

Novel immobilization method of enzymes using a hydrophilic polymer support†

Juta Kobayashi, Yuichiro Mori and Shū Kobayashi*

Received (in Cambridge, UK) 30th June 2006, Accepted 10th August 2006

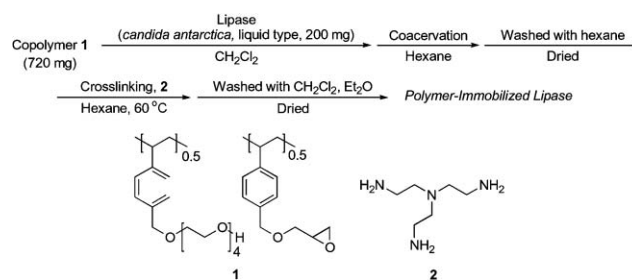
First published as an Advance Article on the web 30th August 2006

DOI: 10.1039/b609335c

A novel immobilization of an enzyme with a hydrophilic polymer support in organic solvents has been developed utilizing the “polymer-incarcerated (PI) method”, which has been used to immobilize metal catalysts; the kinetic resolution of secondary alcohols was found to proceed more smoothly using immobilized lipases (CALB) than free lipases.

Enzymes, which catalyze a variety of reactions and play an indispensable role in living systems, have many specific features, such as highly controlled regio-, stereo- and substrate-specificity. In addition, enzymatic reactions generally proceed under very mild conditions. Therefore, enzymes have found wide application in the pharmaceutical sciences, chemical synthesis and biotechnology, *etc.* Even though a large number of efficient chemical reactions have been well developed, enzymes still play a significant role in providing many useful chemical products.¹

However, the use of enzymes is subject to limitations and drawbacks in some cases. For example, generally speaking, enzymes are relatively expensive, and therefore discarding them after a single use is not economical. Another problem is their general instability towards heat, organic solvents, acids or bases, *etc.* One method to circumvent these issues is to immobilize the enzyme on a suitable supporting medium. Not only does this facilitate easier recovery and reuse of the enzyme, but in many cases, immobilized enzymes show a higher stability than free species. In recent years, a variety of methods for the immobilization of enzymes, such as attachment to prefabricated carriers (by covalent or ionic binding, adsorption, *etc.*) or cross-linkage and entrapment (encapsulation), have been developed.² Among these, entrapment—where covalent bonding, which may alter the enzyme structure and cause loss of activity, is avoided—is an effective method for immobilization. While several types of entrapped enzymes in liposomes, micelles and membranes, *etc.* have been reported,³ we have developed the “Polymer-Incarcerated (PI)” method⁴ in our laboratory for immobilizing a variety of metal catalysts. A representative example of this method is PI-Pd, which exhibits excellent catalytic properties for typical reactions mediated by palladium, such as the Heck reaction, allylic substitution and hydrogenation, *etc.*^{4a,b} Recently, this immobilization method has been successfully applied to microchannel reactors, providing efficient systems for hydrogenation.⁵ Whilst the immobilization of



Scheme 1 Preparation of polymer-immobilized lipase.

enzymes has been widely conducted in aqueous phases, the corresponding immobilization procedure conducted in organic solvents is much less well known. Herein, we describe a new immobilization method for lipase in an organic phase and disclose our results of using the immobilized lipase for the kinetic resolution of secondary alcohols, a well known and representative reaction that is promoted by lipases.

The immobilization procedure is depicted in Scheme 1. Lipase B from *Candida antarctica* (CALB)⁶ was selected as the candidate enzyme and copolymer **1** was chosen as the polymer support. This copolymer contains hydrophilic moieties such as tetraethylene glycol (TEG) and glycidol, and thus hydrophilic interactions between the copolymer and the liquid lipase were expected. First, copolymer **1** was dissolved in CH₂Cl₂, and then the liquid lipase was dispersed into the solution by stirring vigorously. After the lipase had been dispersed, hexane was slowly added. This caused coacervation to occur, forming a precipitate with lipase particles inside the polymer. At this stage, most of the lipase was in the polymer phase. The supernatant was removed by decantation, and then cross-linking was conducted using amine **2** as a cross-linking

Table 1 Reuse of the catalyst

Yield, ^a ee (recovered alcohol) (%)				
1st	2nd	3rd	4th	5th
42, > 99 (42, 99)	42, > 99 (42, 99)	44, > 99 (42, 99)	45, > 99 (44, > 99)	43, > 99 (43, > 99)
30, > 99 ^b	8, > 99 ^b	—	—	—



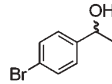
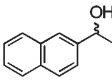


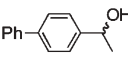
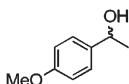
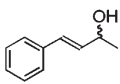
^a Isolated yield. ^b Free liquid lipase (100 mg) was used. The yield and ee of the recovered alcohol were not determined.

Graduate School of Pharmaceutical Sciences, The University of Tokyo, The HFRE Division, ERATO, Japan Science and Technology Agency (JST), Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan.

