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Preparation and characterization of dendritic composite magnetic particles as a novel enzyme immobilization carrier

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Abstract

Three kinds of composite carriers (S-type, M-type, MS-type) were prepared and assessed their application in immobilizing lipase. The S-type carrier was prepared by copolymerization of tetraethyl orthosilicate and a dendritic monomer derived from 3-aminopropyltriethoxysilane and methyl acrylate, and the MS-type carrier by copolymerization of tetraethyl orthosilicate and 3-aminopropyltriethoxysilane or a dendritic monomer on the surface of magnetic microparticle (Fe_3O_4). The carriers were characterized by means of IR spectrum, titration and particle size analyzer. Subsequent immobilization experiments clearly indicated that the MS-type was the best in lipase immobilization among the three types of carriers. Moreover, the immobilized PPL by simple adsorption could be reused without detectable decline for its activity until up to four cycles, while the immobilized PPL by cross-linking could be reused up to five cycles without any loss in activity. Interestingly, hydrolysis of 2-phenyl-1-propyl acetate catalyzed by PPL immobilized on the S-type and MS-type carriers showed a higher enantioselectivity (18–32% up) than that by free PPL. The optimal immobilization conditions of PPL on MS-type carriers were as follows: 50 mg PPL/g; the stirring time: 60 min; pH 7.0; room temperature.

Keywords: Dendritic composite silica; Magnetic microparticle; Immobilization; Porcine pancreas lipase; Recyclable

1. Introduction

As versatile biocatalysts, lipases (EC 3.1.1.3) are widely employed for preparing optically active intermediates [1–4]. Meanwhile, lipases have often been immobilized onto insoluble or solid supports for industrial interest, which can increase their thermal and operational stability, recoverability, recycle and easy separation from the reaction mixture [5–11]. Mineral supports, such as silica [12,13], porous glass beads [14,15], diatomaceous earth [16] and alumina [17,18] were used. The most used supports are ion exchange resins [19,20], organic and inorganic supports [21–24], and biopolymers [25–27]. Recently, adsorption on carriers having the dimension of micro- and nanoparticles has been reported [11,28]. In fact, immobilization of lipases has now become a model system to characterize the novel carrier [29].

In our previous work, we prepared five dendritic composite silicas and assessed their properties by immobilization of porcine pancreas lipase (PPL) [30]. Using the hydrolysis of olive oil as a model reaction, the immobilization efficiency was only 42–79%. In addition, the immobilization lipase was not easy to separate from the reaction mixture by simple filtration.

Herein, we describe an efficient way to improve the separation of the dendritic composite silica carriers by embedding the magnetic Fe_3O_4 microparticles. The novel dendritic silica magnetic Fe_3O_4 composite carriers (MS-type) can be easily separated from the reaction mixture due to its magnetism. Moreover, the immobilization efficiency of PPL on this carrier is much higher than that on the dendritic composite silica.

2. Materials and methods

2.1. Materials

Porcine pancreas lipase was purchased from Sigma Company. All the chemicals used were purchased domestically, and were of analytical grade. Phosphate buffer solution (PBS)

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(0.1 M) was prepared from NaH_2PO_4 and Na_2HPO_4. Silica nanoparticles were commercially available (particle size: 5–30 nm).

2.2. Equipment

Chromatographic analysis was performed by GC 9790 with a chiral column (WCOT FUSED SILICA 25 M × 0.25 MM COATING CP CHIRASIL-DEX CB DF = 0.25). FT-IR spectra were taken using EQVIONX model 55 Fourier from Bruker Company. LB-500 Dynamic light scatting particle size analyzer was from Horiba Company (Japan).

