Abstract

Development and Application of Immobilized Lipase Assembled with Ionic Liquid on Poly(acrylic acid) Beads

Sewon Yeo

School of Chemical and Biological Engineering

The Graduate School

Seoul National University

Immobilized enzymes have attracted much attention due to the characteristics of operational stability, enhanced activity, and reusability as well as simple separation. Most of commercial lipases are immobilized form and are used to convert fatty acids to fatty acid alkyl esters (FEs) via an environment-friendly process. Immobilization of enzymes on resin beads has been utilized as a common tool in biological studies as well as bio-industrial field.

However, factors controlling the stability of enzyme on resin beads have not been fully explored. So, esterification reactions are designed to evaluate how the stability of *Candida antarctica* lipase B (CALB) depends on the hydrophilic/hydrophobic properties with or without ionic liquids (ILs) on poly(acrylic acid) (PA) beads, because the ILs can provide a tunable microenvironment to the lipase. The catalytic activities of the immobilized lipases were compared under several factors such as bead properties, immobilization methods, loading levels of ILs, chain lengths of alkyl group on ILs, and solvents.

From these results, we found that the IL-grafted PA beads which contained more hydrophilic properties (octyl group) showed better catalytic performance than hydrophobic ones (dodecyl group). The use of *tert*-butanol as a solvent was a pivotal factor to maintain the activity of the immobilized lipase. Surprisingly, the single type immobilized lipase, PA-C_8Im-Lipase, (C_8Im loading level, 1.2 mmol/g) showed the best catalytic performance (95% yield) at 50 ^oC even though the network type immobilized lipase was expected to show the best performance. The single type immobilized lipase can be reused with slightly loss of activity.

In conclusion, octyl group provided positive influence to the lipase activity on IL-grafted PA beads without blocking the hydrophobic active site of lipase.

Keywords: Bio-catalysis, Lipase, Esterification, Ionic Liquid-grafted Poly(acrylic acid) Beads.

Student Number: 2012-20960

Contents

List of Abbreviations

ATR	attenuated total reflection
Boc anhydride	di-*tert*-butyl dicarbonate
CALB	lipase B from *Candida Antarctica*
DCE	1,2-dichloroethane
DCM	dichloromethane
DIC	*N,N'*-diisopropylcarbodiimide
DMF	*N,N'*-dimethylformamide
DMSO	dimethylsulfoxide
EA	elementary analysis
EDC	*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride
EDS	energy dispersive spectrometer
FE	fatty acid alcohol ester
FE-SEM	field emission scanning electron microscopy
FT-IR	Fourier transform-infrared spectroscopy
GA	glutaraldehyde
GC	gas chromatography
HOBt	1-hydroxybenzotriazole hydrate anhydrous

IL	ionic liquid
MeOH	methanol
$MgSO_4$	magnesium sulfate
$NaHCO_3$	sodium bicarbonate
NHS	*N*-hydroxysuccinimide
^1H NMR	proton nuclear magnetic resonance spectrometer
PA	poly(acrylic acid)
PG	propylene glycol
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
UV-Vis	ultraviolet-visible spectrophotometer

List of Figures

List of Schemes

List of Tables

Chapter 1. Introduction

1.1 General Introduction of Lipase

1.1.1 Progress of Enzyme Catalysts

Bio-catalysis has been attracted because many industrial processes have been replaced by cost-effective and environment-friendly ones. The advantages of enzymes in chemical processes are less production of waste, energy efficiency and no requirement of protection/activation steps of the functional groups.[1] Enzymatic processes have been utilized in industry to produce specific product.[2] Therefore, various valuable and sustainable chemical products are derived by enzymes from renewable resources with economic and environmental benign chemical processes. For example, enantioselective arylpropionate by arylmalonate decarboxylase-catalyzed reaction have been used in manufacturing anti-inflammatory drug.[3] Polyol esters based on vegetable oils and animal fats by lipase-catalyzed reaction have been used in the lubricant technology,[4] due to renewable and environmentally friendly properties of the natural triglycerides.[5]

1.1.2 Properties of Free Lipase

Lipase-catalyzed transesterification/esterification reactions have been utilized to obtain enantio-pure and value-added compounds in the food, pharmaceutical and fine chemical industries.[6] Among the lipases, *Candida antarctica* lipase B (CALB) gives high selectivity and is relatively stable compared to other lipases.

However, due to the high cost of enzyme, reusability and stability of lipase under the chemical processes are the most concerned factors. Generally, the catalytic efficiency is decreased by high concentration of the alcohol substrates and inhibited by hydrophobic products because transesterification/esterification reactions with alcohols are carried out under the bi-phasic systems.[7,8]

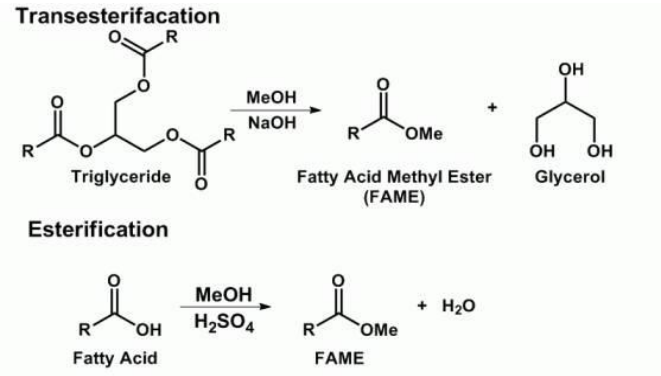


Figure 1. Transesterification and esterification reactions.

1.1.3 Industrially Valuable Ester Compounds

Fatty acid alcohol esters (FEs) as a biodiesel are considered to be valuable chemicals as clean renewable fuels,[9,10] lubricant,[4,5] food,[11,12] and pharmaceutical materials.[6,13] They are produced by the transesterification of vegetable oils and animal fats, or the esterification of fatty acids with alcohols. The synthetic processes are commonly performed with basic or acidic catalysts. When using the basic catalysts, a certain amount of water is produced, which lead to the saponification reaction. This major drawback of conventional industrial processes can be overcome by enzymatic reaction with lipases. Therefore, lipase-catalyzed esterification reactions are one of the significant biochemical processes.[14] Moreover, specific alkyl esters can be synthesized and glycerol as a by-product can be recovered easily.[7]

1.2 Enzyme Immobilization

1.2.1 Strategies for Enzyme Immobilization

Enzyme immobilization is a pivotal step for practical application of enzyme in terms of green and sustainable chemical process. Enzyme as a biocatalyst is usually used under mild conditions such as moderate temperature, pressure and pH in aqueous media. Enzymes require more environmentally friendly conditions that give high rates and selectivity. Conversely, the necessity of relatively mild reaction condition is pointed out as the limitation. In this regard, the immobilization of enzyme can improve its stability and reusability, especially, in organic solvents compared to the free enzyme. Also, it gives the additional advantages of reduction of product contamination by enzyme residues.

The methods of enzyme immobilization can be classified in three ways, binding to a solid support,[15] entrapment[16] and cross-linking.[17] First, binding to a solid support is proceeded by physical adsorption or ionic and covalent bonding. Among these, physical adsorption gives a relatively weak binding force between solid support and enzyme so that it has a leaching problem. Ionic and covalent bondings are stronger than physical adsorption and could prevent the problem. However, during the process of covalent binding,

enzyme deactivation can be occurred. Second, entrapment method fixes the enzyme into porous matrixes such as polymer, silica sol-gel and hollow fiber. The covalent bonding after entrapment is generally used because leakage can occur under only physical entrapment. Third, cross-linking method is conducted with glutaraldehyde to interconnect the enzymes.[18] Cross-linked enzyme has the advantages of highly concentrated loading on the support with high thermostability.[14]

Novozyme 435 is a commercially available lipase B from *Candida Antarctica* (CALB) physically adsorbed on a macroporous acrylic resin. Because of non-covalent bonding, enzyme leaching can be generated by substrates or products with surfactant-like properties.[18,19] In this regard, development of efficient enzyme immobilization methods are still challengeing.

