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Journal of Molecular Catalysis B: Enzymatic 9 (2000) 269–274

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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# Immobilization of lipase on a new inorganic ceramics support, toyonite, and the reactivity and enantioselectivity of the immobilized lipase

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Received 22 September 1999; received in revised form 21 October 1999; accepted 22 October 1999

**Abstract**

A porous ceramics support, Toyonite 200-M (TN-M), for the immobilization of lipases was prepared hydrothermally from the minerals of kaolinite. Compared with some other commercial solid supports, the TN-M one exhibited better stability and higher selectivity for lipase proteins, and lipase PS (*Pseudomonas cepacia*) immobilized on the ceramics support showed higher reactivity for organic substrates than the free crude enzyme. © 2000 Elsevier Science B.V. All rights reserved.

*Keywords:* Immobilization; Inorganic support; Lipase; Acylation; Biotransformation

## 1. Introduction

Lipases have been used frequently in the form of a crude protein extract as an asymmetric catalyst for the preparation of chiral building blocks and enantiomerically pure synthetic and natural products including pheromones and antibiotics [1–3], because they are inexpensive and easy to handle for synthetic organic chemists. Further, the enzymes can accept and transform enantioselectively not only natural substrates but also foreign synthetic substrates in both aqueous solutions and organic solvents under mild conditions. The use of crude lipases, however, often leads to the longer reaction time and/or the lower enantioselectivity and may cause the opposite selectivity due to the presence of several contaminating enzymes [4,5]. Recently, Bristol–Myers Squibb (BMS) lipase (*Pseudomonas* sp. SC13856) and lipase PS were independently immobilized on a solid support, Accurel polypropylene, the reactivity and enantioselectivity of these two immobilized lipases being demonstrated for the enzymatic preparation of a chiral synthon in the semisynthesis of taxol having anti-cancer activity [6]. Several lipases (e.g., *Chromobacterium viscosum*, *Pseudomonas* sp. and *Candida cylindracea* lipases) immobilized in microemulsion-based gels were

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1381-1177/00/$ - see front matter 
PII: S1381-1177(99)00105-8

each used in the enantioselective esterification of racemic alcohols such as (±)-*sec*-phenethyl alcohol (**1**) [7]. A more recent immobilization for lipases required the use of hydrophobic silica gels which were prepared from alkyl-substituted silane precursors [8]. The immobilization with hydrophobic silica matrices was applicable to lipases from different sources, the activity of immobilized lipases being higher than that of the commercially available crude enzymes.

In an effort to increase the reactivity and selectivity of lipases as an asymmetric catalyst by means of immobilization on solid support [9], a kind of inorganic, porous ceramics particles, which we now call Toyonite 200-M (TN-M), was prepared hydrothermally from kaolin minerals under acidic conditions; TN-M support has the average particle size of 155 μm and the surface was treated with an organic coating agent possessing methacryloyloxy group. The group-free Toyonite showed a poor binding ability for proteins in the immobilization of lipase PS described below.

## 2. Experimental

### 2.1. Materials

Racemic alcohols **1**, **2** and **4**–**11** were each purchased from Tokyo Kasei Kogyo or Aldrich. Aromatic alcohol **3** was prepared from the corresponding ketone by reduction with $NaBH_4$ in ethanol. Lipase from *Pseudomonas cepacia* (Amano PS, 30 000 U/g) was used. Analytical samples were prepared by a combination of column chromatography and microvacuum distillation with a Kugelrohr distillation apparatus. All reaction products were fully characterized by their IR and $^1$H-NMR spectral data. IR spectra were determined with a Fourier transform Perkin-Elmer IR spectrometer. $^1$H-NMR spectra were obtained with a Bruker AMX-R400 spectrometer for $CDCl_3$ solutions with $Me_4Si$ as a standard. Gas chromatography was carried out on a Hitachi G-5000 gas chromatograph equipped with different capillary columns (GL Sciences) using He as the carrier gas. Optical rotations were measured on a Horiba SEPA-300 high-sensitivity polarimeter.

