ARTICLE

OBC
www.rsc.org/obc

Poly(ethylene glycol)-lipase complexes catalytically active in fluorous solvents

Tatsuo Maruyama, Takahiro Kotani, Hiroshi Yamamura, Noriho Kamiya and Masahiro Goto*
Department of Applied Chemistry, Graduate School of Engineering, Kyushu University, 6-10-1 Hakozaki, Fukuoka 812-8581, Japan

Received 2nd October 2003, Accepted 7th December 2003
First published as an Advance Article on the web 20th January 2004

Lipase-catalyzed alcoholysis between vinyl cinnamate and benzyl alcohol in fluorous solvents was investigated. This is the first report of a lipase-catalyzed reaction in a fluorous solvent. Forming the poly(ethylene glycol) (PEG)-lipase PL complex enhanced lipase activity over 16-fold over that of native lipase powder. The PEG-lipase PL complex exhibited markedly higher alcoholysis activities in fluorous solvents than in conventional organic solvents such as isooctane and *n*-hexane. The optimum reaction temperature for FC-77 (perfluorooctane) was 55 °C and the optimum pH for the preparation of the PEG-lipase complex was 9.0; similar to the conditions for lipase PL-catalyzed reaction in aqueous solution. The alcoholysis reaction in fluorous solvent requires the addition of a FC77-miscible organic solvent (isooctane) in order to dissolve non-fluorinated substrates. Lipase activity in the fluorous solvent was significantly influenced by the volume ratio of isooctane in the reaction medium. Vinyl cinnamate inhibition of the lipase-catalyzed reaction occurred at a much lower concentration in the fluorous solvent than in isooctane. These results can be explained by the localization of substrates around lipase molecules, induced by adsorption of the substrates to the PEG layer of the PEG-lipase complex.

Introduction

In the last decade, fluorous solvents, especially perfluorocarbons and perfluorinated ethers, have attracted interest as novel reaction or extraction solvents, due to their unique properties. They are nonpolar, inert and immiscible with water. The miscibility of perfluorocarbons with conventional organic solvents such as hexane and isooctane depends strongly on the conditions (temperature *etc.*). Zhu summarized various organic reactions carried out in perfluorocarbons and described the advantages of using perfluorocarbons as a reaction medium.[1] Horvath and Rabai developed a fluorous biphasic system (FBS) as an efficient reaction system with facile separation.[2] They demonstrated that the FBS enabled easy extractive recovery or isolation of fluorous-tagged catalysts and reagents.

Fluorous-tagged techniques have also achieved excellent enzyme-assisted optical resolution performance. Hungerhoff *et al*. reported that combining the lipase-catalyzed enantioselective reaction with extraction of the product by a fluorous phase allowed for the efficient resolution of racemic alcohols.[3] More recently, Beier and O'Hagan studied lipase-catalyzed transesterification (alcoholysis) in homogeneous perfluorocarbon-hydrocarbon solvents followed by the enantiometric partitioning of the products by phase separation.[4] These studies clearly demonstrated the usefulness of coupling fluorous solvents with enzymatic reactions in order to achieve optical resolution. However, no report has discussed lipase activity in perfluorocarbons or in homogeneous perfluorocarbon-hydrocarbon solvents.

In the present study we investigate the lipase-catalyzed alcoholysis in perfluorocarbons and discuss the lipase activity in perfluorocarbons in comparison with that in conventional organic solvents. Since native lipase powder does not exhibit significant activity in fluorous solvents, we used a poly(ethylene glycol)-lipase complex,[5] which exhibits high synthetic activity in fluorous and organic solvents, to examine alcoholysis activity in fluorous solvents.

Results and discussion

In a preliminary investigation, native lipase powder (lipase PS) exhibited extremely low alcoholysis activity for the reaction between vinyl cinnamate and benzyl alcohol in a fluorous solvent (FC-77) at 40 °C (initial reaction rate was 0.156 mmol $(h \cdot g\text{-enzyme})^{-1}$). We then examined non-aqueous enzyme activation methods (enzymology in organic media). One involved using a surfactant (dioleyl *N*-D-glucono-L-glutamate)-coated lipase[6] and another involved using a PEG-lipase complex.[5] The surfactant-coated lipase exhibited 5-fold higher alcoholysis activity, while the PEG-lipase complex showed 16-fold higher alcoholysis activity than the native lipase. These results mean that the lipase preparations, which are catalytically active in conventional organic solvents, are also very effective for enhancing the enzymatic activity in a fluorous solvent. Several lipases in PEG-complex form were tested for alcoholysis in the fluorous solvent (FC-77). Of the five lipases tested in this study, lipase PL exhibited the highest activity (5.0 mmol $(h \cdot g\text{-enzyme})^{-1}$) in FC-77 (Fig. 1). Further experiments were then carried out employing the PEG-lipase PL complex as the biocatalyst.