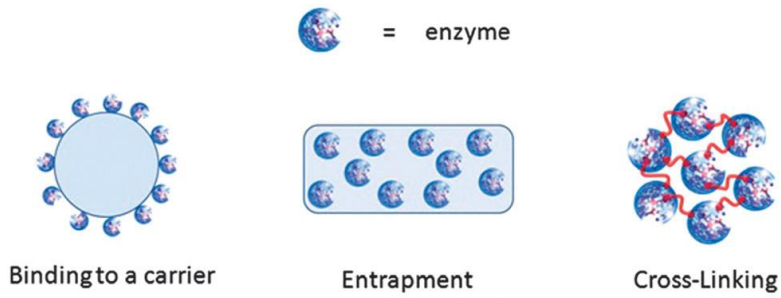


Figure 2. Three methods of enzyme immobilization.[20]

1.2.2 Role of Polymer Supports in Lipases Immobilization

Like the Novozyme 435, polymeric beads are commonly used as a support in enzyme immobilization. They have advantages in easy controlling chemical and physical properties. Several methods of lipase immobilization have been known with polymer beads having capability of controlling their size, porosity, and surface properties.[13,21,22] Among them, controlling surface properties of the beads is a crucial factor for obtaining maximum activity of the immobilizaed lipase. In this regard, the recent paper which showed the effect of ionic liquid (IL) on the surface properties of polymeric substrate has encouraged us to study on the IL grafting.[7]

Poly(acrylic acid) (PA) beads are highly hydrophilic and can offer a desirable microenvironment to lipase by controlling hydrophilic/hydrophobic nature by covalent attachment of alkyl imidazolium groups. It is important that the PA beads can be solvated in a hydrophobic environment because esterification reaction is proceeded in a hydrophobic condition.[20] Consequently, the modification of PA beads with IL is inevitably required.

1.2.3 Polymer Grafting using Ionic Liquids for Enzyme Immobilization

Grafting polymer beads with ILs is one of the methods for modulating polymer properties. ILs are used in bio-catalytic process for biodiesel production and enhance the stability of lipase.[23] Compared to organic solvents, ILs are more hydrophilic, and have good dissolution capacity and high thermal stability. Their properties are easily controlled by changing cations, anions and alkyl chain length, and these can be applied to polymeric beads.[24] These properties of ILs can be also applied to a soluble polymer.[25]

The polymer grafting with ILs can be used to change the physical property of immobilized enzyme, especially the lipases. Long alkyl chain in ILs offers a flexible mobility to enzyme than short alkyl chain. In this way, the physical property of polymer supports can be controlled by considering the advantages and disadvantages of ILs.[20] Also, these properties can be changed to obtain an optimal enzyme activity.

1.3 Research objectives

In this thesis, the catalytic activity of immobilized lipase was enhanced by grafting with ILs. The hydrophilic/hydrophobic properties of PA beads are controlled for giving desirable microenvironment to lipase. To achieve these, PA beads having two loading levels of IL moieties were prepared and their physical properties were compared. After that, the remaining carboxylic acid groups were covalently bonded with CALB using EDC/NHS coupling method. This allows us to compare the activities of various immobilized lipases depending on the amounts of ILs. Additionally, through the glutaraldehyde treatments, lipase was cross-linked to overcome the enzyme leaching problem that can occur in a reusability test. In this method, the amounts of lipase bound to the support can be optimized depending on the properties of support. The activities of immobilized lipase (single type or network type) were compared through hydrolysis and esterification reaction. *p*-Nitrophenyl butyrate was used to measure the hydrolytic activity of the immobilized lipapes. Oleic acid and four kinds of alcohols were used to measure the esterification activity. The reaction was operated under the solvent-free, relatively low temperature.

Chapter 2. Experiments

2.1 General

2.1.1 Materials

Poly(acrylic acid) (PA) beads were obtained from Samsong Polymer Co. (Korea). 1-(3-Aminopropyl)imidazole, 1-butanol, 1-chlorooctane, *N,N'*-diisopropylcarbodiimide (DIC), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), glutaraldehyde (GA), *n*-heptane, methanol (MeOH, HPLC grade), Novozyme 435 (immobilized lipase from *Candida antarctica*), and oleic acid were purchased from Sigma-Aldrich. Di-*tert*-butyl dicarbonate (Boc anhydride) and 1-chlorododecane were purchased from Tokyo Chemical Industry. *N*-Hydroxysuccinimide (NHS) was purchased from Fluka. Cyclohexane, 1,2-dichloroethane (DCE) and propylene glycol (PG) were purchased from Junsei Chemical Co. *Candida antarctica* lipase B (powder form) was obtained from Genofocus Inc. (Korea). 1-Hydroxybenzotriazole hydrate anhydrous (HOBt) was obtained from Bead Tech Inc. (Korea). *tert*-Butanol, dichloromethane (DCM), diethyl ether, *N,N'*-dimethylformamide (DMF), dimethylsulfoxide (DMSO), ethyl acetate (EA), *n*-hexane, magnesium sulfate ($MgSO_4$), methanol (MeOH), 1-propanol,

sodium bicarbonate ($NaHCO_3$), tetrahydrofuran (THF) and trifluoroacetic acid (TFA) were purchased from Daejung Chemicals Co. (Korea). All reagents were used without any further purification.

2.1.2 Instruments

ATR FT-IR spectrophotometer (Nicolet 6700, Thermo Scientific), ^{1}H NMR spectrometer (JNM-LA300, JEOL), UV-Visible spectrophotometer (Optizen 2120UV , Mecasys), elemental analysis (CHNS-932, Leco), field-emission scanning electronic microscopy (FE-SEM, Helios 650) equipped with an energy dispersive spectrometer (EDS), and GC instrument (GC-6500 series, Younglin) equipped with a flame ionization detector (FID) and a DB-5 capillary column (30m × 0.320mm, 0.25μm) were used for characterization and analysis.

2.2 Modification of Poly(acrylic acid) Beads

2.2.1 Preparation of Ionic Liquid-grafted Poly(acrylic acid) Beads

_N_-Boc-1-(3-aminopropyl)imidazole

1-(3-Aminopropyl)imidazole (4.8 mL, 40 mmol) in a round bottom flask was dissolved in THF (100 mL). A $NaHCO_3$ (8.4 g, 100 mmol) aqueous solution (100 mL) was added to 1-(3-aminopropyl)imidazole solution. Then, Boc anhydride (12 mL, 52 mmol) dissolved in THF (50 mL) was added dropwise to the 1-(3-aminopropyl)imidazole solution under magnetic stirring at 0 ^{o}C. After stirring for 4h at room temperature, the progress of the reaction was checked with thin layer chromatography (TLC). The mixture was evaporated in vacuo. The residue mixture was dissolved in EA and extracted with water. Then, the organic phase was dried over $MgSO_4$ and evaporated in vacuo (7.6 g, yield : 85%). ^{1}H NMR (400 MHz, d_6-DMSO, TMS) : δ (ppm) = 7.6 (s, 1H), 7.2 (s, 1H), 7.0 (t, 1H), 6.9 (s, 1H), 4.0 (t, 2H), 2.9 (q, 2H), 1.8 (quin, 2H), 1.4 (s, 9H) (see the appendix).

1-Octyl- and 1-Dodecyl-(3-aminopropyl)imidazolium Chloride

N-Boc-1-(3-Aminopropyl)imidazole (7.6 g, 34 mmol) was dissolved in DCE (100 mL) and stirred at 80 ^{o}C. A solution of 1-chlorooctane or 1-

chlorododecane (1.3 equiv., 7.5 mL) was added dropwise under magnetic stirring. After stirring for 64 h, the progress of the reaction was checked with TLC. The mixture was evaporated in vacuo. The residue mixture was dissolved in MC and extracted with diethyl ether by centrifuging. Centrifugation was conducted five times, and the organic phase was dried in vacuo (8.0 g, yield : 63%). *N*-boc-1-Octyl-3-aminopropylimidazolium chloride. ^{1}H NMR (400 MHz, d_6-DMSO, TMS) : δ (ppm) = 9.4 (s, 1H), 7.9 (d, 3H), 7.0 (t, 1H), 4.6 (t, 2H), 4.2 (t, 2H), 4.1 (t, 2H), 3.4 (s, 6H), 2.9 (q, 3H), 1.9 (q, 3H), 1.4 (s, 13H). *N*-boc-1-Dodecyl-3-aminopropylimidazolium chloride. ^{1}H NMR (400 MHz, d_6-DMSO, TMS) : δ (ppm) = 9.3 (s, 1H), 7.9 (d, 3H), 7.0 (t, 1H), 4.6 (t, 3H), 4.2 (t, 3H), 4.1 (t, 2H), 3.4 (s, 10H), 2.9 (q, 3H), 1.9 (q, 3H), 1.4 (s, 15H) (see the appendix).

