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Development of Magnetically Separable Immobilized Lipase by Using Cellulose Derivatives and Their Application in Enantioselective Esterification of Ibuprofen

Lee, Gowoun[1], Hongil Joo[1], Jungbae Kim[2], and Jung-Heon Lee[1]*

[1]*Department of Chemical Engineering, Chosun University, Kwangju 501-759, Korea*
[2]*Department of Chemical and Biological Engineering, Korea University, Seoul 136-701, Korea*

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Highly active, stable, and magnetically separable immobilized enzymes were developed using carboxymethyl cellulose (CMC) and diethylaminoethyl cellulose DEAE-C; hereafter designated "DEAE" as supporting materials. Iron oxide nanoparticles penetrated the micropores of the supporting materials, rendering them magnetically separable. Lipase (LP) was immobilized on the surface of the supporting materials by using cross-linked enzyme aggregation (CLEA) by glutaraldehyde. The activity of enzyme aggregates coated on DEAE was approximately 2 times higher than that of enzyme aggregates coated on CMC. This is explained by the fact that enzyme aggregates with amine residues are more efficient than those with carboxyl residues. After a 96-h enantioselective ibuprofen esterification reaction, 6% ibuprofen propyl ester was produced from the racemic mixture of ibuprofen by using DEAE-LP, and 2.8% using CMC-LP.

Keywords: Enzyme stabilization, ibuprofen, racemic resolution

Enzyme stability and recovery are the key factors for the development of economically feasible enzyme processes. Several methods have been developed for the stabilization and recycling of enzymes *via* techniques related to nanobiotechnology. Recently, there has been a growing interest in using nanomaterials as carriers for enzyme immobilization [9, 12, 23]. Enzymes were immobilized on the surface of nanoparticles whose functional groups were modified or encapsulated into nano-sized conductive polymer capsules [13] and TiO_2 nanoparticles [21]. A new method has recently been developed for the immobilization of lipases (LPs) in hydrophobic sol-gel-derived silica matrices, with high activity yield and increased stability [2–4].

*Corresponding author
Phone: 82-62-230-7159; Fax: 82-62-230-7226;
E-mail: leejh@chosun.ac.kr

Proteins adsorbed on magnetic nanoparticles can be easily recovered using a magnet and recycled for iterative use such as immobilization carriers [1–3, 8, 11], bioseparation [4, 20], protein concentration [16], drug delivery systems [22], biofuel cell [10], and biomedical purposes [5, 14]. Most magnetic nanospheres consist of a superparamagnetic core embedded in a shell that protects the enzyme from contact with iron oxide. It has been demonstrated that enzymes immobilized on magnetic iron oxide nanoparticles can be easily recovered using a magnet and recycled for iterative uses.

LPs are versatile biocatalysts that can be exploited in many reactions in clinical analysis or for industrial purposes. LP was covalently immobilized on ferromagnetic azide polyethylene terepthalate (Dacron) by Pimentel *et al.* [19]. The immobilization of LP offers several advantages, such as the easy separation of products from the biocatalyst, improvement of enzyme stability, and easy enzyme recovery.

Selecting a suitable supporting medium for enzyme immobilization also increases the enzyme stability without any negative influence on its catalytic activity. In the present work, we demonstrate magnetically separable enzyme-supporting media by using cellulose derivatives. LP molecules were stabilized using developed supporting particles, and their long-term stabilities and recycling activities were measured and compared with those of free LPs. These immobilized enzymes have also been used for the enzymatic resolution of ibuprofen to ibuprofen ester.

Materials and Methods

Chemicals

Lipase from *Candida rugosa* type VII, glutaraldehyde, *p*-nitrophenyl butyrate (*p*NPB), *p*-nitrophenol, and nanoferrite were products of Sigma-Aldrich (St. Louis, MO, U.S.A.). Carboxymethyl cellulose (CMC) and diethylaminoethyl cellulose (DEAE-C; herein after, designated as "DEAE") was also purchased from Sigma-Aldrich.

