

Characterization of *Proteus vulgaris* K80 Lipase Immobilized on Amine-Terminated Magnetic Microparticles

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Received: April 7, 2014
Revised: June 18, 2014
Accepted: July 4, 2014

First published online
July 7, 2014

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pISSN 1017-7825, eISSN 1738-8872

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Proteus vulgaris K80 lipase was expressed in *Escherichia coli* BL21 (DE3) cells and immobilized on amine-terminated magnetic microparticles (Mag-MPs). The immobilization yield and activity retention were 84.15% and 7.87%, respectively. A homology model of lipase K80 was constructed using *P. mirabilis* lipase as the template. Many lysine residues were located on the protein surface, remote from active sites. The biochemical characteristics of immobilized lipase K80 were compared with the soluble free form of lipase K80. The optimum temperature of K80-Mag-MPs was 60°C, which was 20°C higher than that of the soluble form. K80-Mag-MPs also tended to be more stable than the soluble form at elevated temperatures and a broad range of pH. K80-Mag-MP maintained its stable form at up to 40°C and in a pH range of 5.0–10.0, whereas soluble K80 maintained its activity up to 35°C and pH 6.0–10.0. K80-Mag-MPs had broader substrate specificity compared with that of soluble K80. K80-Mag-MPs showed about 80% residual relative activity after five recovery trials. These results indicate the potential benefit of K80-Mag-MPs as a biocatalyst in various industries.

Keywords: Lipase, *Proteus vulgaris*, immobilization, amine-terminated magnetic microparticle

Introduction

Lipase (triacylglycerol acylhydrolase, E.C. 3.1.1.3) catalyzes the hydrolysis, alcoholysis, esterification, and transesterification of carboxylic esters. It functions under aqueous conditions to hydrolyze the ester bonds in triacylglycerol and to liberate fatty acids and glycerol [3]. Lipase is applied widely for biodiesel production, biosensor construction, polymer synthesis, and functional lipid production [5, 11, 13, 16].

Lipase K80 is an extracellular alkaline enzyme produced from the facultative anaerobic bacteria *Proteus vulgaris*, which was isolated from soil collected near a sewage disposal plant in Korea. The lipase gene was cloned and expressed in *Escherichia coli* [6]. The enzyme can be produced at a high yield and it has high specific hydrolytic activity and high stability in various organic solvents [17].

Practical use of lipase presents several technical difficulties, such as contamination of the product with residual enzymatic activity and high cost. As a solution to these

problems, the enzyme is usually used in an immobilized form so it can be recovered easily to reduce the cost and improve product quality. Immobilized enzymes are defined as enzymes that are physically confined or localized to a defined space while retaining their catalytic activities. Adsorption, covalent binding, entrapment, encapsulation, and cross-linking are methods to immobilize lipases [14].

The advantage of using microparticles (MPs) for enzyme immobilization is their ability to support the maximum surface area available to immobilize an enzyme. MPs also show minimal steric hindrance for reactants to access the enzyme's active sites. Furthermore, magnetic MPs (Mag-MPs) can be separated easily from the reaction solution by applying an external magnet. Immobilization is achieved through covalent binding between the enzyme and the particle surface [10].

In this study, lipase K80 was immobilized onto amine-terminated Mag-MPs. We characterized the biochemical properties of lipase K80 and compared them with those of the soluble K80 enzyme.

Materials and Methods

Materials

p-Nitrophenyl esters, tributyrin, tricaprylin, fish oil, coconut oil, olive oil, and soybean oil were purchased from Sigma-Aldrich (St. Louis, MO, USA). Castor oil was acquired from the OCI Co. Ltd. (South Korea). Palm oil was purchased from NewDia (Seoul, South Korea). Waste cooking oil was acquired from recycled family-used oil. BcMag amine-terminated 1 μ m Mag-MPs were purchased from Bioclone Inc. (San Diego, CA, USA).

Production of *P. vulgaris* Lipase K80

The *P. vulgaris* lipase K80 gene [6] was transformed in *E. coli* BL21 (DE3). The transformed cells were cultured in LB medium containing 100 μ g/ml of ampicillin. A seed culture was prepared at 37°C overnight, and the main culture (200 ml) was incubated at 20°C. After the optical density at 600 nm (OD_{600}) reached 0.5, 1 mM isopropyl thio- β -D-galactoside was added and the cells were cultured for an additional 16 h.