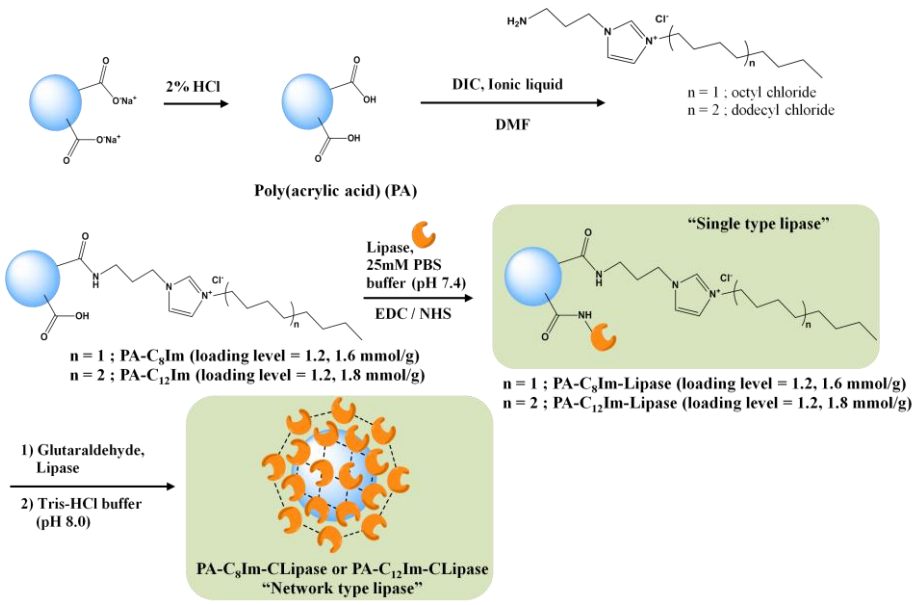
The Boc group was removed by treating with TFA/CH_2Cl_2 (10% v/v) solution for 2 h. After stirring for 2 h, the product was checked with TLC. The mixture was evaporated in vacuo. The residue mixture was dissolved in MeOH and extracted with hexane by centrifuging. Centrifugation was conducted five times, and the organic phase was dried in vacuo (4.2 g, yield : 73%). 1-Octyl-3-aminopropylimidazolium chloride. ^{1}H NMR (400 MHz, d_6-DMSO, TMS) : δ (ppm) = 9.4 (s, 2H), 7.9 (q, 3H), 4.7 (t, 4H), 4.4 (t, 5H), 4.1 (t, 4H), 2.8 (q, 5H), 2.1 (q, 5H). 1-Dodecyl-3-aminopropylimidazolium chloride. ^{1}H NMR (400 MHz, d_6-DMSO, TMS) : δ (ppm) = 9.3 (s, 2H), 7.9 (q, 6H), 4.6 (t, 5H),

4.3 (t, 6H), 4.1 (t, 5H), 2.8 (q, 6H), 2.1 (q, 6H) (see the appendix).

1-Octyl- and 1-Dodecyl-3-propylimidazolium Chloride (IL) Grafted Poly(acrylic acid) Beads

Acid-treated PA beads (2 g, 4 mmol/g) and DMF (120 mL) were added into a 3-neck round bottom flask equipped with reflux condenser and stirred for 20 min at 80 ^{o}C. Then, HOBt (2 equiv., 2.1 g) and DIC (2 equiv., 2.4 g) were added to the solution. After stirring for 2 h, activated PA beads were washed with DMF. Activated PA beads and DMF were put again in a 3-neck round bottom flask equipped with reflux condenser. A solution of the 1-octyl- or 1-dodecyl-3-aminopropylimidazolium chloride (1 equiv., 2.1 g) was added dropwise at 80 ^{o}C. After stirring for overnight, the product was washed with DMF, de-ionized water, and MeOH and dried in vacuo.

Preparation steps of IL-grafted PA beads and enzyme immobilization were shown in Scheme 1.



Scheme 1. Preparation of ionic liquid-grafted PA beads and enzyme immobilization.

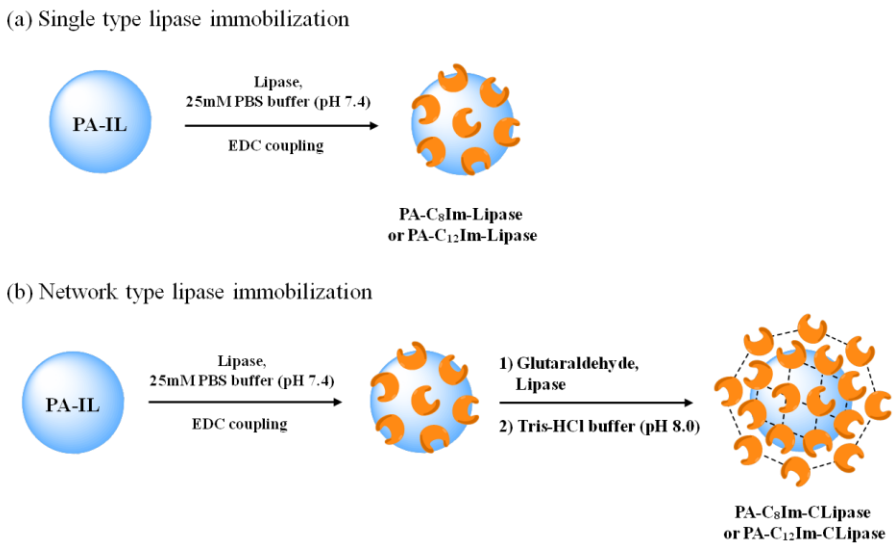
2.2.2 Immobilization of *Candida Antarctica* Lipase B on Ionic Liquid-grafted Poly(acrylic acid) Beads

Single Type Immobilized Lipase

Candida antarctica lipase B was immobilized on poly(acrylic acid)-grafted with 1-octyl-3-aminopropylimidazolium chloride (PA-C_8Im) or 1-dodecyl-3-aminopropylimidazolium chloride (PA-C_{12}Im). Pre-activated PA-C_8Im or PA-C_{12}Im were prepared by addition of EDC (1.2 equiv. of carboxylic acid on PA beads) and NHS (1.2 equiv. of carboxylic acid on PA beads) into PA beads in 25 mM phosphate buffer (pH 7.4). The activation reaction was performed for 6 h at room temperature. After shaking for 6 h, the pre-activated PA-C_8Im or PA-C_{12}Im were filtered and washed with phosphate buffer (25 mM, pH 7.4). Then, the CALB solution (50 % w/w PA bead, 10 mL of phosphate buffer (25 mM), pH 7.4) was added and stirred for 20 h at room temperature. After this, immobilized lipase (PA-C_8Im-Lipase or PA-C_{12}Im-Lipase) was centrifugated and the concentration CALB in the supernatant solution was calculated by BCA protein assay. After determining the CALB concentration, PA-C_8Im-Lipase or PA-C_{12}Im-Lipase were filtered and washed with 25 mM phosphate buffer (pH 7.4), and lyophilized.

Network Type Immobilized Lipase

PA-C_8Im-Lipase or PA-C_{12}Im-Lipase (1 g) were crosslinked with 1% glutaraldehyde solution (10 mL of pH 7.4 25 mM phosphate buffer) for 2 h at room temperature. After shaking for 2 h, the glutaraldehyde treated PA-C_8Im-Lipase or PA-C_{12}Im-Lipase were filtered and washed with 25 mM phosphate buffer (pH 7.4). Then, the CALB solution (50 % w/w PA bead, 10 mL of pH 7.4 25 mM phosphate buffer) was added and stirred for 20 h at room temperature. After filtration and lyophilization, network type immobilized lipase, cross-linked PA-C_8Im-Lipase or PA-C_{12}Im-Lipase (PA-C_8Im-CLipase or PA-C_{12}Im-CLipase) was prepared. Two types of lipase immobilization were shown in Scheme 2.

