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석사학위 청구논문

Enhancing the activities of *Candida antarctica* lipase B by the immobilization on hydroxyapatite and by site directed mutagenesis

Candida antarctica lipase B의 수산화인회석에의 고정화를 통한 반응성 향상과 효소변이를 통한 과가수분해 반응성 향상에 관한 연구

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이 논문을 석사학위 논문으로 제출함

2017년 5월

성신여자대학교 대학원
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전 민 정의 석사학위논문을 인준함

2017년 5월

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논 문 개 요

본 논문은 효소 고정화를 통한 리파제 효소의 반응성 향상에 관한 연구와 효소변이를 통한 반응성 향상에 관한 연구를 다룬다. 먼저 환경친화적인 물질로 알려진 수산화인회석을 담체로 활용한 고정화를 통해 유기 용매 하에서의 리파제 반응성 향상을 수행하였고, 그리고 *Candida antarctica* lipase B (CAL-B)의 효소변이를 통해 과가수분해 반응성 향상을 수행하였다.

수산화인회석은 사람 뼈의 주성분으로 생체 및 환경친화적인 물질이다. 따라서 수산화인회석은 효소고정화용 담체로서 훌륭한 후보물질로 판단되지만, 현재까지 이에 대한 연구가 활발히 수행되지는 않았다. 본 연구에서는 공유결합을 이용한 리파제 고정화를 시도하였다. 먼저 펩티드결합 물질인 *N,N'*-dicyclohexylcarbodiimide (DCC), *N,N'*-diisopropyl carbodiimide (DIC) and *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide (EDC) 을 활용하여 리파제를 직접 수산화인회석에 결합하고자 시도하였다. 그런데, 수산화인회석에 리파제를 직접 결합시키면, 많은 양의 단백질이 결합하지 않는다는 것을 확인하였다 (0.26-0.48 mg/g). 이는 아마도 극성의 수산화인회석과 비극성의 리파제 표면들 사이의 반발력에 기인하는 것으로 판단된다. 따라서 이 문제를 해결하기 위해 6-aminohexanoic acid를 연결화합물로 활용하여 리파제와 수산화인회석 사이의 반발력 감소를 시도하였고, 결과적으로 단백질의 결합 양을 대략 5배 정도 향상시킬 수 있었다 (1.70-2.76 mg/g). 그리고 CAL-B의 경우를 포함하여 *Burkholderia cepacia* lipase (BCL)와 *Candida rugosa* lipase

(CRL)의 고정화된 형태들은, 그렇지 않은 효소형태보다 유기용매 하에서 최대 100배까지 반응성이 증가하였고, 10회의 재사용에도 85% 이상의 반응성이 유지됨을 확인하였다.

CAL-B는 가수분해 효소의 일종이기는 하지만, 과산화수소수를 반응물로 활용한 과가수분해 역시 수행할 수 있음이 알려져 있다. 본 연구에서는 효소변이를 통해 CAL-B의 과가수분해 반응성 향상을 시도하였다. 최근에 CAL-B 가수분해 반응의 반응물인 물분자가 활성자리로 접근하는 통로 존재의 가정이 제안되었다. 본 연구는 이 가정을 기반으로, 과가수분해를 위한 반응물인 과산화수소수 역시 이 통로를 따라 효소 내부로 진입하게 될 것을 가정하였다. 이 가정이 사실이라면, 물통로를 구성하는 아미노산의 변화는 과가수분해 반응성에도 영향을 미칠 수 있을 것으로 기대하였다. 본 연구에서는 분자 모델링을 통해 물통로를 구성하는 아미노산들을 파악하였고, 이들 중 Pro280과 Ala281을 변화대상으로 선정하였다. 선정된 위치에 크기가 다르거나 혹은 보다 극성의 아미노산을 도입시킨 후, 가수분해 반응성과 과가수분해 반응성의 변화를 관찰하였다. Pro280 자리의 변화는 가수분해 또는 과가수분해 반응성의 증가나 감소 정도가 미미하였다. 반면, Ala281의 변이체인 Ala281Ser와 Ala281Thr의 경우, 가수분해 반응성은 각각 2배, 1.2배 가까이 증가하였고, 과가수분해 반응성은 원형 CAL-B의 경우보다 약 2배 정도 향상되는 결과를 확인하였다.

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Chapter 1. Introduction

Enzymes are very useful biocatalysts which can be obtained from natural environments, plant or animals. They help us digest food, produce vital nutrients, and move all our body in our daily life. Enzymes can also accept broad substrate range, and catalyze chemical reactions with high regio-, and enantioselectivity [1]. However, enzymes are easily inactivated. To overcome these problem, several stabilization strategies were used to improve catalytic stability and activity of enzymes such as genetic modification and enzyme immobilization [2]. Especially, enzyme immobilization has been generally regarded as a solution for the stabilizing enzyme, and enabling the reuse and easy separation [3].

Perhydrolysis is the formation of peroxy acids from carboxylic acid and hydrogen peroxide. This reaction is reversible and generally performed by perhydrolase (also called metal-free haloperoxidases) [4]. *Candida antarctica* lipase B (CAL-B) has been known to catalyze the perhydrolysis of carboxylic acid [5-7]. The perhydrolase activity of CAL-B can be enhanced by modification of amino acids of specific channel because water or hydrogen peroxide molecules can enter into this channel.

The goal of this thesis is immobilization of enzyme on hydroxyapatite as supporting material to improve catalytic activity of immobilized enzymes and enhancing perhydrolase activity of recombinant lipase by using site directed mutagenesis.

1.1. Enzyme

Enzymes are efficient biological catalysts. They often exhibit high regio- and enantioselectivity. They accelerate reaction rates by lowering the activation energy. They possess the active site where a substrate binds and the reaction occurs. Substrates should be fitted in the active site similar to a piece of puzzle, and enzymes induced conformational changes to strengthen their binding [8]. Catalytic reactions by enzymes are generally carried out in mild conditions: at low pressure, near neutral pH, and the temperature below 100 °C. Enzymes can be classified into six types according to their reaction catalyzed as seen in Table 1 [9]. Enzymes are used for a wide range of areas such as food, plant, and medical industries [10].

Table 1. The classification of enzymes based on the chemical reactions.

Class	Reaction catalyzed	Examples
1. Oxidoreductase	To catalyze oxidation and reduction reactions	Dehydrogenase, Oxidase
2. Transferase	Transfer of a functional group from one substance to another	Transaminase, Kinase
3. Hydrolase	Formation of two products from a substrate by hydrolysis	Lipase, Amylase, Peptidase
4. Lyase	Non-hydrolytic addition or removal of groups from substrates	Decarboxylase
5. Isomerase	Intramolcule rearrangement	Isomerase, Mutase
6. Ligase	Join together two molecules by synthesis of new bonds	Synthetase

1.1.1. Lipase

Lipases (EC 3.1.1.3) are enzymes that catalyze hydrolysis of lipid. They contain the catalytic triad Ser-Asp/Glu-His, and α/β -hydrolase fold found in esterases and lipases [11]. They can be obtained from microorganism, plants, fungi, and animals. These enzymes have a wide range of substrate and high regio-, and enantioselectivity. The advantages in use of lipases are low cost, no cofactors, and showed high activity and stability in organic solvents.

Lipases have a distinct property called interfacial activation apart from esterases. They possess a lid which consists of amphipathic polypeptide chain [12]. At soluble interface, this lid covered the active site and lipases show low activity. When lipases are exposed to a lipid interface, the lid opens and lipases become active by changing a conformation of enzyme. Then, the activity of lipase increases dramatically and its curve shows a sigmoidal plot (Figure 1) [13].

The most frequently used lipases for organic synthesis are porcine pancreatic lipase (PPL), lipase from *Burkholderia cepacia* (BCL), lipase from *Candida rugosa* (CRL), and lipase B from *Candida antarctica* (CAL-B). In this study, three lipases, CAL-B, BCL and CRL were used in the study of immobilization.

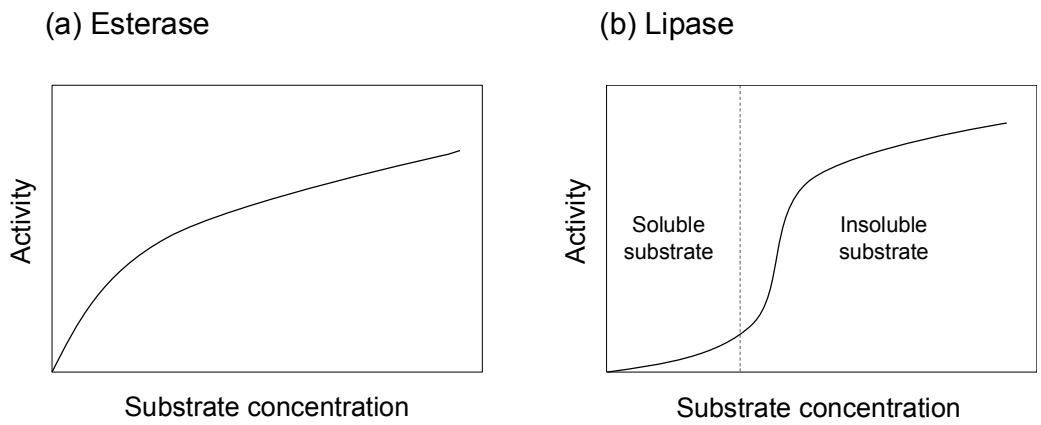


Figure 1. The difference of esterase and lipase. (a) The kinetics of esterase show normal Michaelis–Menten kinetics, but (b) lipase kinetics show a sigmoidal plot due to interfacial activation.

1.1.1.1. Lipase B from *Candida antarctica* (CAL-B)

One of the most useful lipases is CAL-B. CAL-B has high regio- and enantioselectivity toward a wide range of substrates, and the resistance against high temperature. It has 317 amino acids, and the molecular weight is 33 kD. The catalytic triad consists of Ser105, Asp187, and His224 in the active site as shown in Fig. 2. The oxyanion hole consists of Thr40 and Gln106. Since CAL-B has no lid, it shows no interfacial activation [14].

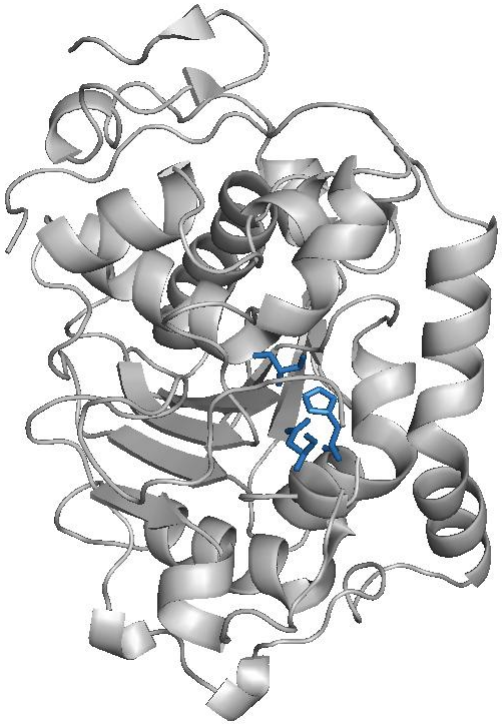


Figure 2. The structure of *Candida antarctica* lipase B (PDB code: 1TCA). The catalytic triad (Ser105, Asp187, and His224) is shown in a stick model (blue) in center.

CAL-B catalyzes acyl-transfer reaction through the ping-pong mechanism. It consists two steps with the acylation and deacylation steps [13]. First, the serine attacks the carbonyl carbon atom of an ester substrate to form the first tetrahedral intermediate (T_d1). The oxyanion is stabilized by Thr40 and Gln106. Then the acyl enzyme is formed by releasing the alcohol product. Second substrate, a water molecule, attacks the acyl enzyme, and then the second tetrahedral intermediate is formed (T_d2). Finally, the carboxylic acid product is released in the deacylation step, and the free enzyme is formed again (Figure 3).

