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Journal of Molecular Catalysis B: Enzymatic 54 (2008) 116–121
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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The use of polyaniline nanofibre as a support for lipase mediated reaction

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Received 9 July 2007; received in revised form 15 November 2007; accepted 15 November 2007
Available online 7 January 2008

Abstract

Highly stable and recoverable polianiline nanofibres are developed for enzyme immobilisation and recovery. *Candida rugosa* lipase (LP) was immobilised onto a polyaniline nanofibre with cross-linking for enzyme aggregation. The optimal LP loading was 5 mg LP/1 mg polyaniline. The stability of the immobilised LP was measured and shown to be high under vigorous shaking at room temperature. This polyaniline nanofibre LP was easily separable with low-speed centrifugation and repeatedly usable. LP immobilised on polyaniline nanofibre demonstrated high stereoselectivity in the kinetic resolution of racemic (*R*,*S*)-ibuprofen and improved the long-term stability as compared to that by the free enzyme, allowing the supported enzyme to be repeatedly used for a series of chiral resolution reactions. The conversion from racemic ibuprofen to a chirally selective compound, a prophilic ester of ibuprofen, was approximately 30% with free LP and approximately 10% with immobilised LP. The enantiomeric excess using immobilised LP after 96 h reaction was 0.884.

Keywords: Lipase; Polyaniline nanofibre; Immobilisation; Stereoselective; Ibuprofen

1. Introduction

An enzyme is produced to catalyse a biochemical reaction and is highly specific with regard to the type of biochemical reaction that it catalyses and to the substrate upon which it acts.

Various nanostructured materials, such as nanofibres, mesoporous materials and magnetic nanoparticles, have been used for protein immobilisation via approaches including adsorption, covalent attachment, protein encapsulation and sophisticated combinations of methods [1–7]. Although a variety of nanostructured materials and enzymes have been designed and tested, both stable and economically feasible enzyme systems are still under development for realising the successful use of enzymes in practical application [8–10]. Nanofibres offer a number of attractive features compared to the other nanostructures which relieves the mass transfer limitation of substrates/products due to their reduced thickness and make it easier to recover and reuse nanofibre than nanoparticles or carbon nanotubes.

An enzyme has many advantages over conventional chemical synthesis due to its specificity. Asymmetric synthesis with enzymes is used to convert a racemic substrate into an unequal amount of chiral product. Lipases (LP) have a great potential for commercial applications due to their stability, enantioselectivity and broad substrate specificity [11]. LP is used for the production of the chiral compound (*R*,*S*)-ibuprofen by enantioselective esterification in organic media or by enantioselective hydrolysis of its chemically synthesized racemic ester [12–15]. Although only 50% production could be achieved through the kinetic resolution with LP, LPs are being employed in pharmaceutical industries to produce large amounts of desired products [16,17].

In this research, highly stable and easily recoverable polyaniline nanofibres were developed for enzyme immobilisation. *Candida rugosa* lipase-immobilised polyaniline nanofibres were prepared to investigate the operational, recycling and storage stability of immobilised LP in batch systems. The catalytic activity for the selective conversion of (*R*,*S*)-ibuprofen with immobilised LP was compared with that of free LP.

2. Materials and methods

2.1. Materials

LP from *C. rugosa* type VII, *p*-nitrophenyl butyrate (pNPB), *p*-nitrophenol, ibuprofen, 1-propanol, isooctane, glutaaldehyde (GA) and aniline were purchased from Sigma (St. Louis, MO).

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doi:10.1016/j.molcatb.2007.11.020

All the chemicals used were analytical-grade, and the solvents were of the highest quality commercially available.

2.2. Preparation of the supporting polyaniline nanofibres

Polyaniline nanofibres were made by rapidly mixed reactions. The 0.1% (weight fraction) of initiator (ammonium peroxy disulfate) and 5 ml aniline monomer solutions (with different weight fraction) in 1 M HCl are rapidly mixed together all at once [18]. Once the initiator molecules are depleted during nanofibre formation, there is no further polymerization that leads to overgrowth. The samples were washed 4 times with distilled water to remove the remaining HCl and stored in a refrigerator for further use.