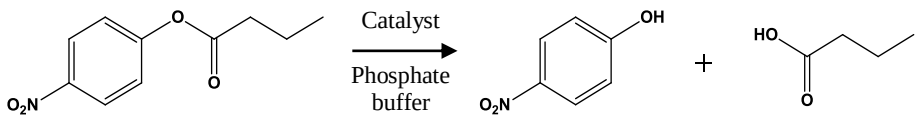


Scheme 2. Two types of lipase immobilization.

2.3 Enzyme Activity Test

2.3.1 Hydrolysis of *p*-Nitrophenyl Butyrate

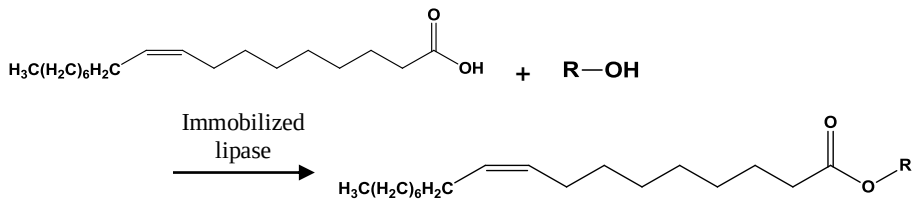
Immobilized lipase (10 mg) was dispersed in 990 μL phosphate buffer (25 mM, pH 7.4) and shaken for 5 min at 35 ^{o}C. Then, 10 μL *p*-nitrophenyl butyrate (20 mM in the final solution) was added to the mixture and shaken for 10 min at 35 ^{o}C. After 10 min of shaking, the reaction mixture was centrifugated for 30 s. The 50 μL of supernatant was diluted with 20-folds of phosphate buffer (25 mM, pH 7.4) and UV absorbance at 410 nm was measured by an UV-Visible spectrophotometer.



Scheme 3. Hydrolysis of *p*-nitrophenyl butyrate by immobilized lipase on the IL-grafted PA beads.

2.3.2 Esterification of Fatty Acids

Fatty acid, alcohol, and immobilized lipase (50 mg) were mixed in a reaction tube. The esterification was carried out for 18 h at 50 ^{o}C. After the reaction, fatty acid ester was produced on upper phase. Then, fatty acid ester (100 μL) was analyzed by gas chromatography. The immobilized lipase in lower phase was recovered by washing with 25 mM phosphate buffer (pH 7.4), methanol, and then lyophilized. The recovered immobilized lipase was reused by the same way as before.



Scheme 4. Esterification of oleic acid with various alcohols by immobilized lipase on the IL-grafted PA beads.

Chapter 3. Results and Discussion

3.1 Preparation and Characterization of Poly(acrylic acid)-supported Lipase

Two types of ILs (C_8Im or C_{12}Im) were coupled on the PA beads by HOBt/DIC coupling method respectively. After ILs were coupled to PA beads, the loading level of IL groups were determined by nitrogen analysis (1.2 and 1.6 mmol for C_8Im/g PA beads; 1.2 and 1.8 mmol for C_{12}Im/g PA beads). The existence of IL groups was confirmed by field emission scanning electron microscopy (FE-SEM) equipped with an energy dispersive spectrometer (EDS). It was confirmed that the IL groups were coupled to PA beads and the morphology of the beads was not changed as shown in Figure 3. The IL groups coupled to PA beads were confirmed by ATR FT-IR analysis. The carbamate and quaternary imidazolium bands of IL-grafted PA beads appeared at about 1160 and 1660 cm^{-1} as shown in Figure 4.

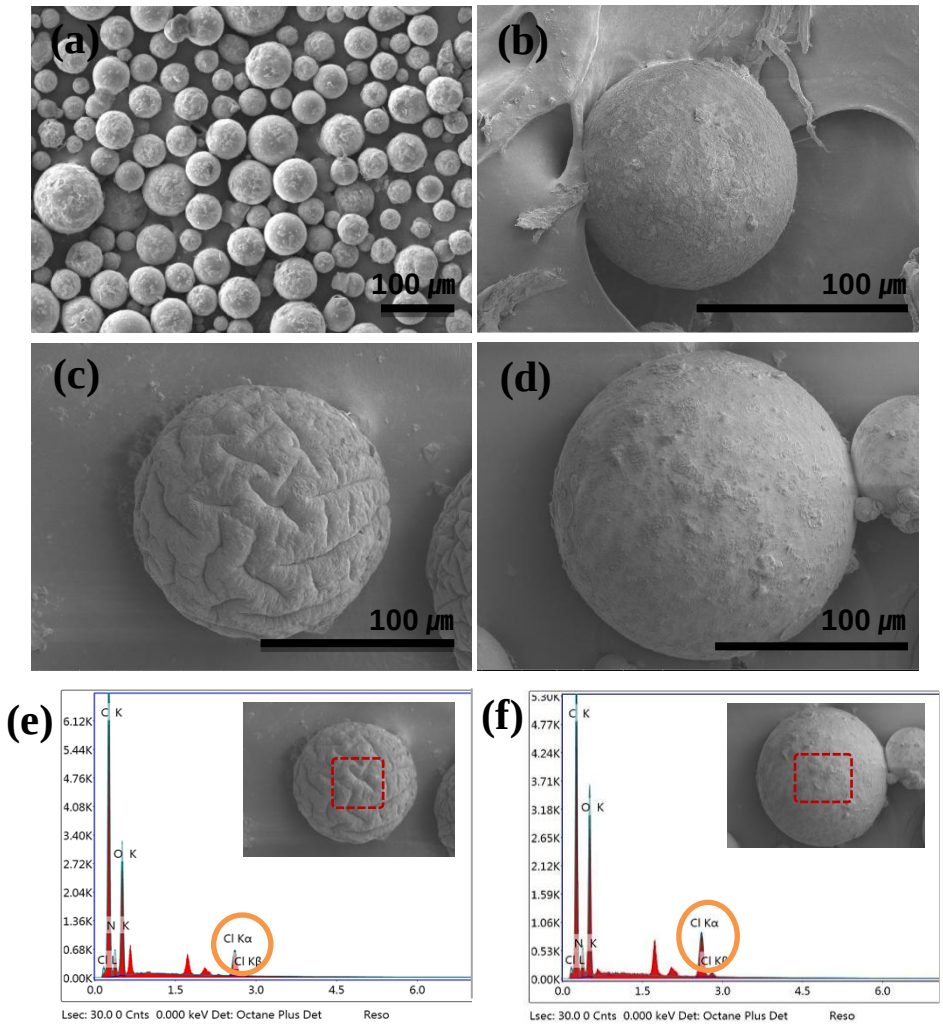


Figure 3. Characterization of ionic liquid-grafted PA beads by FE-SEM analysis (a) PA (salt form), (b) PA, (c) PA-C_8Im (1.2 mmol/g), (d) PA-C_{12}Im (1.8 mmol/g). EDS data of (e) PA-C_8Im (1.2 mmol/g) and (f) PA-C_{12}Im (1.8 mmol/g).