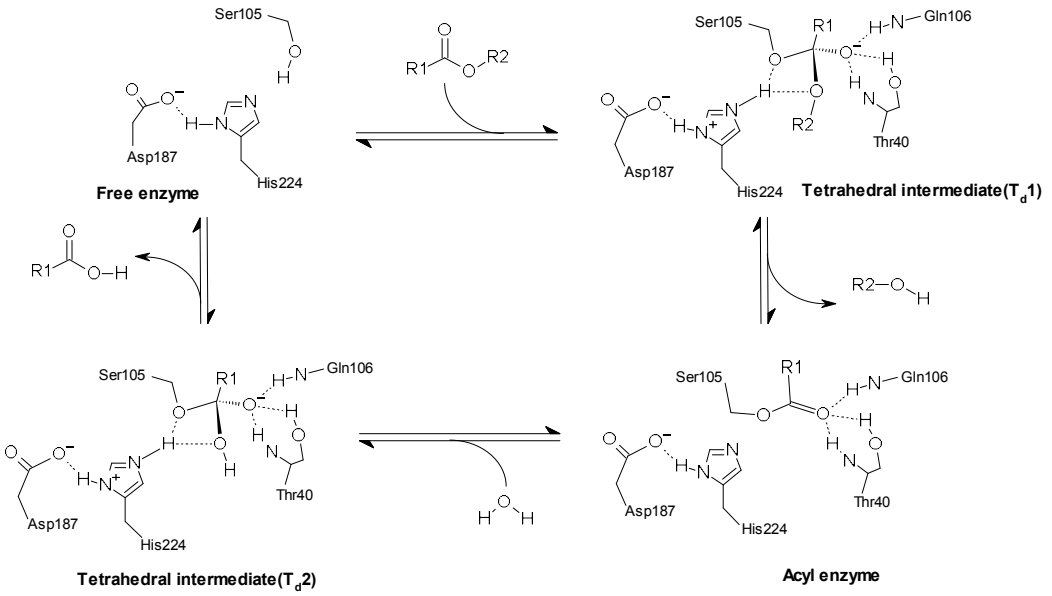


Figure 3. Catalytic mechanism of *Candida antarctica* lipase B through the acylation and deacylation steps.

1.1.1.2. Lipase from *Burkholderia cepacia* (BCL)

BCL has 320 amino acids, and its molecular weight is 33 kD. It is known that the catalytic triad of BCL consists of Ser87, Asp264 and His286, and the oxyanion hole is composed of Leu17 and Gln88 residues. Also, BCL has vital Ca^{2+} ion that stabilizes the three dimensional structure (Figure 4). BCL shows interfacial activation due to the mobile structure, called the lid. The lid covers the active site in water because the exterior of the lid is hydrophilic. When insoluble substrates approached to lipase, the lid opens and lipid/water interface is formed. Then, the activity of lipase increased. That is, the active site is shielded (closed form, inactive) or revealed (open form, active) by moving the lid [15].

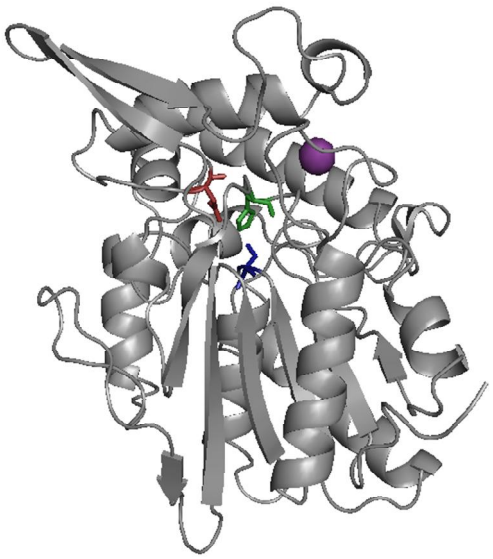


Figure 4. The structure of *Burkholderia cepacia* lipase (PDB code: 3LIP). The catalytic triad consists of Ser87(blue), Asp264(red) and His286(green). Also, BCL has Ca^{2+} ion (purple).

1.1.1.3. Lipase from *Candida rugosa* (CRL)

CRL is prominent biocatalyst, and is also applicable in biotransformations of various products. Since the CRL has also a large lid, it shows interfacial activation similar to BCL. It can produce seven isoforms according to their culture conditions [16]. CRL has 534 amino acids and its gel electrophoresis shows a single protein with a molecular weight of ~60 kD [15].

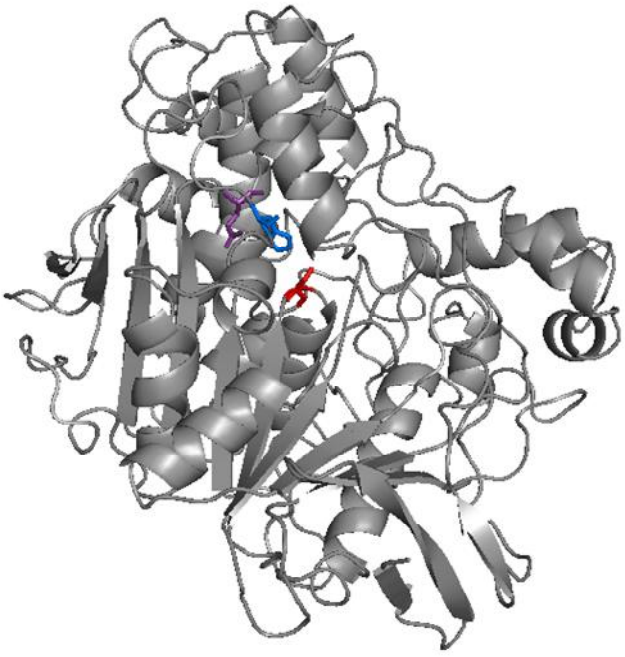


Figure 5. The structure of *Candida rugosa* lipase (PDB code: 1TRH) that displayed in cartoon representation. In center, there are catalytic triad represented by stick. Ser209, Glu341 and His449 are colored red, purple and blue, respectively.

1.1.2. Enzyme immobilization

Enzymes are easily inactivated by heating, strong acid or base. Enzyme immobilization is one of the effective techniques to solve their inactivation problem, and a conventional method for improving enzyme stability in organic solvent and recyclability [17]. In addition, enzyme immobilization is used in a wide range of analytical, medical and industrial applications [18]. For example, glucose biosensor is utilized for measuring blood sugar and sugar contents in food [19]. Besides, a pregnancy test tool is also an application of immobilizing enzyme [20]. The requirements of support materials are non-toxic, highly stable, biologically safe, and environment-friendly. There are five categories, such as adsorption, entrapment, covalent binding, ionic binding and cross-linking in enzyme immobilization (Figure 6). Among these methods, using covalent binding has several benefits. As a covalent bond is the strongest interaction, enzymes will not be detached from the supports [21]. Most commonly used supports are celite, silica, polymers and glass.

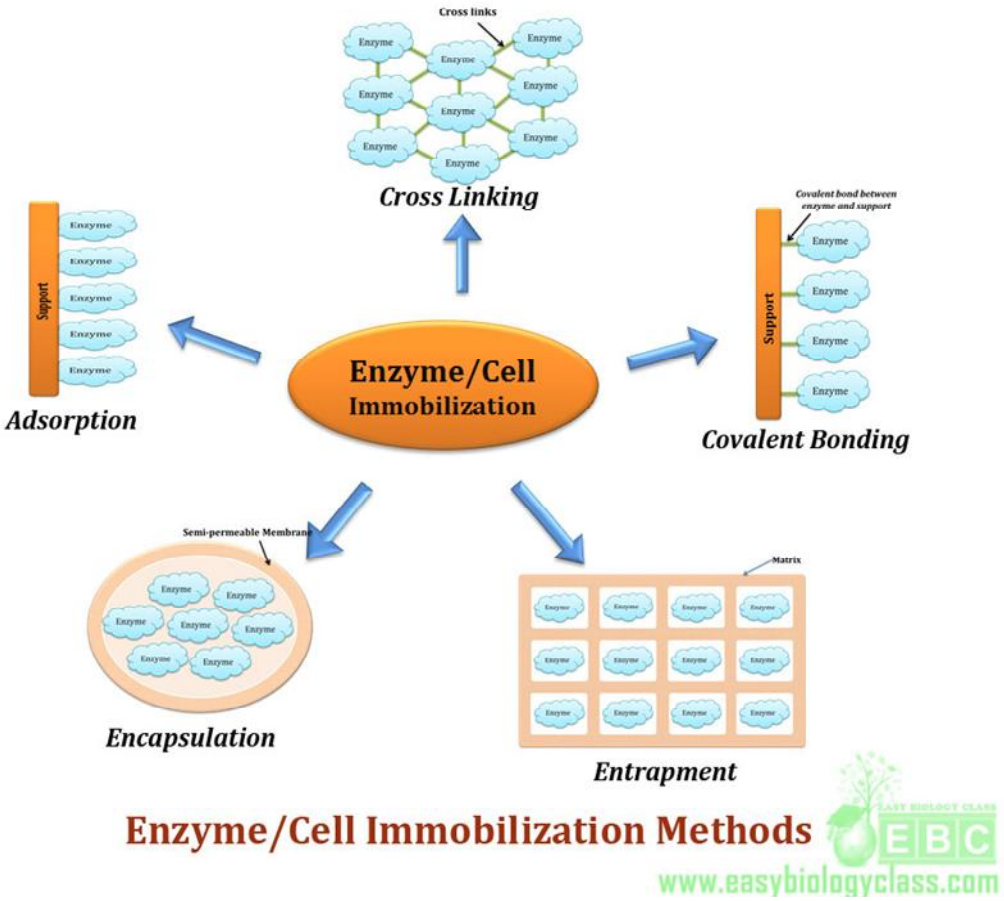


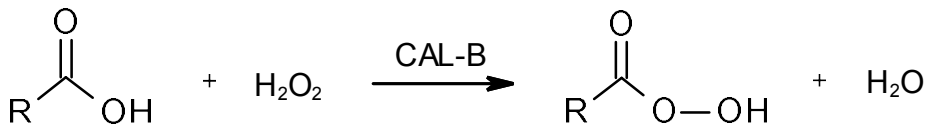
Figure 6. The schematic five methods for immobilization of enzymes to support materials. Adapted from *EASY BIOLOGY CLASS*, Retrieved from http://www.easybiologyclass.com/enzyme-cell-immobilization-techniques.

1.1.2.1. Hydroxyapatite

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is a calcium phosphate ceramic and a major component of human bones and teeth. It has a hexagonal crystal structure and with a stoichiometric Ca/P ratio of 1.67 [22]. In addition, there are several advantages of using hydroxyapatite: biocompatibility, non-toxicity, osteoconductivity, eco-friendly materials and affordable price. Hydroxyapatite can be easily synthesized *in vitro* from calcium and phosphate ions by sol-gel method, wet chemical precipitation methods, biomimetic deposition and electrodeposition [23]. Hydroxyapatite has been studied in many medical applications such as dental implants because it is a very good substitute of bones. In pharmaceutics, it has been used as supporting material in drug-delivery system due to its inherent properties [24, 25].

1.2. Perhydrolase activity

Perhydrolysis is a reaction for the formation of peroxy acid from carboxylic acids and hydrogen peroxide (Scheme 1). Perhydrolysis occurs through the reaction mechanism catalyzed by esterase that a carboxylic acid reacts with serine of active site to form an acyl enzyme intermediate. Then, the hydrogen peroxide reacts with the intermediate forming a peroxy acid. *Candida antarctica* lipase B (CAL-B) has been known to catalyze the perhydrolysis of carboxylic acid more effectively among lipases [26].



Scheme 1. Lipase-mediated perhydrolysis of carboxylic acid with hydrogen peroxide. The carboxylic acid reacts with serine group of active site in CAL-B to form the acyl enzyme. Then peroxy acid was formed by the hydrogen peroxide releasing the water molecules.