Distilled and deionized water were used for preparation of aqueous buffer and solutions.

Preparation of Magnetically Separable Media

For a typical preparation, nanoferrite (100 mg) was introduced with 10 ml of swelled CMC and DEAE. This mixture was agitated vigorously for 2 h at 500 rpm. The mixture was washed with distilled water to remove ferrite nanoparticles that were located outside of the bead. The supernatant was decanted and the sediment was washed with distilled water several times until further free ferrite was observed. Finally, sediments were washed twice with 100 mM phosphate buffer and dried for future use.

Enzyme Immobilization Method

Magnetic porous particles (dry mass 2 mg) were incubated in 1 ml of 20 mM phosphate buffer (pH 6.5) containing 10 mg of LP. The vials were shaken at 200 rpm at room temperature for 30 min, and then moved into a refrigerator for an additional rocking at 30 rpm. After 2 h incubation at 4°C, GA solution was added (final GA concentration was 0.5% w/v), and the mixture was put on a rocker (30 rpm) at 4°C overnight. The enzyme aggregate coating on fibers were transferred to a new glass vial, and washed with 20 mM phosphate buffer (pH 6.5) and 100 mM Tris-HCl (pH 7.9). To cap the unreacted aldehyde groups, magnetic porous particles were incubated in Tris-HCl buffer for 30 min. After capping, magnetic porous particles were washed extensively with 20 mM phosphate buffer (pH 6.5) until no enzymes were observed in the washing solution. Finally, the samples were excessively washed until no enzyme was observed in the washing solution. The enzyme aggregates coated on fibers were stored in 10 mM phosphate (pH 6.5) at 4°C for further use.

Activity and Stability Measurement

The activities of free and immobilized lipases were measured with the product *p*-nitrophenol concentration, which was converted from 0.5 mM *p*NPB substrate by lipase. The reactant mixture consisted of 0.03 ml of 50 mM *p*NPB and 2.97 ml of 20 mM phosphate buffer (pH 6.5). After addition of free and immobilized lipase (10 μl of 0.1 mg/ml free LP and 50 μl of immobilized enzyme mixture), the activity was measured from the increase in the absorbance per minute at 400 nm. We determined the product concentration from the calibration curve between the absorbance reading and the *p*-nitrophenol concentration. The reaction mixture solution was shaken at 200 rpm. At the definite interval, 100 μl of aliquot was mixed with 900 μl of sodium phosphate buffer in a cuvette to measure the absorbance changes.

Chromatography Analysis

Gas chromatography was performed using a GC-2010 gas chromatography system (Shimadzu, Japan) equipped with a flame ionization detector (FID) and a DB-5MS column (60 m×0.25 mm× 0.25 μm). The injector temperature was maintained at 300°C, the detector temperature at 350°C, and the oven temperature at 180°C. The carrier gas used was nitrogen with an airflow rate of 12 ml/min. An external standard method was used to quantify the ester formed.