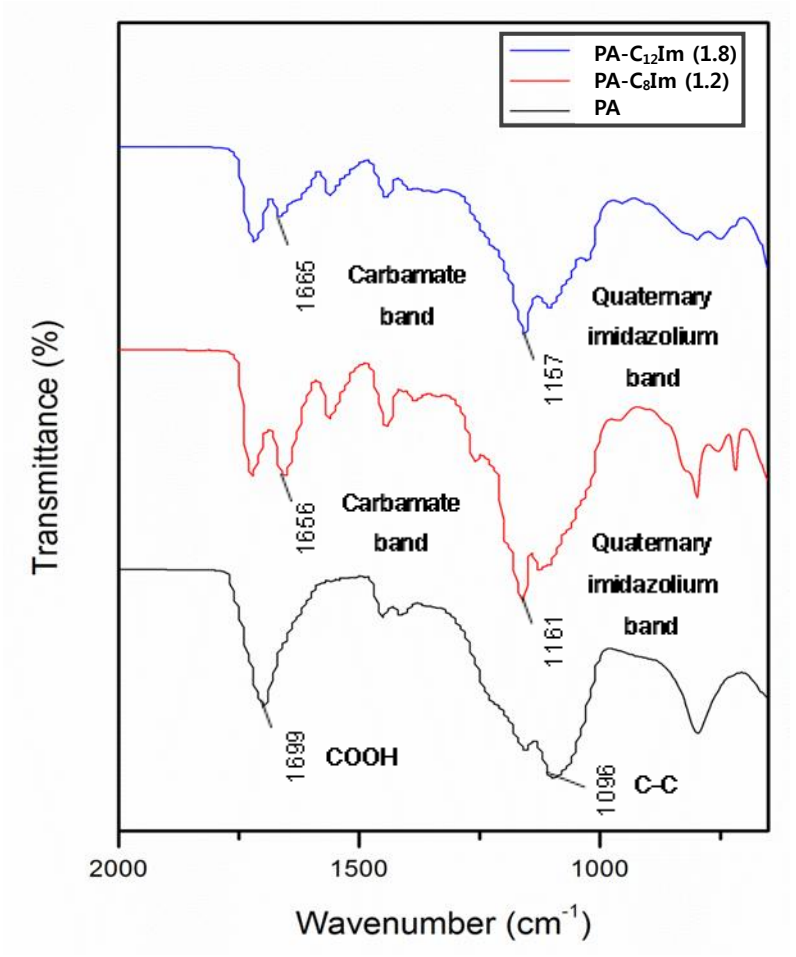


Figure 4. ATR FT-IR spectra of the PA beads (black), PA-C_8Im beads (1.2 mmol/g, red) and PA-C_{12}Im beads (1.8 mmol/g, blue).

The swelling properties of PA beads have a tendency to become hydrophobic one when the beads are changed to IL-grafted PA beads. To figure out hydrophilic/hydrophobic properties of PA and IL-grafted PA beads, the swelling properties in various solvents were screened (Table 1). We confirmed from the swelling properties that the high swelling of IL-grafted PA beads was observed in the solvent having high polarity index. However, except PA-C_{12}Im beads of 1.2 mmol/g loading, it did not follow our expectation because IL-grafted PA beads presented only a charged hydrophobic property at the bead surface that is not much swollen in the tested solvents.

Table 1. Swelling Properties of the PA Beads and IL-grafted PA Beads in Various Solvents

Support	Loading level of ionic liquid (mmol/g)	Vol/Mass (mL/g beads)					
		Water	DMSO	DMF	MeOH	THF	DCM
PA[a]	-	22	26	26	24	8	6
PA-C_8Im[b]	1.2	3.2	6	4	3.2	2	4
	1.6	10	10	6	10	3.2	6
PA-C_{12}Im[c]	1.2	36	32	22	12	3.2	6
	1.8	3.2	3.2	4	4	2	4

[a]PA : poly(acrylic acid) beads, [b]PA-C_8Im : 1-octyl-3-propylimidazolium chloride-grafted poly(acrylic acid) beads, [c]PA-C_{12}Im : 1-dodecyl-3-propylimidazolium chloride-grafted poly(acrylic acid) beads

3.2 Catalytic Activities of the IL-grafted PA Beads

3.2.1 Hydrolysis of *p*-Nitrophenyl Butyrate

The activity of immobilized lipases was measured by hydrolysis of *p*-nitrophenyl butyrate, which was followed by the absorbance of *p*-nitrophenol at 410 nm as shown Figure 5. Table 2 showed the activity results of eight types of the immobilized lipase. The immobilized lipases of the network type showed better hydrolytic performance (Min. 47.5 % in activity), compared to the immobilized lipase of the single type (Min. 26.0 % in activity). The immobilized lipases containing C_{12}Im group showed better activity (74.4 %) than those of C_8Im group. From these results, we found that the hydrolytic performance was increased as the loading amount of lipase was increased.

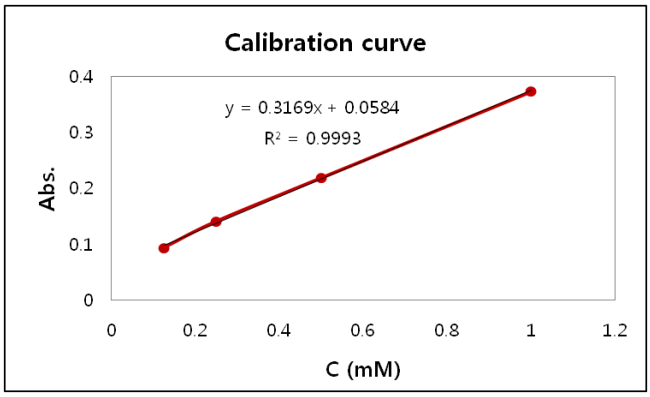
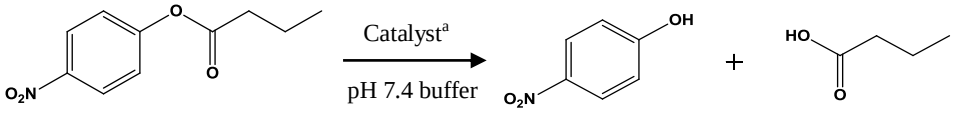


Figure 5. Calibration curve of *p*-nitrophenol measured at 410 nm.

Table 2. Hydrolytic Activity of Immobilized Lipases



***p*-Nitrophenyl butyrate** ***p*-Nitrophenol**

Catalyst	Loading level of IL-moieties on PA beads (mmol/g)	Activity (U^b/mg of beads)
None	-	N.A
Free lipase	-	72.7[c]
PA-C_8Im	1.2 1.6	N.A
PA-C_{12}Im	1.2 1.8	N.A
PA-C_8Im-Lipase	1.2 1.6	41.9 26.0
PA-C_{12}Im-Lipase	1.2 1.8	60.7 74.1
PA-C_8Im-CLipase	1.2 1.6	65.7 47.5
PA-C_{12}Im-CLipase	1.2 1.8	74.4 69.8

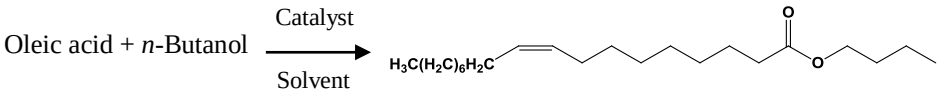
[a]Reaction conditions : catalyst (10 mg), *p*-nitrophenyl butyrate (0.2 mM), phosphate buffer (25 mM, pH 7.4), 35 ^oC, 10 min. [b]U (Unit) : One unit of enzyme activity was defined as the amount of enzyme which catalyzed the production of 1 μmol *p*-nitrophenol per minute. [c]Activity of free lipase was confirmed by U/mg of protein. Determined by UV-Vis spectroscopy at 410 nm.

3.2.2 Esterification Activity of Immobilized Lipases

The esterification of oleic acid with alcohols by immobilized lipases was evaluated in various organic solvents. Hexane, heptane and cyclohexane were selected as the solvent because they were known to give good activity in lipase catalyzed esterification.[26] *tert*-Butanol as a co-solvent has been used because it dissolves both alcohol and oleic acid, and improved the yield of biodiesel production by lipase.[27] Moreover, *tert*-butanol does not react with oleic acid by lipase. Among these solvent systems, hexane/*tert*-butanol and *tert*-butanol showed the best performance (47 % yield) compared to other hydrophobic solvents, such as heptane and cyclohexane as shown in Table 3. According to this result, *tert*-butanol was selected as the solvent.

.