1.3. Outline of this thesis

This thesis deals with two studies including immobilization of lipase on hydroxyapatite and enhancing the perhydrolase activity of CAL-B. In chapter 2, several lipases such as CAL-B, BCL, and CRL were covalently immobilized on hydroxyapatite. For efficient immobilization of lipase, specific compound was introduced as a linker between hydroxyapatite and lipases. Also, the three peptide coupling reagents (DCC, DIC and EDC) were used when immobilizing lipase on linker-conjugated hydroxyapatite compounds. The specific activity of immobilized lipases are measured in transesterification toward 1-phenylethanol. The specific activity of immobilized CAL-B was higher than free-form lipase by a factor of ~100. It was verified that the crystallinities of hydroxyapatite were not altered during immobilizing process by SEM, XRD, and IR analysis. Chapter 3 describes that the perhydrolase activity was improved by modification of enzyme. It was assumed that a hydrogen peroxide, which is larger than water molecule, can enter a water channel in CAL-B. If the residue composed of this channel was modified by different amino acids, the perhydrolase activity was potentially affected. As a result, Ala281 residue was chosen as a target mutant. Several mutations of Ala281 residue showed higher perhydrolase activity than wild-type enzyme up to 2-fold. Also, selected mutants was performed epoxidation of styrene. According to comparison of their conversion, the conversion of Ala281Thr was higher than that of wild-type enzyme.

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Chapter 2. Enhancing catalytic activity of lipases in organic solvents by immobilization on hydroxyapatite

Abstract : Hydroxyapatite (HA) has been used for immobilization of enzymes as supporting materials. After hydroxyapatite was activated by carbodiimide activation reagents, lipase B from *Candida antarctica* (CAL-B), lipase from *Burkholderia cepacia* (BCL) and lipase from *Candida rugosa* (CRL) were covalently immobilized on hydroxyapatite. However, only small amount of lipase has been bound on hydroxyapatite. Presumably, the hydrophobic surface of lipase unfavorably interacts with the polar surface of hydroxyapatite. Thus, we introduced 6-aminohexanoic acid as a linker during immobilization process in order to maintain a reasonable distance between hydroxyapatite and lipases. The crystallinities of lipase-conjugated hydroxyapatite were not altered during immobilization process. The specific activities for the reaction of (±)-1-phenylethanol with vinyl butyrate are up to 100-fold higher than those of free enzyme. Also, the lipase-conjugated hydroxyapatite can be reused, and the activities were retained more than 85% except for CRL after ten-time recycling.

Introduction

Hydroxyapatite is a major component of biological bone systems, and thus considered as a completely biocompatible and environmentally benign material [1]. Hydroxyapatite is chemically composed of calcium ions, phosphates, and hydroxides to form $Ca_5(PO_4)_3(OH)$, and can be prepared by a simple procedure *in vitro*. Many proteins bind to hydroxyapatite during bone growth, and stronger binding of proteins is required in many bio-applications [2]. Enzymes can be immobilized to different carriers by cross linking, entrapment, adsorption, and covalent or ionic binding. Among these methods, covalent binding have several advantages. The strength of covalent binding is so strong that no detachment occurs from the support. The formation of covalent bond reduces conformational flexibility, thus enzyme are rarely denaturated. In addition, immobilized enzyme can be used in any medium [3].

Generally the surface of hydroxyapatite is polar, whereas that of lipases is non-polar. For that reason, the studies about the immobilization of lipase on hydroxyapatite are rarely reported. Thus, it was needed the introduction of a specific compound as a linker during immobilization process to decrease the repulsive force between hydroxyapatite and lipase. The linker compound is required to provide appropriate space between lipase and hydroxyapatite [4]. Herein, we report a facile covalent immobilization of lipase on hydroxyapatite with introduction of a linker compound, and an application to improve the activity of lipase in organic solvent.

Results and Discussion

Immobilization of enhanced green fluorescent protein (EGFP) on hydroxyapatite and selection of proper activating reagents

Enhanced green fluorescent protein (EGFP) is widely used since it can be easily tracked by its fluorescent property. The EGFP was expressed, purified and analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) as shown Fig. 1.

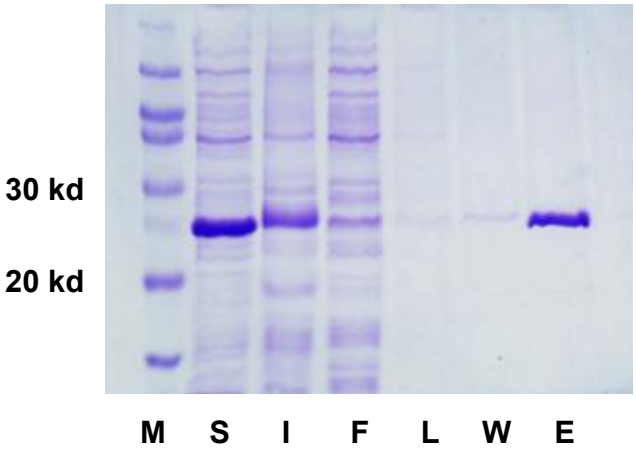
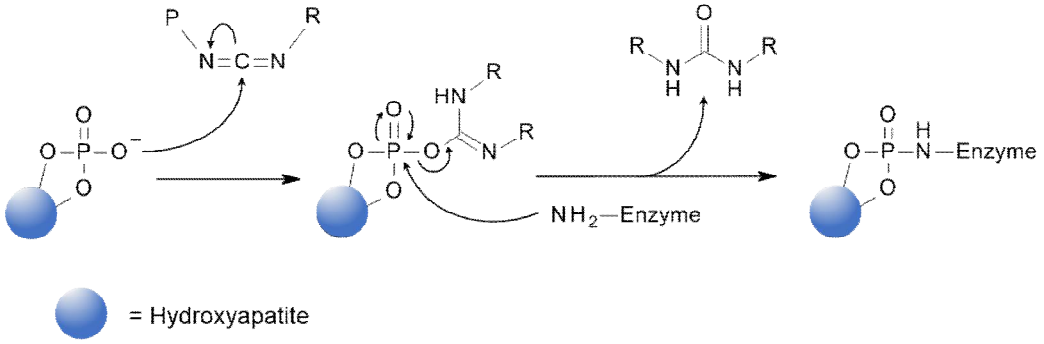


Figure 1. SDS-PAGE analysis of EGFP. SDS-PAGE was performed on a 12% polyacrylamide gel and stained using the Coomassie brilliant blue. M, molecular weight marker; S, soluble fraction; I, insoluble fraction; F, flow through fraction; L, lysis buffer fraction; W, wash buffer fraction; E, elution buffer fraction.

First, the EGFP was immobilized on hydroxyapatite by using three peptide coupling reagents such as *N,N'*-dicyclohexylcarbodiimide (DCC), *N,N'*-diisopropyl

carbodiimide (DIC), and *N*−ethyl−*N*′−(3−dimethylaminopropyl) carbodiimide (EDC). The phosphate groups unoccupied by calcium ions of hydroxyapatite was activated by coupling reagents, and this can be used for formation of a phosphoamide bond between a phosphate and amino groups of protein. The mechanism of activation and immobilization procedure is shown in Scheme 1. The deprotonated carbonyl oxygen of hydroxyapatite attacks carbon of coupling reagent, forming the covalent bond. The amine group of protein is binded to phosphorus atom, and the peptide bond between enzyme and hydroxyapatite is formed releasing urea derivatives [5, 6].



Scheme 1. Mechanism of activation by carbodiimide coupling reagents.

Although most of the proteins were conjugated well by each coupling reagent, the amount of binding EGFP was highest in the case of EDC used (Table 1). Among the peptide coupling reagents, DIC was determined to use in immobilization process due to its low

cost in comparison with EDC. The EGFP-conjugated hydroxyapatite was then examined by a fluorescent microscope (Figure 2). The hydroxyapatite which was immobilized with EGFP was seen fluorescent green color.

Table 1. The amount of binding EGFP on hydroxyapatite directly using peptide coupling reagents.

peptide coupling reagents	amount of binding enzyme ($mg \cdot g^{-1}$)	efficiency (%)
DCC	6.88	76
DIC	8.22	91
EDC	8.62	95

[a]The concentration of EGFP : 1.81 mg/ml

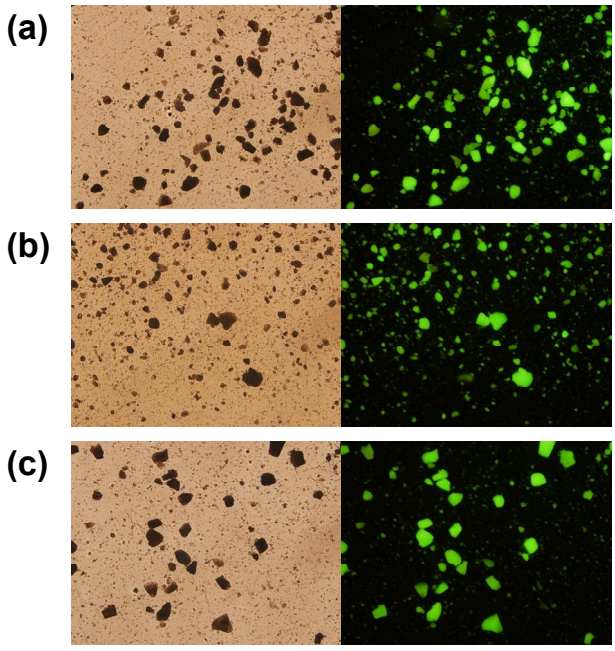


Figure 2. Optical and fluorescence microscopic images of immobilized samples activated by (a) DCC, (b) DIC and (c) EDC.

Conjugation of CRL on hydroxyapatite with 6-aminohexanoic acid as a linker

Lipase from *Candida rugosa* (CRL) was chosen as a model lipase because CRL is an inexpensive enzyme and one of the most commonly used enzymes for industrial use [7]. First, CRL was directly conjugated with three peptide coupling reagents. However, only small amount of CRL was conjugated unlike the case of EGFP (Table 2). The hydrophobic surface of CRL probably does not interact well with the surface of hydroxyapatite (HA). Next, CRL (10 mL, 0.83 mg/ml) was immobilized on HA and HA-(6-AmHAc) by using DCC, DIC and EDC. The amount of CRL on HA-(6-AmHAc) increased ten times compared to that of CRL on HA when using EDC. From these result, it was determined to use EDC during immobilization process between lipase and HA. Thus, the overall immobilization process is shown in Scheme 2.

6-aminohexanoic acid (6-AmHAc) as a linker compound was introduced between lipase and HA. To investigate whether or not the immobilization of 6-AmHAc on HA was made, ^{1}H NMR was conducted from D_2O solutions with DCl (20% w/w in D_2O, 99.5%). It was confirmed that 6-AmHAc was conjugated on HA by ^{1}H NMR analysis (Figure 3). There are a few difference of ppm value due to DCl.

Table 2. The amount of binding CRL when using three peptide coupling reagents.

	peptide coupling reagents	amount of binding enzyme (mg g^{-1})
HA-CRL	DCC	0.31
	DIC	0.26
	EDC	0.48
HA-(6-AmHAc)-CRL	DCC	1.70
	DIC	2.51
	EDC	2.76

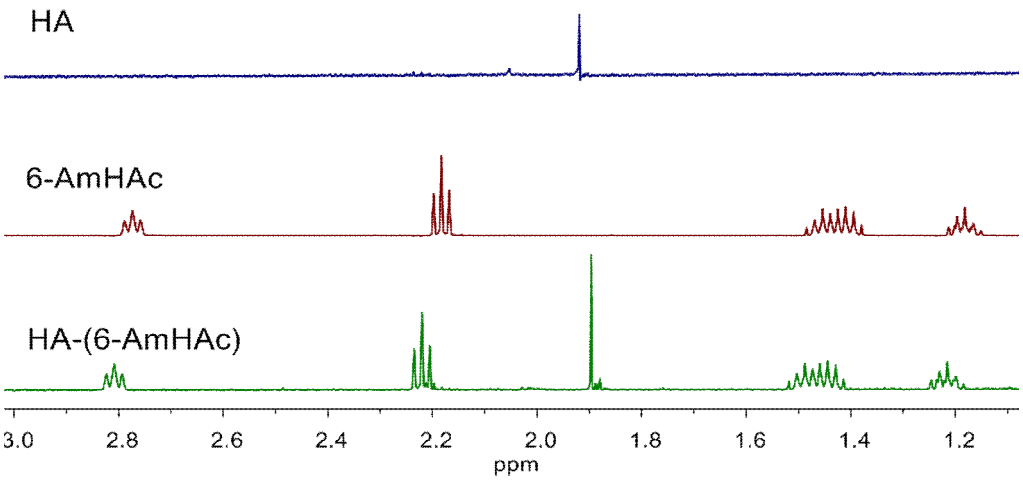
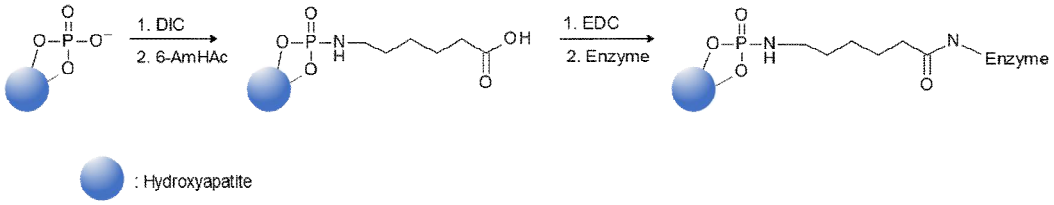


Figure 3. ^{1}H NMR spectra of the conjugation of 6-AmHAc on hydroxyapatite (HA) compared to HA and 6-AmHAc.