Fig. 2 depicts the time courses of alcoholysis catalyzed by the PEG-lipase PL complex in FC-77 and in isooctane. The enzymatic reaction in FC-77 proceeded 10-fold faster than that in isooctane. In isooctane, the reaction rate appears to gradually reduce over time. This implies that the PEG-lipase complex was

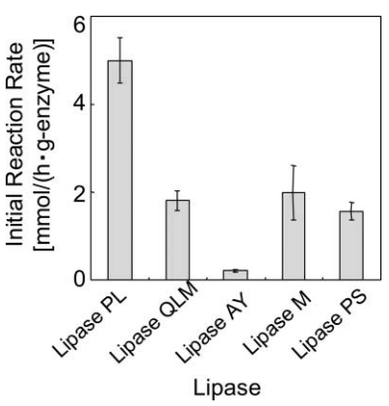

Fig. 1 Alcoholysis activities of various lipases complexed with poly(ethylene glycol) in FC-77. Alcoholysis reactions were carried out between vinyl cinnamate (1 mM) and benzyl alcohol (1 mM) at 40 °C using lipase (0.1 mg ml^{-1}).

DOI: 10.1039/b312212c

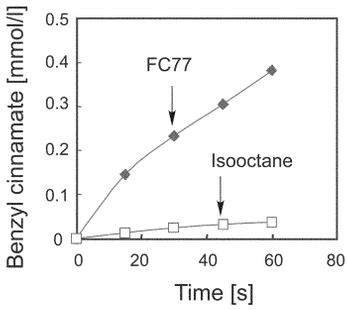

Fig. 2 Time courses of benzyl cinnamate production by lipase-catalyzed alcoholysis in FC-77 and in isooctane. Reaction conditions were the same as those given in Fig. 1.

relatively stable in the fluorous solvent, while some denaturation of the lipase occurred in isooctane at 40 °C over the course of a few hours.

Lipase activities in various fluorous and organic solvents are summarized in Fig. 3. Lipase activities in fluorous solvents were remarkably high compared with those in organic solvents. In general, the selection of solvents available for an enzymatic reaction in organic media is severely constrained; because some organic solvents have inherent ability to deactivate enzymes. Laane *et al*. reported that enzymatic activity in organic solvents is related to the hydrophobicity (such as log *P*) of the organic solvents.[7] They concluded that solvents with a log *P* greater than four (*i.e.* hydrophobic solvents) tend to maintain high enzymatic activity. Since the solubility of water in the fluorous solvents tested here is less than 13 ppm, the fluorous solvents are very hydrophobic. This hydrophobicity is one of the reasons why the lipase exhibited markedly high activity in the fluorous solvents. It should be noted that we did not find any significant differences between the lipase activities in the fluorous solvents, even though the properties of the fluorous solvents tested varied.[8] For example, the viscosity of FC-77 at 25 °C is 1.3 cp, which is 2-fold higher than that of FC-72. The boiling points of FC-72, FC-77 and FC-3255 are 56 °C, 97 °C and 101 °C, respectively.

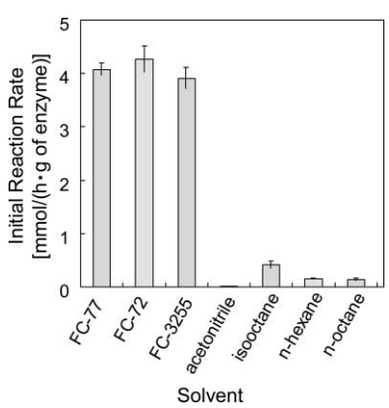

Fig. 3 Alcoholysis activities of the PEG-lipase PL complex in fluorous solvents and in ordinary organic solvents. Reaction conditions were the same as those given in Fig. 1.

