type CAL-B possesses isoleucine at the 189 residue. Isoleucine contains non polar aliphatic carbon chain as the side chain, which could not act as a hydrogen bond acceptor. Substitution of this residue by a hydrogen-bond acceptor or a polar group clearly improved the amidase activity of CAL-B.

Table 8. Initial rates (μmol min^{-1} mg^{-1}) of hydrolysis of *p*-nitrophenylacetate and *p*-nitroacetanilide

Enzyme	*p*-nitrophenylacetate		*p*-nitroacetanilide	
	Initial rate	Relative rate	Initial rate	Relative rate
template (5D_CAL-B)	4.3 ± 0.4	1	(0.85 ±0.23) × 10^{-5}	1
I189M	3.3 ± 0.3	0.8	(5.4 ± 0.4) × 10^{-5}	6.4
I189D	0.91 ± 0.03	0.2	(2.7 ±0.3) × 10^{-5}	3.2
I189Q	1.5 ± 0.1	0.3	(9.6 ± 0.8) × 10^{-5}	11.3

Acylation of 1-phenylethanol and 1-phenylethanamine

Methionine contains a sulfur atom. Although methionine is regarded as a hydrophobic amino acid,[34] it has been reported that methionine often accepts a hydrogen bond in proteins.[35] Indeed, about 10% of sulfur atoms of methionine in proteins are capable of serving as a hydrogen bond acceptor.[36] To identify the potential role of the sulfur atom for enhancing amidation rate as the oxygen atom of methoxyacetate in BCL-catalyzed amidation, we performed aminolysis by a commercial immobilized CAL-B (Novozym 435) using methyl butyrate, methyl thioacetate, and methyl methoxyacetate as an acyl donor (Scheme 7). As in the BCL-catalyzed amidation, using methyl methoxyacetate as an acyl donor increased the initial rate by a factor of about 100 compared to using methyl butyrate. Interestingly, utilizing methylthioacetate also increased the initial rate by about seven times compared to butyrate. This result is similar to the previous report (Table 9).[37]



Scheme 7. Acylation of 1-phenylethanol and 1-phenylethanamine

Table 9. Initial rates of acylation of 1-phenylethanamine by Novozym435

Acyl donor	Initial rate ($\mu mol\ min^{-1}\ mg^{-1}$)	Relative rate
Methyl butyrate	0.016 ± 0.0012	1
Methyl methoxyacetate	1.56 ± 0.049	96
Methyl methylthioacetate	0.11 ± 0.0068	6.8

HPLC analyses

HPLC analyses clearly showed that the reaction by I189Q mutant enzyme produce much more *p*-nitroaniline product than that by the template enzyme (Figure 10).