2.3. Enzyme immobilisation method

Polyaniline nanofibres (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. Following incubation for 2 h at 4 °C, GA solution was added (at a final GA concentration of 0.5%, w/v), and the mixture was placed on a rocker (30 rpm) at 25 °C for 2 h. The enzyme aggregate-coated nanofibres were transferred into 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, the nanofibres were incubated in Tris–HCl buffer for 30 min. Following capping, the nanofibres were excessively washed with 20 mM phosphate buffer (pH 6.5) until no enzyme was released into the washing solution. The enzyme aggregate-coated nanofibres were then stored in 10 mM phosphate buffer (pH 6.5) at 4 °C for further use.

2.4. Protein assay

The protein concentration was determined by the Lowry method using the Folin reagent, and the absorbance was measured at 595 nm on a spectrophotometer [19]. The protein concentration was determined by using the Bio-Rad protein assay kit (Hercules, CA) with bovine serum albumin as a standard.

2.5. Scanning electron microscopy (SEM)

SEM images were obtained by using a Hitachi S-4800 scanning electron microscope (Hitachi, Tokyo, Japan). Images were obtained before and after LP immobilisation. The SEM images of the samples were digitized under the following conditions: files, 1280 × 960 pixel; voltage, 15 kV; probe size, 20 nm; and magnification, 10,000×. The photographs represent a magnification of 10,000×.

2.6. Activity and stability measurement

The activities of free and immobilised LPs were measured with the product *p*-nitrophenol concentration which was converted from 0.7 mM pNPB substrate by LP. The reactant mixture consisted of 0.03 ml of 50 mM pNPB and 2.97 ml of 20 mM phosphate buffer (pH 6.5). After addition of free and immobilised LP, the activity was measured from the increase in the absorbance per minute at 400 nm. We determined 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, a 100 μl aliquot was mixed with 900 μl sodium phosphate buffer in a cuvette to measure the changes in absorbance.

2.7. 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. The enantiomers of the unreacted substrate were measured with HPLC using a chiral column (Chiral-AGP, Chrom Tech) capable of separating the *R*- and *S*-enantiomers without previous derivatization. The mobile phase was 0.01 M phosphate buffer, pH 7.0 (99%), with added methanol (1%). The column temperature was 40 °C and the maximum pressure 400 bar. The compounds were detected with UV detector at 230 nm. The enantioselectivity was represented by enantiomeric ratio (*E*) calculated with the conversion (*c*) and the enantiomeric excess (ee).

$$E = \frac{\ln[1 - c(1 + \mathrm{ee})]}{\ln[1 - c(1 - \mathrm{ee})]}$$

where

$$\mathrm{ee} = \frac{([S] - [R])}{([S] + [R])} \quad (1)$$

[*S*] and [*R*] represent the concentration of the *S*- and *R*- enantiomer of ibuprofen ester, respectively.

2.8. Process development for biotransformation

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 LP enzyme solution (3% water in isooctane) in 5 ml 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 immobilised LP. 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 immobilised LP was separated by low-speed centrifugation and successfully recovered from the reaction mixture. The amount of ester (conversion degree) formed during the reaction was determined by gas chromatography.

3. Results and discussion

3.1. SEM image analysis

Pure polyaniline nanofibres have been developed by the rapid mixing of aniline solution in 1N HCl with ammonium peroxydisulphate (Fig. 1(a)). The nanofibres are interconnected and the porosity depends on aniline concentration. The typical size of the nanofibres is approximately 50–200 nm (Fig. 1(b)). The formation of nanopores by nanofibre cross-linking is observed particularly when the rapid polymerization of polyaniline occurs with vigorous mixing. The analysis of scanning electron microscope images was used to determine the enzyme coating on the polyaniline nanofibre. The SEM image after LP immobilisation shows that the surface of the polyaniline nanofibre is covered with LP and also revealed aggregated shapes (Fig. 1(c)).

3.2. Stability analysis of free and immobilised enzyme

The immobilised LP catalytic stability was investigated by the continuous incubation of LP in a sodium phosphate buffer under vigorous shaking (200 rpm) conditions at room temperature. At the predetermined time, the relative activity was calculated from the ratio of the remaining catalytic activity to the initial catalytic activity. After each measurement of the catalytic activity, the immobilised LP was washed thrice with a buffer to eliminate all residual substrates and products. The leaching of LP was also monitored by measuring the protein contents in the aqueous buffer solution at each time point.