Scheme 2. The overall immobilization process.

Structural analyses of hydroxyapatite

The SEM images showed the analyses of commercial hydroxyapatite and lipase-conjugated hydroxyapatite. The analyses indicated that the morphology of hydroxyapatite particles was not altered and the diameter was about 50-100 nm. The morphology of immobilized lipase on hydroxyapatite using DIC (Figure 4) and EDC (Figure 5) was similar compared to commercial hydroxyapatite.

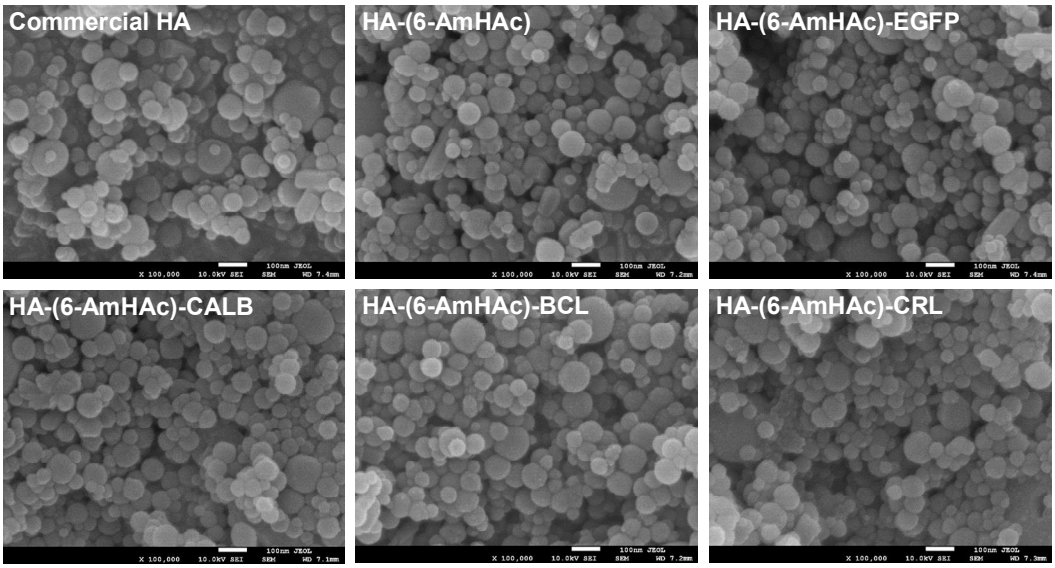


Figure 4. SEM images of commercial and lipase-conjugated HA with 6-AmHAc using DIC. The scale bar = 100 nm.

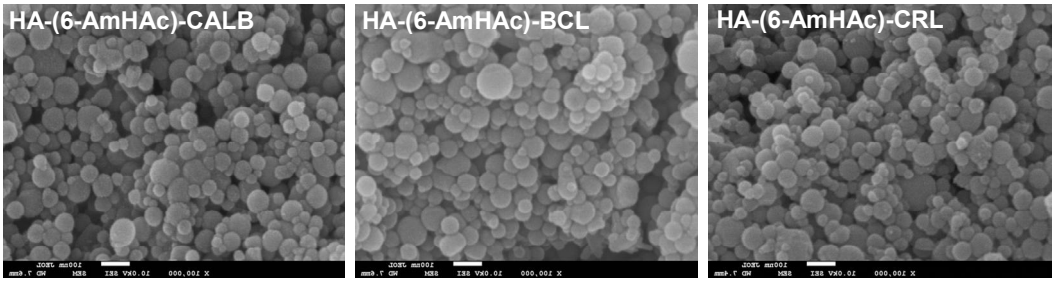


Figure 5. SEM images of commercial and lipase-conjugated HA with 6-AmHAc using EDC. The scale bar = 100 nm.

The X-ray diffraction (XRD) analyses are shown in Fig. 6. XRD spectra were obtained from 5 to 90° of 2θ at 1 sec/step. The major peaks at 26° , 29° , 32° -34° , 40° of 2θ displayed similar patterns from commercial hydroxyapatite to CRL-immobilized hydroxyapatite. These peaks are also matched with XRD data of previously reported crystalline hydroxyapatite [8].

The IR spectra of the lipase-conjugated hydroxyapatite are shown in the Fig. 7. They all have similar patterns through all frequencies. Major peaks of phosphate group (PO_4^{3-}) were observed at 1020 cm^{-1}, 600 cm^{-1} and 560 cm^{-1}. A medium size peak at 1080 cm^{-1} and 960 cm^{-1} are also the vibrations of PO_4^{3-} groups. Also, a small size peak showed vibrations of PO_4^{3-} groups at 470 cm^{-1}. The absorption band at 1530 cm^{-1} indicated carboxylate group (COO^-) attributed from 6-AmHAc in the spectrum of HA-(6-AmHAc) [2, 9].

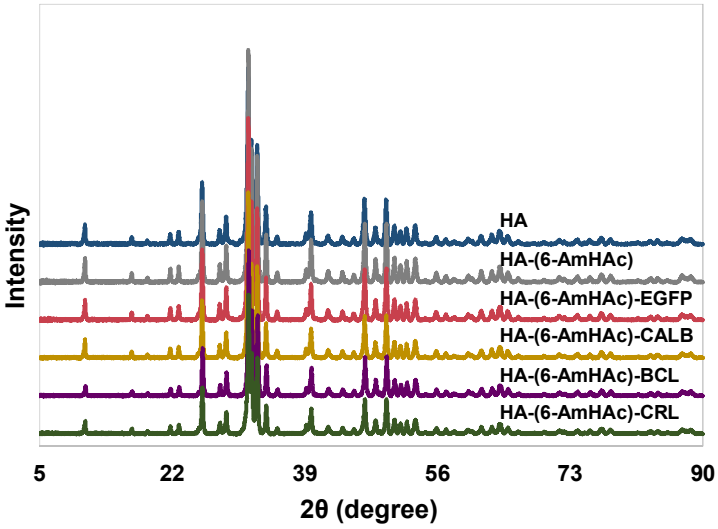


Figure 6. XRD analysis of commercial HA and lipase-conjugated HA. Their crystallinities were not altered during immobilizing process.

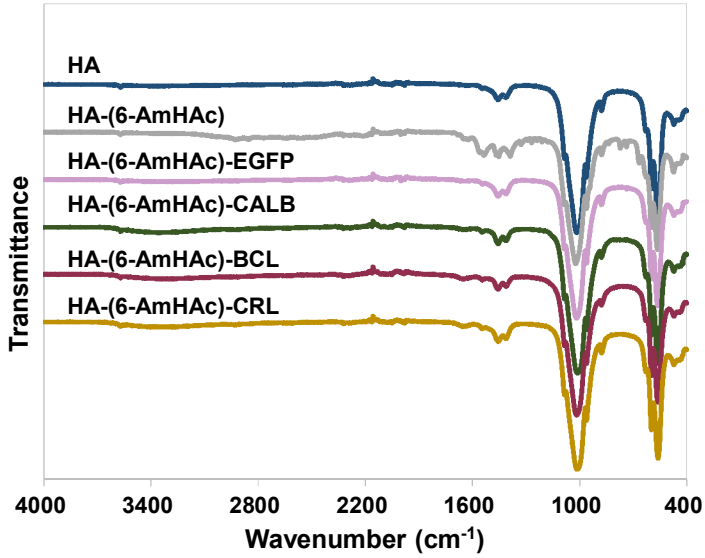
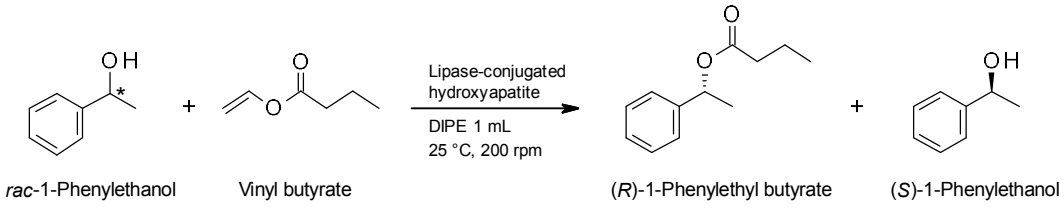


Figure 7. IR spectrum of comparison to commercial HA and lipase-conjugated HA according to a type of lipases.

Measurement of specific activity and enantioselectivity of lipase-immobilized on hydroxyapatite

To evaluate the catalytic activity of immobilized enzymes (CAL-B, BCL and CRL), we compared the activity in transesterification of (±)-1-phenylethanol with vinyl butyrate (Scheme 3). The specific activity of free enzymes was compared with those of the immobilized enzymes [10, 11].



Scheme 3. Transesterification toward (*R*)-1-phenylethanol with vinyl butyrate from racemate. DIPE = Diisopropyl ether.

All enzyme forms exhibited high enantioselectivity (E ≥ 200) toward (*R*)-1-phenylethanol. The specific activity of the immobilized CAL-B is 100-fold higher compared to that of the free form of CAL-B. Likewise, the specific activity of immobilized BCL and CRL is about 80- and 50-fold higher than those of free enzymes, respectively (Table 3).

Table 3. Specific activity and enantiomeric ratio (E) of immobilized enzyme compared to free enzyme.

Enzyme	specific activity (μmol min^{-1} mg^{-1})	E
Free CAL-B	1.41 ± 0.26	>200
HA-(6-AmHAc)-CAL-B	(1.94 ± 0.28) × 10^2	>200
Free BCL	0.54 ± 0.15	>200
HA-(6-AmHAc)-BCL	(4.65 ± 0.41) × 10^1	>200
Free CRL	0.30 ± 0.12	>200
HA-(6-AmHAc)-CRL	(1.46 ± 0.14) × 10^1	>200

[a]The amount of immobilized lipase : CAL-B (4.50 mg/g), BCL (1.46 mg/g), CRL (1.43 mg/g)

The product of transesterification was analyzed by gas chromatography (GC). GC chromatogram are shown in Fig. 8. To obtain the initial reaction rate, the reactions should finished before its conversion reached 5%. The reactions were carried out three times to confirm their reproducibility.

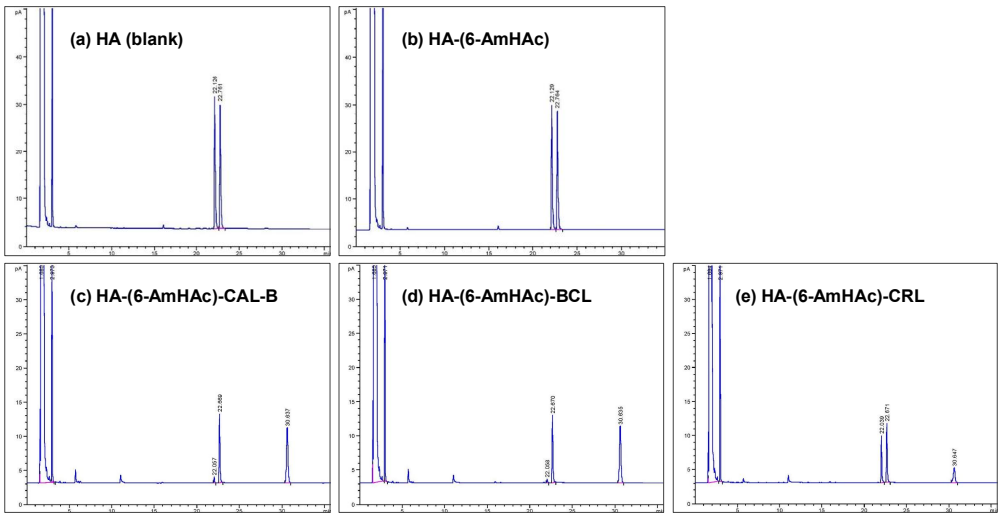


Figure 8. GC chromatogram of transesterification for lipase-conjugated HA after 24 h reaction.