The cultured cells were harvested by centrifugation (6,000 $\times g$, 10 min) and resuspended in 5 ml of distilled water. The cells were lysed with an ultrasonicator, and the soluble fraction was obtained by centrifugation (10,000 $\times g$, 10 min). This cell-free extract was utilized for immobilization.

Homology Modeling

The amino acid sequence of *P. vulgaris* lipase K80 (GenBank Accession No. AAB01071.1) was submitted to the SWISS-MODEL homology modeling server [1, 2], and modeling was performed in the automatic mode. The homology model was based on the crystal structure (PDB code: 4GW3) of *P. mirabilis* lipase with 80% amino acid identity [9].

Immobilization of Lipase K80

Amine-terminated Mag-MPs were prepared with 100 mM EDTA (pH 7.0) solution to reduce their ionic strength. Then, the amine-terminated Mag-MPs (6 mg) were mixed with lipase K80 solution (1.98 mg lipase in 200 μ l) and incubated at 4°C for 2 h on a rotary shaker. The enzyme precipitate coating immobilization technique (Fig. 1) was used to precipitate lipase K80 for 30 min using ammonium sulfate (50% saturation). Glutaraldehyde (GA) was then added at a final concentration of 0.1% (v/v) at 4°C, using a rotary shaker overnight. After washing with 10 mM phosphate buffer (pH 7.0), the unbound aldehyde groups of GA were blocked in 100 mM Tris-HCl buffer (pH 7.4), and the sample was washed with 10 mM phosphate buffer (pH 7.0) and stored at 4°C until use. Lipase immobilization yield (η) was defined as

$$\eta(\%) = \frac{U_A - U_E}{U_A} \times 100$$

where U_A is the activity of soluble lipase K80 and U_E is that of the supernatant after immobilization. Activity retention (R) of the immobilized enzyme was calculated as

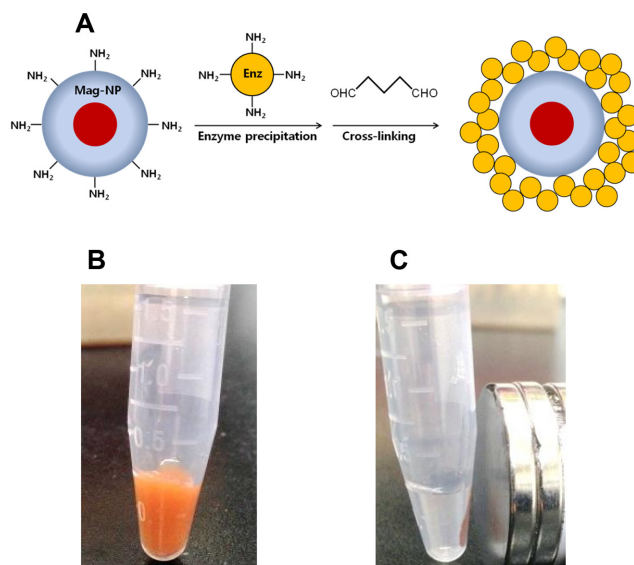


Fig. 1. Immobilization of lipase K80.

(A) Schematic representations of enzyme precipitate coating onto Mag-MP [8]. Immobilized K80 suspension (B) and aggregate (C) are shown.

$$R(\%) = \frac{U_H}{U_A - U_E} \times 100$$

where U_H is the activity of the immobilized enzyme.

Measurement of Immobilized Enzyme Activity

Activities of free and immobilized lipase K80 were measured by hydrolysis of *p*-nitrophenyl caprylate (pNPC) at 35°C in 50 mM Tris-HCl buffer (pH 8.0) for 3 min. The concentration of the hydrolysis product, *p*-nitrophenol (pNP), was measured using a spectrophotometer at 405 nm. One unit of lipases activity was defined as the amount of lipases required to release 1 μ mol of pNP under the assay conditions.

Effect of Temperature on Lipase Activity and Stability

The optimal temperatures for both forms of lipase were determined by assaying their activity toward pNPC at various temperatures (20–85°C), using spectrophotometry. Lipase stability at various temperatures was determined by pre-treatment of the enzyme at 20–60°C for 30 min, and activity was measured under standard conditions.