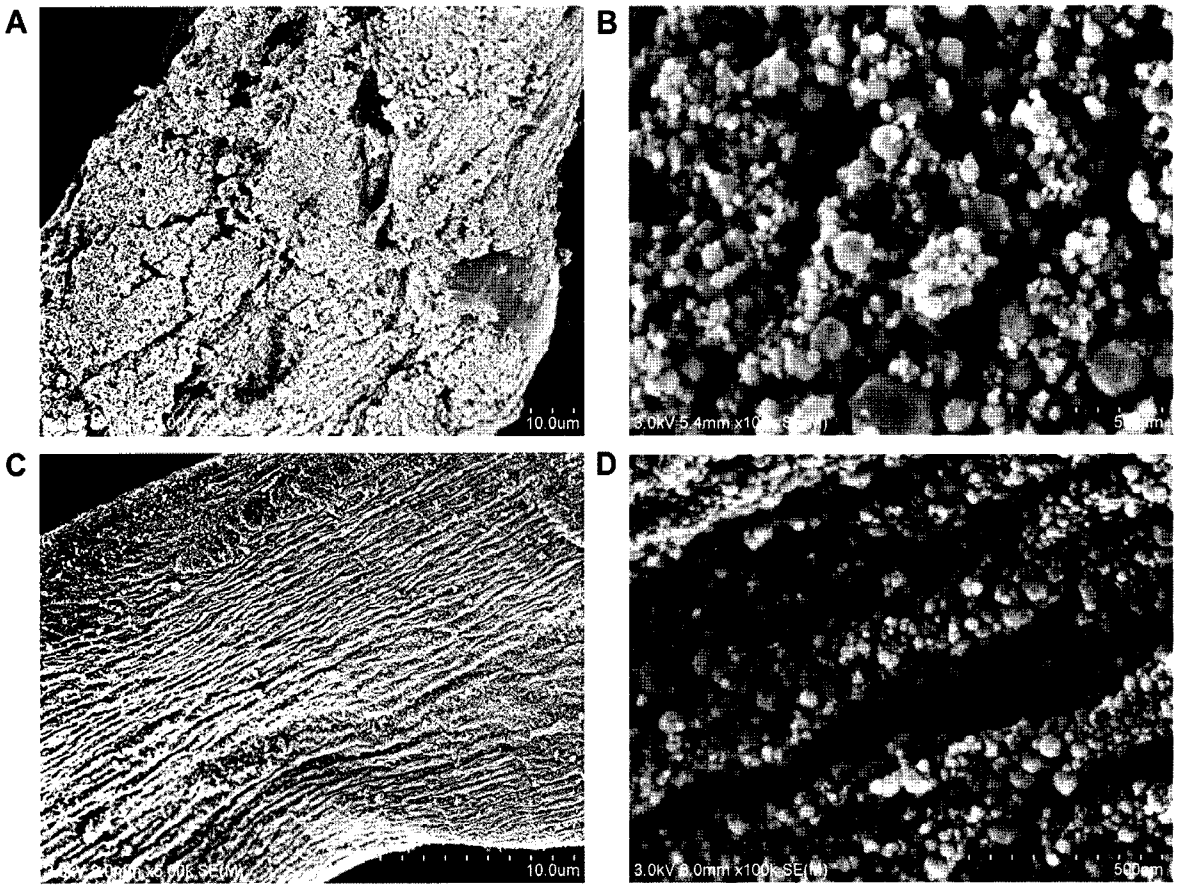

Fig. 1. SEM images of CMC-immobilized enzymes (**A**, **B**) and DEAE-immobilized enzymes (**C**, **D**).

Ibuprofen Esterification Using Immobilized Lipase

The changes in the concentrations of the reactant ibuprofen and its ester product were measured over a period of 96 h. The reaction system comprised an aqueous lipase enzyme solution (3% water in isooctane) in 5 ml of isooctane as an organic solvent. The reactant mixture comprised 10 mM ibuprofen and 100 mM propanol. The batch reaction was performed with orbital stirring at 150 rpm. Samples were obtained from the aqueous/organic emulsion at regular time intervals and analyzed by GC.

The mixture was incubated in a shaking incubator at 37°C and 150 rpm. The reaction was initiated by the addition of free and immobilized lipase. All experiments were carried out in duplicate. At specific sampling times, 0.1 ml of each sample was retrieved for GC analysis. At the end of the reaction, the immobilized lipase was separated by magnets and successfully recovered from the reaction mixture. The amount of ester (conversion degree) formed during the reaction was determined by gas chromatography.

Protein Assay

The protein concentration was determined with the Lowry method with the Folin reagent, and the absorbance was read at 660 nm with a spectrophotometer [15]. The protein concentration was determined using a Bio-Rad protein assay kit (Hercules, CA, U.S.A.) with bovine serum albumin (BSA) as a standard.

