ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 3 (1997) 303–309

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Methods for the immobilization of lipases and their use for ester synthesis

Alexander E. Ivanov [a], Manfred P. Schneider [b,*]

[a] *Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Miklukho-Maklaya 16 / 10, 117871 Moscow, Russia*
[b] *Bergische Universität Gesamthochschule Wuppertal, Gauss-Strasse 20, 42097 Wuppertal, Germany*

Received 6 December 1996; accepted 14 March 1997

Abstract

The lipase from *Pseudomonas fluorescens* was immobilized onto five different carriers: celite, octyl-silica, aminopropyl-silica, gluterdialdehyde-activated silica and Eupergit C250L. Activities and operational stabilities of the prepared catalysts were compared using the enantioselective acylation of (R,S)-1-phenylethanol by vinyl acetate as acyl donor and *t*-butylmethyl ether with variable water content (0.038–0.97% v/v) as reaction medium. The above carriers provide catalysts with widely different specific activities ranging from excellent 25 mmol/h mg protein (celite) to 0.07 mmol/h mg protein (glutardialdehyde-activated silica) on the lower end. The lipase immobilized onto Eupergit C250L exhibited the best operational stability among the catalysts studied. It retained 30% of its initial activity after 11 cycles of application, each with a duration between 2 and 6 h.

Keywords: Lipase immobilization; Ester synthesis; *Pseudomonas fluorescens*

1. Introduction

Lipases (EC. 3.1.1.3) are widely used for the solution of many synthetic organic problems. These enzymes catalyze both the hydrolysis of esters in largely aqueous media [1] and their synthesis under low water conditions [2,3]. Due to economical considerations their application on an industrial scale requires their immobilization and thus re-usability.

Although many techniques and carriers have been employed for immobilization of lipases, the activities and operational stabilities of the resultant catalysts, exhibited in ester synthesis, were rarely studied and compared in a systematic way. Quantification of catalytic activity was performed for lipases immobilized onto silicas and porous glass [4–6], celite [5,7], nylon [8], polypropylene [9,10] and ion-exchange resins [9,11–13]. The widely different substrates, supports and enzymes employed led to an enormous amount of quantitative data, which, however, can not be directly compared. A conclusion or evaluation of the most promising immobilization technique is thus extremely difficult. In this sense we felt, that a systematic study, in which only one parameter, i.e. the type of immobilization, is varied should be of considerable interest and highly warranted.

* Corresponding author.

PII S1381-1177(97)00012-X

In order to evaluate the enzyme activity and operational stability of various immobilized lipase preparations, we employed the enantioselective esterification of (R,S)-1-phenylethanol in the presence of vinyl acetate as an acyldonor under standardized conditions and the lipase from *Pseudomonas fluorescens* as biocatalyst. This reaction was studied in great detail previously [14,15] and is used for kinetic resolution of secondary alcohols of this type on a preparative scale. In the present study a wide variety of immobilization techniques such as adsorption (celite, alkyl-silica), covalent attachment to γ-aminopropyl- and glutardialdehyde-activated silicas and oxyrane activated polymers (Eupergit C250L) are investigated.

The successful preparation of a highly active catalyst with high operational stability could be of considerable practical significance for the preparation of enantiomerically pure secondary alcohols (e.g. building blocks for pharmaceuticals) on an industrial scale.

2. Experimental

2.1. Materials

The lipase from *Pseudomonas fluorescens* and Eupergit C250L were provided by RÖHM, Pharma Polymers, Darmstadt, Germany. Celite 545 is a product of Bayer AG, Germany. Porous silica XWP-1500 of particle diameter 16–23 μm, average pore diameter 1500 Å and specific pore volume 0.7 cm^3/g, was a gift from Diagen, Hilden, Germany. Glutardialdehyde (25% aqueous solution) was obtained from E. Merck, Germany. *t*-Butylmethyl ether, vinyl acetate, 1-phenylethanol, γ-aminopropyltriethoxy silane, triolein and Triton X-100 are the products of Fluka Chemie AG, Buchs, Switzerland.