E-mail: skobayas@mol.f.u-tokyo.ac.jp; Fax: +81 3 5684 0634

† Electronic supplementary information (ESI) available: Experimental section. See DOI: 10.1039/b609335c

Table 2 Kinetic resolution of secondary alcohols

Substrate			$\xrightarrow[\text{Et}_2\text{O}, 25^\circ\text{C}, 24\text{h}]{\text{Polymer-Immobilized Lipase, vinyl acetate (5.0 eq.)}}$			Product + Unreacted Alcohol		
Substrate	Ester		Substrate	Ester		Substrate	Ester	
	Yield ^a	ee (%)		Yield ^a	ee (%)		Yield ^a	ee (%)
	17	> 99		42	> 99		40	> 99
	46	> 99		44	> 99		3	n.d. ^b
	47	> 99		45	98		43	98

^a Isolated yield. ^b Not determined.

agent at 60 °C in hexane. After 12 h, the supernatant was again decanted off and the residue washed with organic solvents such as dichloromethane, diethyl ether or ethyl acetate. The residue was then dried *in vacuo*, giving a polymer-immobilized lipase (242 mg for 1 reaction). The loading level of the lipase was estimated indirectly as follows: First, the organic washing solutions used during the immobilization process were combined, extracted with water and the amount of protein in the water phase determined by Lowry's method.⁷ Next, the loading of the immobilized lipase was calculated by subtracting the amount of protein found in the aqueous phase from the total amount of protein initially used. Using this method, the loading was estimated to be 72.5 mg protein g⁻¹, indicating that more than 90% of the lipase had been immobilized onto the polymer.

Using the polymer-immobilized lipase obtained, we next examined the kinetic resolution of secondary alcohols. Racemic 1-phenylethyl alcohol was used as a starting substrate to assess the activity and reusability of the immobilised enzyme. The reaction was conducted in diethyl ether at 25 °C for 24 h using vinyl acetate as an acetylating agent. The results are shown in Table 1. During the reaction, the immobilized lipase dispersed into the reaction mixture, and the reaction proceeded to give both the product and the recovered alcohol in good yields with excellent enantioselectivity. After the reaction was complete, the organic liquid phase was collected by decantation, the residue washed with organic solvents, and the remaining immobilized polymer dried and used for the next trial. In this way, it was found that the polymer-immobilized lipase could be recovered and reused at least five times without loss of activity. As a control experiment, the free lipase before immobilization was used and the reaction conducted under the identical conditions. This reaction proceeded sluggishly, which might be ascribed to insufficient contact between the lipase and the substrate due to the liquid lipase remaining at the bottom of the flask during the reaction. After decantation and washing with organic solvents, the lipase was recovered and reused, as in

the case of the immobilized lipase. The second use of free lipase gave less acetylated product, implying that lipase was deactivated by organic solvents and that protection of an enzyme by a polymer support might take effect in the case of an immobilized lipase.

Encouraged by these results, we next examined other substrates (Table 2). The reaction proceeded with various kinds of secondary alcohols. For example, those having naphthyl groups, several substituted benzene groups or cyclic alcohols gave good resolution, with the exception of 1-phenyl-1-propanol. The reaction rate depended on the substrate: some were complete within several hours while others required longer reaction times.

In summary, a new immobilization method of lipase in organic phases has been developed utilizing a hydrophilic polymer support. The immobilization method is based on the PI method, which has previously been used to immobilize metal catalysts. The immobilized lipase exhibits high enzymatic activity, and efficient kinetic resolution of secondary alcohols has been attained. Recovery and reuse of the immobilized lipase is possible, implying a higher stability against organic solvents than free lipase. Further applications of the immobilization method to other enzymes and/or microchannel reactors are under investigation.

Notes and references

- E. García-Urdiales, I. Alfonso and V. Gotor, *Chem. Rev.*, 2005, **105**, 313 and references cited therein.
- (a) B. P. Sharma, L. F. Bailey and R. A. Messing, *Angew. Chem., Int. Ed. Engl.*, 1982, **21**, 837; (b) *Chiral Catalyst Immobilization and Recycling*, ed. D. E. De Vos, I. F. J. Vankelecom and P. A. Jacobs, Wiley-VCH, Weinheim, 2000, vol. 5, p. 97. For several examples of lipase immobilization, see: (c) R. A. Wisdom, P. Dunnill and M. D. Lilly, *Enzyme Microb. Technol.*, 1985, **7**, 567; (d) C. J. Gray, J. S. Narang and S. A. Barker, *Enzyme Microb. Technol.*, 1990, **12**, 800; (e) J. M. Moreno, M. J. Hernaiz, J. M. Sánchez-Montero, J. V. Sinisterra, M. T. Bustos, M. E. Sánchez and J. F. Bello, *J. Mol. Catal. B: Enzym.*, 1997, **2**, 177; (f) A. E. Ivanov and M. P. Schneider, *J. Mol. Catal. B: Enzym.*, 1997, **3**, 303; (g) J. M. Moreno, M. Arroyo, M. J. Hernaiz and J. V. Sinisterra, *Enzyme Microb. Technol.*, 1997, **21**, 553; (h) J. M. S. Rocha, M. H. Gil and F. A. P. Garcia, *J. Biotechnol.*, 1998, **66**, 61; (i) M. Kamori, T. Hori,