### 2.2. Immobilization of lipase PS on Toyonite

A suspension of crude lipase PS (2.5 g) in a phosphate buffer (10 ml, pH = 7) was stirred for some time at room temperature. After filtration through Celite, to the filtrate was added Toyonite (200 mg) and the mixture was then stirred for 10–15 h at room temperature. The mixture was filtered, and the resulting lipase-supporting Toyonite (TN-M PS) was dried and stored under suitable conditions.

### 2.3. Lipase-catalyzed acylation

A mixture of each racemic alcohol **2**–**11**, TN-M PS (or free PS), vinyl acetate and dry diethyl ether was stirred for the required period (Table 3). The reaction mixture was filtered through Celite, and the filtrate was washed with brine, dried over anhydrous $Na_2SO_4$ and concentrated. Purification of each product by column chromatography on silica gel gave chiral acetates and alcohols, which were characterized on the basis of their spectral data. According to this procedure (use of TN-M PS), the following acetates and alcohols were obtained.

(*R*)-**2a**, $[\alpha]_D^{20}$ +99.22° ($c$ = 1.65, $CHCl_3$) {for corresponding alcohol (*R*)-**2** obtained by alkaline hydrolysis of (*R*)-**2a**, $[\alpha]_D^{20}$ +50.68° ($c$ = 2.25, $CHCl_3$)}; IR $\nu_{max}$ (neat) ($cm^{-1}$): 2981, 2936, 1735, 1516, 1372, 1243, 1036, 832; $^1$H-NMR $\delta$: 1.51 (3H, d, $J$ = 6.4 Hz), 2.04 (3H, s), 3.78 (3H, s), 5.84 (1H, q, $J$ = 6.4 Hz), 6.86 (1H, d, $J$ = 8 Hz), 7.28 (1H, d, $J$ = 8 Hz). (*S*)-**2**, $[\alpha]_D^{20}$ −43.57° ($c$ = 1.99, $CHCl_3$).

(*R*)-**3a**, $[\alpha]_D^{20}$ +90.37° ($c$ = 1.31, $CHCl_3$) {for corresponding alcohol (*R*)-**3** prepared from (*R*)-**3a**, $[\alpha]_D^{20}$ +52.11° ($c$ = 1.65, $CHCl_3$)}; IR $\nu_{max}$ (neat) ($cm^{-1}$): 2983, 2934, 1740, 1519, 1373, 1240, 1048, 843; $^1$H-NMR $\delta$: 1.50 (3H,

d, $J = 6.5$ Hz), 2.06 (3H, s), 2.32 (3H, s), 5.84 (1H, q, $J = 6.5$ Hz), 7.14 (1H, d, $J = 8$ Hz), 7.24 (1H, d, $J = 8$ Hz). (*S*)-**3**, $[\alpha]_D^{20}$ −47.56° ($c = 1.74$, $CHCl_3$).

(*R*)-**4a**, $[\alpha]_D^{20}$ +98.26° ($c = 1.12$, $CHCl_3$) {for corresponding alcohol (*R*)-**4**, $[\alpha]_D^{20}$ +48.11° ($c = 2.05$, $CHCl_3$)}; IR $\nu_{max}$ (neat) ($cm^{-1}$): 2982, 2932, 1740, 1496, 1372, 1240, 1066, 827; $^1$H-NMR $\delta$: 1.50 (3H, d, $J = 6.5$ Hz), 2.06 (3H, s), 5.83 (1H, q, $J = 6.5$ Hz), 7.28 (4H, m). (*S*)-**4**, $[\alpha]_D^{20}$ −42.48° ($c = 2.97$, $CHCl_3$).