2.3. Preparation of composite carriers

2.3.1. Preparation of dendritic composite silica carriers (S-type)

2.3.1.1. Preparation of S-1 carrier. The S-type carriers were prepared according to the procedures reported previously [30]. Into a three-necked flask was added 8.3 ml (0.033 mol) of 3-aminopropyltriethoxysilane and 26.0 ml of methanol under nitrogen atmosphere. The mixture was stirred for 2 h. Then 12.0 ml (0.13 mol) of methyl acrylate (MA) was added, the solution was kept in a dark place and stirred vigorously for 48 h at room temperature. The solution was evaporated under reduced pressure, and the residue weighed 10.8 g (crude product A). To this residue, 56.0 ml (0.25 mol) of tetraethyl orthosilicate and 50.0 ml of 1% NaF aqueous solution was added with continuous stirring at room temperature. After 48 h, the white precipitate was filtered out, washed twice with ethanol, and dried. The obtained white powder weighed 30.6 g (S-1 carrier). Its main IR peaks appear at (ν, cm^{-1}): 1730 (COOR), 1629 (C–N), 1588 (N–H), 1090 (Si–O).

2.3.1.2. Preparation of S-2 carrier. A mixture of 10.8 g of crude product A, 13.0 ml (0.26 mol) of ethylenediamine (EDA) and 14.0 ml of absolute methanol was stirred vigorously for 48 h at room temperature. This mixture was then evaporated under reduced pressure, giving 11.8 g of colorless liquid (crude product B). The crude product B was mixed with 56.0 ml of tetraethyl orthosilicate and 50.0 ml of 1% NaF aqueous solution with continuous stirring for 48 h at room temperature, affording 31.2 g of white powder (S-2 carrier). Its main IR peaks appear at (ν, cm^{-1}): 3415 (NH_2), 1656 (CONH) and 1571 (NH), 1059 (Si–O).

2.3.1.3. Preparation of S-3 carrier. A mixture of 12.0 ml (0.13 mol) of MA and 26.0 ml of methanol was added to the 11.8 g of crude product B, and stirred with continuous stirring for 48 h at room temperature. The mixture was evaporated under reduced pressure, providing 15.7 g of colorless oil (crude product C). The crude product C was polymerized with a mixture of 56.0 ml of tetraethyl orthosilicate and 50.0 ml of 1% NaF aqueous solution, forming 34.9 g of white powder (S-3 carrier). The main IR peaks of the S-3 carrier appear at (ν, cm^{-1}): 1742 (COOR), 1654 (CONH) and 1570 (NH), 1084 (Si–O).

Fig. 1. The formation scheme of MS-1 carrier.

2.3.2. Preparation of magnetic carriers (M-type)

According to the literature method [31,32], a mixture of 500 ml of $FeCl_2$ aqueous solution (0.2 mol/l) and 500 ml of $FeCl_3$ aqueous solution (0.3 mol/l) was added to a flask containing 5.0 g of stearic acid. Then the mixture was stirred vigorously for a while, and 200 ml of 0.4 mol/l NaOH aqueous solution was dropped into the flask (2 drops/s), until the pH of the solution became 12. A black precipitate appeared. The precipitate was filtered, washed twice by ethyl acetate, and dried. The final Fe_3O_4 powder weighted 28.3 g (M_1 carrier).

2.3.3. Preparation of composite magnetic carrier (MS-type)

2.3.3.1. Preparation of MS-1. Two grams of Fe_3O_4 powder was suspended in 100 ml of distilled water in a 250 ml flask. A mixture of 5.0 ml (0.02 mol) of 3-aminopropyltriethoxysilane, 15 ml of methanol and 5 ml of 1% NaF aqueous solution was stirring for 5 min. Then 30 ml of tetraethyl orthosilicate was dropped slowly into the flask and stirred vigorously for 48 h at room temperature. The precipitate was collected, washed in turn by ethanol and distilled water, and dried. The powdered MS-1 carrier weighed 13.6 g (outlined in Fig. 1). The IR spectral data of the MS-1 carrier (ν, cm^{-1}): 3432, 1636 and 1545 (NH_2), 1069 (Si–O).

2.3.3.2. Preparation of MS-2 carrier. MS-2 carrier was prepared according to the preparation procedures of MS-1 carrier. Copolymerization of 2.0 g of Fe_3O_4 powder, 30 ml of tetraethyl orthosilicate, 9 ml of the crude product A in 15 ml of methanol and 5 ml of 1% NaF aqueous solution provided 15.0 g of the MS-2 carrier (Fig. 2). The IR spectral data of the MS-2 carriers (ν, cm^{-1}): 3438, 1732 (COOR), 1073 (Si–O).