Effect of pH on Lipase Activity and Stability

The optimal pHs of both forms of lipase were determined by assaying their activity toward pNPC at various pHs (7.0–11.0), using spectrophotometry. The stability of the immobilized lipase under various pHs was determined by pretreating the enzyme in 50 mM NaCH₂COOH buffer (pH 4.0–6.0), 50 mM KH₂PO₄-K₂HPO₄ buffer (pH 6.0–8.0), 50 mM Tris-HCl buffer (pH 8.0–9.0), and

glycine-KCl-KOH buffer (pH 9.0–11.0) for 30 min. Thereafter, the activity was measured under standard conditions.

Substrate Specificity

The hydrolysis rates of various substrates, including tributyrin, tricaprylin, olive oil, castor oil, fish oil, soybean oil, palm oil, coconut oil, and waste cooking oil, were measured using the pH STAT method at 35°C [7]. Oil emulsions containing 1% oils and 1% gum arabic were prepared in a Waring blender at maximum speed for 2 min. A lipase suspension (50 µl) was added to the substrate emulsion solution (20 ml) that was previously adjusted to pH 8.0 by adding 10 mM NaOH solution. The release rate of free fatty acids was measured at 30°C for 5 min with a 718 Titrino pH titrator (Metrohm, Herisau, Switzerland). The amount of enzyme catalyzing the release of 1 µmol of fatty acid per minute was defined as one unit.

The hydrolysis rates of various synthetic substrates, including *p*NP-acetate (C₂), *p*NP-butyrate (C₄), *p*NP-caproate (C₆), *p*NP-caprylate (C₈), *p*NP-caprate (C₁₀), and *p*NP-laurate (C₁₂), were measured using an established spectrophotometric method. A different assay method was used for *p*NP-laurate (C₁₂), *p*NP-myristate (C₁₄), *p*NP-palmitate (C₁₆), and *p*NP-stearate (C₁₈) [11]. Lipase solutions were added to 0.88 ml of reaction buffer containing 50 mM Tris-HCl (pH 8.0), 0.1% gum arabic, and 0.2% deoxycholate. After a 3 min incubation at 35°C, the reaction was initiated by adding 0.1 ml of 8 mM substrate solubilized in isopropanol, followed by a 3 min incubation at 35°C. The reaction was stopped by adding 0.5 ml of 3 M HCl. After centrifugation at 12,000 rpm for 2 min, 333 µl of the supernatant was mixed with 1 ml of 2 M NaOH, and the OD_{420 nm} was measured.

Recovery Assay

The immobilized K80 was recovered by applying an external magnet for 2 min and resuspending the enzyme in 10 mM phosphate buffer (pH 7.0) for use in the next assay. This recovery process was repeated 10 times to evaluate the magnet efficiency to recover immobilized K80.

Results and Discussion

Immobilization of Lipase K80

Immobilization yield was determined by the immobilization method and the amount of soluble enzyme used. We mixed 200 µl (6 mg) of Mag-MPs and 8 mg of protein (1.98 mg of

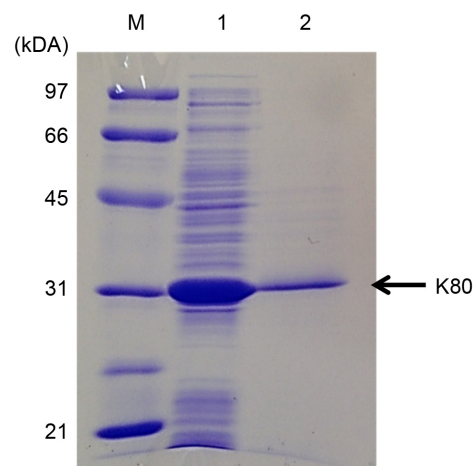


Fig. 2. SDS-PAGE of supernatant after immobilization.

Lane M, standard proteins; lane 1, *E. coli* cell-free extract; lane 2, supernatant after immobilization reaction. The size of lipase K80 is about 31 kDa.

lipase). The immobilization yield of lipase K80 was as high as 75.45% (Table 1). This result was supported by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis result (Fig. 2) showing a relatively small amount of protein left in the supernatant. One important factor to increase the binding capacity of Mag-MPs was the ammonium sulfate precipitation process. Because Mag-MPs tend to precipitate at the bottom of the tube under static conditions, precipitating protein has a greater chance for binding. Another important factor was the glutaraldehyde treatment, which led to the formation of multiple layers of lipase K80 protein onto a single Mag-MP (Fig. 1). Activity retention of the lipase K80 enzyme after immobilization was 8.2% (Table 1). Enzyme activity after immobilization depends mostly on the enzyme adsorption and crosslinking efficiency. Additionally, it may be determined by the accessibility of enzyme molecules within pores and by the diffusion velocity of substrate molecules [15].