Table 3. Esterification Activity of Immobilized Lipase in Various Solvents



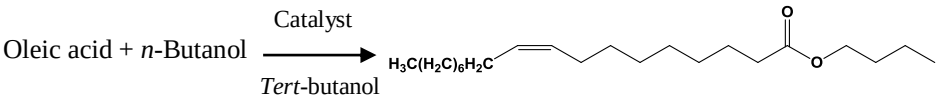
Catalyst[a]	Loading level of IL-moieties on PA beads (mmol/g)	Solvent	Yields[b] (%)
PA-C_8Im-Lipase	1.2	Hexane	47
		Heptane	45
		Cyclohexane	25
		tert-Butanol	47

[a]Reaction conditions : catalyst (50 mg), oleic acid (0.1 mmol), *n*-butanol (1 mmol), solvent : *tert*-butanol (co-solvent) = 8:1, 50 ^{o}C, 18 h. [b]Determined by GC analysis.

Esterification of oleic acid with *n*-butanol, as a control, was chosen to study the catalytic activities of immobilized lipases. Immobilized lipase without the IL moieties (PA-Lipase) showed low activity of esterification, and gave 17 % yield (Table 4). From this, IL moieties proved to be an important factor as a stabilizer of lipase in organic solvent.

PA-C_8Im-Lipase (1.2) showed relatively higher performance (47 % after 18 h) in the esterification than PA-C_{12}Im-Lipase catalysts. This observation can be explained by the previous report that enzymatic activity was decreased as the alkyl chain length of ILs was increased.[28] The similar tendency was observed by our immobilized lipase with relatively hydrophilic anions (Cl^-, BF_4^-). However, the activity of immobilized lipase was increased as the alkyl chain length of ILs was increased when the immobilized lipase contained hydrophobic anions (PF_6^-, NTf_2^-).[27,29] Interestingly, immobilized lipase in network types gave negative effect on the activity probably because crowded lipase molecules led to diffusion limitation and difficulty accessibility of substrate.[18,20] Based on the results, the PA-C_8Im-Lipase (1.2) was selected as an optimal catalyst and used in esterification reactions with four kinds of alcohols.

Table 4. Esterification Activity of Immobilized Lipase on the IL-grafted PA Beads

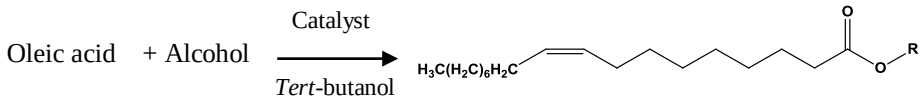


Catalyst[a]	Loading level of IL-moieties on PA beads (mmol/g)	Yields[b] (%)
None	-	2
PA-C_8Im	1.2	7
	1.6	3
PA-C_{12}Im	1.2	4
	1.8	3
PA-Lipase	-	17
PA-C_8Im-Lipase	1.2	47
	1.6	37
	2.2	28
PA-C_{12}Im-Lipase	1.2	29
	1.8	27
PA-C_8Im-CLipase	1.2	16
	1.6	36
PA-C_{12}Im-CLipase	1.2	10
	1.8	8

[a]Reaction conditions: catalyst (50 mg), oleic acid (0.1 mmol), *n*-butanol (1 mmol), *tert*-butanol (400 μL), 50 °C, 18 h. [b]Determined by GC analysis.

With four different kinds of alcohols, the yield of methyl oleate was the highest (95 %) as shown in Table 5. Furthermore, when propylene glycol was used as an alcohol substrate our immobilized lipase gave diester in 80 % yield. The high yield might be due to the fact that *tert*-butanol gives an effective miscibility to all the reactants of alcohol, oleic acid, and immobilized lipase. Also, ILs afford stable microenvironments and protect the immobilized lipase from direct influence of high temperature.

Table 5. Esterification of Oleic Acid with Various Alcohols by Immobilized Lipase



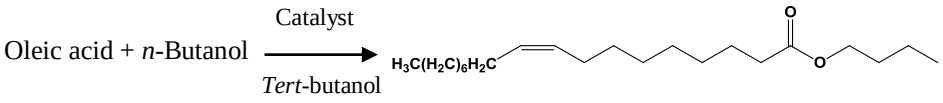
Catalyst[a]	Loading level of ionic liquid (mmol/g)	Alcohol	Yields[c] (%)
PA-C_8Im-Lipase	1.2	Methanol[b]	95
		1-Propanol	85
		1-Butanol	81
		Propylene glycol	80

[a]Reaction conditions : catalyst (50 mg), oleic acid (0.1 mmol), alcohol (1 mmol), *tert*-butanol (400 μL), 50 °C, 63 h. [b]2.5 mmol was used. [c]Determined by GC analysis.

3.2.3 Reusability of Immobilized Lipase

To evaluate the reusability the recovered immobilized lipase was reused several times for the same esterification reaction. Under the same conditions, the yield of ester was slightly decreased from 81 % to 62 % in second cycle, and much more decreased in third cycle (52 % in Table 6). One of the reasons for the decreased activity is assumed to be that lipase might be deactivated by the alcohols during esterification and lose the binding activity to the substrates. Based on these results, we concluded that immobilized lipase containing IL moieties should be improved further to increase the resistance against organic solvents and thermal stability.

Table 6. Reusability Result of Immobilized Lipase in Esterification



Catalyst[a]	Yield[b] (%)		
	1st	2nd	3rd
PA-C_8Im-Lipase (1.2)	81	62	52

[a]Reaction conditions : catalyst (50 mg), oleic acid (0.1 mmol), *n*-butanol (1 mmol), *tert*-butanol (400 μL), 50 °C, 63h. [b]Determined by GC analysis.

Conclusion

Eight types of IL-grafted PA beads were prepared and used for immobilization of lipase. The activity of immobilized lipase was confirmed by the hydrolysis of *p*-nitrophenyl butyrate. Efficiency of esterification reactions were compared according to the loading level and alkyl chain length of ILs. From the results, we confirmed that the IL moieties are essential to accelerate the lipase-catalyzed reaction when CALB was immobilized on PA beads. Compared to the immobilized lipase without IL moieties, the catalytic activity of the PA-C_8Im-Lipase was turned out to be the most effective in esterification reactions. These observations are in good agreement with previous results from other groups. In addition, *tert*-butanol as a solvent was a pivotal factor to maintain the activity of the lipase. These results demonstrate that the relatively hydrophilic supports can provide positive influence to the activity of lipase without blocking its hydrophobic active site. Our catalysts showed a good performance in the esterification of four kinds of alcohols without using nonpolar solvents and gave a reusable advantage suitable as industrial catalyst.

References

(1) Wohlgemuth, R. *Current opinion in biotechnology* **2010**, *21*, 713.

(2) Broering, J. M.; Hill, E. M.; Hallett, J. P.; Liotta, C. L.; Eckert, C. A.; Bommarius, A. S. *Angewandte Chemie* **2006**, *118*, 4786.

(3) Ijima, Y.; Matoishi, K.; Terao, Y.; Doi, N.; Yanagawa, H.; Ohta, H. *Chemical communications* **2005**, 877.

(4) Gryglewicz, S.; Piechocki, W.; Gryglewicz, G. *Bioresource technology* **2003**, *87*, 35.

(5) Arngek, A.; Vitintin, J. *Journal of Synthetic Lubrication* **2000**, *16*, 281.

(6) Munoz Solano, D.; Hoyos, P.; Hernaiz, M. J.; Alcantara, A. R.; Sanchez-Montero, J. M. *Bioresource technology* **2012**, *115*, 196.

(7) Lozano, P.; Garcia-Verdugo, E.; Bernal, J. M.; Izquierdo, D. F.; Burguete, M. I.; Sanchez-Gomez, G.; Luis, S. V. *ChemSusChem* **2012**, *5*, 790.

(8) Rodrigues, R. C.; Ortiz, C.; Berenguer-Murcia, A.; Torres, R.; Fernandez-Lafuente, R. *Chemical Society reviews* **2013**, *42*, 6290.

(9) Halim, S. F. A.; Harun Kamaruddin, A. *Process Biochemistry* **2008**, *43*, 1436.

(10) Salis, A.; Pinna, M.; Monduzzi, M.; Solinas, V. *Journal of*

Molecular Catalysis B: Enzymatic **2008**, *54*, 19.