2.2. Immobilization of lipase onto solid supports: Eupergit C250L

200 mg of crude lipase was dissolved in 5 ml of 0.1 M potassium phosphate buffer solution (PBS, pH 8.5) by stirring the suspension with a magnetic stirrer in an ice bath for 1 h. The solution was filtered off and diluted with distilled water up to 20 ml. The protein concentration in the filtrate was determined by the method of Bradford et al. [16], indicating that only 0.5 mg of protein was contained in 200 mg of the crude enzyme preparation. Oxyrane beads Eupergit C250L (2.5 g) and 6% hydrogen peroxide (0.2 ml) was added to the enzyme solution, mixed and left for 4 days at room temperature. The thus immobilized lipase was washed with distilled water and kept in 0.1 M PBS (pH 7.5), containing 2% isopropanol and 0.02% sodium azide at 8°C.

2.3. Celite and octyl-silica

Octyl-silica was prepared by treatment of polyacrylate-coated silica with octylamine according to the method described elsewhere [17]. 200 mg of crude lipase was dissolved in 0.01 M PBS (pH 7.5), filtered off and diluted as described above. Celite 545 or octyl-silica (2.5 g) was added to the enzyme solution, the mixture was shaken for 10–15 min and dried under reduced pressure on a rotavapor at 40°C. The catalyst was stored as a dry material at 8°C.

2.4. γ-Aminopropyl-silica

γ-Aminopropylsilica was prepared by chemical modification of XWP-1500 silica gel. For this 10 g of the silica was treated with 2% v/v γ-aminopropyltriethoxy silane solution (100 ml) in boiling toluene for 10 h, washed by acetone, water, acetone and dried in an air flow. γ-Aminopropylsilica (2.5 g) was added to the solution of the crude lipase (200 mg) in 10 ml 0.01 M phosphate buffer solution (pH 6.5) and shaken for 5 min. 150 mg of N(dimethylaminopropyl)-N′-ethylcarbodimide hydrochloride in 2 ml of the phosphate buffer was added. The mixture was slowly shaken for 4 h at room temperature and then left overnight at 8°C. The catalyst was washed with 1 M NaCl in 0.01 M

PBS, pH 6.5 and stored in PBS, containing 2% isopropanol and 0.02% sodium azide at 8°C.

2.5. Glutardialdehyde-activated silica

Glutardialdehyde-activated silica was prepared by soaking γ-aminopropylsilica (2.5 g) in a mixture of 2 ml 25% aqueous glutardialdehyde and 18 ml 0.1 M PBS (pH 7.5) for 1 h. The carrier was washed with 1 × 100 ml water followed by 100 ml PBS on a sintered glass filter. 200 mg of crude lipase was dissolved in PBS and filtered off as described above. Glutardialdehyde-activated silica was then mixed with the enzyme solution and left at room temperature overnight under slow shaking. Then the prepared catalyst was washed by PBS and stored in this buffer, containing 2% isopropanol and 0.02% sodium azide at 8°C.

2.6. Reduction of the water content of immobilized lipase preparations

Reduction of the water content of the catalysts was achieved using the method developed by RÖHM, Pharma Polymers (Darmstadt, Germany). For this, preparations of the lipase immobilized onto aminopropylsilica, glutardialdehyde-activated silica and Eupergit C250L were washed while shaken with 0.01 M PBS (pH 7.5), then washed twice by mixtures of PBS with acetone (40:60% v/v), followed by two washings with mixtures of PBS with acetone (5:95% v/v). Each washing was carried out for 20 min. The catalysts were transferred onto the sintered-glass filters and the supernatants were removed by filtration. The resultant catalysts were dried in an air flow and stored at 8°C.