RESULTS AND DISCUSSION

SEM Images of Immobilized Enzymes

LP-coated diethylaminoethyl cellulose (DEAE) and carboxymethyl cellulose (CMC) fibers were observed in SEM analysis (Fig. 1). The images revealed nanoparticles of approximately 10–100 nm that covered the surface of fibers, and cavities were observed between the nanoparticles. Magnetic particles and enzymes were aggregated with the fibers. The DEAE surface was uniformly coated with the enzymes (Fig. 1C); however, that of CMC was rough (Fig. 1A) and was not coated locally with nanoparticles and enzymes. Aggregated magnetic particles and enzymes were tightly attached to the media.

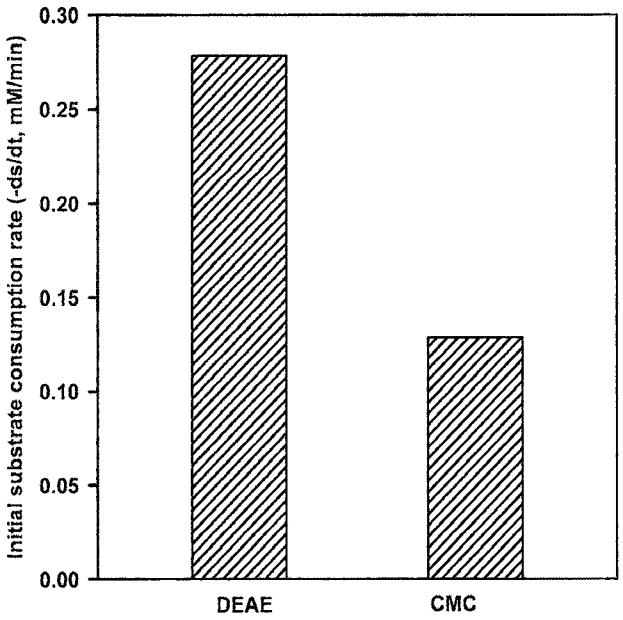

Fig. 2. Effect of supporting particles on the activity of immobilized enzyme (So=0.6 mM).
The initial activities of enzyme aggregates coated on DEAE and CMC. The activity was measured using the hydrolysis of *p*NPB in an aqueous buffer and normalized to the total mass of fibers after final washing.

Effect of Supporting Media on Enzyme Activity

The magnetic nanoparticles and enzymes were simultaneously entrapped in porous DEAE and CMC fibers, resulting in magnetically separable and heterogeneous biocatalysis. The activities of magnetic microporous enzymes were investigated by incubating the enzymes in an aqueous buffer solution containing 0.6 mM *p*-nitrophenyl benzoate (*p*NPB). Fig. 2 shows the initial substrate degradation rate of the LP-coated DEAE and LP-coated CMC under the same reaction conditions. DEAE contains diethyl amine moieties that can form covalent bonds with primary carboxyl groups on the enzyme and with glutaraldehyde, whereas CMC contains carboxyl moieties that can form covalent bonds with primary amine groups on the enzyme. The initial activity of the enzyme aggregates coated on DEAE was approximately 2 times higher than that of the enzyme aggregates coated on CMC. This is explained by the fact that enzyme aggregates with amine residues possess greater capacity than those with carboxyl residues.