The effect of the reaction temperature on lipase activity in FC-77 was studied (Fig. 4). The optimum temperature in FC-77 was 55 °C, while that for the hydrolysis reaction in an aqueous buffer is 50 °C (data provided by enzyme manufacturer). Many researchers have reported that the optimum reaction temperature in non-aqueous enzymology is often higher than that in aqueous buffer.[9] However, in the present work, not much difference was observed between the optimum temperatures in fluorous solvent and in aqueous buffer, even though the lipase molecule was coated with PEG-complex in the fluorous solvent.

In non-aqueous enzymology, enzyme preparation is of great significance in obtaining an active biocatalyst in non-aqueous

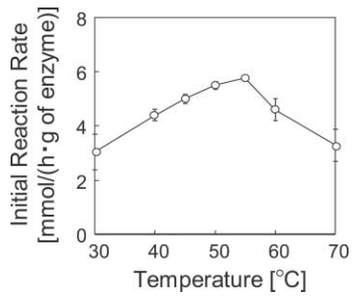

Fig. 4 Effect of reaction temperature on the activity of PEG-lipase PL in FC-77. Reaction conditions were the same as those given in Fig. 1.

media. In particular, enzymes are known to remember the buffer pH used during preparation, and an enzyme prepared at an optimum pH exhibits maximal activity in a non-aqueous solvent. This is called "pH memory".[10] In this work we investigated the effect of the buffer pH used during preparation on subsequent lipase activity in a fluorous solvent (Fig. 5). While native lipase PL showed maximal activity around pH 8.5 in the aqueous solution (data provided by enzyme manufacturer), the buffer with pH 9 provided the highest activity for the PEG-lipase PL complex in FC-77. That is, we found that "pH memory" is observed in the lipase-catalyzed reaction in the fluorous solvent as well as in other organic solvents.

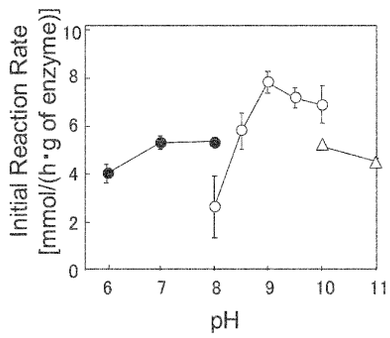

Fig. 5 Effect of pH during PEG-lipase complex preparation on alcoholysis activity in FC-77. The buffers employed are KH_2PO_4–NaOH buffer (pH 6–8, ●), borate buffer (pH 8-10, ○) and carbonate buffer (pH 10-11, △). The temperature of the alcoholysis reaction was set at 55 °C and other reaction conditions were the same as those given in Fig. 1.

The present reaction medium consisted of 99 vol% fluorous solvent and 1 vol% isooctane. The substrates (vinyl cinnamate and benzyl alcohol) were not fluorinated and were not soluble in pure fluorous solvents. A small volume of isooctane was, therefore, added to dissolve the non-fluorinated substrates. The effect of the volume ratio of isooctane on the lipase activity in FC-77 was examined (Fig. 6). The isooctane ratio was varied from 1% to 100%, and the lipase activity in FC-77 increased with decreasing isooctane ratio. This result implicitly suggests

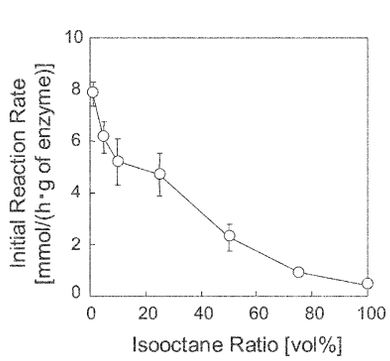

Fig. 6 Effect of the volume ratio of isooctane on the activity of PEG-lipase complex in FC-77. Reaction conditions were the same as those given in Fig. 1.

either the concentration effect of substrates that is induced by localization of the substrates in a fluorous solvent or inhibition by isooctane. Many papers have, however, described lipase-catalyzed reactions using isooctane that showed no inhibition of the enzymatic reactions by the isooctane co-solvent.[11]