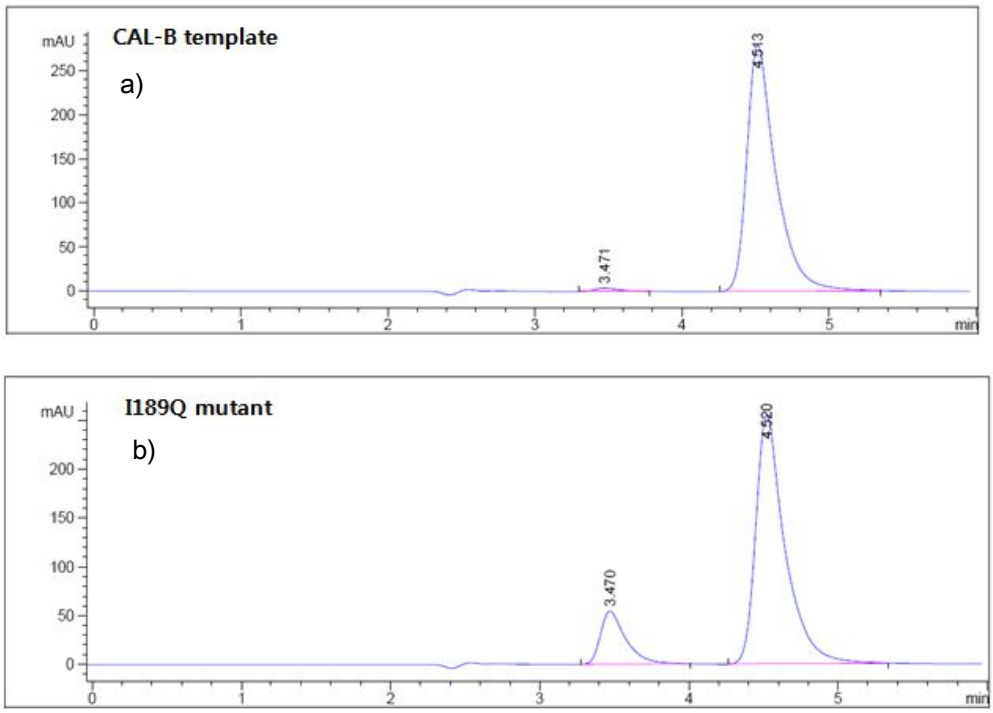


Figure 10. HPLC analyses of the hydrolysis of *p*-nitroacetanilide by the CAL-B template and I189Q mutant enzymes. a) the reaction by the CAL-B template enzyme. b) the reaction by the I189Q mutant enzyme. The product, *p*-nitroaniline, and the reactant, *p*-nitroacetanilide, were shown at 3.47 min and 4.52 min, respectively. The reaction by the I189Q mutant enzyme produced much more products.

Chapter 4. Conclusion

Previous research suggested that introducing a residue for hydrogen bonding construction or electronic interaction with the amide nitrogen atom at the transition state could be an important factor to improve the amidase activity of lipase. The current experimental result clearly showed that introduction of a hydrogen bond acceptor or a polar group increases the reaction rates of amide hydrolysis presumably through assistance of stabilization of the transition state and could provide a clue for the molecular basis of different activities between lipase and serine-protease.

Among designed mutants, A281E showed the highest esterase activity and I189Q has the highest activity toward amide hydrolysis with the initial rate increased by a factor of 11. These results clearly indicate that introduction of residue acting as a hydrogen-bond acceptor is a key to enhancing amidase activity of CAL-B.

Reference

1) Beilen, J. B. V.; Li, Zhi. *Curr. Opin. Biotechnol.* 2002, *13*, 338-344.

2) ExPASy Proteomics Server. http://www.expasy.org/enzyme (accessed Nov 2009).

3) Cygler, M.; Schrag, J. D.; Sussman, J. L.; Harel, M.; Silman, I.; Gentry, M. K.; Doctor, B. P. *Protein Sci.* 1993, *2*, 366-382.

4) Macrae, A. R. *JAOCS*, 1983, *60*, 243-246.

5) Bornscheuer, U. T.; Kazlauskas, R. J. Availability, structure and properties. Hydrolases in organic synthesis, 2nd ed.; Wiley-VCH: New york. 2006; p 1.

6) Nakagawa, Y.; Hasegawa, A.; Hiratake, J.; Sakata, K. *Protein engineering Design & selection*. 2007, *20*, 339-346.

7) Jaeger, K. E.; Eggert, T. *Curr. Opin. Biotechnol.* 2002, *13*, 390 - 397.

8) Jaegera, K. E.; Ransacb, S.; Dijkstrab, B. W.; Colsonc, C.; van Heuveld, M.; Missetd, O. *FEMS Microbiology Reviews.* 1994, *15*, 29-63.

9) Hedstrom, L. *Chem. Rev.* 2002, *102*, 4501-4523.

10) Rawlings, N. D.; Barrett, A. J. *MEROPS*, 2000, *28*, 323-325

11) Dodson, G.; Wlodawr, A. *Trands Biochen. Sci.* 1998, *23*, 347-352.

12) Steitz, T. A.; Shulman, R. G. *Annu. Rev. Biophys. Bioeng.* 1982, *11*, 419-444.

13) Blank, K.; Norfill, J.; Gumpp, H.; Gaub, H. E. *Journal of Biotechnology*. 2006, *125*, 474-483.

14) Uppenberg, J.; Ohrner, N.; Norin, M.; Hult, K.; Kleywegt, G. J.; Patkar, S.; Waagen, V.; Anthonsen, T.; Jones, T. A. *Biochemistry* 1995, *51*, 16838-16851.

15) Mugnusson, A. Rational redesign of *Candida antarctica* lipase B. Ph. D. Thesis, KTH, Sweden, 2005

16) Otten, L. G.; Quax, W. J. *Biomol. Eng.* 2005, *22*, 1-9.