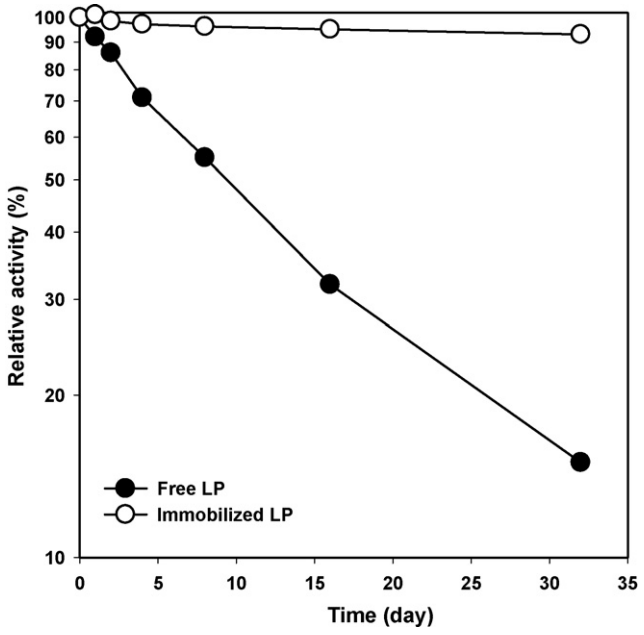

Fig. 2. The stabilities of free LP and LP-aggregate coating on polyaniline nanofibres in an aqueous buffer solution (20 mM phosphate, pH 6.5) on vigorous shaking conditions (200 rpm). Following each measurement of the catalytic activity, the sample was washed thrice in excess buffer solution to remove both the residual substrate and product. The relative activity was calculated from the ratio of the residual activity at each time point to the initial activity.

Fig. 2 shows the relative activities over a longer time period. The activity of free LP rapidly decreased due to denatura-

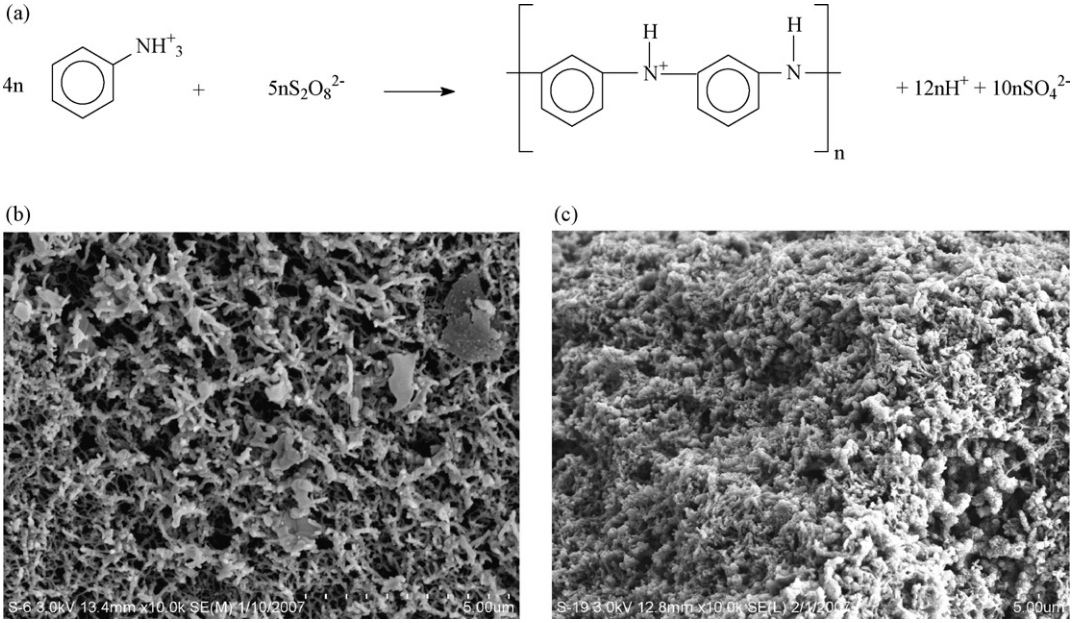

Fig. 1. SEM images of polyaniline nanofibres measuring several hundred nanometers in diameter. The scale bars shown in the figures are 5 μm (a) before LP immobilisation (b) after LP immobilisation.

