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ORIGINAL PAPER

Enantioselective behavior of lipases from *Aspergillus niger* immobilized in different supports

Vania Castriani Fernandes da Silva · Fabiano Jares Contesini · Patrícia de Oliveira Carvalho

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Abstract Considering the extraordinary microbial diversity and importance of fungi as enzyme producers, the search for new biocatalysts with special characteristics and possible applications in biocatalysis is of great interest. Here, we report the performance in the resolution of racemic ibuprofen of a native enantioselective lipase from *Aspergillus niger*, free and immobilized in five types of support (Accurel EP-100, Amberlite MB-1, