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Immobilization of lipase from *Burkholderia cepacia* into calcium carbonate microcapsule and its use for enzymatic reactions in organic and aqueous media

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ABSTRACT

We disclosed a useful procedure for the immobilization of a lipase from *Burkholderia cepacia* based on its encapsulation into vaterite calcium carbonate microcapsule. The immobilized enzyme had an adequate activity for the hydrolysis of 4-nitrophenyl acetate, which was preserved over 2 years in storage at 4 °C. The enantioselective esterification of (±)-1-phenylethanol with vinyl acetate using the immobilized lipase smoothly progressed to afford the corresponding optically active compounds, where the enzyme was easily recovered for reuse. Although the hydrolysis of (±)-2-acetoxyhexyl tosylate in aqueous solution was catalyzed by the enzyme, the phase transition of calcium carbonate from vaterite to poorly porous calcite during the reaction prevented the access of the reactant to the active site of the lipase, resulting in the significant decrease of the activity of the recovered enzyme. However, the treatment of the immobilized enzyme with zinc chloride solution inhibited the phase transition under the reaction conditions. The enzyme after the zinc treatment was recyclable for the hydrolysis of the substrate without significant decrease of the reactivity even after the uses in aqueous media.

1. Introduction

In our previous studies, we have reported on the enzymatic enantioselective hydrolysis of racemic 1,2-diol monotosylate derivatives with lipase PS (from *Burkholderia cepacia*, Amano Enzyme Inc.; Scheme 1) [1–4]. Since the molecular length of the R substituent group does not affect the enantioselelctivity and the gram-scale reactions smoothly proceeded, this method is applicable to the practical preparations of the various optically active 1,2-diol monotosylates. However, the enzymes used in the previous reactions were seriously wasted after the reactions, although the amount of the enzyme used (75 mg) was much lower than that of the substrate (ca. 300 mg, 1 mmol) [1–3]. Then, we are now focusing on the improvements that enable the recovery and recycling of the enzyme in order to establish the more effective and environmentally benign procedures.

Immobilization of enzymes is a typical and important technology for easy treatment, recovery and recycling of biocatalysts in practical reaction processes [5–7]. In particular, the immobilization of lipases, which are utilized not only to obtain optically active molecules in fine chemistry as mentioned above but also to produce various organic compounds such as agrochemical, pharmaceutical, food, biodiesel fuel and so on, in industrial chemistry, has significant advantages for achieving environmentally benign production and reducing their process costs [8–11]. Therefore, a number of studies for the immobilization of lipases have been developed. Among these procedures, the entrapment within a polymer lattice and the physical adsorption onto support materials are the representative techniques so far. Especially, in the latter case, some lipases immobilized onto diatomaceous earth and ceramics as the inorganic support matrices are easily obtained from commercial sources, and those immobilized lipases are useful for increasing the reactivity of transesterification in organic solvents and for easy recycling of the enzymes from organic solvents. However, the immobilized lipases by the physical adsorption are not suitable for the hydrolytic reactions in aqueous media, because the leaching of the lipases from the support matrices can easily occur under the reaction conditions to result in the decisive loss of the hydrolytic activity in the recovered materials.

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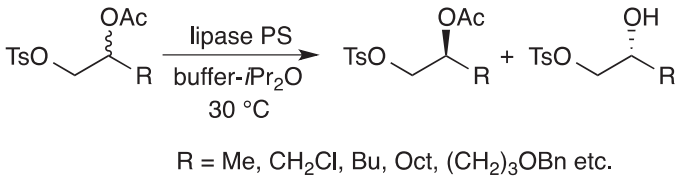


Scheme 1. Enantioselective hydrolysis of 1,2-diol monotosylate derivatives using lipase PS.

Calcium carbonate is a well-known biocompatible and environmentally-friendly material appropriate for an inorganic support matrix of enzyme immobilization, and some encapsulation techniques of proteins and enzymes have been studied [12–19]. Since calcium carbonate materials are generally non-porous to be unsuitable for the adsorption and inclusion of proteins, the additional components and processes such as carboxymethyl cellulose [15], silane coupling reagent [16], layer-by-layer techniques [17,18] and gold nanoparticles [19] are often required for immobilizing enzymes. Recently, porous calcium carbonate materials are prepared and utilized for the fixation of various molecules and proteins, where fine nanoparticles of calcium carbonate in amorphous [20] or calcite [21] are used. Lipases are also immobilized onto calcium carbonate related materials [22–25], and an immobilized lipase from *Rhizopus oryzae* onto calcium carbonate by adsorption was an active and stable catalyst, which could be used repeatedly in *n*-hexane solution [22]. However, a lipase-immobilized calcium carbonate material is reported to be difficult to recover in the case of olive oil hydrolysis in the presence of considerable amount of water [24], despite lipases are very useful for the reactions even in aqueous solutions. Thus, the further improvements of the lipase immobilization into calcium carbonate are desired especially for the utilization in aqueous solutions.