2.7. Immobilized lipase: Determination of esterification activity

350 mg of the corresponding immobilized lipase was washed with *t*-butylmethyl ether (20 ml) and then incubated with 10 mmol of racemic 1-phenylethanol and 30 mmol vinyl acetate in 20 ml *t*-butylmethyl ether. The suspensions contained in 100 ml Erlenmeyer flasks were agitated on a reciprocal shaker (200 rpm) at a temperature of 21–22°C. In the course of the reaction aliquots (0.5 ml) were taken and the beads were removed by centrifugation. The supernatants of these aliquots were analyzed by gas chromatography (GS) using a FRAPTOVAP Series 4160 Carlo Erba Strumentazione instrument equipped with a glass capillary column OV-1701, the temperature gradient proceeded from 120 to 150°C in 15 min. Identification of the substrates and the product were performed by comparing the GS retention times to those of the individual samples.

Quantification of 1-phenylethanol and 1-phenylethyl acetate was performed by registration the GC peak areas by means of the Hewlett Packard HP 3394A Integrator. The initial rate of the ester synthesis was calculated from 2–3 subsequent measurements of the 1-phenylethyl acetate content in the reaction mixture.

2.8. Evaluation of operational stability of immobilized lipase

350 mg of the immobilized lipase, which was used for determination of the specific activity in ester synthesis, was recovered by filtration on a sintered glass filter, washed using 1 × 50 ml acetone containing 5% v/v of distilled water, dried in an air flow and subsequently under vacuum. The thus recovered catalyst was stored in dry form in a sealed flask. With this material repeated esterifications were carried out with average incubation times of 2–6 h, corresponding to conversions of phenylethanol from 1–10%. In all cases the catalyst was recovered and handled as described above.

2.9. Immobilized lipases: Determination of hydrolytic activity

1 g of triolein and 30 ml of Triton X-100 were mixed by magnetic stirring at 50–60°C. 200 ml of 1 M NaCl previously heated to

50–60°C was added to the above mixture in portions of ca. 50 ml. The thus prepared substrate solution was cooled to room temperature. Aliquots of 25 ml of this solution were placed into the thermostated unit (21°C) of a Radiometer Copenhagen TTT 80 Titrator. 0.5 ml of a wet precipitate of the corresponding immobilized enzyme was added to the substrate solution. Titration of oleic acid, liberated as a result of enzymatic hydrolysis, was performed by 0.01 M NaOH at pH 7.0. The initial rate of the reaction was recorded for 10–15 min. The immobilized enzyme was filtered off from the reaction mixture using a sintered glass filter, washed with 12% Triton X-100–0.86 M NaCl solution (20–30 ml) and introduced into the next cycle of hydrolysis carried out with a fresh portion of substrate. Enzymatic activities of immobilized preparations expressed in μmol/min g (or U/g) were calculated from the initial rates by accounting for the different bulk densities of the carriers (5.2 ml/g dry carrier for Eupergit C250L and 2.0 ml/g for silica).

2.10. Determination of the water content in t-butylmethyl ether

The water contents in the dried and water-saturated solvents (0.038 and 0.97% v/v, respectively) were determined by Karl Fischer titration performed with the Mettler DL 40RC Memotitrator. In order to reduce the water content in *t*-butylmethyl ether, the solvent was distilled over $LiAlH_4$. To saturate the solvent with water, 2 ml of distilled water was added to 100 ml of the solvent and shaken overnight. The excess water was removed using a separatory funnel.

3. Results and discussion

It had been shown previously, that the enzymatic esterification of (R,S)-1-phenylethanol in the presence of the lipase from *Pseudomonas fluorescens* leads to (R)-acetate and (S)-alcohol (Eq. 1) of high optical purity (> 99% ee) [14]. Due to its inherently high enantioselectivity the reaction comes close to a still stand after ca. 50% conversion (Fig. 1). As obvious from Fig. 1 and supported by the calculated initial rates of esterifications summarized in Table 1, these transformations are highly dependent both regarding the carrier and the type of immobilization.