(*R*)-**5a**, $[\alpha]_D^{20}$ +101.91° ($c = 2.32$, $CHCl_3$) {for corresponding alcohol (*R*)-**5**, $[\alpha]_D^{20}$ +45.64° ($c = 1.08$, $CHCl_3$)}; IR $\nu_{max}$ (neat) ($cm^{-1}$): 2983, 2933, 1736, 1492, 1371, 1239, 1072, 823; $^1$H-NMR $\delta$: 1.51 (3H, d, $J = 6.5$ Hz), 2.06 (3H, s), 5.83 (1H, q, $J = 6.5$ Hz), 7.23 (2H, d, $J = 8.4$ Hz), 7.44 (2H, d, $J = 8.4$ Hz). (*S*)-**5**, $[\alpha]_D^{20}$ −31.54° ($c = 2.17$, $CHCl_3$).

(*R*)-**6a**, $[\alpha]_D^{20}$ +114.51° ($c = 5.14$, $CHCl_3$) {for corresponding alcohol (*R*)-**6**, $[\alpha]_D^{20}$ +42.45° ($c = 3.74$, $CHCl_3$)}; IR $\nu_{max}$ (neat) ($cm^{-1}$): 3058, 2983, 2935, 1740, 1449, 1371, 1240, 1064, 860, 821; $^1$H-NMR $\delta$: 1.63 (3H, d, $J =$ 6.5 Hz), 2.10 (3H, s), 6.06 (1H, q, $J = 6.5$ HZ), 7.45–7.50 (3H, m), 7.80–7.84 (4H, m). (*S*)-**6**, $[\alpha]_D^{20}$ −45.46° ($c = 3.71$, $CHCl_3$).

(*R*)-**7a**, $[\alpha]_D^{20}$ +4.46° ($c = 5.02$, *n*-pentane) {for corresponding alcohol (*R*)-**7**, $[\alpha]_D^{20}$ −5.12° ($c = 3.55$, *n*-pentane). (*S*)-**7**, $[\alpha]_D^{20}$ +4.68° ($c$ = 3.50, *n*-pentane).

(*R*)-**8a**, $[\alpha]_D^{20}$ −2.92° ($c = 1.95$, *n*-pentane) {for corresponding alcohol (*R*)-**8**, $[\alpha]_D^{20}$ −5.80° ($c = 2.41$, *n*-pentane)}. (*S*)-**8**, $[\alpha]_D^{20}$ +7.78° ($c$ = 3.08, *n*-pentane).

(*R*)-**9a**, $[\alpha]_D^{20}$ −3.47° ($c = 2.70$, *n*-pentane) {for corresponding alcohol (*R*)-**9**, $[\alpha]_D^{20}$ −7.07° ($c = 3.59$, *n*-pentane)}; IR $\nu_{max}$ (neat) ($cm^{-1}$): 2957, 2932, 1741, 1461, 1373, 1245, 1124, 1021, 950; $^1$H-NMR $\delta$: 0.88 (3H, t, $J = 6.7$ Hz), 1.20 (3H, d, $J = 6.1$ Hz), 1.28–1.52 (10H, m), 2.02 (3H, s), 4.89 (1H, m). (*S*)-**9**, $[\alpha]_D^{20}$ +10.07° ($c = 2.61$, *n*-pentane).

(*R*)-**10a**, $[\alpha]_D^{20}$ −9.39° ($c = 3.18$, *n*-pentane) {for corresponding alcohol (*R*)-**10**, $[\alpha]_D^{20}$ −13.68° ($c = 3.93$, *n*-pentane)}. (*S*)-**10**, $[\alpha]_D^{20}$ +11.34° ($c = 1.41$, *n*-pentane).

(*S*)-**11a**, $[\alpha]_D^{20}$ −2.26° ($c = 1.61$, *n*-pentane) {for corresponding alcohol (*S*)-**11**, $[\alpha]_D^{20}$ +5.10° ($c = 1.80$, *n*-pentane)}. (*R*)-**11**, $[\alpha]_D^{20}$ −2.54° ($c = 1.84$, *n*-pentane).