We have also studied the preparations and the applications of calcium carbonate materials prepared by using water/oil/water emulsion (interfacial reaction method) [26]. These calcium carbonate materials (microcapsules) are spherical and hollow, and its crystalline form is a metastable vaterite phase [27,28]. Recently, we found a simple and effective procedure of the protein encapsulation that the phase transformation of the vaterite calcium carbonate microcapsule to stable calcite phase in aqueous solutions with dissolved proteins achieved the inclusion of a wide variety of proteins into calcite calcium carbonate [29,30]. In this paper, we wish to report our successful approaches to immobilize lipase PS into vaterite calcium carbonate microcapsule using the phase transformation technique for the enzymatic enantioselective transesterification of racemic 1-phenylethanol (**1**) in organic media and the hydrolysis of racemic 2-acetoxyhexyl tosylate (**2**) in aqueous media. Furthermore, for improving the recyclability of the catalyst in the reaction of aqueous solutions, the treatment of calcium carbonate with zinc chloride to maintain the calcium carbonate in the vaterite crystalline phase was also described.

2. Experimental

2.1. Materials

The chemicals and proteins employed here are commercial available and listed as follows. $(NH_4)_2CO_3$, $CaCl_2{\cdot}2H_2O$, and 0.05 M Tris–HCl buffer (pH 7.6), 1 M Tris–HCl buffer (pH 7.0), (±)-1-phenylethanol (**1**) and 4-nitrophenyl acetate were obtained from Wako Pure Chemical Industries. Tween 85, vinyl acetate (monomer) and *n*-hexane were purchased from Kanto Chemical Co. Inc. Lipase PS from *B. cepacia* (534641) and lipase PS-IM (immobilized on diatomaceous earth, 709603) were obtained from Sigma–Aldrich Co. LLC. A substrate (±)-2-acetoxyhexyl tosylate (**2**) was synthesized by the procedure mentioned in our previous paper [3]. E. Merck Kieselgel 60 F_{254} Art.5715 was used for analytical TLC. Preparative TLC was performed on E. Merck Kieselgel 60 F_{254} Art.5744. Column chromatography was performed with Silica Gel 60N (63–210 μm, Kanto Chemical Co. Inc.). All other solvents were also obtained from commercially sources and were used without further purification.

2.2. Preparation of vaterite calcium carbonate microcapsule (μCap)

Vaterite calcium carbonate microcapsules (μCap) were prepared by a described method in our recent papers [28,29]. A typical procedure is as follows: an aqueous solution (32 mL) of 9.23 g (96 mmol) of $(NH_4)_2CO_3$ was mixed with a *n*-hexane solution (48 mL) of 1.0 g of Tween 85, and the resulting solution was emulsified with 8000 rpm for 1 min using a homogenizer (IKA-T25 digital ULTRA-TURRAX) with shaft generator S25N-25 F to form water/oil (W/O) emulsion. This W/O emulsion was poured into another aqueous solution (640 mL) of 28.2 g (192 mmol) of $CaCl_2{\cdot}H_2O$ in one portion at 30 °C. The final solution was stirred for 300 rpm at the same temperature for 5 min, and the white precipitate yielded was filtered, and washed with 1 L of deionized water twice and with 100 mL of methanol. Finally, the power samples were dried at 80 °C for 12 h.

2.3. Encapsulation of lipase PS into μCap

This process is generally analogous to reported one [29]. To a solution of lipase PS (1.0 g; 0.2 g per gram of μCap, 2630 units for the enzymatic activity) in Tris–HCl buffer (pH 7.0 or 7.6, 200 mL) was added 5 g of μCap. The resulting solution with μCap solid was stood at room temperature for 4 days. This solid was filtered, washed with enough amounts of fresh deionized water, and dried at room temperature for a few days (4.3 g; 731 units). The crystal phase and the morphology of these calcium carbonate materials (lipase PS μCap) were analyzed by XRD measurement and SEM observation, respectively. The presence of the enzyme in these calcium carbonate materials was ascertained by diffuse reflection UV spectroscopic analysis. For the enzymatic activity, see Section 2.6.