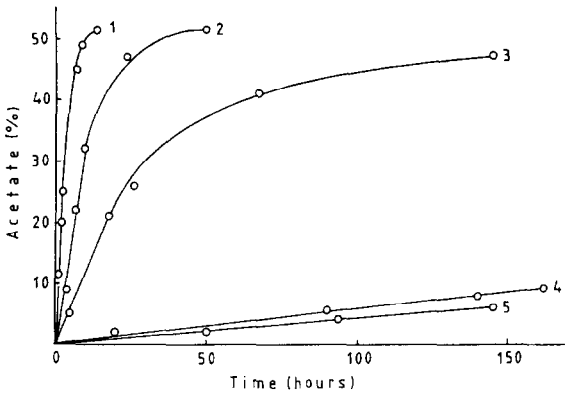
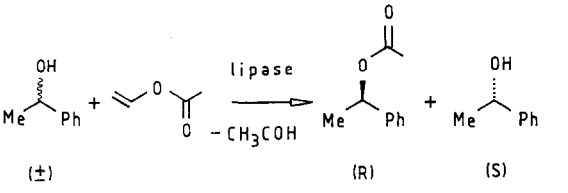


Fig. 1. Kinetics of 1-phenylethanol acetylation catalysed by preparations of lipase immobilized onto the solid carriers: celite (1), octylsilica (2), Eupergit C250L (3), aminopropylsilica (4) and glutardialdehyde-activated silica (5).



(1)

Table 1
Initial rates of the ester synthesis obtained with lipase immobilized onto various carriers and non-immobilized lipase (mmol/h mg $_{protein}$). The loadings of crude enzyme are equal on each carrier (see Section 2)

Carrier	Initial rate
Celite	25
Octylsilica	4.3
Non-immobilized	3.3
Eupergit C250L	1.9
Aminopropylsilica	0.09
Glutardialdehyde-activated silica	0.07

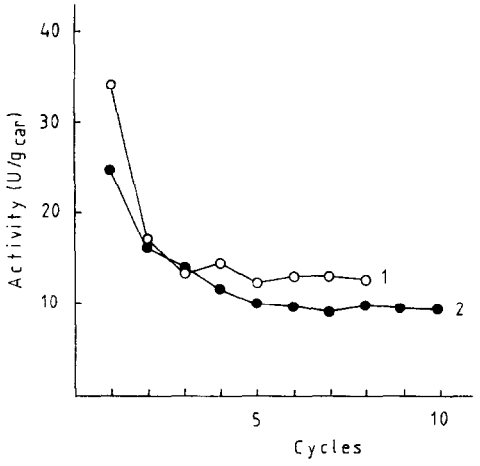


Fig. 2. Activity of lipase immobilized onto glutardialdehyde-activated silica (1) and Eupergit C250L (2) registered in several repeated cycles of triolein hydrolysis.

3.1. Immobilization efficiencies and hydrolytic activities

Due to the employed experimental procedure of adsorptive immobilization onto celite and octylsilica (see above) and the fact that no lipase activity could be detected in the medium after immobilization onto aminopropylsilica, glutardialdehyde-activated silica and Eupergit C250L, it can be assumed with confidence that all immobilizations were quantitative. This is also supported by the fact that the hydrolytic activities of different covalently immobilized enzyme preparations, determined in several repeated cycles using glutardialdehyde-activated silica and Eupergit C250L, were almost identical as shown in Fig. 2. On the other hand, the descending profiles of the activities displayed in the initial cycles of triolein hydrolysis are probably due to desorption of non-covalently bound portions of lipase washed out by the highly concentrated solution of Triton X-100 [18].

3.2. Esterification activities

In contrast, the same pair of immobilizates display widely different rates of esterification activities (compare Fig. 1 and Table 1) with a factor of 25 in favour of Eupergit C250L. Interestingly, wide-pore silica gel chemically modified with different organic grafts (octyl groups for sample 2, aminopropyls for sample 4 and glutardialdehyde for sample 5, Fig. 1) provides the catalysts exhibiting very different initial rates of esterification. The higher initial rate observed with octyl-silica may be ascribed to its higher hydrophobicity [6], what agrees well to the concept of Mattiason et al. [19]. They found an inverse relationship between the esterification activity of α-chymotrypsin immobilized onto different carriers and the ability of immobilizates to extract water from the organic solvent (aquaphilicity) [20].