Recycling of lipase-conjugated hydroxyapatite

Recycling experiments was performed by ten times re-use of each lipase-conjugated hydroxyapatite. As shown in the Fig. 9, the relative conversion of CAL-B- and BCL-conjugated hydroxyapatite was maintained above 90% conversion after ten-time recycling, while the conversion of CRL-conjugated hydroxyapatite decreased gradually to 80%. The enantioselectivity of three immobilized lipases was maintained ($E \geq 200$).

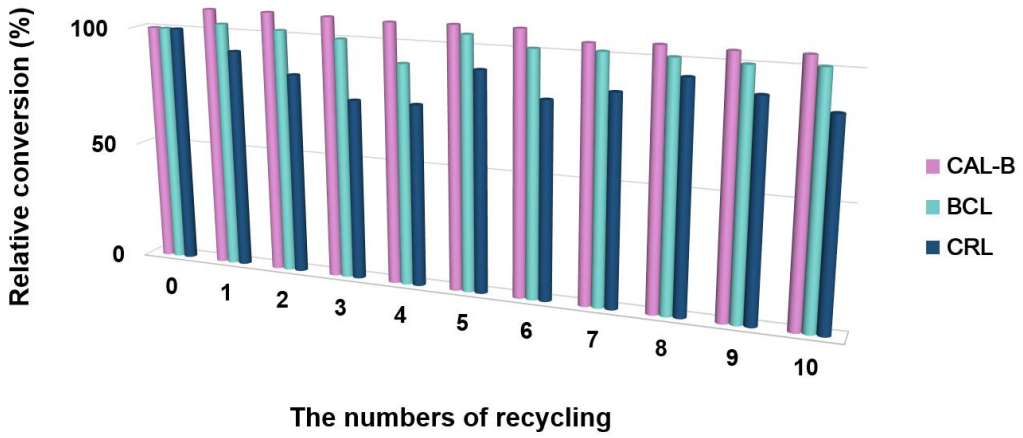


Figure 9. Recycling of the lipase-conjugated hydroxyapatite.

Experimental Section

General Methods

Chemicals were purchased from Acros or Sigma-Aldrich. Lipase from *Burkholderia cepacia* (Lipase PS) was obtained from Amano Enzyme Inc. (Japan). Lipase B from *Candida antarctica* was purchased from Novozymes. Lipase from *Candida rugosa* was purchased from Sigma-Aldrich. Gas Chromatography was analyzed by Agilent 6890N with a chiral capillary column (Cyclosil-B 30 m × 0.25 mm). The FE-SEM images were obtained by JSM-7500F (Jeol, Japan).

Expression and purification of EGFP

To 100 mL of LB medium, *E. coli* overnight culture (1 mL) and ampicillin (100 μL) were added. The LB medium was

incubated at 37 °C and 200 rpm until OD_{600} reached to 0.5. Then, addition of IPTG (100 mM, 1 mL) led to protein expression, and the expression culture was incubated at 20 °C and 200 rpm overnight. The cells were harvested by centrifugation (4 °C, 20 min, 10,000 rpm) and the supernatant was discarded. The cell pellet was resuspended in the lysis buffer (3 mL, NaH_2PO_4, 50 mM; NaCl, 300 mM; imidazole, 10 mM; pH 8.0 adjusted with NaOH) and then a sonication (30 kHz, 6 times of 10 sec pulse with 10 sec interval) was applied. The cells were frozen and then thawed. The solution was sheared by passing the lysates through a needle repeatedly. After centrifugation (4 °C, 10 min, 10,000 rpm), the cell pellet and the supernatant (3 mL) was separated. The supernatant showed green fluorescence color.

The expressed EGFP was purified by affinity chromatography as follows. First, Ni-NTA agarose resin (1 mL, 50% w/v slurry, QIAGEN Inc.) was added to the supernatant and the mixture was stirred at 4 °C for 1 h. The mixture was loaded on a Poly-Prep column (Bio-Rad) and drained. Then the mixture was washed twice with the lysis buffer (5 mL) and three times with the wash buffer (5 mL, NaH_2PO_4, 50 mM; NaCl, 300 mM; imidazole, 20 mM; pH 8.0 adjusted with NaOH). The His_6-tag protein was eluted three times with the elution buffer (1 mL, NaH_2PO_4, 50 mM; NaCl, 300 mM; imidazole, 250 mM; pH 8.0 adjusted with NaOH).

Immobilization of lipase on hydroxyapatite using DIC or DCC

Five hundred milligrams of hydroxyapatite (HA) was activated by DIC (500 μL) in dichloromethane (5 mL) at 25 °C for 1 h with shaking. The activated compounds were washed three times with dichloromethane (5 mL). After addition of 6-aminohexanoic acid (6-AmHAc, 500 mg) and dichloromethane (5 mL), the suspension was shaken at 25 °C for 24 h. And then, the mixture was washed three times with dichloromethane (5 mL).

The HA-(6-AmHAc) was activated then by DIC or DCC. DIC (0.5 mmol) in dichloromethane (10 mL) were mixed with the HA-(6-AmHAc), and the mixture was shaken for 20 min at 25 °C. DCC (0.5 mmol) and 1,6-hexanediamine (100 mg, 1% w/v) was added to the mixture in dichloromethane (10 mL). Then, the mixture was incubated at 10 °C for 4 h. The activated HA-(6-AmHAc) was washed three times by dichloromethane (5 mL), acetone (5 mL), and water (5 mL) in sequence. An enzyme solution was added to the mixture and then incubated at 4 °C for 24 h. The measurement of amount of binding enzyme was conducted by microplate reader (Molecular Devices, SpectraMax 190) with initial and final concentration of enzyme.

Immobilization of lipase on hydroxyapatite using EDC

Five hundred milligrams of hydroxyapatite (HA) was activated by DIC (500 μL) in dichloromethane (5 mL) at 25 °C for 1 h with shaking. The activated compounds were washed three times

with dichloromethane (5 mL). After addition of 6-AmHAc (500 mg) and dichloromethane (5 mL), the suspension was shaken at 25 °C for 24 h. And then, the mixture was washed three times with dichloromethane (5 mL).

The HA-(6-AmHAc) was activated by EDC. EDC (0.5 mmol) and MES buffer (10 mL, 100 mM, pH 5.0) were mixed with the HA-(6-AmHAc) and shaken for 20 min at 25 °C. The activated HA-(6-AmHAc) was washed three times by BES buffer (10 mL, 5 mM, pH 7.2). An enzyme solution was added to the mixture and then incubated at 4 °C for 24 h.

Determination of binding amounts of enzymes

The binding amount of lipase was determined by the Bradford assay [12]. The initial and final concentration of enzymes were determined by measuring at 595 nm after mixing with the dye solution (Bio-Rad).

Transesterification of (±)-1-phenylethanol with vinyl butyrate

Activity of the enzymes were measured in the transesterification of (±)-1-phenylethanol with vinyl butyrate. The immobilized BCL (50 mg), CRL (50 mg), or CAL-B (5 mg) was mixed with (±)-1-phenylethanol (0.05 mmol) and vinyl butyrate (0.15 mmol) in diisopropyl ether (DIPE, 1 mL). The reaction mixture was shaken at 25 °C and 200 rpm. The samples (20 μL) were retrieved with 5 or 10 min intervals for 60 min and analyzed

by gas chromatography (GC): Initial column temperature started 80 °C for 5 min and ramped up to 120 °C at a rate of 2.5 °C/min. Then, it lasted at 120 °C for 15 min. (*R*)-phenylethanol and (*S*)-phenylethanol were detected at 22.0 and 22.6 min, respectively. Also, (*R*)-phenylethyl butanoate and (*S*)-phenylethyl butanoate were detected at 30.6 and 31.8 min, respectively.

Recycling of lipase-conjugated hydroxyapatite with (±)-1-phenylethanol in transesterification

In 1.5 mL eppendorf tube, (±)-1-phenylethanol (0.05 mmol) and vinyl butyrate (0.15 mmol) were added to diisopropyl ether (1 mL) with lipase-conjugated hydroxyapatite (50 mg) or CAL-B (5 mg). The reaction was incubated at 25 °C for 24 h. After the first reaction finished, lipase-conjugated hydroxyapatite was washed three times with diisopropyl ether (1 mL). Then, the washed lipase-conjugated hydroxyapatite was added to a solution of (±)-1-phenylethanol (0.05 mmol) and vinyl butyrate (0.15 mmol) in diisopropyl ether (1 mL).

Scanning Electron Microscope (SEM)

The FE-SEM images were obtained by JSM-7500F (Jeol, Japan). Before the analyzing by SEM, powder samples were coated with platinum on a carbon tape. The accelerating voltage was operated at 10 kV and the emission current was adjusted to 16 μA.

X-ray diffraction (XRD)

XRD patterns were obtained by using a Bruker D8 Focus with an X-ray tube with a Cu target with operating at 40 kV and 40 mA.

Infrared spectroscopy (IR)

IR was obtained by using Thermo Scientific Nicolet iS50 FT-IR spectrometer. The investigation was performed in the wavenumber range from 400 cm^{-1} to 4000 cm^{-1} to obtain the functional groups of hydroxyapatite.

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Chapter 3. Enhancing perhydrolase activity of *Candida antarctica* lipase B by modification of a potential water channel

Abstract : Although *Candida antarctica* lipase B (CAL-B) is one of hydrolases, it is known that CAL-B can perform perhydrolysis. Also, the existence of a water channel in CAL-B was hypothesized recently. Thus, we expected that hydrogen peroxide, which is a reactant in perhydrolysis, can also enter to active site through the channel. The perhydrolase activity was enhanced by modifying the amino acid of the water channel. The Pro280 and Ala281 residues were selected as target residue. Whereas the Pro280 residue did not influence on he perhydrolase activity, the Ala281 residue affected both the hydrolase and perhydrolase activity. The hydrolase activities of Ala281Ser, Ala281Thr, and Ala281Leu were increased than that of wild-type enzyme up to 2-fold. The perhydrolase activities of them was 1.5-, 2-, and 1.7-fold higher compared to that of wild-type enzyme, respectively.

Introduction

Candida antarctica lipase B (CAL-B) is a widely used biocatalyst, and it has high enantioselectivity, thermostability and substrate specificity. CAL-B has been reported consistently its numerous applications [1]. Although CAL-B is one of hydrolases which catalyze hydrolysis and lipid, it is also known to catalyze the perhydrolysis of carboxylic acid and ester [2]. The perhydrolysis is the formation of peroxycarboxylic acid from carboxylic acid with hydrogen peroxide and perhydrolase (metal-free haloperoxidases) [3, 4]. The peroxycarboxylic acid is used oxidant for alkenes in epoxidation to form epoxide [5]. Epoxides are used as vital intermediate in pharmaceutical industries, thus it is regarded as a highlighted material [6].

Many enzymes have specific channels which allow a small substrates to enter the active site [7]. Recently, a water channel that water molecules can enter into the active site in CAL-B was newly hypothesized by Hult et al. [8]. We assumed that hydrogen peroxide can also enter to the channel, and the perhydrolase activity can be influenced by altering the size or polarity of the water channel. Several amino acids were introduced into that residues. A large hydrophobic amino acids would probably block the channel, and polar amino acids would interact with hydrogen peroxide more favorably. Furthermore, lipase-mediated epoxidation is eco-friendly method because enzymes are used to form epoxide. The epoxide is useful

compound generated by peroxycarboxylic acid with styrene as a alkene [9, 10]. We also carried out epoxidation to compare the conversion of mutant and wild-type CAL-B. In this work, we confirmed that the enhancing of the perhydrolase activity of the Ala281 mutants by modification of a water channel.