(11) Dhake, K. P.; Thakare, D. D.; Bhanage, B. M. *Flavour and Fragrance Journal* **2013**, *28*, 71.

(12) Claon, P. A.; Akoh, C. C. *Enzyme Microb Tech* **1994**, *16*, 835.

(13) Petri, A.; Marconcini, P.; Salvadori, P. *Journal of Molecular Catalysis B: Enzymatic* **2005**, *32*, 219.

(14) Stergiou, P. Y.; Foukis, A.; Filippou, M.; Koukouritaki, M.; Parapouli, M.; Theodorou, L. G.; Hatziloukas, E.; Afendra, A.; Pandey, A.; Papamichael, E. M. *Biotechnology advances* **2013**.

(15) Chen, B.; Pernodet, N.; Rafailovich, M. H.; Bakhtina, A.; Gross, R. A. *Langmuir : the ACS journal of surfaces and colloids* **2008**, *24*, 13457.

(16) Ursoiu, A.; Paul, C.; Kurtan, T.; Peter, F. *Molecules* **2012**, *17*, 13045.

(17) Margolin, A. L.; Navia, M. A. *Angew Chem Int Edit* **2001**, *40*, 2204.

(18) Sheldon, R. A.; van Pelt, S. *Chemical Society reviews* **2013**, *42*, 6223.

(19) Kirk, O.; Christensen, M. W. *Organic Process Research & Development* **2002**, *6*, 446.

(20) Cantone, S.; Ferrario, V.; Corici, L.; Ebert, C.; Fattor, D.; Spizzo, P.; Gardossi, L. *Chemical Society reviews* **2013**, *42*, 6262.

(21) Katchalski-Katzira, E.; Kraemer, D. M. *Journal of Molecular Catalysis B: Enzymatic* **2000**, *10*, 157.

(22) J.Felipe Díaz; Jr, K. J. B. *Journal of Molecular Catalysis B:*

Enzymatic **1996**, *2*, 115.

(23) Gamba, M.; Lapis, A. A. M.; Dupont, J. *Advanced Synthesis & Catalysis* **2008**, *350*, 160.

(24) de los Ríos, A. P.; van Rantwijk, F.; Sheldon, R. A. *Green Chemistry* **2012**, *14*, 1584.

(25) Cho, H.-J.; Lee, S.-M.; Jung, S.; Lee, T.-K.; Yoon, H.-J.; Lee, Y.-S. *Tetrahedron Letters* **2011**, *52*, 1459.

(26) Shaw, J. F.; Lo, S. *J Am Oil Chem Soc* **1994**, *71*, 715.

(27) De Diego, T.; Manjón, A.; Lozano, P.; Vaultier, M.; Iborra, J. L. *Green Chemistry* **2011**, *13*, 444.

(28) Persson, M.; Bornscheuer, U. T. *Journal of Molecular Catalysis B: Enzymatic* **2003**, *22*, 21.

(29) De Diego, T.; Lozano, P.; Abad, M. A.; Steffensky, K.; Vaultier, M.; Iborra, J. L. *Journal of biotechnology* **2009**, *140*, 234.

초록

고정화 된 효소는 일반적인 효소에 비해서 분리가 간편할 뿐만 아니라 반응의 안정성, 향상된 반응성과 재사용성에서 이점을 갖는 생체 촉매이다. 특히 고정화 된 리파아제를 사용한 지방산 에스터의 합성은 환경 친화적인 반응이라는 점에서 많은 관심을 받고 있다. *Candida antarctica* lipase B (CALB)는 산업에서 쓰이는 리파아제의 한 종류로써, 용해성을 가지는 리파아제에 비해 상대적으로 넓은 범위의 pH와 온도에서 안정하여 고정화 효소 형태로서 에스터화 반응에 많이 쓰인다.

본 논문에서는 환경 친화적인 고정화 생체 촉매를 개발하는데 있어서 이온성 분자를 폴리아크릴산 고분자 비드에 공유 결합시키는 방법을 사용하였다. 이온성 액체 부분에 알킬기를 커플링함으로써 고분자 지지체의 소수성기를 조절하였으며, 리파아제를 안정화시키는 미세 환경을 제공하여 고정화된 리파아제의 활성을 증대시키고자 하였다. 폴리아크릴산 비드는 높은 친수성을 보이나, 알킬화된 이온성 액체가 그래프팅됨으로써 양쪽성을 띄게 되어 고정화된 리파아제가 최적의 활성을 유지할 수 있게 하였다.

이렇게 이온성 액체가 도입된 폴리아크릴산 비드에 리파아제를 고

정화하여, 에스터화 반응의 촉매로 이용하였다. 또한 3차 부탄올을 용매로 사용함으로써 극성을 띄는 부산물이 리파아제의 활성 부위를 차단하는 것을 막을 수 있었다. 특히, 리파아제를 단일 코팅시킨 1-옥틸-3-프로필이미다졸륨이 그래프트 된 폴리아크릴산 비드를 촉매로 사용, 메틸 올레이트를 합성하였을 때 50도에서 95%의 좋은 수율을 나타내었다. 또한 3회 재사용함으로써 재사용의 가능성을 확인할 수 있었다.

주요어: 생체 촉매 반응, 리파아제, 에스터화, 이온성 액체가 도입된 폴리아크릴산 비드

학번: 2012-20960

Appendix

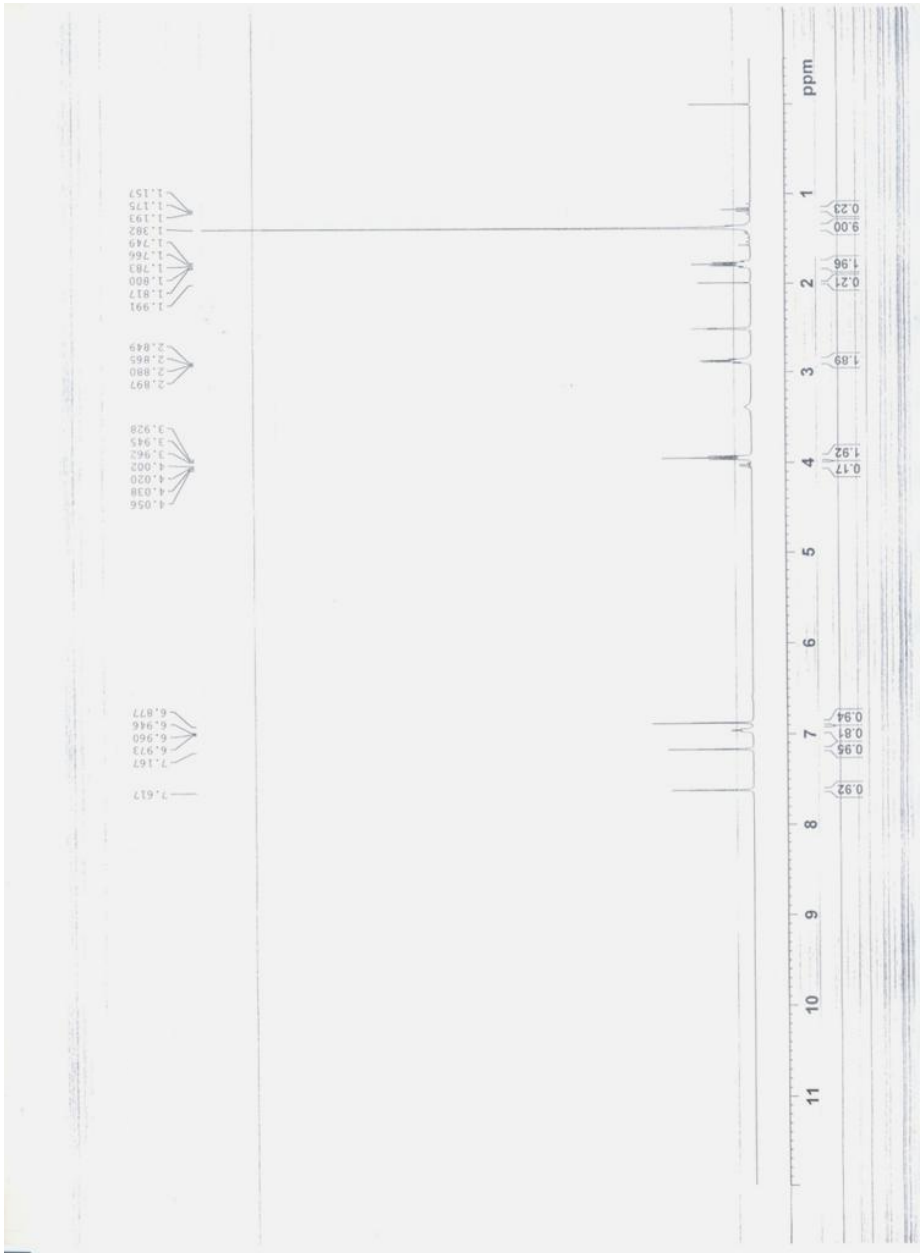


Figure A.1. [1]H NMR data of *N*-boc-1-(3-aminopropyl)imidazole.