Clearly, the highest esterification activity was observed for the lipase immobilized onto celite. The esterification activity increased by 6–7 fold more active catalyst compared to the non-immobilized enzyme (see Table 1). There are precedences for this in the literature, e.g. for α-chymotrypsin [20], other lipases [5,7,22], or lipase from *Candida cylindracea* immobilized onto Nylon particles activated with glutardialdehyde [8]: the rate of ester synthesis was enhanced by a factor of 1.8 after immobilization.

3.3. Water content

In many previous studies the water content in a given reaction mixture was shown to be an

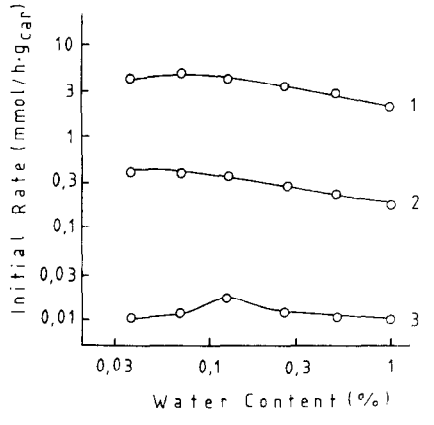


Fig. 3. Initial rate of 1-phenylethanol acetylation as a function of the water content in the organic solvent, obtained for lipase immobilized onto Celite (1), Eupergit C250L(2), glutardialdehyde-activated silica (3).

important factor in lipase catalyzed ester synthesis [2,3,11,21]. In this study we did not investigate aquaphilicities of the different carriers, but instead compared the initial rates of ester synthesis registered in *t*-butylmethyl ether (*t*-BME) of different water content (Fig. 3). While the relative esterification activities remain largely unchanged for the different immobilizates (compare Fig. 1), the rates of esterification of a given carrier system are rather independent from the water content ranging from 0.038% v/v to 0.97% with somewhat higher rates being observed at low water concentration, presumably due to the ester hydrolysis at higher water concentrations.

3.4. Operational stability

When comparing the catalysts intended for preparative or industrial use, characterization of their operational stabilities is of extreme importance. In Fig. 4 the initial rates of esterification for three different immobilizates (celite, octylsilica and Eupergit C250L) are plotted against the number of reaction cycles. It is interesting to note that the lipase immobilized on celite retains its high activity for about 7 cycles, while the most of its activity (> 99%) is lost after 10 cycles. In contrast, the moderate activity of the lipase immobilized on Eupergit C250L is accompanied by much higher operational stability retaining an almost constant level of 30% of its initial activity even after 11 cycles.

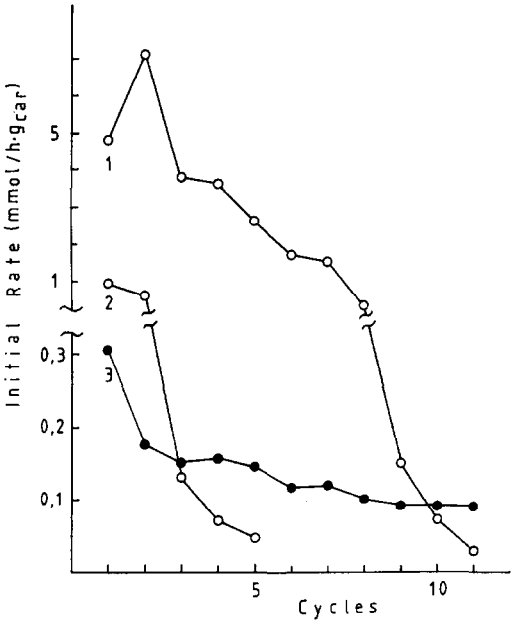


Fig. 4. Operational stability of lipase immobilized onto Celite (1), octylsilica (2), Eupergit C250L (3). Initial rate of esterification catalysed by 80 mg crude lipase (the amount corresponding to 1 g of any carrier, see Section 2) equals to 0.63 mmol/h.