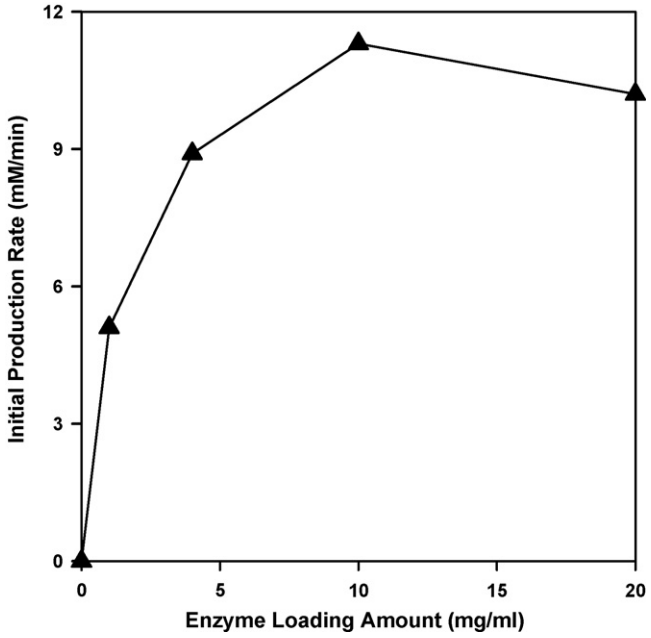

Fig. 3. The effect of enzyme loading on the immobilisation of LP on polyaniline nanofibre. Various amounts of LP (1, 2, 4, 10 and 20 mg) were dissolved in 1 ml of distilled water. Polyaniline nanofibre (2 mg) was added, and immobilisation was performed using 0.5% glutaraldehyde. The immobilised enzyme was washed successively with 20 mM phosphate buffer (pH 6.5) until no proteins were released.

tion while the adsorbed and covalently attached LP showed an improvement in the enzyme stability. The immobilised LP on the polyaniline nanofibre exhibited enhanced catalytic stability as compared to that of free LP. A small decrease in the activity of immobilised LP was observed during the early period due to the detachment of LP from polyaniline; however, after 4 days, not much of a decrease was observed. While free LP is inactivated rapidly by deactivation, immobilised LP exhibited approximately a 10% decrease in the LP activity after 32-day incubation at room temperature, indicating that the half-life of immobilised LP activity had greatly improved.

3.3. Effect of enzyme loading on LP activity

The effect of enzyme loading on the immobilised enzyme activity was studied for different enzyme concentrations. The immobilised enzyme activity increased corresponding to enzyme loading up to 10 mg (Fig. 3). However, an increase in enzyme loading hampered the increasing rate of activity due to an increase in the inhibition of substrate diffusion by enzyme aggregation.

3.4. Effect of temperature on immobilised enzyme activity

With cross-linked enzyme aggregation, the stability of the enzyme and its thermostability were greatly enhanced. The initial reaction rate for different temperatures was measured in order to investigate the effect of temperature on enzyme