17) Bornscheuer, U. T.; Pohl, M. *Biocatal. Biotransform.* **2001**, *5*, 137-143.

18) Beilen, J. B. V.; Li, Zhi. *Curr. Opin. Biotechnol.* **2002**, *13*, 338-344.

19) Magnusson, A.; Hult, K.; Holmquist, M. *J. Am. Chem. Soc.* **2001**, *123*, 4354-4355.

20) van den Heuvel, R. H.; H.; Fraaige, M. W.; Ferrer, M.; Mattevi, A.; van Berkel, W. J. H. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 9455-9460.

21) Rotticci, D.; Rotticci-Mulder, J. C.; Denman, S.; Norin, T.; Hult, K. *ChemBioChem.* **2001**, *2*, 766-770.

22) Petrounia, I. P.; Arnold, F. H. *Curr. Opin. Biotechnol.* **2000**, *11*, 325-330

23) Liebeton, K.; Zonta, A.; Schimossek, K.; Nardini, M.; Lang, D.; Dijkstra, B. W.; Reetz, M. T.; Jaeger, K. E. *Chemi. & Biol.*. **2000**, *7,* 709-718.

24) Chen, Z.; Zhao, H. *Encyclopedia of Chemical Processing* **2006**, *10*, 2467-2477.

25) Reetz, M. T. *Angew. Chem. Int. Ed.* **2001**, *40*, 284-310.

26) Wang, C.; Oh, M. K.; Liao, J. C. *Biotechnol Prog.* **2000**, *16,* 922-926.

27) Chen, R. *TRENDS Biotechnol.* **2001**, *19*, 13-14.

28) Reetz, MT. *Proc Natl Acad Sci USA.* **2004**, *101*, 5716-5722

29) Brady, L.; Brzozowski, A. M.; Derewenda, Z. S.; Dodson, E.; Dodson, G.; Tolley, S.; Turkenburg, J. P.; Christiansen, L.; Huge-Jensen, B.; Norskov, L.; Thim, L.; Menge, U. *Nature*, **1990**, *343*, 767-770.

30) Henke, E.; Bornscheuer, U. T. *Anal. Chem.* **2003**, *75*, 255-260.

31) Klibanov, A. M. *Science,* **1983**, *219*, 722-727

32) Cammenberg, M.; Hult, K.; Park, S. *ChemBioChem.* **2006**, *7*, 745-1749.

33) Balkenhohl, F.; Ditrich, K.; Hauer, B.; Ladner, W.; *Prakt, J. Chem./Chem-Ztg.* **1997**, *339*, 381-384.

34) Kyte, J.; Doolittle, R. F. *J. Mol. Biol.* **1982**, *5*, 105-32.

35) Wennmohs, F.; Staemmler, V.; Schindler, M. *J. Chem. Phys.* **2003**, *119*, 3208-3218.

36) Zhou, P.; Tian, F.; Lv, F.; Shang, Z. *Proteins.* **2008**, *76*, 151-163.

37) Ismail, H.; Liu,, R. M.; van Rantwijk, F.; Sheldon, R. A. *Adv. Synth. Catal.* 2008, *350*. 1511-1516.

ABSTRACT

Rational design for improving amidase activity of *Candida antarctica* lipase B

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Because lipases have high reactivity, selectivity, and stability in organic solvent, they are used in industrial and academic areas. In addition, lipases are relatively inexpensive and commercially available. Lipases have the catalytic triad, Asp-His-Ser, which is similar to that of serine proteases. However, most lipases cannot hydrolyze amide bonds although serine-proteases catalyze hydrolysis of amide bonds. This phenomenon is not yet fully understood and comprehending its molecular basis remains a challenge for researchers. For this reason, lipases have a limit to produce chiral amines, which is an important building block in pharmaceutical industry. Therefore, research to create lipase variants containing high activity toward chiral amines is important in academia and industry.

This thesis deals with rational design for improving amidase activity of *Candida antarctica* lipase B, and understanding different activities lipase and serine-protease. Based on the assumption of the

previous study, a residue, such as Met, Asp, Glu, Gln, was introduced near the active site to provide a hydrogen bond with the amide nitrogen atom. Total ten mutant enzymes were created. Among them, the I189Q mutant enzyme showed eleven-fold faster hydrolysis activity toward *p*-nitroacetanilide than the wild-type enzyme.