2.4. Zinc chloride treatment of μCap encapsulating lipase PS

To a solution of zinc chloride (0.46 g) in 1 M Tris–HCl buffer (pH 7.0, 96 mL) was added 2.4 g (408 units) of μCap encapsulating lipase PS (lipase PS μCap). After leaving the solution to stand for 1 day, the solid samples were filtered, washed with enough amounts of fresh deionized water, and dried at room temperature for a few days (1.7 g; 68 units).

2.5. Characterization of materials

X-ray diffraction patterns were recorded using Mac Science MXP3V diffraction meter with Ni filtered Cu Kα radiation (λ = 0.15406 nm). Scanning electron microscopy (SEM) images were measured using JEOL JSM-6390 microscope apparatus. For SEM observation, gold was deposited on the each sample using an ion sputtering device (JEOL JFC-1500). Powder UV spectrum measurement was carried out using JASCO V-550 spectrometer with an integrating sphere for diffuse reflectance UV spectroscopy. Kubelka–Munk functions were plotted against the wavelength.

2.6. Catalytic tests of enzymes by hydrolysis of 4-nitorophenyl acetate

Hydrolytic activities of enzymes were performed by measuring the increase of 4-nitrophenol produced by the hydrolysis of 4-nitrophenyl acetate. To a 50 mL-Erlenmyer flask containing 0.1 M Tris–HCl buffer (pH 7.0, 10 mL) were added a 2 M acetone solution of 4-nitrophenyl acetate (100 μL). In the case of commercially available lipase PS, a 100 μL-portion of the enzyme solution in 0.1 M Tris–HCl buffer (pH 7.0, 5.0 mg particle in 1 mL buffer) was added to the mixture, and the solution was shaken with 100 min^{-1} at 25 °C. On the other hand, in the case of the immobilized lipase PS μCap, 5.0 mg of the enzyme and 100 μL of 0.1 M Tris–HCl buffer (pH 7.0) were added, and the reaction was carried out in the same way as mentioned above. After 4, 8, and 12 min, a 50 μL-portion of the mixture was added to a cuvette containing 0.1 M Tris–HCl buffer (pH 7.0, 950 μL), and the absorbance at 405 nm was immediately measured by a Shimadzu PharmaSpec UV-1700 (UV/Vis spectrophotometer). One unit of hydrolytic activity was defined as the amount of enzyme necessary to release 1 mmol of 4-nitrophenol per minute under the reaction conditions.

2.7. Determination of protein content

The amount of protein immobilized was determined by subtracting the amount of protein in supernatant of the immobilization suspension from the total amount of protein offered for immobilization. Protein assay was performed by the Bradford method using bovine serum albumin (BSA) as the standard [31].

2.8. Immobilization efficiency

Immobilization yield (%) and expressed yield (%) were calculated using the following equations [32]:

$$\text{Immobilization yield}\,(\%) = \frac{\text{Total protein offered} - \text{Protein in the supernatant of the immobilization}}{\text{Total protein offered}} \times 100$$

$$\text{Expressed yield}\,(\%) = \frac{\text{Actual activity of the derivative}}{\text{Expected activity considering the immobilized enzyme}} \times 100$$

2.9. Typical procedure for the enantioselective esterification of (±)-1-phenylethanol (**1**) with lipase PS in organic media

To a 20-mL recovery flask were added 122 mg (1.00 mmol, sub. conc. 250 mM) of (±)-**1** and 4.0 mL of vinyl acetate. After the addition of lipase PS μCap (200 mg, 34 U), the mixture was stirred for 24 h at 30 °C. The mixture was filtered through a filter paper, and the recovered enzyme was washed with ethyl acetate. Evaporation under reduced pressure and purification by column chromatography on silica gel (*n*-hexane/ethyl acetate = 4/1) afforded (*S*)-**1** (49 mg, 40%, 98% ee) and (*R*)-1-phenylethyl acetate (**3**, 60 mg, 36%, 99.0% ee). The spectral data were in full agreement with those of commercial sources. The ees of the resulting (*S*)-**1** and (*R*)-**3** were determined by GC analysis, and the absolute configuration of **1** was confirmed by comparing the retention time with that in our previous paper [33]. GC conditions: column, CP-Cyclodextrin-B-236-M19 (Chrompack), 0.25 mm × 50 m; injection, 140 °C; detection, 140 °C; oven, 120 °C; carrier gas, He; head pressure, 2.4 kg/cm^2; retention time, **1**: 14.5 (*R*) and 14.7 (*S*) min, **3**: 14.7 (*S*) and 15.2 (*R*) min. The reaction conversions and *E* values in Tables 2–4 were calculated using $ee_s/(ee_s + ee_p)$ and using $\ln[(1 - conv.)(1 - ee_s)]/\ln[(1 - conv.)(1 + ee_s)]$, respectively [34].