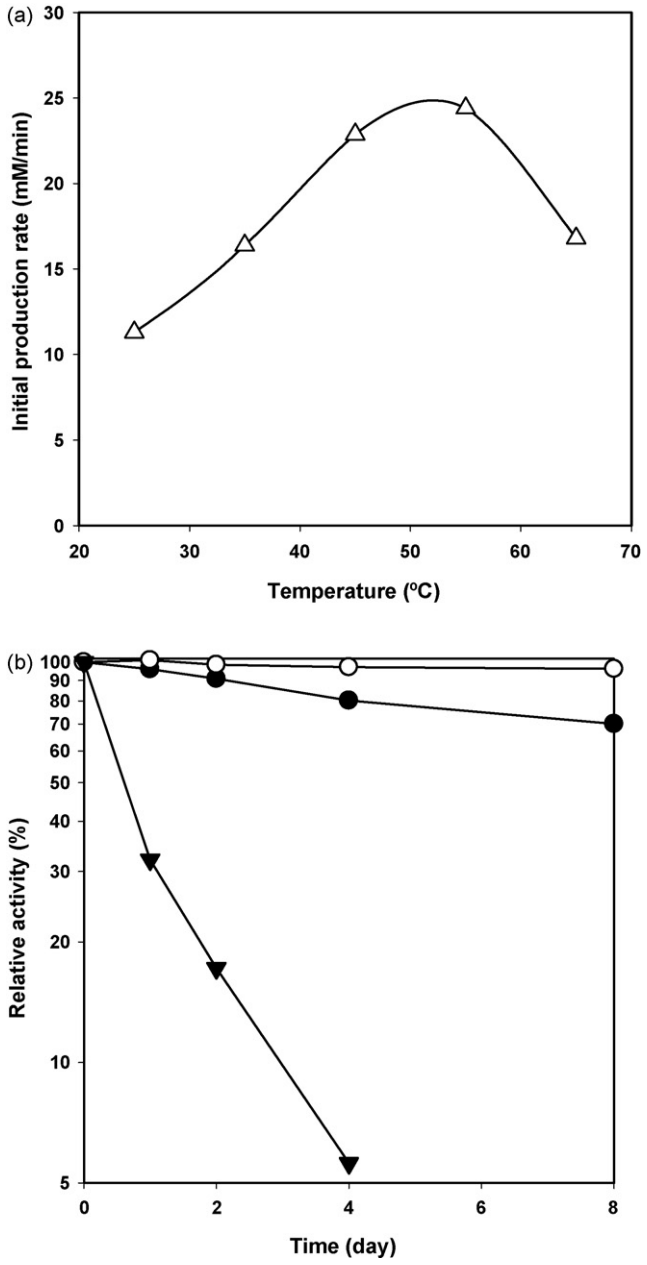

Fig. 4. The effect of temperature on the activity (a, △) and stability (b) of immobilised LP. The activity of immobilised LP rapidly increased corresponding with the temperature; however, the stability of LP at high temperature (at 55 °C, ▼) was very low relative to that at 25 °C (◯) and 37 °C (●).

activity. Homogeneous enzyme-coated polyaniline nanofibres were used to check the effect of temperature on LP activity. LP activity was strongly dependent on temperature and demonstrated a maximum activity at 45–55 °C. Researchers have reported that the optimal temperature for the LP reaction is approximately 40–45 °C [20,21]. Due to the aggregation of cross-linked enzymes, these enzymes were more stable than the free enzymes and the initial enzyme reaction rate increased corresponding to the temperature. Further, significant enzyme deactivation due to high temperature (55 °C) was observed (Fig. 4(b)).

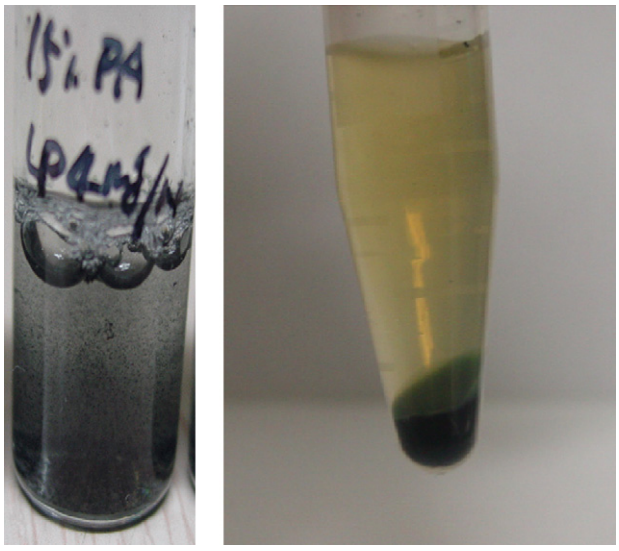

Fig. 5. The recovery of the immobilised enzymes after the reaction. The enzymes were separated by low-speed centrifugation.

3.5. Effect of recycling on immobilised enzyme activity

Recycling enzymes with separable immobilised LP has proven to be an efficient way to recover coated enzymes. A separable enzyme-coated nanofibre was developed for the recovery of enzymes after the batch reaction. Immobilised LP prepared with the polyaniline nanofibre was efficiently separated from the reactant by low-speed centrifugation (5000 × *g*) in a reactor as shown in Fig. 4. For each cycle, the buffer was decanted after separation, and the same amount of new buffer was added (Fig. 5). The immobilised LP clearly stabilised activity for many iterative cycles of enzymatic reactions and separation with low-speed centrifugation. For example, 92% of the initial activity was preserved after 20 iterative cycles of enzyme reaction and separation. These results suggested that on recycling, the enzyme remained active for repeated use. It is likely that enzyme recovery and reuse during the LP reaction could lead to reductions in the enzyme cost during LP-related biotechnology processes.