The IR and $^1$H-NMR spectra of (*S*)-alcohols **2**, **4** and **5** were identical with those of their commercially available racemic compounds [10]. The IR and $^1$H-NMR spectra of (*S*)-**3** were consistent with those of racemic **3** prepared by $NaBH_4$ reduction of the corresponding ketone [10]. Spectral data of (*S*)-**6** were consistent with those reported previously for the racemic sample [11]. The IR and $^1$H-NMR spectra of chiral alcohols **7**–**11** and esters **7a**, **8a**, **10a** and **11a** were identical with those reported previously [5].

Similar acylations of (±)-**1** with immobilized PS lipases were carried out in the absence of diethyl ether as described in Table 2.

Table 1
Immobilization of lipase PS on solid supports
Each immobilization was carried out by the procedures described in the text.

| Solid support | Residual protein (mg/ml)[a] | Lipase residual activity (U/ml)[b] | Rate of immobilized protein (%) | Rate of immobilized lipase (%) |
|---|---|---|---|---|
| Toyonite 200-M | 9.8 | 45 | 12.5 | 99.4 |
| Celite | 10.4 | 5000 | 7.2 | 32.4 |
| Glass beads (MIS-10) | 10.2 | 5300 | 8.9 | 28.4 |
| Amberlite (XAD-7) | 5.5 | 1600 | 50.9 | 78.4 |

[a] Protein remaining in the enzyme solution after immobilization. The initial amount of protein before immobilization was 11.2 mg/ml. Protein was determined by Lowry's method [14].

[b] Activity of the lipase remaining in the enzyme solution after immobilization. The initial activity before immobilization was 7400 U/ml. Activity was determined by the hydrolysis of olive oil according to JIS method (K 0601, 1988).

(*R*)-**1a** (use of TN-M PS), $[\alpha]_D^{20}$ +107.89° ($c = 6.30$, $CHCl_3$) {for corresponding alcohol (*R*)-**1** prepared by alkaline hydrolysis of (*R*)-**1a**, $[\alpha]_D^{20}$ +62.01° ($c = 4.12$, $CHCl_3$)}; IR $\nu_{max}$ (neat) ($cm^{-1}$): 3034, 2983, 2935, 1740, 1496, 1455, 1372, 1240, 1210, 945, 856; $^1$H-NMR $\delta$: 1.54 (3H, d, $J = 6.6$ Hz), 2.07 (3H, s), 5.87 (1H, q, $J = 6.6$ Hz), 7.35 (5H, m). (*S*)-**1**, $[\alpha]_D^{20}$ −53.21° ($c = 5.20$, $CHCl_3$).

The $^1$H-NMR spectrum of chiral alcohol **1** was consistent with that of the commercially available racemic compound and that reported previously [12].

## 3. Results and discussion

To evaluate the ability of TN-M and other solid supports to concentrate lipase proteins, the immobilization of lipase PS on each support was carried out. The lipase (lipase Amano PS, *P. cepacia*) is one of the most popular lipases utilized in organic synthesis. As shown in Table 1, the residual activity of lipase PS — that is, the activity of lipase PS remaining in the filtrate, which was each obtained in the procedure of immobilization on four different solid supports — was examined by the hydrolysis of olive oil. Among each filtrate for these solid supports including glass beads and synthetic resins, the filtrate for TN-M displayed the lowest PS residual activity. The results indicate that the binding ability for lipase proteins of TN-M is higher than that of other solid supports. Table 1 also shows that the amount of immobilized lipase is not always proportional to that of immobilized protein. The data for the lipase PS-catalyzed acylation of **1** in Table 2 show that the esterification activity of TN-M PS lipase is greater than that of other three immobilized PS ones. In order to obtain more information concerning the enzymatic activity of TN-M PS lipase, the consecutive use of the lipase was performed for the acylation of racemic **1** by a column reactor; after 12 days' continuous use, TN-M PS maintained about 70% of the initial catalytic activity (Fig. 1).