On the other hand, the recovered lipase PS μCap (156 mg) was dried under reduced pressure, and was examined for the next run. The reuse of the recovered enzyme was performed on the basis of keeping the mass ratio of catalyst/substrate in the first run. The esterification of (±)-**1** using lipase PS-IM (40 U, 20 mg) was carried out by means of the same procedure.

2.10. Typical procedure for the enantioselective hydrolysis of (±)-2-acetoxyhexyl tosylate (**2**) with lipase PS in aqueous media

To a 200-mL Erlenmeyer flask containing 126 mg (0.400 mmol; sub. conc., 10 mM) of (±)-**2** was added 4 mL of diisopropyl ether and 36 mL of 0.1 M Tris–HCl buffer (pH 7.6). To the mixture was added 600 mg (24 U) of lipase PS μCap treated with zinc chloride solution, and the solution was incubated for 24 h at 30 °C. Centrifugation of the mixture with 8500 rpm for 5 min gave a supernatant and precipitate. After re-suspension of the precipitate in 0.1 M Tris–HCl buffer (pH 7.6), the mixture was subjected to the next centrifugation. The products were extracted from the combined supernatant with ethyl acetate (×3), washed with brine, and dried over Na_2SO_4. Evaporation and purification by column chromatography on silica gel (*n*-hexane/ethyl acetate = 3/1) afforded (*S*)-**2** (80 mg, 64%, 46% ee) and (*R*)-2-hydroxyhexyl tosylate (**4**, 31 mg, 28%, 99.7% ee). The spectral data were in full agreement with those reported [1,3]. The ees of the resulting (*S*)-**2** and (*R*)-**4** were determined by HPLC analysis, and the absolute configurations were also confirmed by comparing the retention times with those reported [1,3]. HPLC conditions: column, CHIRALCEL AD-H (Daicel Chemical Industries, Ltd.); eluent, *n*-hexane/2-propanol = 90/10; flow rate, 0.5 mL/min; 254 nm; temperature, 25 °C; retention time, **2**: 18 (*S*) and 19.5 (*R*) min, **4**: 32 (*R*) and 43 (*S*) min.

On the other hand, the recovered lipase PS μCap (540 mg) as the precipitate was dried under reduced pressure, and was examined for the next run. The reuse of the recovered enzyme was performed on the basis of keeping the mass ratio of catalyst/substrate in the first run. The hydrolyses of (±)-**2** using lipase PS-IM (60 U, 30 mg) and lipase PS μCap (50 U, 300 mg) were carried out by means of the same procedure, while in the case of lipase PS-IM the enzyme was recovered by filtration with a filter paper.

3. Results and discussion

3.1. Encapsulation of lipase into calcium carbonate microcapsule (μCap) and its catalytic activity

The immobilization of lipase into calcium carbonate was carried out by the analogous process to the phase transformation method we reported before [29]. A calcium carbonate microcapsule (μCap) was immersed in a Tris–HCl buffer solution dissolving lipase PS. The recovered solid was washed thoroughly with deionized water and dried. The encapsulation of lipase in this sample (lipase PS μCap) was ascertained by a diffuse reflectance UV spectroscopic analysis. As shown in Fig. 1, a typical UV absorption of lipase at about 280 nm in wavelength was observed in the lipase PS μCap, while no absorption around 280 nm was found in the μCap before the encapsulation treatment (not shown here) [29]. Since the lipase PS μCap sample was thoroughly washed with water, the immobilized lipase in μCap must be strongly incorporated into the calcium carbonate matrix. Fig. 2 shows the X-ray diffraction patterns of two calcium carbonate samples, the original μCap and the lipase PS μCap. The XRD pattern of the original μCap shows a typical vaterite one, and the