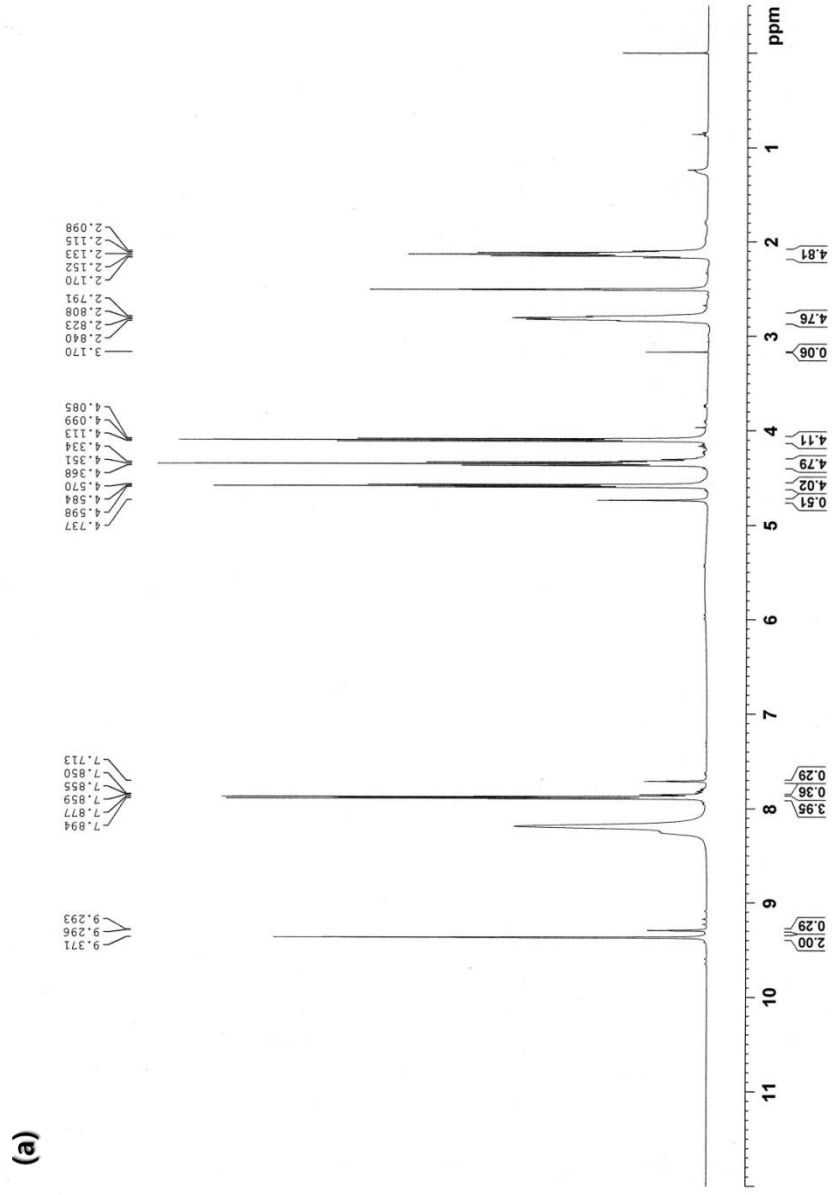


Figure A.2. [1]H NMR data of (a) 1-octyl-3-aminopropylimidazolium chloride, and (b) 1-dodecyl-3-aminopropylimidazolium chloride.

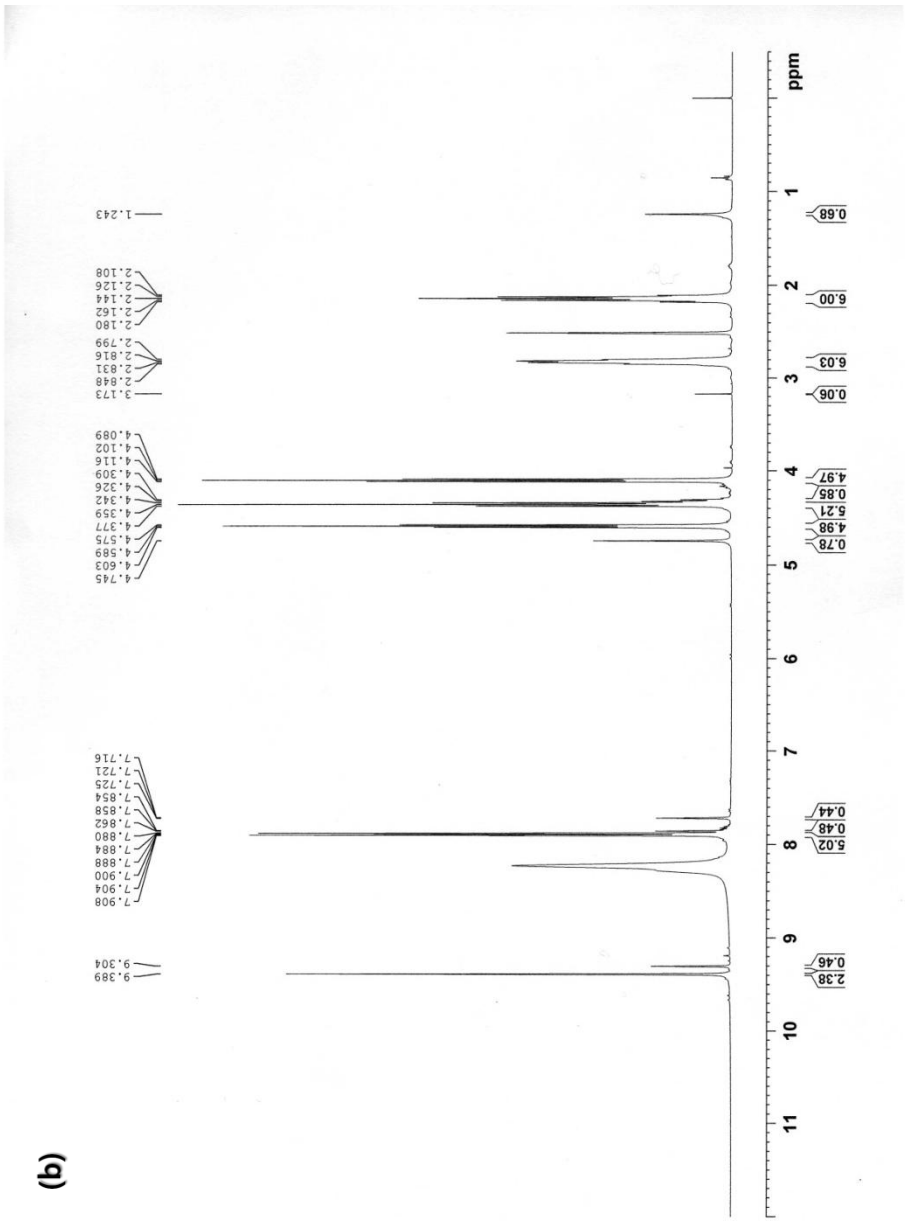


Figure A.2. [1]H NMR data of (a) 1-octyl-3-aminopropylimidazolium chloride, and (b) 1-dodecyl-3-aminopropylimidazolium chloride.