Results and Discussion

Selection of mutation sites

Recently, Hult and coworkers reported that a water channel exists in CAL-B aside from the entrance for a substrate. We performed molecular modeling to identify the channel (Figure 1). First, the residues which are composed of the water channel were identified: Pro280, Ala281, Thr40, Gly41, Thr42, and Trp104. Different sized or more polar amino acids were introduced to the residues. A large hydrophobic amino acid would block the channel, or a small and polar amino acid help the hydrogen peroxide easily enter to this channel. Thus, the Ala281 residue which affects the perhydrolase activity were chosen.

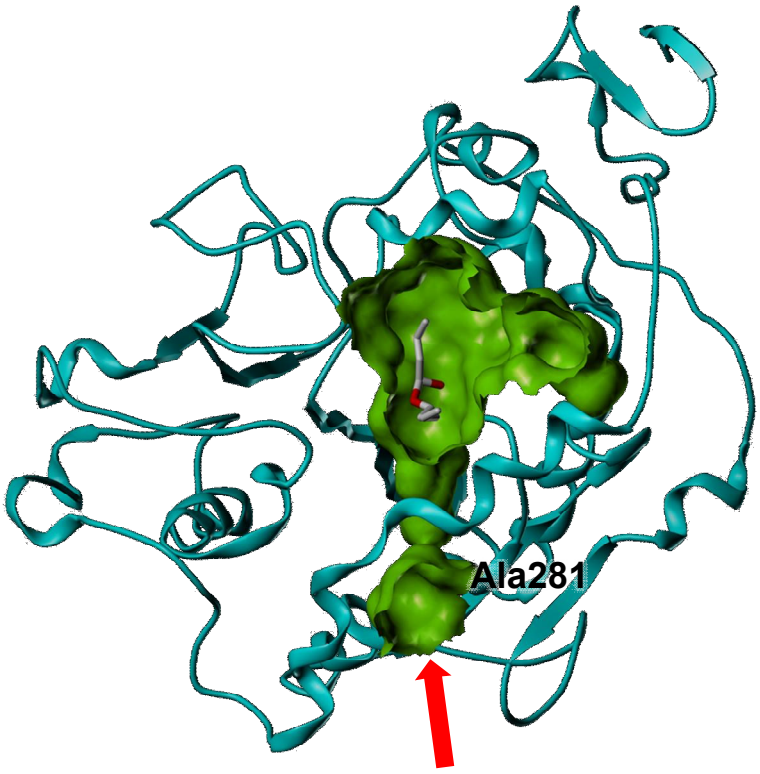
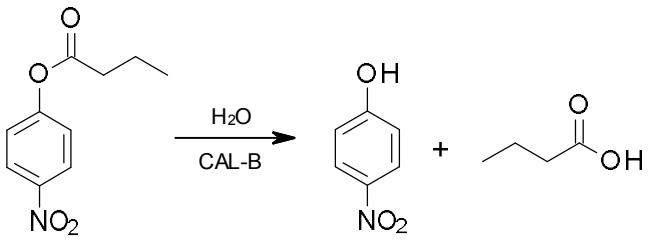


Figure 1. The expected access route of water and hydrogen peroxide to active site of CAL-B (red arrow).

Measurement of hydrolytic activity of mutant and wild-type CAL-B

To determine the effect of the mutation on the catalytic machinery of CAL-B, the specific activity of mutant enzyme was compared to that of wild-type enzyme. *p*-Nitrophenyl butyrate was chosen as a substrate to compare hydrolysis activity (Scheme 1) [11]. As shown in Table 1, the mutants at the Pro280 residue showed no significant increase in specific activity. However, the Ala281 residue was considered to effect hydrolytic activity in CAL-B. When the Ala281 mutant enzymes were substituted by hydrophilic amino acid such as aspartic acid (D) and glutamic acid (E), the specific activity was increased up to 3 compared to wild-type enzyme.



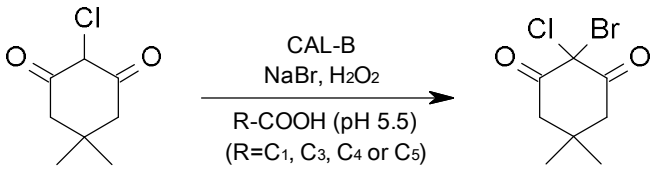
Scheme 1. Model reaction to compare hydrolytic activity.

Table 1. The specific activities of CAL-B mutants compared to that of wild-type in hydrolysis.

Entry	Enzyme	Specific activity ($\mu mol\ min^{-1}\ mg^{-1}$)	Relative activity (%)
1	wild-type	9.72×10^{1}	100
2	P280D	7.28×10^{1}	76
3	P280E	5.30×10^{1}	55
4	P280T	2.88×10^{1}	30
5	P280G	4.72×10^{1}	49
6	P280S	5.20×10^{1}	54
7	P280L	1.65×10^{1}	17
8	P280I	8.81×10^{0}	9
9	P280V	9.63×10^{0}	10
10	**A281D**	2.43×10^{2}	**250**
11	**A281E**	2.80×10^{2}	**289**
12	**A281M**	1.79×10^{2}	**184**
13	**A281S**	1.95×10^{2}	**201**
14	**A281T**	1.12×10^{2}	**115**
15	**A281L**	1.91×10^{2}	**197**
16	A281I	4.60×10^{1}	47
17	A281V	3.13×10^{1}	32
18	**A281K**	2.27×10^{2}	**233**
19	T40S	4.80×10^{1}	49
20	G41P	1.80×10^{0}	2
21	T42S	9.06×10^{1}	93
22	W104H	1.62×10^{1}	17

Modification of the monochlorodimedone assay

A typical assay for measuring the perhydrolase activity is the monochlorodimedone (MCD) assay, and acetic acid is used as a substrate in this assay. However, it was found that the poor binding of acetic acid with CAL-B was observed due to low sensitivity of the MCD assay. Thus, we investigated the effects of butyric acid, pentanoic acid, and hexanoic acid on the assay to find an appropriate substrate (Scheme 2). The sensitivity between carboxylic acid as a substrate and the active site of CAL-B was determined by slope of the initial rate. When the concentration of buffer was adjusted same, 1 M pentanoate buffer had the highest sensitivity with CAL-B (Fig. 2(a)). Thus, the pentanoate buffer was selected as a substrate for the MCD assay. The further studies were carried out to determine the effect of concentration level including 0.2, 0.4, 0.6, 0.8, and 1.0 M. Among these concentrations, 0.6 M pentanoate buffer was showed the highest sensitivity with CAL-B (Fig. 2(b)). Hence, 0.6 M pentanoate buffer was used as a substrate during the further MCD assay.



Scheme 2. The scheme of monochlorodimedone (MCD) assay.

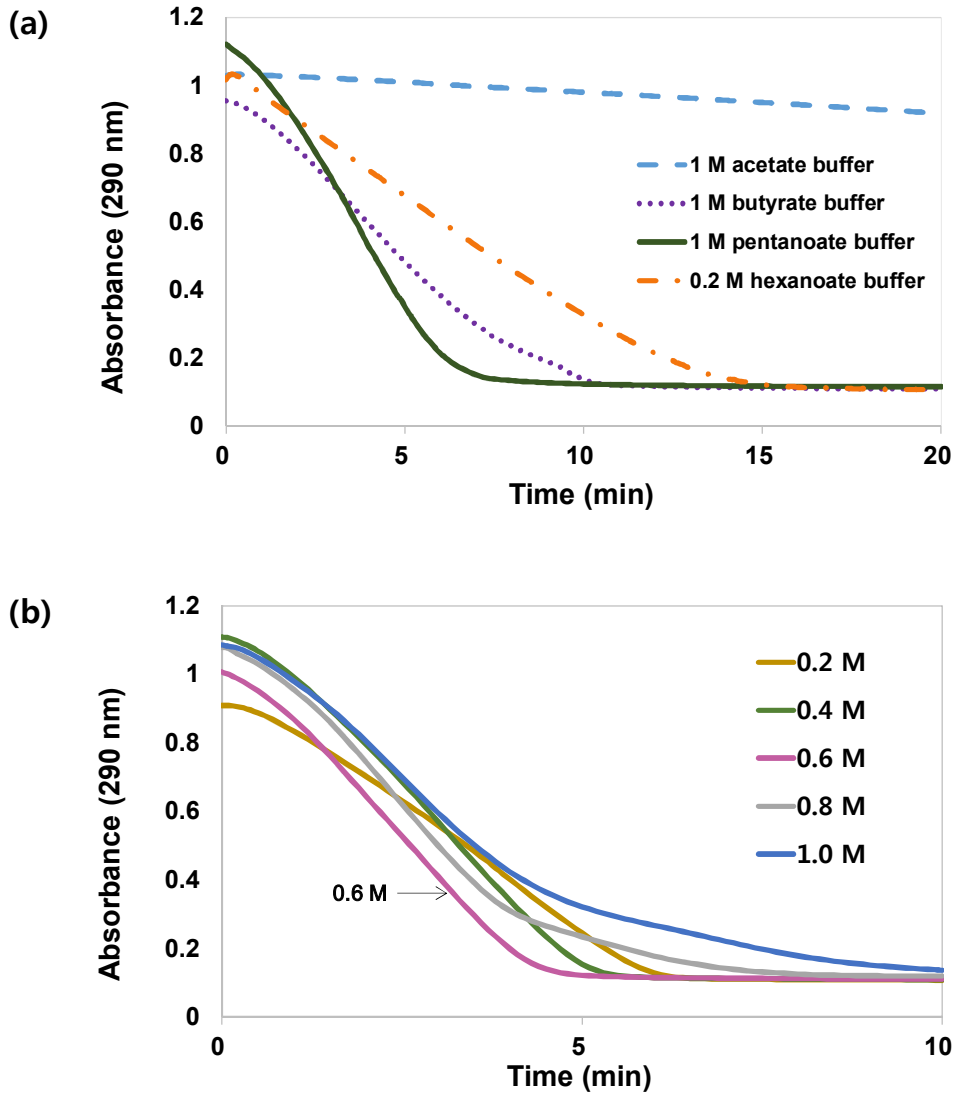


Figure 2. The comparison of MCD assay (a) by changing carboxylic acid, and (b) by varying concentration of pentanoate buffer.

Measurement of perhydrolase activity of CAL-B enzymes

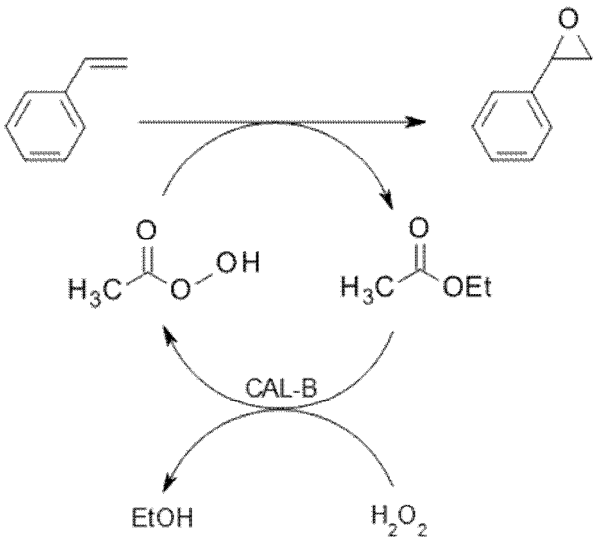
The perhydrolase activity was measured by the MCD assay [12]. To calculate the specific activities of the wild-type and mutation enzyme, the initial reaction rates were obtained from 10% of absorbance. Among all of mutations, Ala281Ser, Ala281Thr, and Ala281Leu had higher perhydrolase activities because the polar and hydrophilic amino acids reacted with hydrogen peroxide instead of hydrophobic alanine. They also had higher hydrolytic activities compared to wild-type enzyme. Although the hydrolytic activity of Ala281Glu was higher, the perhydrolase activity of that was lower than that of wild-type enzyme (Table 2).

Table 2. The specific activities of CAL-B mutants compared to that of wild-type in perhydrolysis.