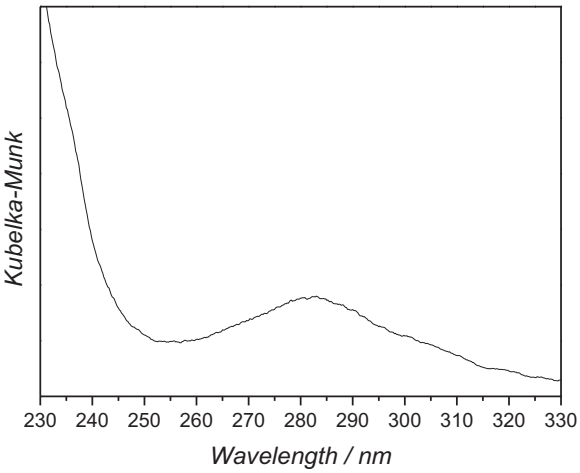


Fig. 1. Diffuse reflectance UV spectrum of lipase-immobilized calcium carbonate (lipase PS μCap).

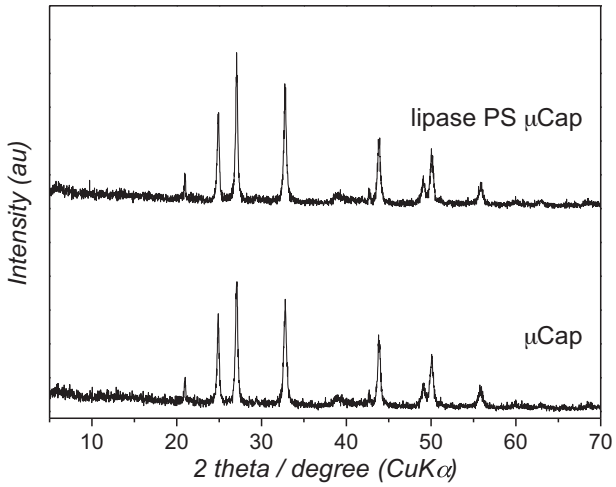
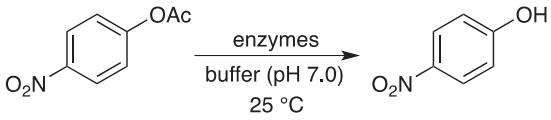


Fig. 2. X-ray diffraction patterns of μCap and lipase PS μCap.

pattern of the lipase PS μCap is approximately identical to that of the μCap. These results illustrated that the crystalline phase of the lipase PS μCap was still metastable vaterite and no phase transition occurred. Therefore, the lipase enzyme was successfully included into vaterite calcium carbonate even when the phase transition did not progressed. From the SEM images shown in Fig. 3, no significant

Table 1
Catalytic activity of the enzymes for the hydrolysis of 4-nitrophenyl acetate.[a].



Entry	Enzyme	Hydrolytic activity (U/mg particle)
1	Lipase PS	2.63
2	Lipase PS-IM	2.00
3	Lipase PS μCap	0.17
4[b]	Lipase PS μCap	0.21

[a] Conditions: 4-nitrophenyl acetate (20 mM) and lipases in 0.1 M Tris–HCl buffer (pH 7.0) were shaken at 25 °C.
[b] After storage for 2 years at 4 °C.

change of the morphology of these samples were found between the μCap (Fig. 3A) and the lipase PS μCap (Fig. 3B).

Next, we examined to confirm the catalytic activity of the lipase PS μCap and the efficiency of the encapsulation process by comparison of the hydrolytic ability with the original lipase PS and the commercially available lipase PS-IM immobilized by physical adsorption on diatomaceous earth. At first, the hydrolysis of 4-nitrophenyl acetate was examined, and the results are summarized in Table 1. From the results shown in Table 1, we definitely confirmed that the newly immobilized lipase PS μCap had the hydrolytic activity for 4-nitrophenyl acetate (0.17 U/mg particle, entry 3). The immobilization yield and expressed activity were 49.0% and 56.7%, respectively. The immobilization did not affect the stability of the original enzyme, which is essentially highly stable, and the hydrolytic ability of lipase PS μCap could be preserved even after storage for 2 years at 4 °C (0.21 U/mg particle, entry 4). The residual lipase PS in the supernatant of the immobilization suspension (1376 U) can be reused in the further encapsulation process to not be wasted as claimed in our previous paper [29].