Homology Modeling of K80 Lipase

A homology model of K80 lipase was constructed to

Table 1. Immobilization yield and activity retention of lipase K80.

Immobilization time (h)	Residual lipase activity in supernatant (U) ^a	Immobilization yield (η, %)	Immobilized enzyme activity (U) ^b	Activity retention (R, %)
0	3,962	-	-	-
20	975	75.45 ± 7.79	245 ± 15.89	8.2 ± 0.29

^aHydrolytic activity of soluble lipase K80 in the supernatant was measured using *p*NPC method.

^bHydrolytic activity of immobilized lipase K80 on beads was measured using *p*NPC method.

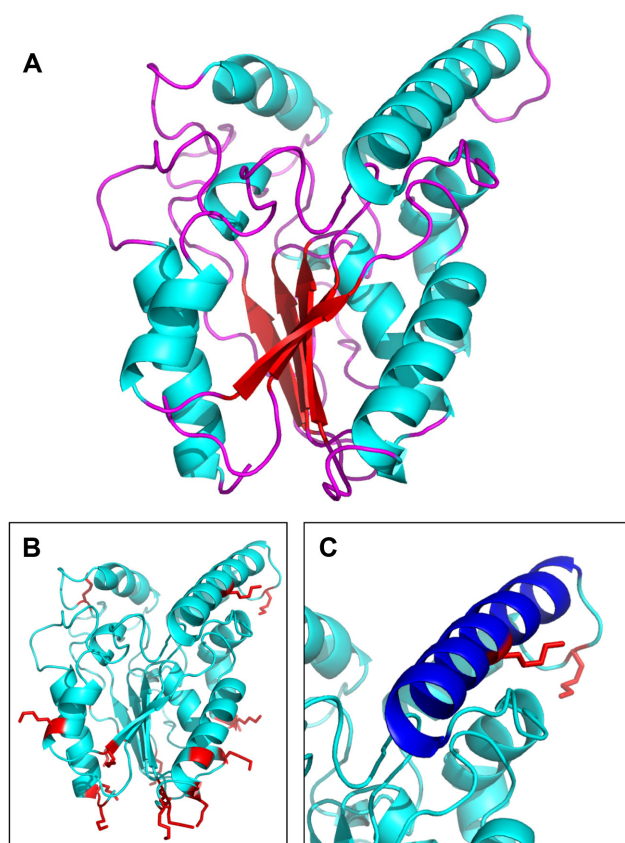


Fig. 3. Homology model of lipase K80.

(A) The homology model of lipase K80 was constructed using the SWISS-MODEL program based on the *P. mirabilis* lipase as a template structure (4GW3). α -Helix (cyan), β -sheet (red), and loop (magenta) are shown in the structure. (B) The backbone structure is shown (cyan). Lysine residues are marked with red color. (C) Active site and lid helix (blue) are shown.

explain the cross-linking mechanism. The X-ray crystal structure of *P. mirabilis* lipase has been elucidated (4GW3) [9]. *P. mirabilis* lipase contained the α/β -hydrolase fold of typical lipases. Protein sequence alignment of *P. mirabilis* lipase and lipase K80 showed 80% identity.

The homology model of lipase K80 (Fig. 3A) was constructed through the SWISS-MODEL homology modeling server using the *P. mirabilis* structure as the template. *P. mirabilis* lipase contains the typical catalytic triad (Ser79, Asp232, and His254) and the oxyanion hole is composed of His14 and Gly15 [9]. The corresponding sequences of K80 lipase are highly conserved. The sequence alignment and conserved catalytic machinery suggest that the crystal structure of *P. mirabilis* lipase affords an appropriate template for constructing a homology model of K80 lipase.

In fact, the homology model of K80 lipase superimposed well with the *P. mirabilis* lipase structure and possessed the low energy of the backbone and most side chains.

Lipase K80 had 19 Lys residues in a total of 290 amino acid residues. Their ϵ -amino groups made imine bonds with the aldehyde groups of glutaraldehyde. According to the homology model, almost all Lys residues were located on the surface of the protein (Fig. 3B), which could be used for inter- and intra-protein cross-linking. The active site pocket had no Lys residues, and the lid helix had one Lys residue in the middle (Fig. 3C). The Lys residue seemed to have little effect on the covalent cross-linking process.