Entry	Enzyme	Specific activity ($\mu mol\ min^{-1}\ mg^{-1}$)	Relative activity (%)
1	wild-type	1.05×10^2	100
2	P280D	6.44×10^1	62
3	P280E	6.18×10^1	59
4	P280T	6.76×10^1	65
5	P280G	6.52×10^1	62
6	P280S	5.82×10^1	56
7	P280L	3.73×10^1	36
8	P280I	7.04×10^1	67
9	P280V	6.02×10^1	58
10	**A281D**	1.32×10^2	**126**
11	A281E	8.84×10^1	85
12	**A281M**	1.32×10^2	**127**
13	**A281S**	1.63×10^2	**156**
14	**A281T**	2.21×10^2	**212**
15	**A281L**	1.77×10^2	**169**
16	A281I	1.19×10^2	113
17	**A281V**	1.54×10^2	**147**
18	A281K	1.01×10^2	96
19	T40S	5.66×10^1	54
20	G41P	2.40×10^1	23
21	T42S	1.16×10^2	111
22	W104H	9.27×10^1	89

Epoxidation of styrene by Ala281 mutants

From the MCD assay experiment, it was confirmed that the perhydrolase activity of Ala281Ser, Ala281Thr and Ala281Leu mutants are higher than wild-type enzyme. The wild-type and three mutant enzymes was conducted epoxidation of styrene as alkene substrate in ethyl acetate to form styrene oxide (Scheme 3). In previous study, ethyl acetate was used as a solvent and carboxylic acid substrate because it exhibited higher conversion than other solvent [13]. The conversions were measured with the help of hexadecane as an internal standard which does not involve in the reaction. The production of epoxidation was determined by GC [9, 14].



Scheme 3. Chemoenzymatic epoxidation toward styrene with hydrogen peroxide. Racemic styrene oxide products are formed.

The lipases are easily inactivated due to high concentration of hydrogen peroxide. Thus, the hydrogen peroxide was added gradually to the reaction mixture over several hours to avoid lipase deactivation and to obtain higher conversions. One molar equivalent hydrogen peroxide was added to the mixture at every 24-h interval for 7 days (Figure 3). The conversion of wild-type enzyme was showed with 23% after 48 h. The Ala281Thr and Ala281Leu mutants had higher conversion than wild-type enzyme after 48 h. However, the Ala281Ser mutant had the low conversion compared to wild-type enzyme.

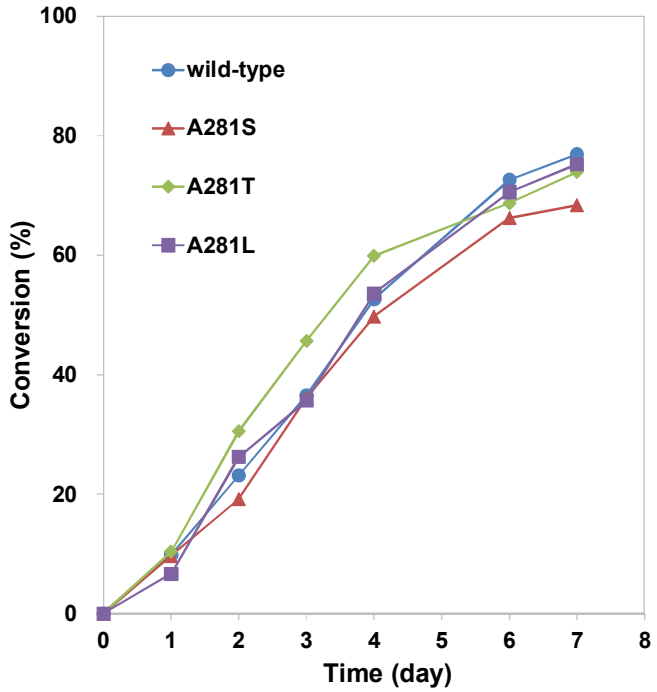


Figure 3. The comparison of conversion over reaction time.

It was expected that the perhydrolase activity of Ala281Leu would be lower than wild-type enzyme because large hydrophobic amino acids would block the water channel. However, we did not find out the reason why the activity of Ala281Leu mutant is higher than that of the wild-type enzyme. Most mutants usually have low stability than wild-type enzyme. Even if an initial rate of mutant enzyme is higher, the conversion might be slightly lowered as reaction time prolonged. Thus, further studies are needed from these results.

Experimental Section

General methods

Chemicals were purchased from Acros or Sigma-Aldrich. The vector (pBAD/gIIIa) was purchased from Invitrogen Korea (Seoul, Korea). DNA sequencing was conducted by Solgent Co. (Daejeon, Korea). The Ni-NTA agarose resin was purchased from QIAGEN. ^{1}H NMR spectrum was recorded from D_2O solutions on a Varian 500 MHz spectrometer.

Site directed mutagenesis

Using the opt2_5D CAL-B template [15], the mutant was created by overlap PCR method with the mutagenesis primers, as shown Table 3. The mutant gene was digested with *Nco* I and *Sal* I and inserted into pBADgIIIa. The plasmid of the mutant was

transformed into *E. coli* (Top10) [16].

Table 3. Primers for mutagenesis.

T40, G41, T42, W104 residue	
F_5D-CALB_T40S	5'-TACCGGGCAGCGGTACCACTGGCC-3'
R_5D-CALB_T40S	5'-TACCGCTGCCCGGTACCAGCAG-3'
F_5D-CALB_G41P	5'-TACCGGGCACCCCGACCACTGG-3'
R_5D-CALB_G41P	5'-AGTGGTCGGGGTGCCCGGTACCAG-3'
F_5D-CALB_T42S	5'-GCACCGGTAGCACTGGCCCGCAGTCT-3'
R_5D-CALB_T42S	5'-GCCAGTGCTACCGGTGCCCGGTAC-3'
F_5D-CALB_W104H	5'-CTGCCAGTGCTGACCCATTCCCAGGGTGGTCTG-3'
R_5D-CALB_W104H	5'-CAGACCACCCTGGGAATGGGTCAGCACTGGCAG-3'

P280 residue	
F_5D-CALB_P280D	5'-GCGGCTCTGCTGGCAGATGCTGCAGCTGCAATT-3'
R_5D-CALB_P280D	5'-CAGCATCTGCCAGCAGAGCCGC-3'
F_5D-CALB_P280E	5'-GCGGCTCTGCTGGCAGAAGCTGCAGCTGCAATT-3'
R_5D-CALB_P280E	5'-CAGCTTCTGCCAGCAGAGCCGC-3'
F_5D-CALB_P280T	5'-GCGGCTCTGCTGGCAACCGCTGCAGCTGCAATT-3'
R_5D-CALB_P280T	5'-CAGCGGTTGCCAGCAGAGCCGC-3'
F_5D-CALB_P280G	5'-GCGGCTCTGCTGGCAGGCGCTGCAGCTGCAATT-3'
R_5D-CALB_P280G	5'-CAGCGCCTGCCAGCAGAGCCGC-3'
F_5D-CALB_P280S	5'-GCGGCTCTGCTGGCAAGCGCTGCAGCTGCAATT-3'
R_5D-CALB_P280S	5'-CAGCGCTTGCCAGCAGAGCCGC-3'
F_5D-CALB_P280L	5'-GCGGCTCTGCTGGCACTGGCTGCAGCTGCAATT-3'
R_5D-CALB_P280L	5'-CAGCCAGTGCCAGCAGAGCCGC-3'
F_5D-CALB_P280I	5'-GCGGCTCTGCTGGCAATTGCTGCAGCTGCAATT-3'
R_5D-CALB_P280I	5'-CAGCAATTGCCAGCAGAGCCGC-3'
F_5D-CALB_P280V	5'-GCGGCTCTGCTGGCAGTGGCTGCAGCTGCAATT-3'
R_5D-CALB_P280V	5'-CAGCCACTGCCAGCAGAGCCGC-3'

Table 3 continued.

A281 residue	
F_5D-CALB_A281D	5'-GCGGCTCTGCTGGCACCGGATGCAGCTGCAATTGTT-3'
R_5D-CALB_A281D	5'-AACAATTGCAGCTGCATCCGGTGCCAGCAGAGCCGC-3'
F_5D-CALB_A281E	5'-GCGGCTCTGCTGGCACCGGAAGCAGCTGCAATTGTT-3'
R_5D-CALB_A281E	5'-AACAATTGCAGCTGCTTCCGGTGCCAGCAGAGCCGC-3'
F_5D-CALB_A281M	5'-GCGGCTCTGCTGGCACCGATGGCAGCTGCAATTGTT-3'
R_5D-CALB_A281M	5'-AACAATTGCAGCTGCCATCGGTGCCAGCAGAGCCGC-3'
F_5D-CALB_A281S	5'-CTGGCACCGAGCGCAGCTGCAATTGT-3'
R_5D-CALB_A281S	5'-CTGCGCTCGGTGCCAGCAGAGCC-3'
F_5D-CALB_A281T	5'-GCTCTGCTGGCACCGACCGCAGCTGCAATTGTT-3'
R_5D-CALB_A281T	5'-CTGCGGTCGGTGCCAGCAGAGCC-3'
F_5D-CALB_A281L	5'-GCTCTGCTGGCACCGCTGGCAGCTGCAATTGTTGCG-3'
R_5D-CALB_A281L	5'-CGCAACAATTGCAGCTGCCAGCGGTGCCAGCAGAGC-3'
F_5D-CALB_A281I	5'-GCTCTGCTGGCACCGATTGCAGCTGCAATTGTT-3'
R_5D-CALB_A281I	5'-CTGCAATCGGTGCCAGCAGAGCC-3'
F_5D-CALB_A281V	5'-GCTCTGCTGGCACCGGTGGCAGCTGCAATTGTT-3'
R_5D-CALB_A281V	5'-CTGCCACCGGTGCCAGCAGAGCC-3'
F_5D-CALB_A281K	5'-GCTCTGCTGGCACCGAAAGCAGCTGCAATTGTTGCG-3'
R_5D-CALB_A281K	5'-CGCAACAATTGCAGCTGCTTTCGGTGCCAGCAGAGC-3'

Protein expression and purification of CAL-B

The protein expression and purification method are referred by Blank et al. [11].

Determination of th amount of the enzymes

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

Measurement of hydrolytic activity of mutant and wild-type CAL-B toward *p*-nitrophenol butyrate

To make an assay solution for hydrolysis, acetonitrile (MeCN, 870 μL) and *p*-nitrophenyl butyrate (*p*-NPBu, 20 μL, 200 mM in MeCN) were added to BES buffer (11,110 μL, 5 mM, pH 7.2). After mixing the enzyme solution (5 μL) with assay solution (100 μL), the hydrolysis was measured for 5 min at 404 nm using microplate reader (Molecular Devices, SpectraMax 190). The activity was calculated according to the method of Janes et al. [17] where $\Delta\varepsilon$ = 18,000 $M^{-1}cm^{-1}$.

Assay conditions for measurement of the perhydrolase activity

Enzyme assay for the perhydrolase activity were conducted by monochlorodimedone (MCD) changing carbon length of carboxylic acid which used as buffer. The MCD has been used to detect peroxy acid at 290 nm. The MCD assay solution contained MCD (0.18 mM), 30% wt H_2O_2 (90 mM), NaBr (90 mM in distilled water) in acetate, butyrate, pentanoate and hexanoate buffer (pH 5.5). The total reaction volume is 100 μL of assay solution (95 μL) and enzyme solution (5 μL), so the perhydrolysis was observed at 290 nm in microplate reader. The graphs of the perhydrolase activity were monitored decline tendency due to depletion of the substrate (MCD). The activity was calculated according to the method of Bernhardt et al. [12] where $\Delta\varepsilon$ = 19,900 $M^{-1}cm^{-1}$.

Immobilization of enzymes on celite by adsorption

Enzyme (2 mg) and sucrose (60 mg) were added to a suspension of celite (1 g) in BES buffer (5 mL, 5 mM, pH 7.2). The solution was incubated and shaken at 25 °C and 200 rpm for 10 min. Then, the suspension was poured onto a weighing dish and dried under air with stirring.