3.2. Enantioselective transesterification of (±)-1-phenylethanol (*1*) with lipase PS μCap

We next evaluated the enantioselectivity of the lipase PS μCap by the examination of transesterification of (±)-**1** using vinyl acetate for 24 h at 30 °C as the representative reaction in organic media (Table 2). The same reaction with the lipase PS-IM was also attempted in order to compare the reactivity and the enantioselelctivity between them. Entries 1 and 2 showed good enantioselectivities of the lipase PS-IM, and it could be reused after the first run. The transesterification using the lipase PS μCap also proceeded with excellent enantioselectivities to afford optically active compounds (*S*)-**1** and (*R*)-**3** with high ee (entries 3–5). It was

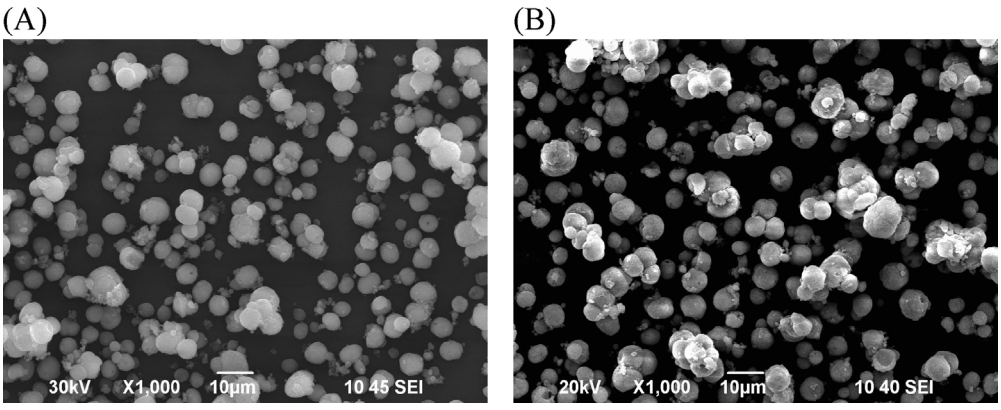
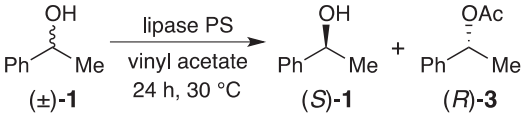


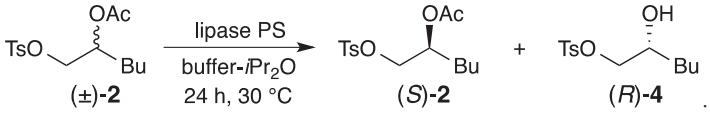
Fig. 3. SEM images of (A) μCap and (B) lipase PS μCap.

Table 2
Enantioselective esterification of (±)-1-phenylethanol (**1**) using lipase PS.



Entry	Lipase PS	Run	Alcohol **1**		Acetate **3**		Conv.	*E* value
			Yield (%)	ee (%)	Yield (%)	ee (%)		
1	IM (40 U)	1st	40	99.6	36	98	0.50	>200
2		2nd	34	99.1	37	99.3	0.50	>200
3	μCap (34 U)	1st	40	98	36	99.0	0.50	>200
4		2nd	39	93	39	99.4	0.48	>200
5		3rd	41	69	28	99.5	0.41	>200

Table 3
Enantioselective hydrolysis of (±)-2-acetoxyhexyl tosylate (**2**) using lipase PS.



Entry	Lipase PS	Run	Acetate **2**		Alcohol **4**		Conv.	*E* value
			Yield (%)	ee (%)	Yield (%)	ee (%)		
1	IM (60 U)	1st	48	74	34	99.0	0.43	>200
2		2nd	83	6	6	95	0.06	41
3	μCap (50 U)	1st	50	66	34	99.0	0.40	>200
4		2nd	58	40	26	98	0.29	146
5		3rd	82	8	6	97	0.08	71

also confirmed that the lipase PS μCap was reusable in essentially the same way as that with the lipase PS-IM.

3.3. Enantioselective hydrolysis of (±)-2-acetoxyhexyl tosylate (**2**) with lipase PS μCap

We next focused on the hydrolysis of (±)-**2** with the lipase PS μCap in aqueous media according to the essential plan of this study. The reactions were carried out in 0.1 M Tris–HCl buffer (pH 7.6) containing 10% diisopropyl ether for 24 h at 30 °C, and the results were summarized in Table 3. In the case of the lipase PS-IM, the first reaction proceeded with a good reactivity and enantioselectivity (entry 1). However, the recovery of the enzyme was difficult by centrifugation, because the small particles of diatomaceous earth as support could not precipitate completely. Therefore, the filtration of the enzyme material that was more time-consuming than centrifugation was required and the recovered ratio was low (ca. 50%). As expected, the decrease of the enzymatic activity took place by the leaching of the hydrophilic lipase PS, and the second reaction using the recovered material scarcely proceeded (entry 2). On the other hand, the lipase PS μCap also catalyzed the enantioselective hydrolysis of (±)-**2** to give (*S*)-**2** and (*R*)-**4** (entry 3). It is noteworthy that the immobilized enzyme was easily precipitated to be recovered by centrifugation. Furthermore, the second reaction using the recovered material moderately proceeded (conv. 0.29; entry 4) unlike that in the case of the lipase PS-IM. Thus, the recyclability of the lipase PS μCap was ascertained. However, the enzymatic reactivity significantly decreased in the following third reaction (entry 5).