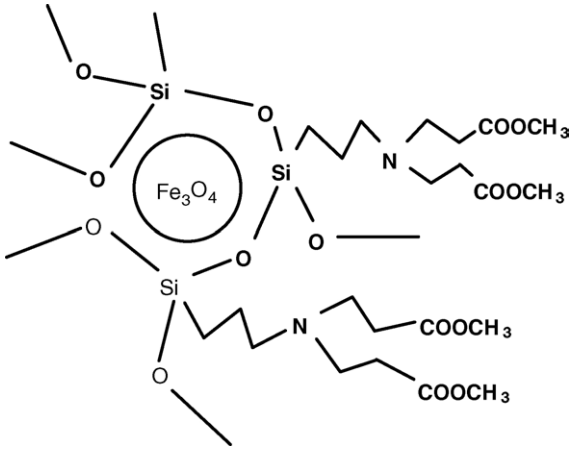

Fig. 2. MS-2 carrier.

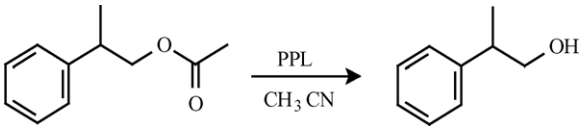

Fig. 3. Hydrolysis of 2-phenyl-1-propyl acetate.

2.4. Enzyme activity assay

In order to determine the activity of free and immobilized PPL, two different model reactions have been adopted. One is the hydrolysis of olive oil [30]. One unit of the activity of immobilized PPL is defined as 1 μmol of the fatty acid produced by the catalysis of 1 g immobilized lipase in 1 min under the assay conditions. Another is the hydrolysis of 2-phenyl-1-propyl acetate [33]. In this test reaction, the relative activity x is defined as υ (immobilized enzyme)/[υ (commercial enzyme) − υ(filtrate)], where υ is the initial rate of the reaction (in $\mu mol\,h^{-1}\,mg\ lipase^{-1}$) (Fig. 3).

2.5. PPL immobilization

2.5.1. Simple adsorption

Certain amount of PPL was added to 25 ml of phosphate buffer solution and stirred until all the PPL was dissolved. To this solution was added certain amounts of the carriers (SiO_2 nanoparticles, S-1, S-3, M-type and MS-type), and stirred for several minutes at room temperature. The immobilized PPL was separated by centrifugation and washed with phosphate buffer. All portions of centrifugation were retained for the determination of protein concentration and enzymatic activity. The immobilized PPL was dried in vacuum.

2.5.2. Cross-linking with glutaraldehyde

To 100 ml of 10% glutaraldehyde was added 4.4 g of the S-2 or MS-1 carriers, and stirred for 24 h at room temperature. Then washed with distilled water, filtered, dried. The immobilization of PPL with cross-linking carrier was done according to the method above.

2.6. Hydrolysis of 2-phenyl-1-propyl acetate catalyzed by immobilized PPL

The immobilized PPL was added into phosphate buffer solution (0.1 M, pH 7.0, 10 ml). The acetate (20 mg) in acetonitrile (0.1 ml) was added and the mixture was stirred at 30 °C for a period. Then the reaction was quenched by addition of acetone. After centrifugation (4000 rpm, 10 min), the supernatant was separated. Aqueous layer was extracted twice with ethyl acetate, and the combined organic layer was dried over anhydrous sodium sulfate. The solvent was evaporated under the reduced pressure, and the residue analyzed by GC.

2.7. Particle size analysis of the composite magnetic carriers

One milligram of the M_1 carrier was added to a 150 ml of conical flask containing 100 ml of ethanol. The mixture was sonicated for 1 h under 10 kHz. The particle size was measured by means of the dynamic light scattering particle size analyzer. The particle size of the MS-2 carrier was measured in the same way.

3. Results and discussion

3.1. Immobilization of PPL on dendritic composite silica carriers (S-type)

The immobilization of PPL with three S-type carriers has been evaluated in comparison with commercial silica nanoparticles. Activity of the immobilized lipase was estimated by using the hydrolysis of 2-phenyl-1-propyl acetate as a model reaction [33]. The hydrolysis was carried out by mixing 20 mg of 2-phenyl-1-propyl acetate, 40 mg of free PPL or 40 mg of PPL on 500 mg of the S-type carrier, 10 ml of pH 7.0 PBS buffer and 0.1 ml of acetonitrile at 30 °C. The concerned results were shown in Table 1.