CAL-B-mediated epoxidation by hydrogen peroxide in ethyl acetate

Styrene (0.05 mmol), 30% aq. H_2O_2 (0.30 mmol), hexadecane (5 μL) as internal standard and ethyl acetate (1 mL) were mixed with immobilized CAL-B (100 mg) in 4-mL vial. The reaction mixture was shaken at 25 °C and 200 rpm. The 30% wt hydrogen peroxide was added in 6 portions with 24-h interval. The samples (50 μL) of the reaction mixture were added to *tert*-Butyl methyl ether (MTBE, 1.2 mL) containing sodium sulfite (50 mg). After centrifuged, the supernatant was filtered and collected in a 2-mL vial. The products were analyzed by gas chromatography. GC conditions : initial column temperature started 40 °C for 5 min, ramped up to 120 °C at a rate of 1 °C/min and then held at 120 °C for 5 min. Next, ramp up to 170 °C at a rate of 3 °C/min and lasted 170 °C for 1 min. To obtain conversion value, the areas of the starting material and internal standard peaks were compared. The starting material was detected at 19.3 min. (*R*)-styrene oxide and (*S*)-styrene oxide were detected at 48.6 and 49.7 min, respectively. Also, the internal standard was shown at 99.9 min.

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Appendix

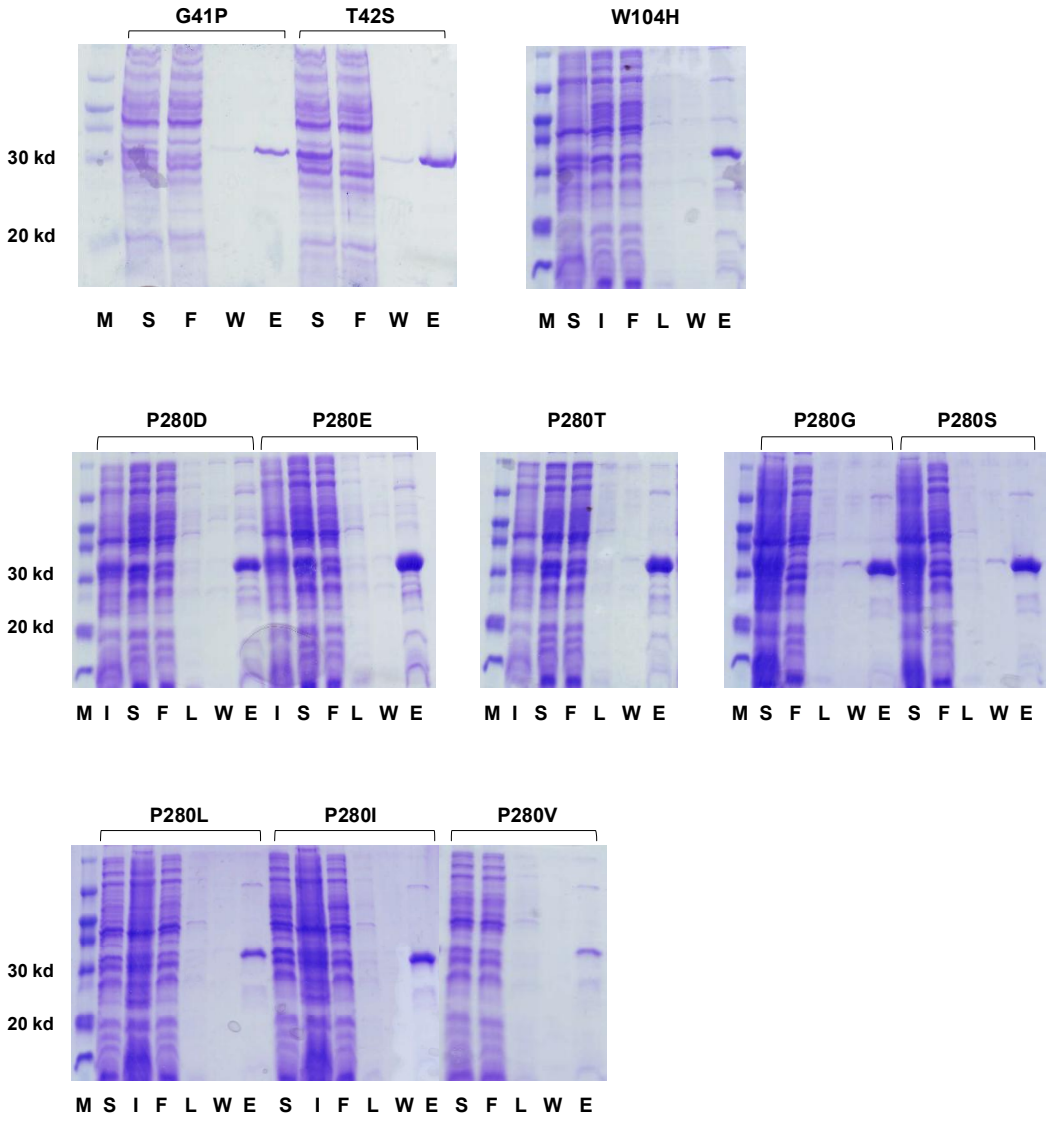

Figure 1. SDS-PAGE analyses of CAL-B mutants: G41P, T42S, W104H, and Pro280 residue. M, molecular weight marker; S, soluble fraction; I, insoluble fraction; F, flow through fraction; L, lysis buffer fraction; W, wash buffer fraction; E, elution buffer fraction.

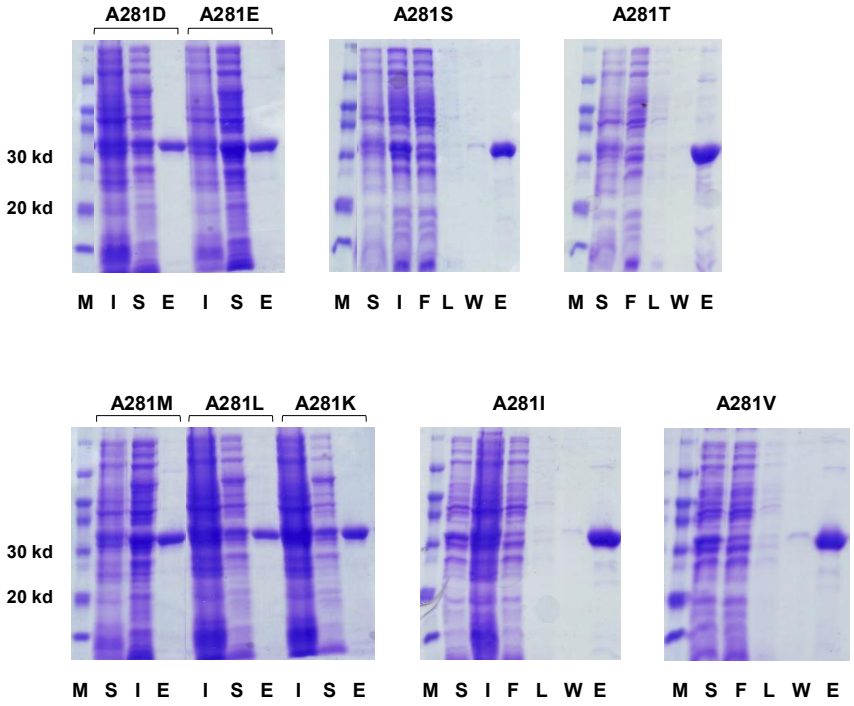

Figure 1 continued. SDS-PAGE analyses of mutants of the A281 residue. M, molecular weight marker; S, soluble fraction; I, insoluble fraction; F, flow through fraction; L, lysis buffer fraction; W, wash buffer fraction; E, elution buffer fraction.

Conclusion and Summary

Lipases are one of the useful biocatalysts because they exhibit high regio- and enantioselectivity, and can accept various substrates (lipids, alcohols, acids and esters). Their catalytic reaction can be performed under mild conditions. However, lipases often do not exhibit enough activity to be required in their application due to their properties of denaturation easily. In this thesis, two approaches have been described for improving the activity of lipase.

First, the lipases were covalently immobilized on hydroxyapatite by a simple chemical treatment using DCC, DIC, or EDC. Immobilization is widely used for improving the activity and reusability of biocatalysts. Although hydroxyapatite is an excellent candidate as a supporting material in enzyme immobilization because it is biocompatible and eco-friendly, its use for lipase immobilization has been rarely reported. Probably, a facile method for the immobilization of lipases has not been developed to date because the properties of the surfaces of hydroxyapatite and lipase are fairly distinct and a repulsive interaction between them is presented. Thus, a linker compound was required to diminish the repulsive interaction. The linker-assisted process increased the binding amount of lipases up to five times. Moreover, the immobilized lipases, such as CAL-B, BCL, and CRL, showed up to

100-fold higher specific activity compared to the free-form lipases. In addition, the ten-time recycled immobilized lipases still retained more than 85% of their activity. This suggests that hydroxyapatite can be used as an excellent supporting material and also that a simple and practical process for covalent conjugation is provided.

In the second study, the enhancement of the perhydrolase activity of CAL-B was investigated. The residues that are composed of the water channel of CAL-B were identified to be substituted by a polar amino acid. Among the candidate residues, altering the Ala281 residue exhibited the greatest effect on the hydrolase and perhydrolase activities. The perhydrolase activities of the Ala281Ser and Ala281Thr mutant enzymes were twice higher than that of the wild-type CAL-B. Moreover, the epoxidation of styrene with three mutant enzymes, such as the Ala281Ser, Ala281Thr, and Ala281Leu mutant enzymes, were performed and compared with the wild-type CAL-B. The Ala281Thr exhibited higher initial rate than the wild-type CAL-B. This indicates that an introduction of a polar residue near the water channel presumably assists the access of hydrogen peroxide into the active site and thus increases the reaction rate.

ABSTRACT

Enhancing the activities of *Candida antarctica* lipase B by the immobilization on hydroxyapatite and by site directed mutagenesis

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This thesis deals with improving transesterification activity of lipase by immobilization and enhancing the perhydrolase activity by protein engineering. First, lipase was conjugated on hydroxyapatite, which is known as an environmentally-friendly material. Second, *Candida antarctica* lipase B (CAL-B) was modified by site directed mutagenesis to increase the perhydrolase activity.

Hydroxyapatite as a component of the human bone systems is a biological and environmentally benign substance. Thus, hydroxyapatite is considered an excellent candidate supporting material for enzyme immobilization. However, the application of

hydroxyapatite for enzyme immobilization have been rarely reported. In this work, the covalent immobilization of lipase on hydroxyapatite has been achieved. A peptide coupling reagent, such as *N*,*N*'-dicyclohexylcarbodiimide (DCC), *N*,*N*'-diisopropylcarbodiimide (DIC), and *N*-ethyl-*N*' -(3-dimethylaminopropyl)carbodiimide (EDC), was used for direct conjugation of *Candida rugosa* lipase (CRL) as a model lipase on hydroxyapatite. As CRL was conjugated directly, it was found that CRL was not immobilized sufficiently on hydroxyapatite (0.26-0.48 mg/g). Probably, it is due to a repulsive interaction between the polar surface of hydroxyapatite and the non-polar surface of lipase. To resolve this problem, 6-aminohexanoic acid (6-AmHAc) was used as a linker. The amount of binding lipase on hydroxyapatite increased up to five times using EDC (1.70-2.76 mg/g). Moreover, the activities of the immobilized CRL, CAL-B, and BCL (*Burkholderia cepacia* lipase) were compared with those of the corresponding free forms. The immobilized lipases exhibited higher activity up to 100 times. In addition, the immobilized lipases maintained up to 85-99% of their activities after ten-time recycling.

It is known that CAL-B catalyzes not only hydrolysis but also perhydrolysis, which is a reaction using hydrogen peroxide as a reactant. In this study, enhancing the perhydrolase activity of CAL-B was achieved by protein engineering. Recently, the existence of the water channel, which the water molecule can enter to the active site,

was proposed. Based on this hypothesis, it was assumed that hydrogen peroxide can also enter to the active site through the water channel in perhydrolysis. If it is true, the modification of the amino acids near the water channel possibly affects the perhydrolysis activity of CAL-B. The residues near the water channel were identified by computer modeling. The Pro280 and Ala281 residues were selected as target residue for the modification. After the residues of the channel were replaced with different sized or polar amino acid, the hydrolysis and perhydrolysis activities were compared. There were no significant effects by modifying the Pro280 residue on both activities for hydrolysis and perhydrolysis. In contrast, the hydrolysis activities of the Ala281Ser and Ala281Thr mutant enzymes were 2- and 1.2-fold higher, respectively, and the perhydrolysis activities of them were twice higher compared to that of wild-type enzyme.