The reactivity and enantioselectivity of TN-M PS lipase were evaluated in the biotransformation of 10 aromatic and aliphatic secondary alcohols (Fig. 2 and Table 3). The reactivity of

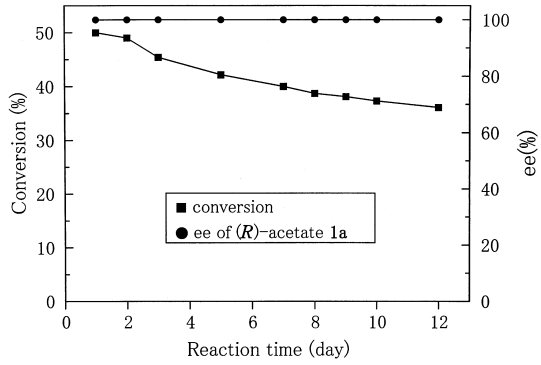
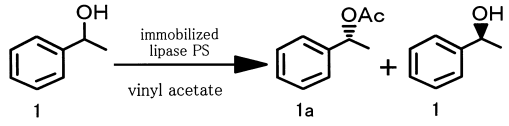



Fig. 1. Acylation of (±)-**1** by column reactor containing TN-M PS lipase. TN-M PS lipase (50 mg) was packed in a column reactor of 1 ml, and the reaction was run at 25°C by consecutively passing a mixture of (±)-**1** (1.2 g) and vinyl acetate (4.5 g) at SV (space velocity) = 15 through the column. About 0.4 kg of (*R*)-acetate **1a** with almost 100% ee was produced by 12 days' continuous acylation. The ee was estimated as described previously [5]. Conversions (%) were determined as described in Table 3.

Table 2
Activity of immobilized PS lipase in the acylation of (±)-**1**
Reactions were run using immobilized lipase PS (50 mg), (±)-**1** (1.2 g), and vinyl acetate (4.5 g) at 25°C.




| Solid support | Protein on support (mg/ml support)[a] | Initial activity (U/ml support)[b] | Specific activity (U/mg protein)[c] |
|---|---|---|---|
| Toyonite 200-M | 35 | 1303 | 37.2 |
| Celite | 20 | 151 | 7.6 |
| Glass beads (MIS-10) | 25 | 20 | 0.8 |
| Amberlite (XAD-7) | 143 | 55 | 0.4 |

[a] Determined by Lowry's method [14].
[b] Determined on the basis of the amount of the acetate **1a** produced during the first 20 min.
[c] Initial activity/protein on support.

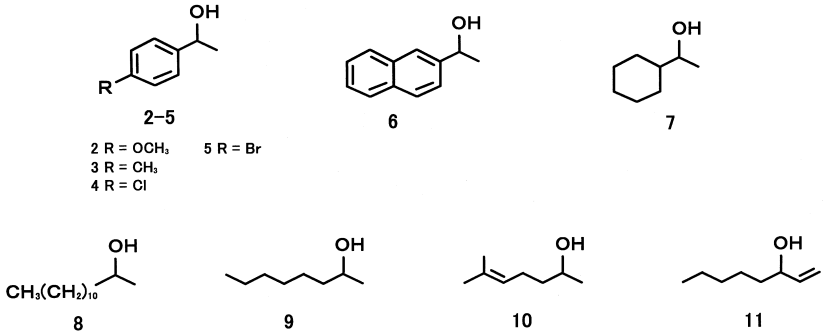



Fig. 2. Synthetic substrates used in the acylation with TN-M PS and free PS lipases.