3.6. Batch conversion of ibuprofen with free and immobilised LP

A (*S*)-prophilic ester of ibuprofen was synthesized via immobilised LP-catalysed esterification between racemic ibuprofen and propanol in the organic solvent isooctane. The performance of an immobilised enzyme is known to be relatively lower than that of the free enzyme. The production rate of the (*S*)-prophilic ester of ibuprofen by the immobilised enzyme was 35% of that by the free enzyme due to the decrease in the catalytic activity caused by enzyme aggregation (Fig. 6). Approximately 1 mM of (*S*)-prophilic ibuprofen ester was produced by LP-coated polyaniline nanofibre. Fig. 6(B) shows the time-course profile of the enantiomeric excess. With the increase of conversion, the enantiomeric excess and ratio were decreased [22]. Enantiomeric excesses of free and immobilised LPs were 0.884 and 0.802, respectively.

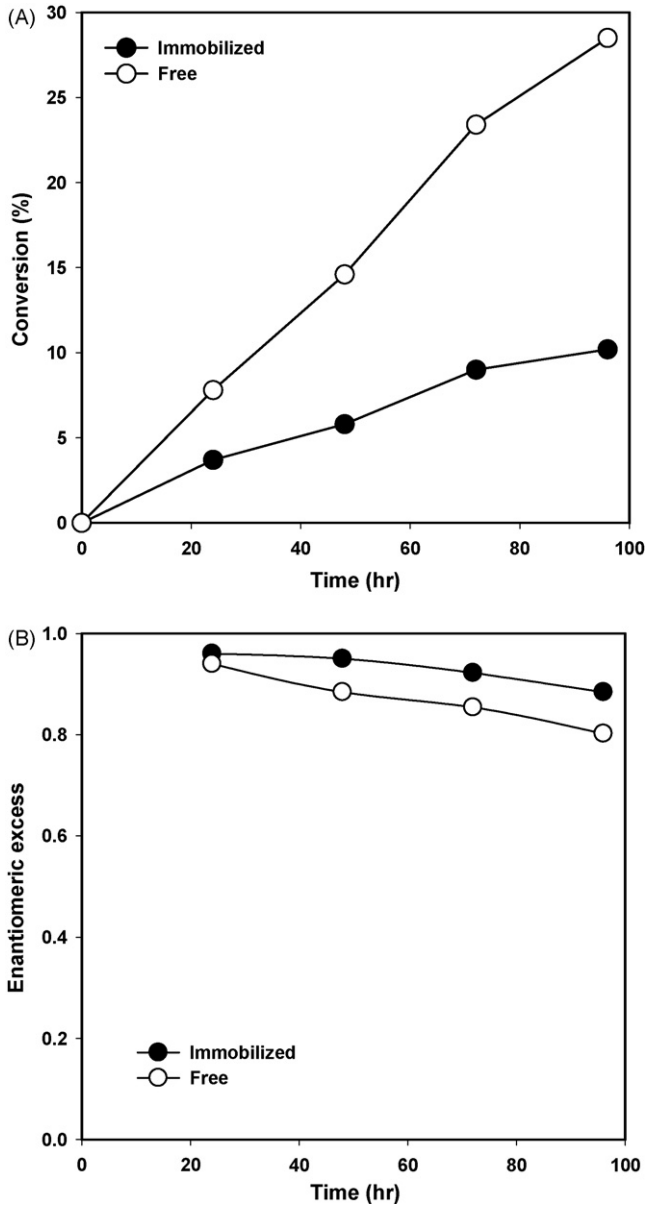

Fig. 6. The effect of enzymes on the time-course conversions of (*R*)- and (*S*)-ibuprofen (10 mM): free LP (5 mg/ml), immobilised LP (5 mg polyaniline/ml), 37 °C, and 3% water in isooctane; (A) conversion, (B) enantiomeric excess, and (C) enantiomeric ratio. The hollow and filled symbols are the conversions with free and immobilised enzymes, respectively.

4. Conclusion

The LP-immobilised nanofibre was developed for the transformation of ibuprofen to the prophilic ester of ibuprofen. A highly active and stable immobilised LP was developed and applied to an enantioselective esterification process to validate its biocatalytic efficiency. Recycling enzymes with separable immobilised LP has proven to be an efficient way to recover the coated enzyme, and 95% of the initial activity was preserved after 20 iterative cycles of enzyme reaction and separation. The chirally selective pharmaceutical compound, the prophilic ester of ibuprofen, was successfully synthesized from racemic ibuprofen by using the immobilised enzyme.

Acknowledgment

This work is supported by Chosun University Research Fund 2005.

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