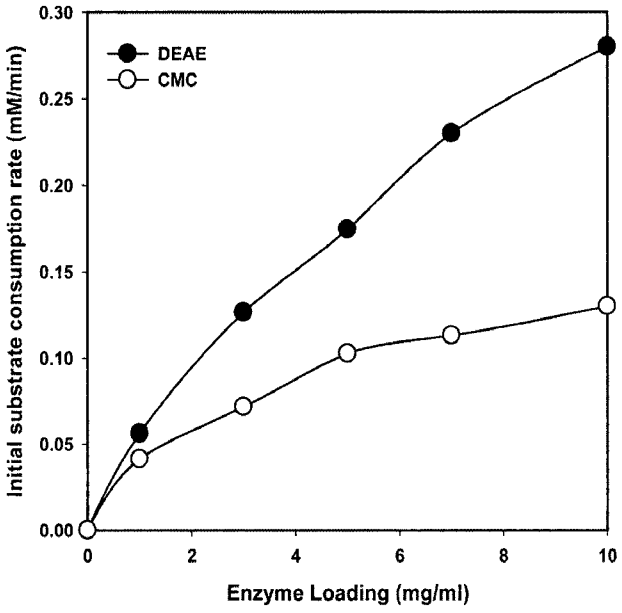

Fig. 3. Effect of enzyme loading on the immobilization of LP on CMC and DEAE.
Various amounts of LP (1, 3, 5, 7, and 10 mg) were dissolved in 1 ml of distilled water. Magnetically separable fibers (2 mg) were added, and immobilization was performed using 0.5% glutaraldehyde. The immobilized enzyme was successively washed with 20 mM phosphate buffer (pH 6.5) until no proteins were released into the solution.

Table 1. Catalytic activity of DEAE immobilized and CMC immobilized LPs.

	V_m (1/min)	K_m (mM)
DEAE	0.931	1.451
CMC	0.761	3.201

The LP activity was determined by the hydrolysis of *p*NPB (0.02–0.7 mM) in an aqueous buffer (20 mM phosphate, pH 6.5) at room temperature (30°C). Kinetic constants were obtained by using software (Enzyme Kinetics Pro from ChemSW, Farifield, CA, U.S.A.) that performs nonlinear regression based on the least-square method.

Effect of Enzyme Loading on Activity

The effect of enzyme loading on the activity of immobilized enzyme was measured. Enzyme loading was changed from 1 mg to 10 mg for immobilization, and the activity was measured; this is illustrated in Fig. 3. The activity of the enzyme immobilized on CMC showed a saturation curve with increased loading amount. However, enzyme activity with DEAE increased with enzyme loading until 10 mg (Fig. 3). With low enzyme loading, the activity difference between DEAE and CMC was small owing to high adsorption capacity; however, this difference increased with the loading amounts. This result showed that the immobilization capacity of DEAE was greater than that of CMC, which is shown in the SEM images. The amine residues of DEAE enhance the cross-linked aggregation of enzymes to a greater extent as compared with the carboxyl residues of CMC.

Immobilized Enzyme Kinetics

Kinetic parameters were estimated using the Michaelis-Menten equation with a change in the initial substrate *p*NPB concentration. The substrate concentration was changed from 0.02 to 0.7 mM, and the activity was measured with the initial substrate consumption rate. The kinetic constants (V_m and K_m) were obtained *via* linear regression based on the least-square method (Table 1). The amount of protein bound to the DEAE surface (2.07 mg) was 28% more than that bound to the CMC surface (1.62 mg). Hence, amine residues are better supporting functional residues than carboxylic acid residues. The maximum enzyme reaction constant of DEAE was 22% higher, and the apparent binding constant (K_m) was approximately half that of CMC. This suggests that CMC-immobilized enzymes have higher mass transfer resistance for the substrate. The reduced V_m can be interpreted as the small number of enzyme active sites due to the enzyme loading amount.

Enzyme Recovery by Using Magnets

Enzyme recycling by using magnets has proven to be an efficient method of recovering coated enzymes. Enzyme-coated magnetically separable fibers were developed for enzyme recovery after batch reactions [6, 7, 17]. Immobilized LP prepared using microporous magnetic particles was efficiently separated from a reactant in a reactor, as shown in Fig. 4. Immobilized enzymes were used for several cycles of activity measurement performed as described above. Between each assay, they were washed twice with a buffer (2 ml). At each cycle, the initial reaction rate was measured, and its relative activity was determined with the slope decrease. After 30 cycles, the activity of the recovered immobilized enzyme was 92% of its original activity. The limited amount of activity decreased after each cycle.

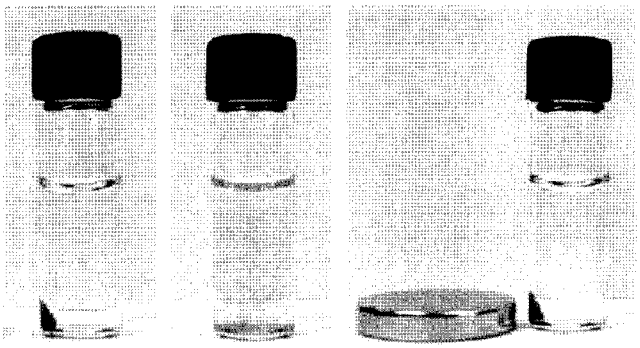

Fig. 4. Recovery of LP aggregate-coated magnetic microporous particles (DEAE) from the reaction solution by using magnets.