- Y. Yamashita, Y. Hirose and Y. Naoshima, *J. Mol. Catal. B: Enzym.*, 2000, **9**, 269; (j) H. F. de Castro, R. de Lima and I. C. Roberto, *Biotechnol. Prog.*, 2001, **17**, 1061; (k) E. Dumitriu, F. Secundo, J. Patarin and I. Fecheté, *J. Mol. Catal. B: Enzym.*, 2003, **22**, 119; (l) F. M. Gomes, E. B. Pereira and H. F. de Castro, *Biomacromolecules*, 2004, **5**, 17; (m) P. Ye, Z. K. Xu, A. F. Che, J. Wu and P. Seta, *Biomaterials*, 2005, **26**, 6394.
- 3 (a) S. Sato, T. Murakata, M. Ochifuji, M. Fukushima and T. Suzuki, *J. Chem. Eng. Jpn.*, 1994, **6**, 27; (b) R. J. Russell, M. V. Pishko, A. L. Simonian and J. R. Wild, *Anal. Chem.*, 1999, **71**, 4909; (c) S. Cosnier, S. Szunerits, R. S. Marks, A. Novoa, L. Puech, E. Perez and I. R-Lattes, *Electrochem. Commun.*, 2000, **2**, 851; (d) F. Caruso, D. Trau, H. Möhwald and R. Renneberg, *Langmuir*, 2000, **16**, 1485; (e) M. Winterhalter, C. Hilty, S. M. Bezrukov, C. Nardin, W. Meier and D. Fournier, *Talanta*, 2001, **55**, 965; (f) S. S. Betigeri and S. H. Neau, *Biomaterials*, 2002, **23**, 3627; (g) G. E. Lawson, Y. Lee and A. Singh, *Langmuir*, 2003, **19**, 6401; (h) F. Panzavolta, S. Soro, R. D'Amato, C. Palocci, E. Cernia and M. V. Russo, *J. Mol. Catal. B: Enzym.*, 2005, **32**, 67.
- 4 (a) R. Akiyama and S. Kobayashi, *J. Am. Chem. Soc.*, 2003, **125**, 3412; (b) K. Okamoto, R. Akiyama, H. Yoshida, T. Yoshida and S. Kobayashi, *J. Am. Chem. Soc.*, 2005, **127**, 2125; (c) S. Kobayashi, H. Miyamura, R. Akiyama and T. Ishida, *J. Am. Chem. Soc.*, 2005, **127**, 9251; (d) H. Miyamura, R. Akiyama, T. Ishida, R. Matsubara, M. Takeuchi and S. Kobayashi, *Tetrahedron*, 2005, **61**, 12177.
- 5 (a) J. Kobayashi, Y. Mori, K. Okamoto, R. Akiyama, M. Ueno, T. Kitamori and S. Kobayashi, *Science*, 2004, **304**, 1305; (b) J. Kobayashi, Y. Mori and S. Kobayashi, *Chem. Commun.*, 2005, 2567; (c) J. Kobayashi, Y. Mori and S. Kobayashi, *Adv. Synth. Catal.*, 2005, **347**, 1889.
- 6 O. Kirk and M. W. Christensen, *Org. Process Res. Dev.*, 2002, **6**, 446.
- 7 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 1951, **193**, 265.

		<p>Comments received from just a few of the thousands of satisfied RSC authors and referees who have used ReSource - the online portal helping you through every step of the publication process.</p> <p>authors benefit from a user-friendly electronic submission process, manuscript tracking facilities, online proof collection, free pdf reprints, and can review all aspects of their publishing history</p> <p>referees can download articles, submit reports, monitor the outcome of reviewed manuscripts, and check and update their personal profile</p> <p>NEW!! We have added a number of enhancements to ReSource, to improve your publishing experience even further.</p> <p>New features include:</p> <ul style="list-style-type: none"> ● the facility for authors to save manuscript submissions at key stages in the process (handy for those juggling a hectic research schedule) ● checklists and support notes (with useful hints, tips and reminders) ● and a fresh new look (so that you can more easily see what you have done and need to do next) <p>Go online today and find out more.</p> <p style="text-align: right;"><small>Registered Charity No. 207890</small></p>
	<p>'I wish the others were as easy to use.'</p>	
<p>'ReSource is the best online submission system of any publisher.'</p>	<p style="writing-mode: vertical-rl; transform: rotate(180deg);"><small>08530626</small></p>	

RSC Publishing
www.rsc.org/resource