Considering these facts, we investigated the effects of substrate concentration on lipase activity in FC-77 and in isooctane (Fig. 7). Lipase activity in both solvents was strongly influenced by the substrate concentrations. In FC-77, lipase activity sharply increased with increasing substrate concentrations. However, when the substrate concentrations exceeded a certain value (10 mM for vinyl cinnamate and 1 mM for benzyl alcohol), the activity started to decrease. Chulalaksananukul *et al*. demonstrated that a lipase-catalyzed esterification in an organic solvent proceeds by a Ping-Pong Bi–Bi mechanism with inhibition by excess alcohol.[12] In the present study, we confirmed inhibition of lipase activity by both benzyl alcohol and vinyl cinnamate in the fluorous solvent. Fig. 7b depicts the effects of substrate concentrations on lipase activity in isooctane. As in the fluorous solvent, lipase activity in isooctane depends on substrate concentrations, but the maximum activity was obtained at a concentration of 200 mM vinyl cinnamate, which is much higher than that in the fluorous solvent. These results indicate that the substrates were localized around lipase molecules in the fluorous solvent, which resulted in a much higher substrate concentration in the environment of the lipase molecules than the apparent one.

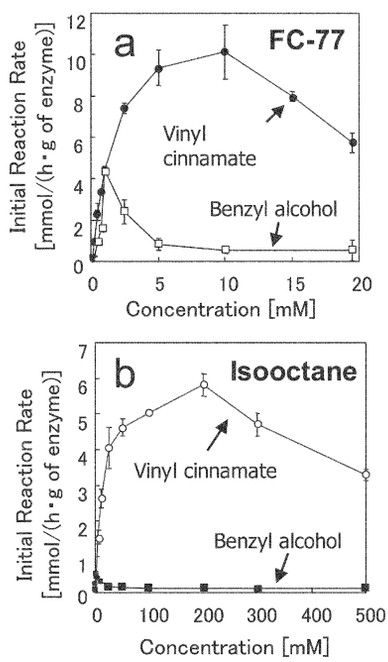

Fig. 7 Effect of substrate concentration on activity of PEG-lipase complex in FC-77 (a) and in isooctane (b). Reaction conditions were the same as those given in Fig. 1, except for the isooctane ratio (which was 10 vol%).

The fluorous reaction medium containing the substrates appeared homogeneous, except for the PEG-lipase complex. If the substrates are localized in the fluorous reaction medium, there are two possibilities for this localization. One is isooctane-in-FC-77 microemulsions where isooctane droplets dissolve the substrates at high concentrations, and the other is adsorption of the substrate to the PEG-lipase complex. To ascertain whether or not a microemulsion formed, fluorescent measurement of pyrene was carried out. Pyrene above 1 mM has intermolecular excimer emission at a wavelength of 475 nm (excitation 345 nm) and is dissolved in isooctane much more readily than in FC-77. One milliliter of isooctane containing 1 mM of pyrene was added to 99 ml of FC-77, followed by fluorescent measurements. We did not observe excimer emission of pyrene, while isooctane containing 1 mM of pyrene exhibited emission at 475 nm (data not shown). This indicated that an isooctane-in-FC-77 microemulsion did not form in the reaction medium.

We then examined the adsorption of substrates and product to PEG (PEG control). Since the PEG-lipase complex could hydrolyse the substrate and the product, the PEG control, which was prepared without lipase by the same procedure as the PEG-lipase complex, was used for the adsorption tests. Fig. 8 shows that the substrates and the product employed here were all adsorbed to the PEG control, both in FC-77 and in isooctane. In particular, the adsorption of benzyl alcohol and benzyl cinnamate was much greater in FC-77 than in isooctane, which would be due to the differing solubilities of these components in the different solvents. The adsorption experiments revealed that the substrates in FC-77 were localized at the PEG layer of the PEG-lipase complex, and suggest that the high activity of the PEG-lipase complex and the results in Fig. 7 can be explained by this localization.

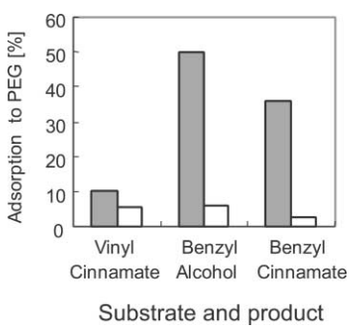

Fig. 8 Adsorption of vinyl cinnamate, benzyl alcohol and benzyl cinnamate on PEG control (see Experimental section for details) suspended in FC-77 (filled bars) and in isooctane (open bars). Substrate and product concentrations were 1 mM, and the concentration of the PEG control was 9 mg ml^{-1}.