Effects of Temperature and pH on Lipase Activity and Stability

Lipase K80 was isolated from *P. vulgaris* that grew optimally in a facultative anaerobic environment at 37–42°C [12]. We tried to examine the effect of temperature on the soluble and immobilized enzymes. The soluble form of this enzyme showed optimum activity at 40°C. However, a 20°C shift in the optimum temperature was observed after enzyme immobilization (Fig. 4A). This result may be caused partly by an increase in enzyme thermostability.

As shown in Fig. 4A, the enzyme activity increased significantly for every 10°C increase in temperature. Assuming the enzyme is stable at high temperatures, the productivity of the reaction can be increased significantly by operating the reaction at a relatively high temperature. Consequently, thermostability is a desirable characteristic of lipases [13]. The thermostabilities of soluble and immobilized K80 were determined by measuring residual activities after a 30 min incubation. As shown in Fig. 4B, soluble K80 maintained its stability at up to 35°C and started losing its activity slowly at 40°C. At 50°C, the enzyme activity decreased rapidly until it was totally inactivated at 60°C. In contrast, the immobilized K80 was stable up to 40°C before its activity started to drop slowly at 45°C. Moreover, immobilized K80 retained noticeable activity at 65°C. A different result occurred between these two conditions because thermostability is influenced by environmental factors, such as the presence of metal ions from the Mag-MPs. In addition, the immobilization process can increase the thermostability of an enzyme through covalent cross-linking [4].

The optimum pH was 9.0 for both the soluble and immobilized K80 lipases (Fig. 4C). Soluble K80 maintained its activity of >80% at pHs of 6–11 (Fig. 4D), whereas the immobilized enzyme maintained activity of >80% at pHs of

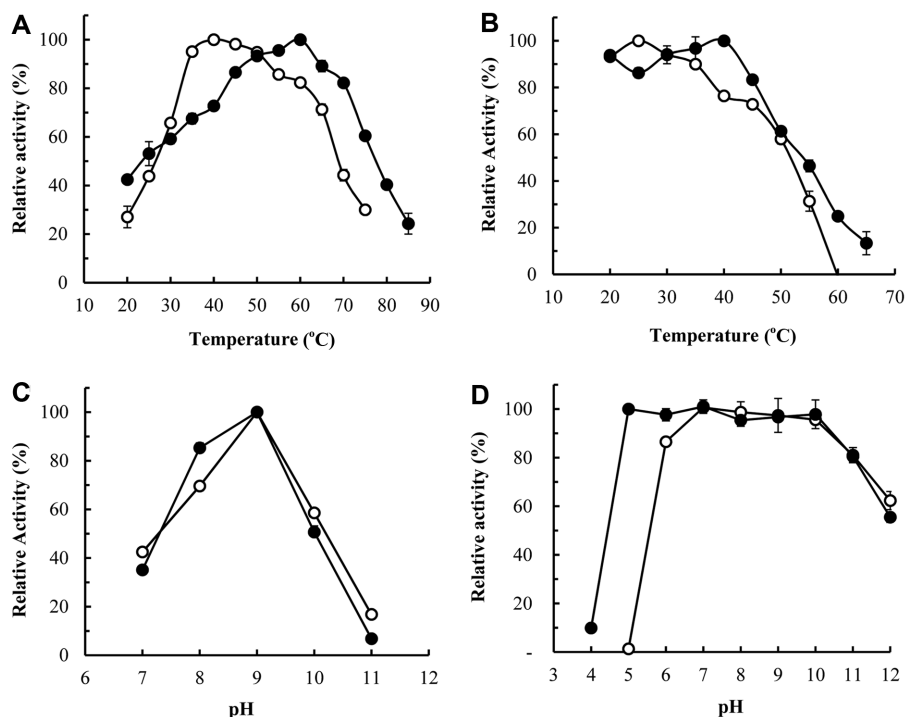


Fig. 4. Effects of temperature and pH on enzyme activity and stability.

Lipase K80 was assayed at various temperature (A) and pH values (C). For stability test, lipase K80 was assayed after incubation of the reaction mixture at various temperatures (B) and pH (D) for 30 min. The error bar indicates the SD from three independent experiments. Open circles represent the soluble form and close circles represent the immobilized form of lipase K80.