From Table 1, it can be seen that the immobilization efficiency of all the S-type carriers was higher than that of commercial silica nanoparticles, wherein S-1 and S-3 carriers better than S-2 carrier. The relative activity decreases along with the order of S-1, S-2 and S-3. These results show that the surface functional groups and the chain length of the dendritic unit have considerable effect on the immobilization properties of the S-type carriers. Among the three carriers, the best is the S-1 carrier. Thus, we think it is no longer necessary to prepare the composite silica carrier with much longer dendritic chain than S-3 carrier.

Moreover, the enantioselective hydrolysis of racemic 2-phenyl-1-propyl acetate was done, respectively, by using free and the immobilized lipases as the catalysts. The conversion and the enantiomeric excess of the product were given in Table 1 (entries 4–6). Comparing the results listed in entries 5 and 6, it can be seen that the e.e. value of the product was raised by 18% under the same conversions when the immobilized PPL on the S-1 carrier was used instead of free PPL. The similar phenomenon was also observed in the cases of PPL immobilized on other S-type carriers.

Cyclic use of the immobilized PPL on the S-type carrier was further examined. A marked decrease in activity was observed for these immobilized PPL obtained by simple absorption. However, this disadvantage can be easily overcome by cross-linking with glutaraldehyde. In fact, the PPL cross-linked on the S-2 carrier shows excellent property for cyclic use. As shown in

Table 1
Properties of the immobilized PPL on S-type carriers

Entry	Parameters	Immobilization carrier					
		None	SiO_2	S-1	S-2	S-3	M_1
1	Immobilization efficiency (%)	–	59	77	65	79	99
2	Relative activity (%)	–	87	86	74	67	34
3	Activity reclaim (%)	–	51	66	48	53	34
4	Time (h)	1.5	3	3	3	3	3
5	Conversion (%)	46	35	47	34	34	20
6	e.e. (%)	52	70	70	82	82	84

Table 2
Cyclic use of immobilized PPL on S-2 carrier at 30 °C

Cyclic times	Conversion (%)	e.e. (%)
First	34	82
Second	40	77
Third	36	81
Fourth	41	77
Fifth	43	77

Table 3
The content of amino group on carriers' surface

Carriers	Content of amino group (mmol/g)
MS-1	1.354
MS-2	0.975

Table 2, no detectable loss in the activity was observed after the immobilized PPL was cycled fifth times. This result indicates that a covalent bonding exists between PPL and the S-2 carrier in this case.

3.2. Immobilization of PPL on the magnetic carrier (M-type)

The immobilization of PPL on magnetic Fe_3O_4 carrier (M_1 carrier) was carried out by simple absorption. The enzymatic estimate was also performed using the hydrolysis of 2-phenyl-1-propyl acetate as the model reaction. The immobilization efficiency, the relative activity and the activity reclaim were determined in the same manner, and the data were given in Table 1. The results clearly indicate that the relative activity of the immobilized PPL on M_1 carrier is quite low (34%), although the immobilization efficiency of PPL very high (up to 99%). In addition, a detectable increase in e.e. value is also observed in the catalytic hydrolysis of PPL immobilized on M_1 carrier.

The high immobilization efficiency and good separability of M_1 carrier promoted us to prepare a kind of novel dendritic silica-magnetic Fe_3O_4 composite carrier that could possess most of the advantages of S-type and M-type carriers. In fact, two dendritic silica magnetic composite carriers have been prepared by enwrapping a thin layer of dendritic composite silica on the surface of magnetic microparticle, and the detailed procedures were described in Section 2.

3.3. Immobilization of PPL on the dendritic silica magnetic composite carriers (MS-type)

3.3.1. Characterization of the dendritic composite magnetic carriers (MS-type)

3.3.1.1. Particle size analysis of the magnetic particles carriers. The particle size analysis of magnetic microparticle and dendritic silica magnetic composite carriers was done by means of dynamic light scattering particle size analyzer. The average diameter of the M_1 microparticle was 1.8848 μm (Fig. 4(A)). Two peaks were detected in the distribution of the particle size, and their diameters were distributed at 0.6–1 and 3–4 μm. On the other hand, the average diameter of particles of the MS-2 carrier was 2.3441 μm. As shown in Fig. 4(B), the main distribution of the particle size was at 0.7–1.2 and 5–6 μm. The increase extent in particle diameter indicated that the Fe_3O_4 particles have been efficiently enwrapped into the dendritic composite silica.

3.3.1.2. Content of MS carrier's surface groups. According to the method of acid base titration, we have determined the content of amino groups distributed on the surface of MS carriers. The results listed in Table 3 clearly indicate there are an abundance of amino groups on the surface of both of the MS-type carriers.

3.3.2. Optimal conditions for immobilizing PPL on the MS-type carriers

The optimal conditions for immobilizing PPL with the similar S-type carriers have been investigated in our early work [30]. Here, we will focus our attention to optimize the immobilizing conditions for the MS-type carriers. The enzymatic estimate was done using the hydrolysis of olive oil as a model reaction. The activity of PPL was determined at 37 °C.

3.3.2.1. Effect of lipase amounts on immobilization efficiency. The amount of PPL immobilized on carrier was limited. In order to find the optimal amount, we tested the immobilization of various amounts of PPL on 1.0 g of the MS-2 carrier. The relationship of PPL amounts with immobilization efficiency was shown in Fig. 5.

When the amount of PPL changed from 25 to 125 mg, the immobilization efficiency decreased in turn. On the other hand, the activity of immobilized PPL was raised with increasing PPL amount, and reached 728 U/g when 50 mg/g of PPL was used.

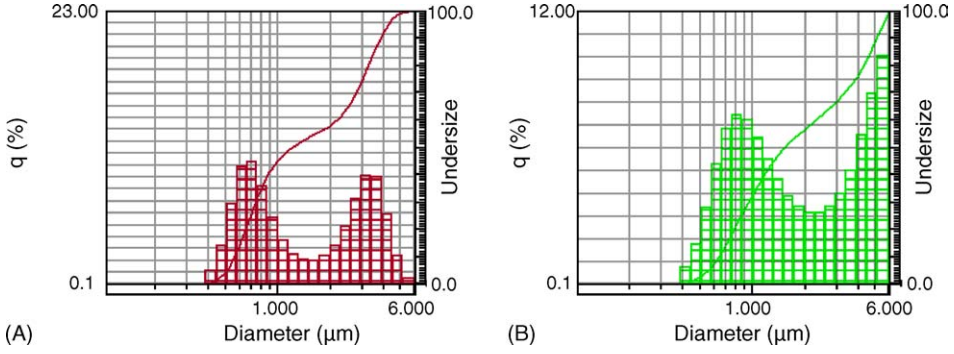

Fig. 4. Distribution of carrier's particle size: (A) M_1 carrier and (B) MS-2 carrier.

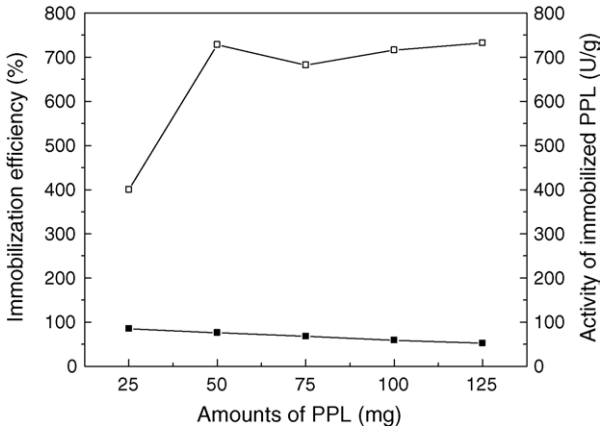

Fig. 5. Effect of lipase amounts on the immobilization efficiency (stirring time: 30 min). (□) Activity of immobilized PPL and (■) immobilization efficiency.

Based on the results, we realized that the optimal amount of PPL was 50 mg/g.