Conclusions

Lipase-catalyzed alcoholysis between vinyl cinnamate and benzyl alcohol was investigated in fluorous solvents. Lipase PL in a complex form with PEG 20 000 was highly active in fluorous solvents. The optimum conditions for the lipase-catalyzed reaction in the fluorous solvents were similar to those in aqueous solution. pH memory was also observed in lipase in fluorous solvents. Fluorous solvents are hydrophobic, but are unlikely to dissolve non-fluorinated substrates, which induced adsorption of the substrates to the PEG layer, and could explain the higher activity achieved for lipases in fluorous solvents. The present study suggests that methodologies for enzyme reactions in ordinary organic solvents are also applicable to those in fluorous solvents.

Experimental

Lipase PL (from *Alcaligence sp*.) and lipase QLM (from *Alcaligence sp.*) were kindly supplied by Meito Sangyo Co. Ltd. (Nagoya, Japan). Lipase AY (from *Candida rugosa*), lipase M (from *Mucor javanicus*) and lipase PS (from *Pseudomonas cepacia*) were kindly provided by Amano Enzyme Inc (Nagoya, Japan). FC-72 (perfluorohexane), FC-77 (perfluorooctane) and FC-3255 (perfluorooctane) were generously donated by Sumitomo 3M Co. Ltd. Benzyl cinnamate, vinyl cinnamate and benzyl alcohol were purchased from Tokyo Chemical Industry, Japan. All other chemicals were purchased from Wako Pure Chemicals Ltd., Japan.

Preparation of poly(ethylene glycol) (PEG)-lipase complexes

The preparation of the PEG-lipase complex has been described previously.[5] Each lipase (10 mg) and poly(ethylene glycol)

20 000 (PEG) were dissolved in phosphate buffer (5 ml, pH 7.0) with a typical molar ratio (PEG : lipase) of 10. In order to effectively coat the surfaces of lipase molecules with PEG[13] and provide an oil–water interface for lipase,[14] toluene (13 ml) was added to the lipase solution, which was then homogenized at 20 000 rpm for 3 min using an Ultra-Turrax T25 in an ice bath, to prepare water-in-oil (w/o) emulsions. The w/o emulsions were immediately frozen in liquid nitrogen, followed by lyophilization for 24 h using a freeze-dryer. The PEG-lipase complexes were obtained as white powders.

Enzymatic reaction in perfluorocarbons and conventional organic solvents

Alcoholysis between vinyl cinnamate (1 mM) and benzyl alcohol (1 mM) to irreversibly produce benzyl cinnamate and acetaldehyde was typically conducted using PEG-lipase complexes (0.1 mg of original enzyme per 1 ml of a reaction medium) at 40 °C in the fluorous and organic solvents. The PEG-lipase complex (containing 0.1 mg of original enzyme) was added to 0.99 ml of a fluorous solvent. The reaction was started by the addition of an isooctane solution (10 μl) containing vinyl cinnamate (100 mM) and benzyl alcohol (100 mM) to the fluorous solvent. Aliquots were periodically withdrawn from the reaction medium and filtered through a 0.5 μm filter (Millex, Millipore Co.). 1 ml of acetonitrile was added to the filtrate to extract substrates and products into the acetonitrile phase. The reaction product (benzyl cinnamate) was monitored at 254 nm using an HPLC system (JASCO 2000 series) equipped with a 4.6 × 250 mm column packed with 5 μm ODS. An acetonitrile : water : acetic acid (80 : 20 : 0.1) solvent system was used as the mobile phase, at a flow rate of 1.0 ml min^{-1}. The initial reaction rate was taken to be the initial rate of benzyl cinnamate production.

The effect of the volume ratio of isooctane in the reaction medium on the enzymatic activity was investigated as follows: Isooctane/FC-77 mixtures with various isooctane ratios were prepared. Enzymatic reactions were started by the addition of isooctane solution (10 μl) containing the dissolved substrate (100 mM each) to 0.99 ml of isooctane/FC-77 mixtures containing the PEG-lipase complex (with 0.1 mg of original enzyme).