Stability of Immobilized Enzymes

Using immobilized LP prepared with magnetically separable DEAE and CMC, we measured the long-term stability

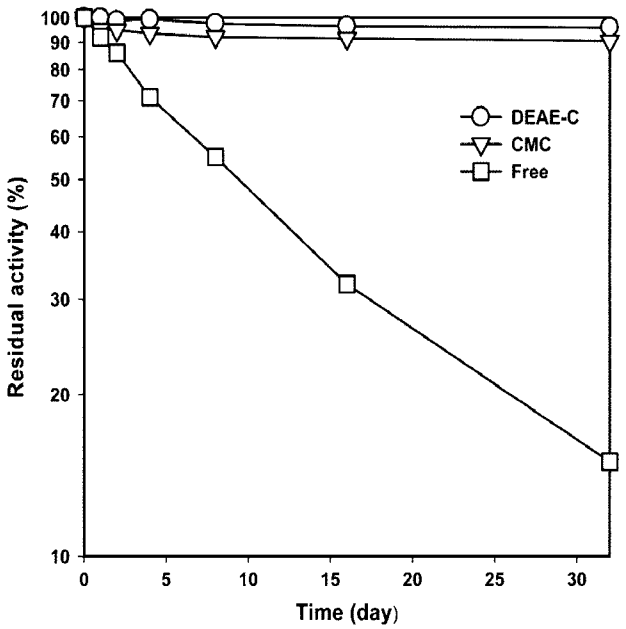

Fig. 5. The stabilities of free LP and LP aggregates coated magnetically separable DEAE and CMC in an aqueous buffer solution (20 mM phosphate, pH 6.5) at room temperature (25°C) under shaking conditions (200 rpm).

Immediately after each activity measurement, the sample was washed 3 times in excess buffer solution to remove both substrate and product. The relative activity was calculated from the ratio of residual activity at each time point to the initial activity.

under rigorous shaking conditions at 200 rpm. Fig. 5 shows that the LP immobilized using DEAE and CMC showed improved catalytic stability as compared with free LP. Immobilized LP with DEAE retained its stability for 4 days with no decrease in activity and showed 90% stability after a 30-day incubation period under room temperature conditions at 200 rpm. The developed immobilized enzyme was highly stable as compared with that with a covalent attachment, which showed 80% residual activity in 80 h [24]. The high stability of immobilized LP is due to a decreased denaturation rate attributed to cross-linked enzyme aggregation (CLEA).

Effect of Temperature on Enzyme Activity

The activities of LP immobilized with DEAE and CMC were measured at 3 different temperatures. Enzyme activity increased with temperature; however, LP activity decreased at high temperatures because of enzyme deactivation. The activity of DEAE-LP was higher than that of CMC-LP; this indicates that the enzyme coating characteristics are better with DEAE than with CMC (Fig. 6). At high temperatures, DEAE and CMC expanded, and the covalent bonds between the supporting materials and enzymes loosened. The liberated enzyme was rapidly deactivated owing to the high temperature.

Effect of Temperature on Enzyme Stability

LP stabilization by the covalent conjugation of DEAE and CMC could be explained by the increased rigidity of the active enzyme conformation due to the formation of intramolecular cross-links of enzymes with CMC and DEAE. The deactivation rate of CMC-LP was higher than that of DEAE-LP because of its weak covalent bonds (Fig. 7). DEAE-LP was highly stable at room temperature, and its original activity was maintained over a 6-day incubation period. The activity of CMC-LP was slightly decreased at room temperature, and its stability rapidly decreased with increased temperature [18]. As shown in Fig. 1, LPs are strongly attached to DEAE rather than to CMC.