TN-M PS can be generally greater in the acylation of the synthetic substrates **2**–**11** than that of the free enzyme powder. Each acylation of aromatic alcohols **2**–**6** with TN-M PS reached 47–52% conversion at about 15 min, while similar reactions with free PS required 1 h to reach almost the same conversions. Enzymatic acylations of alicyclic alcohol **7**, saturated alcohols **8**, **9**, and unsaturated alcohols **10**, **11** with the immobilized lipase PS proceeded at a faster rate than did similar ones with free PS lipase. As exemplified in Table 3, TN-M PS lipase

Table 3
Reactivity and enantioselectivity of TN-M PS and free PS lipases in the acylation of secondary alcohols


| Alcohol | TN-M PS[a] | | | | Free PS[b] | | | |
|---|---|---|---|---|---|---|---|---|
| | Conversion (%)[c] (time, min) | ee (%)[d] | | $E$[e] | Conversion (%)[c] (time, h) | ee (%)[d] | | $E$[e] |
| | | Ester | Alcohol | | | Ester | Alcohol | |
| 2 | 49(15) | 98 | 86 | 270 | 48(1) | 93 | 88 | 76 |
| 3 | 47(15) | > 99 | 86 | > 300 | 47(1) | > 99 | 84 | > 300 |
| 4 | 49(15) | > 99 | 88 | > 300 | 51(1) | > 99 | 92 | > 300 |
| 5 | 47(15) | > 99 | 78 | > 300 | 49(1) | > 99 | 84 | > 300 |
| 6 | 52(20) | 90 | 97 | 80 | 51(1) | 95 | 90 | 130 |
| 7 | 57(20) | 92 | 98 | 110 | 49(1) | 97 | 81 | 140 |
| 8 | 67(20) | 71 | 89 | 17 | 48(1) | 82 | 68 | 21 |
| 9 | 70(20) | 56 | 93 | 12 | 57(1) | 69 | 76 | 12 |
| 10 | 50(20) | 88 | 75 | 36 | 47(2) | 89 | 66 | 33 |
| 11 | 42(15) | 63 | 40 | 7 | 49(2) | 58 | 61 | 6 |

[a] PS protein (25.4 mg/g support = 35 mg/ml support) was determined according to Lowry et al. [14]. Reactions were carried out in diethyl ether (15 ml) at 30°C using TN-M PS (2 g), each racemic alcohol (0.5 g), and vinyl acetate (1 g).

[b] The commercial lipase powder was assumed to contain about 2% of PS protein. Reactions were conducted as described for TN-M PS, except for the use of free PS (2.5 g).

[c] All reaction products were analyzed by capillary GLC (TC-WAX 30 m × 0.25 mm). Conversions (%) were determined on the basis of the peak areas of substrates and products.

[d] The ee of esters **2a**–**11a** and alcohols **2**–**11** was determined as described previously [5,10].

[e] Calculated according to Chen et al. [15].

gave comparable enantioselectivity to the free enzyme against some secondary alcohol substrates. The present TN-M PS lipase was therefore available without loss of the reactivity and the enantioselectivity for synthetic substrates.

From above results, we recognized that the Toyonite, a kind of porous ceramics, is a new inorganic support which can concentrate selectively lipase proteins from the crude enzyme. Although the mechanism for immobilization including the effect of organic coating agent is not yet clear, the higher reactivity of TN-M PS lipase compared to the corresponding free lipase suggests the stability of the immobilized lipase and especially the presence of moderately purified PS lipase supported on the surface of TN-M [8,13]. The difference of the size of holes on the Toyonite surface and the choice of organic coating agent make it possible to control the binding of the inorganic support to lipases and may improve the thermal stability and the chemical and physical resistance of the lipases immobilized on Toyonite.

## Acknowledgements

We wish to thank Isao Matsugeta and Yasutaka Hamaguchi for their contribution on lipase-catalyzed biotransformations. We are also grateful to Dr. Makoto Kamezawa (Konan Chemical Industry, Japan) for his advice and discussion.

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