Data reported in the literature on operational stability of immobilized lipases, used for ester synthesis, are scarce. The *Candida cylindracea* lipase, immobilized onto glutardialdehyde-activated nylon support, retained 25% activity after 5 cycles of ethyl propionate synthesis [8]. The *Mucor miehei* lipase, immobilized onto microporous anion exchanger, retained from 40 to 70% activity after 10 cycles of isopropylidene glycerol oleyl ester synthesis [11], depending on oleic acid/isopropylidene glycerol ratio in the reaction mixture. From the above experiments, the lipase immobilized on Eupergit C250L clearly shows the highest operational stability and would be chosen as catalyst for synthetic applications. In view of the higher esterification activities of the celite-immobilized lipase it would be attractive to attempt an increase of the operational stability of this enzyme preparation. Experiments of this nature are underway in our laboratory.

Acknowledgements

We appreciate the financial support of this work by Volkswagen Foundation, Germany (Grant No. I/69913). We are grateful to Dr. Dieter Kraemer (RÖHM, Darmstadt, Germany) for fruitful discussions and to Mrs. Claudia Berkhout (Wuppertal) for valuable practical assistance.

References

[1] F.H. Mattson, R.A. Volpenheim, J. Lipid. Res. 10 (1969) 271–276.

[2] A. Zaks, A.M. Klibanov, Proc. Natl. Acad. Sci. USA 82 (1985) 3192.
[3] A. Zaks, A.M. Klibanov, Science 224 (1984) 1249.
[4] J. Lavayre, J. Baratti, Biotechnol. Bioeng. 24 (1982) 1007.
[5] C. Marlot, G. Langrand, C. Trioantaphylides, J. Baratti, Biotechnol. Lett. 7 (1985) 647.
[6] M. Norin, J. Boutelje, E. Holmberg, K. Hult, Appl. Microbiol. Biotechnol. 28 (1988) 527.
[7] T. Yamane, T. Ichiryu, M. Nagata, A. Ueno, S. Shimizu, Biotechnol. Bioeng. 36 (1990) 1063.
[8] G. Carta, J.L. Gainer, A.H. Benton, Biotechnol. Bioeng. 37 (1991) 1004.
[9] R.H. Valivety, P.J. Halling, A.R. Macrae, Prog. Biotechnol. 8 (1992) 549.
[10] M.M. Hoq, H. Tagami, T. Yamane, S. Shimizu, Agric. Biol. Chem. 49 (1985) 335.
[11] I.C. Omar, H. Saeki, N. Nishio, S. Nagai, Biotechnol. Lett. 11 (1989) 161.
[12] T.B. Nielsen, M. Hashida, H. Shimoto, Novo Industri A/S, Den, PCT Int. Appl. WO8901032 A1, 9 Feb 1989, Chem. Abs. 111, 152171e.
[13] P. Eigtved, Novo Industri A/S, Den, PCT Int. Appl. WO8902916 A1, 6 Apr 1989, Chem. Abs. 111, 92861t.
[14] K. Laumen, D. Breitgoff, M.P. Schneider, J. Chem. Soc. Chem. Commun. 22 (1988) 1459.
[15] U. Goergens, M.P. Schneider, J. Chem. Soc. Chem. Commun. (1991) 1064.
[16] M.M. Bradford, Anal. Biochem. 86 (1978) 142–146.
[17] A.E. Ivanov, L.V. Verkhovskaya, S.N. Khilko, V.P. Zubov, Bioorgan. Khim. 16 (1990) 1028.
[18] T. Boller, A. Flemmisch., D.M. Kraemer, Proceedings of the 6th European Congress on Biotechnology, Florence, Italy, June 1993.
[19] B. Mattiasson, P. Adlercreutz, Trends Biotechnol. 9 (1991) 394.
[20] M. Reslow, P. Adlercreutz, B. Mattiasson, Eur. J. Biochem. 172 (1988) 573.
[21] H.L. Goderis, G. Ampe, M.P. Feyten, B.L. Foeuve, W.M. Guffens, S.M. Van Cauwenbergh, P.P. Tobback, Biotechnol. Bioeng. 30 (1987) 258.
[22] A. Millqvist, P. Adlercreutz, B. Mattiasson, Enzyme Microb. Technol. 16 (1994) 1042.