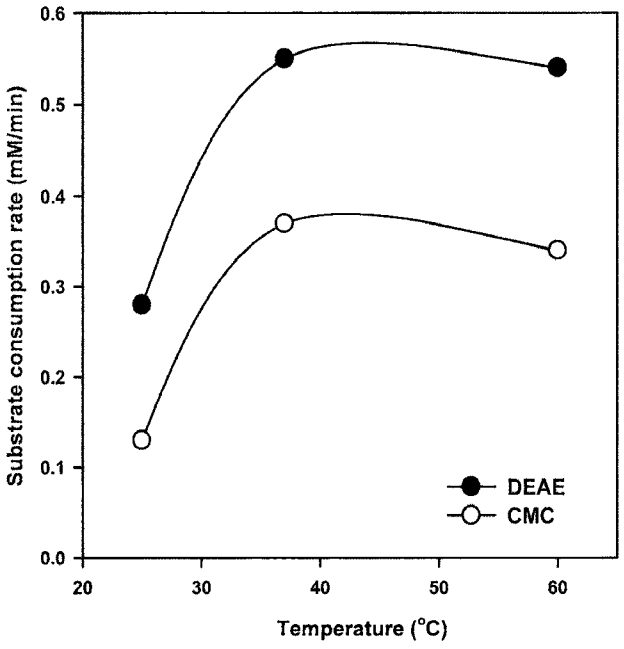

Fig. 6. Effect of temperature on the activity of immobilized LP.
The activity of immobilized LP rapidly increased with temperature. However, the activity decreased at high temperatures owing to enzyme deactivation.

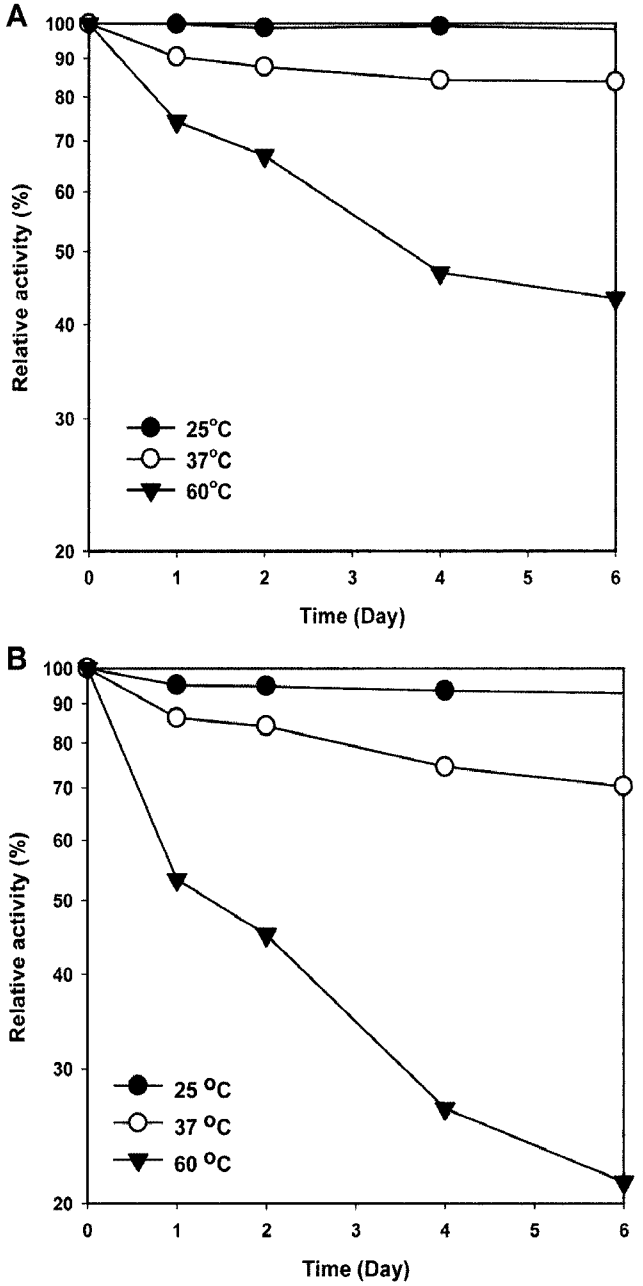

Fig. 7. Effect of temperature on the stability of LP immobilized on DEAE (**A**) and CMC (**B**).
The stability of LP at high temperatures is very low as compared with that at low temperatures.