3.3.2.2. Effect of adsorption time. The immobilization efficiency was also affected to some extent by the immobilizing time of PPL. As shown in Fig. 6, the immobilization efficiency changed with the stirring time, and became the highest after 60–90 min. The adsorption was limited when the stirring time was too short. Thus, the optimal adsorption time was considered to be about 60 min.

3.3.2.3. pH effect. Fig. 7 showed the effect of pH on the relative activity of the free and immobilized lipases. The maximum relative activity was discerned when the immobilization of PPL was executed at pH 7.0, indicating that the optimal pH of the immobilized PPL was 7.0. This is inconsistent with the optimal pH value of free PPL, which was disclosed to 6.5. The deviation in the optimal pH value of the immobilized lipase could be attributed to the existence of an abundance of amino groups on the carriers.

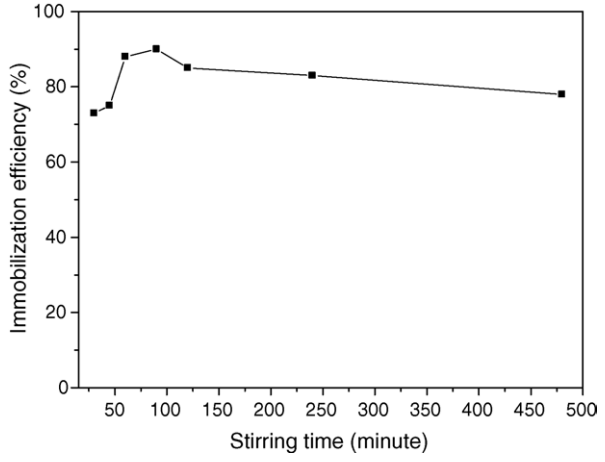

Fig. 6. Effect of adsorption time on the immobilization efficiency (50 mg of PPL used).

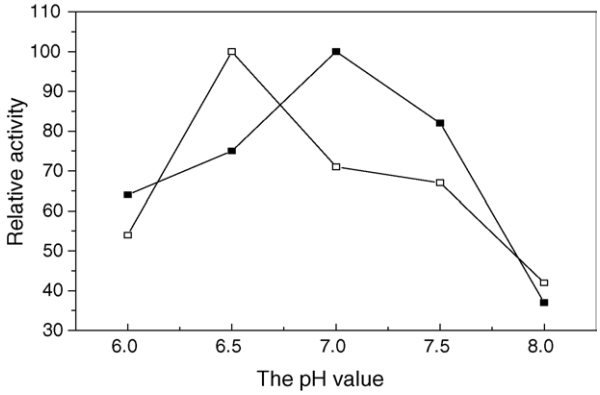

Fig. 7. Effect of the pH value on activity of PPL (50 mg of PPL, stirring for 60 min). (□) Free PPL and (■) immobilized PPL.

3.3.2.4. Effect of adsorption temperature. The relationship of the immobilization efficiency with adsorption temperature was shown in Fig. 8. The higher the adsorption temperature, the lower the immobilization efficiency was. The highest immobilization efficiency was observed when the immobilization was carried out at room temperature.

3.3.2.5. Thermal stability. Effect of the temperature on the activity of free and the immobilized lipases was determined in the temperature range of 30–70 °C. The results were shown in Fig. 9. For free lipase, the optimal activity appeared at 35 °C, and was completely lost at 55 °C. For the immobilized lipase, the optimal activity appeared at 37 °C, and 40% of the activity was maintained at 70 °C. Therefore, thermal stability of the immobilized lipase was much higher than that of free lipase.

3.3.3. Catalytic hydrolysis of 2-phenyl-1-propyl acetate

The immobilization property of the MS-type carriers was also evaluated by the hydrolysis of 2-phenyl-1-propyl acetate. The hydrolysis was done by mixing 20 mg of 2-phenyl-1-propyl acetate, 40 mg of free PPL or 40 mg of PPL on 500 mg of the MS-type carriers, 10 ml of pH 7.0 PBS buffer and 0.1 ml of acetonitrile at 30 °C. The results were shown in Table 4.