The UV absorption of lipase around 280 nm in wavelength was still observed in the spectrum of the lipase PS μCap after the three times use, where the enzymatic activity was poor. This observation indicated that the leaching of lipase from μCap was not responsible for the crucial decrease of the enzymatic ability in the third round use of the lipase PS μCap. Fig. 4 illustrated the variation of the XRD pattern with the recycling use of the lipase PS μCap. The main peak of calcite at 29.4 in 2 theta degree increased remarkably after the first use and the phase transition from vaterite to calcite was completed in the lipase PS μCap after the three times use. It seems that the poor porosity of the calcite crystal (specific surface area less than 1 m^2/g [28,29]) definitively prevented the access of the reactants to the enzymatic site of lipase in the lipase PS μCap in the case of the third reaction. The phase transition of calcium carbonate from vaterite to calcite is generally progressed by the dissolutions of Ca^{2+} and CO_3^{2-} ions of vaterite into the aqueous solution and by their recrystallization to calcite [35]. In the case of the enzymatic reaction in aqueous media, this phase transition was also accompanied in the aqueous solution (Table 3), while no dissolution of calcium carbonate inhibited the phase transition in the

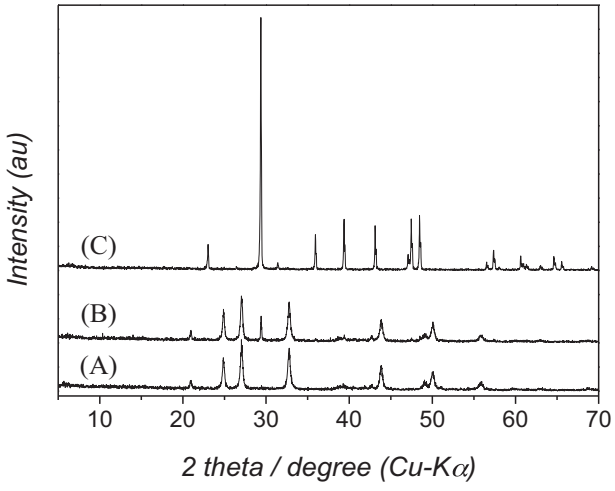


Fig. 4. The variation of the XRD pattern with the recycling use of lipase PS μCap; (A) lipase PS μCap before the first reaction, (B) after the first reaction, (C) after the third reaction.

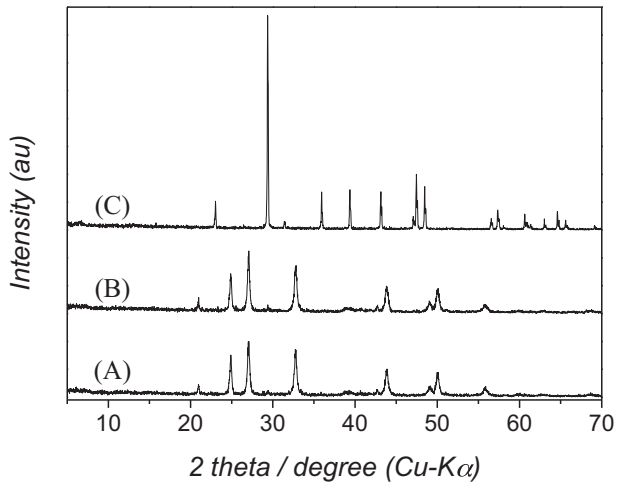


Fig. 5. X-ray diffraction patterns of μCap after soaking treatment for 7 days in 1 M Tris–HCl buffer solution with zinc chloride (A), in 1 M Tris–HCl buffer solution with zinc chloride and the following one in 1 M Tris–HCl buffer solution without zinc chloride (B), and in 1 M Tris–HCl buffer solution without zinc chloride (C).

case of the reactions in organic media (Table 2). The calcite crystal formed in the sample after the first reaction might accelerate the phase transition because the calcite particle served seed crystals. It is presumable that the complete phase transition to calcite conclusively prevented the enzyme reaction of the lipase PS μCap by the poor accessibility of the reactants after the three times use.