Batch Conversion of Ibuprofen Using DEAE-LP and CMC-LP

We performed a systematic study on the enantioselective resolution of ibuprofen by using immobilized LP, using isooctane as a solvent and propanol as the esterification agent. The reaction system comprised 25 mg of immobilized LP in 5 ml of isooctane (3% water in isooctane) as an organic solvent. The reactant mixture comprised 10 mM ibuprofen and 100 mM propanol. The (*R*)-propyl ester of ibuprofen was synthesized *via* an immobilized LP-catalyzed esterification reaction between racemic ibuprofen and propanol in the presence of the organic solvent isooctane. The batch reaction was performed with orbital stirring at 150 rpm. LPs immobilized on magnetically separable CMC and DEAE catalysed the transesterification of (*R*)-ibuprofen by propanol. The rate of (*R*)-propyl ester production was higher when DEAE-LP was used than when CMC-LP was used (Fig. 8). Based on the changes in the V_m value with different supporting media, the production rate was approximately 2 times higher with DEAE-LP than with CMC-LP. After 96 h, approximately 6% racemic ibuprofen was converted to (*R*)-propyl ibuprofen ester following a reaction that used LP-coated magnetically separable DEAE, whereas only 2.8% was converted when CMC-LP was used.

Magnetically separable supporting particles have been successfully developed and are excellent candidates for enzyme immobilization. The developed materials have been used for LP immobilization to produce more stable and recyclable immobilized enzymes. Immobilized enzymes with DEAE, which has diethyl amine residues, showed better enzyme activity and stability than those with CMC, which has carboxyl residues. Both immobilized enzymes showed enhanced enzyme stability under rigorous shaking conditions. The propyl ester of ibuprofen was successfully synthesized using immobilized LP, and the production rate with DEAE-LP was approximately twice as high as that with CMC-LP.

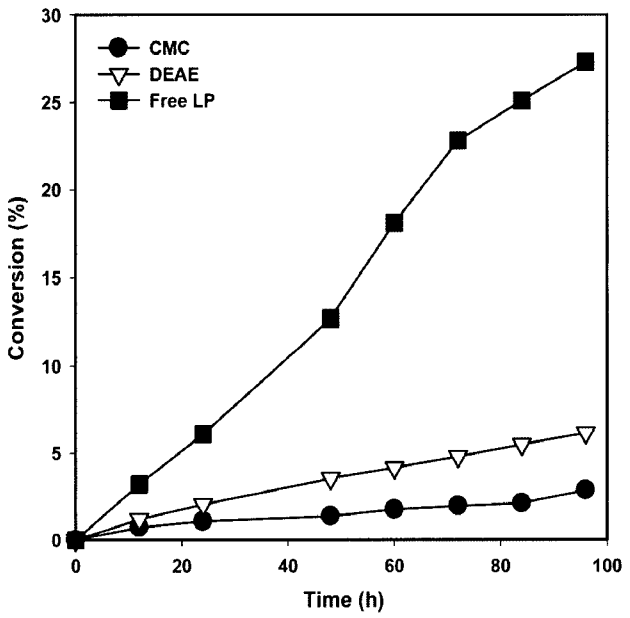

Fig. 8. Effect of enzymes on the time-course conversions of (*R*)- and (*S*)-ibuprofens (10 mM): 5 mg/ml free LP, immobilized LP (5 mg of immobilized enzyme/ml) at 37°C, and 3% water in isooctane.
The hollow and filled symbols represent conversions by using dispersed and bundled enzyme-immobilized fibers, respectively.

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