It was noteworthy that slight variation of the immobilization efficiency of the MS-type carrier appeared according to the different model reactions. The highest immobilization effi-

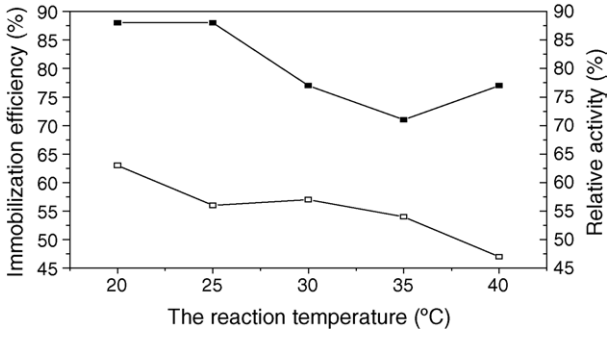

Fig. 8. Effect of adsorption temperature on the immobilization lipase activity (50 mg of PPL, stirring for 60 min). (□) Relative activity and (■) immobilization efficiency.

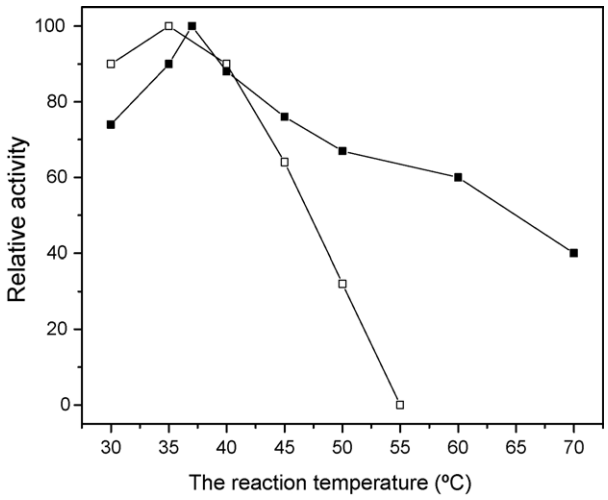

Fig. 9. Effect of temperature on the activity of the free and immobilized lipases. (□) Free PPL and (■) immobilized PPL.

Table 4
Immobilization of PPL on MS-type carriers

Entry	Parameters	Immobilization carrier		
		None	MS-1	MS-2
1	Immobilization efficiency (%)	–	99	98
2	Relative activity (%)	–	75	68
3	Activity reclaim (%)	–	75	66
4	Time (h)	1.5	3	3
5	Conversion (%)	46	46	43
6	e.e. (%)	52	79	77

ciency was about 90% based on the hydrolysis of olive oil, but about 98% on the hydrolysis of 2-phenyl-1-propyl acetate. In spite of the difference, the immobilization efficiency of both of MS-type carriers was obviously higher than that of S-type carriers (Table 1, entry 1). In addition, the relative activity of PPL immobilized on the MS-type carriers was comparable to that on the S-type carriers. The immobilized lipase on MS-type carriers has the advantage that it was easily separated from the reaction mixture by simple filtration. Moreover, the e.e. value of the hydrolysis was enhanced nearly 30% (Table 4, entry 6) when the hydrolysis was catalyzed by the immobilized PPL on the MS-type carriers instead of free PPL.

Cyclic reuse of the immobilized PPL on the MS-1 carrier was further examined. No detectable decrease in activity occurred until the immobilized lipase was recycled fifth times (Table 5). This demonstrates that MS-type carrier is much better than S-type carrier.

Table 5
Cyclic use of immobilized PPL on MS-1 carrier at 30 °C

Cyclic times	Conversion (%)	e.e. (%)
First	43	77
Second	45	73
Third	42	77
Fourth	39	79
Fifth	25	72

The cross-linking immobilization of PPL on MS-2 was also carried out. The corresponding experiments demonstrated this immobilized PPL was recyclable. Its activity did not decrease at all while the immobilization enzyme was reused for fifth times.

4. Conclusion

We considered that the MS-type magnetic carrier is a novel valuable carrier for immobilization of enzyme. It has the following advantages: (1) high immobilization efficiency, (2) high relative activity of immobilized lipase, (3) easy recovery and (4) recycle use.

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