3.4. Zinc treatment of lipase PS μCap and the reactivity with the enzyme in aqueous phase

The preservation of vaterite crystalline structure of the lipase PS μCap during the catalytic reaction in aqueous media is expected to maintain the enzymatic activity of the lipase and to create a reusable immobilized lipase catalyst employable even in aqueous solution. It is reported that the treatment of calcium carbonate with zinc chloride suppresses the phase transition of vaterite by the isomorphic substitution from calcium to zinc [36]. Then, we examined the modification of the lipase PS μCap by the zinc treatment for preventing the phase transition. At the beginning, the μCap (no inclusion of lipase) in vaterite phase was soaked for 7 days in the 1 M Tris–HCl buffer solution with zinc chloride. The variations of XRD pattern of the resulting calcium carbonate materials are shown in Fig. 5. In the XRD pattern of this sample, the peaks of vaterite were still strong and the weak calcite peak at 29.4 in 2 theta degree was found even after the soaking (Fig. 5, line A) to indicate that the phase transition scarcely progressed. This zinc treated sample was further soaked in another 1 M Tris–HCl buffer solution without zinc chloride for 7 days. The XRD pattern of the resulting sample was approximately the same as the sample before this treatment (Fig. 5, line B), revealing that the zinc treatment inhibited the phase transition even in aqueous solution containing no zinc ion. On the other hand, when the μCap without the zinc treatment was added to the buffer solution without zinc chloride, the complete phase transition to calcite was observed (Fig. 5, line C). Thus, it is ascertained that the treatment of zinc chloride efficiently prevented the phase transition of vaterite to calcite. Then, the lipase PS μCap was soaked for 1 day in the zinc chloride solution. The XRD pattern of this sample was approximately similar to the original lipase PS μCap. In addition, no phase transition of this zinc-treated lipase PS μCap occurred even after the soaking to 1 M Tris–HCl buffer solution (without zinc chloride).

Finally, we demonstrated the hydrolysis of (±)-**2** using the lipase PS μCap treated with zinc chloride solution (Table 4). As expected,

Table 4
Enantioselective hydrolysis of (±)-**2** using lipase PS μCap treated with zinc chloride solution.[a]

Entry	Run	Acetate **2**		Alcohol **4**		Conv.	*E* value
		Yield (%)	ee (%)	Yield (%)	ee (%)		
1	1st	64	46	28	99.7	0.32	>200
2	2nd	69	37	27	99.0	0.27	>200
3	3rd	70	31	24	98	0.24	134
4	4th	69	34	24	99.5	0.25	>200
5	5th	89	17	19	98	0.15	117

[a] In the 1st reaction, 24 units of the enzyme was used.

the reactions from the first to the forth run enantioselectively proceeded without noticeable decrease of the conversion. Although the conversion of the fifth run was approximately half of that of the first run, the enantioselectivities of the enzyme sufficiently remained in the recycle process. Thus, we finally found that the zinc-treated lipase PS μCap was a recyclable immobilized lipase catalyst even in aqueous solution, although further improvements are still required.

4. Conclusions

In summary, we have easily prepared a new type of an immobilized lipase based on encapsulation into vaterite calcium carbonate microcapsule. It was found that the immobilized enzyme (lipase PS μCap) was useful both for enantioselective transesterification of **1** in organic media and for enantioselective hydrolysis of **2** even in aqueous media. This new material, the lipase PS μCap, was recyclable for the reactions both in organic media and aqueous media, different from the lipase PS-IM. In these reactions, the lipase PS μCap also indicated the high stability against the use of organic solvents such as ethyl acetate and diisopropyl ether. However, the phase transition of vaterite to calcite inhibited the interaction between the lipase and the reactant, and finally deactivated it after three times use. This deactivation was substantially overcome by the treatment of the lipase PS μCap with zinc chloride solution for preventing the phase transition by the isomorphic substitution from calcium to zinc. We finally accomplished the reuse of the immobilized lipase for the hydrolysis of **2** in aqueous media. Our immobilization procedure has the advantages not only for facile preparation but also for an easy recovery and convenient recycling even in aqueous media. Lipase PS is one of the representative enzymes, which can hydrolyze various kinds of compounds [37–39], and our methods described here are also potentially useful for the many hydrolytic reactions already reported.

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