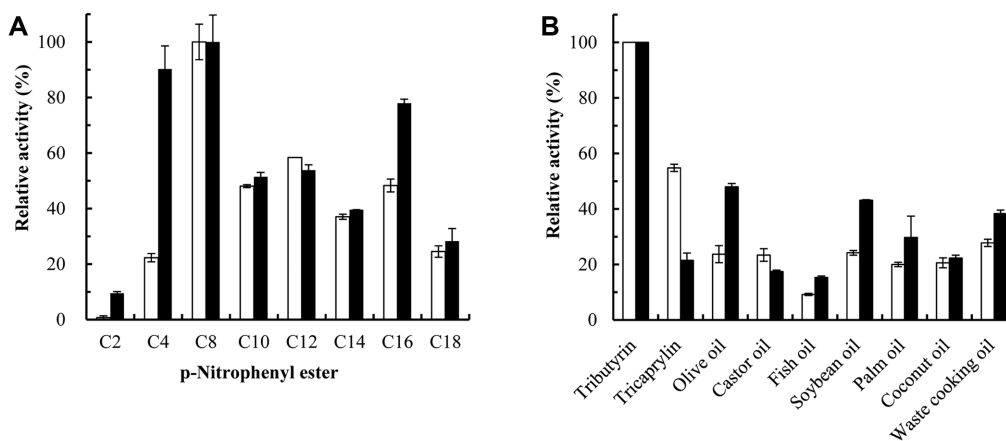


Fig. 5. Substrate specificities of soluble and immobilized lipase K80 toward various *p*-nitrophenyl esters (A) and triglycerides (B).

The error bar indicates the SD derived from three independent experiments. White bars denote the soluble form and black bars denote the immobilized form of lipase K80.

5–11 (Fig. 4D). These results indicate the stability of both enzymes over a wide range of pHs, particularly under alkaline conditions.

Substrate Specificity

Hydrolyzing activities toward various *p*-nitrophenyl esters with different acyl-chain lengths (C₂–C₁₈) and oil

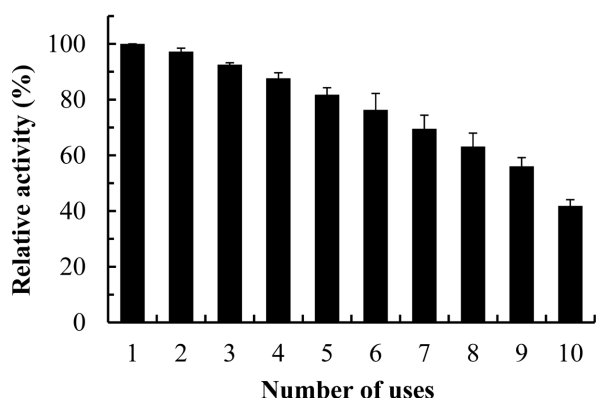


Fig. 6. Reusability of immobilized lipase K80.

Lipase activity toward *p*NPC was assayed after several recovery steps. The error bar indicates the SD derived from three independent experiments.

types were measured to examine the substrate specificity of the K80 lipases. As shown in Fig. 5A, both enzymes had their highest activity toward the *p*NPC (C_8) substrate. Immobilized lipase K80 had relatively high activity toward C_4 and C_{16} in comparison with that of soluble K80.

Both enzymes showed their highest activity toward tributyrin (Fig. 5B). They hydrolyzed various oils, such as tributyrin, tricaprylin, olive oil, castor oil, fish oil, soybean oil, palm oil, coconut oil, and waste cooking oil, with somewhat different hydrolytic activities.

Recovery Assay

One of the most interesting ways to use immobilized enzymes is through their ability to be recovered and used multiple times [14]. In this study, the recovery ability of immobilized K80 was evaluated by repeating the *p*NPC assay 10 times. The immobilized K80 activity decreased gradually but retained >80% of initial activity after five reactions and about 42% after 10 reactions (Fig. 6). This result suggests that the recovery method using an external magnet was very efficient, and that this immobilization method could reduce enzyme cost in an industrial process.

In conclusion, lipase K80 was successfully immobilized and biochemically characterized. The immobilized form of lipase K80 had a higher optimum temperature in comparison with that of the soluble form. Furthermore, the immobilized K80 was more stable in a broad range of temperatures and pHs. The immobilized K80 also had efficient recovery activity even after five reactions. These results indicate the potential benefit of using immobilized

K80 as a biocatalyst in various industries in comparison with its soluble form.

Acknowledgments

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korea Government (MSIP) (2014R1A2A2A01006978).

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