The effects of the substrate concentrations on the enzymatic activity in fluorous solvent were investigated using 1 ml solutions of FC-77 containing 0.1 ml of isooctane and various concentrations of the substrates. When the concentration of one substrate was varied, that of the other substrate was fixed at 1 mM.

Adsorption of the substrates and the product to PEG-lipase complex

To examine the adsorption of benzyl alcohol, vinyl cinnamate and benzyl cinnamate to the PEG-lipase complex, PEG complex without lipase (abbreviated as PEG control) was prepared by the same preparation procedure as the PEG-lipase complex, except that lipase was not added. PEG control (9 mg, a 10-fold amount of the PEG-lipase complex for the reaction) was added to 3 different 1 ml portions of FC-77 containing 10 μl of isooctane and 1 mM of each substrate or product. The mixture was incubated for 30 min at 55 °C and then filtered through a 0.5 μm filter. The free vinyl cinnamate and benzyl cinnamate in FC-77 were extracted into 1 ml of acetonitrile. The acetonitrile phase was then analyzed by HPLC to determine the concentration of each compound. The free benzyl alcohol in FC-77 was extracted into 1 ml of *n*-hexane, followed by GC analysis (Hewlett Packard HP 6890 equipped with a capillary HP-FFAP column (Agilent Technologies, Palo Alto, CA) and a flame-ionization detector).

Acknowledgements

This work was partly supported by Kyushu University Interdisciplinary Programs in Education and Projects I Research Development. We would like to thank Meito Sangyo Co. Ltd., Amano Pharmaceutical Co. Ltd. and Sumitomo 3M Co. Ltd. for generously providing the lipases and perfluorocarbons.

References

1 D. W. Zhu, *Synthesis*, 1993, **10**, 953–954.
2 I. T. Horvath and J. Rabai, *Science*, 1994, **266**, 72–75.
3 (*a*) B. Hungerhoff, H. Sonnenschein and F. Theil, *J. Org. Chem.*, 2002, **67**, 1781–1785; (*b*) B. Hungerhoff, H. Sonnenschein and F. Theil, *Angew. Chem. Int. Ed.*, 2001, **40**, 2492–2494.
4 P. Beier and D. O'Hagan, *Chem. Commun.*, 2002, 1680–1681.
5 T. Maruyama, S. Nagasawa and M. Goto, *Biotechnol. Lett.*, 2002, **24**, 1341–1345.
6 N. Kamiya, M. Goto and F. Nakashio, *Biotechnol. Prog.*, 1995, **11**, 270–275.
7 C. Laane, S. Boeren, K. Vos and C. Veeger, *Biotechnol. Bioeng.*, 1987, **30**, 81–87.
8 http://cms.3m.com/cms/US/en/2-178/iilzRFS/view.jhtml.
9 A. Zaks and A. M. Klibanov, *Science*, 1984, **224**, 1249–1251.
10 A. M. Klibanov, *Nature*, 1995, **374**, 596–596.
11 *Enzymatic Reactions in Organic Media*, ed. A. M. P. Koskinen and A. M. Klibanov, Blackie Academic & Professional, Glasgow, 1996.
12 (*a*) W. Chulalaksananukul, J. S. Condor, P. Delorme and R. M. Willmot, *FEBS Lett.*, 1990, **276**, 181–184; (*b*) A. E. M. Janssen, B. J. Sjursnes, A. V. Vakurov and P. J. Halling, *Enzyme Microb. Technol.*, 1999, **24**, 463–470.
13 S. Okazaki, N. Kamiya, K. Abe, M. Goto and F. Nakashio, *Biotechnol. Bioeng.*, 1997, **55**, 455–460.
14 (*a*) I. Mingarro, C. Abad and L. Braco, *Proc. Natl. Acad. Sci. USA*, 1995, **92**, 3308–3312; (*b*) T. Maruyama, M. Nakajima, S. Ichikawa, H. Nabetani, S. Furusaki and M. Seki, *J. Am. Oil. Chem. Soc.*, 2000, **77**, 1121–1126; (*c*) T. Maruyama, M. Nakajima and M. Seki, *J. Biosci. Bioeng.*, 2001, **92**, 242–247.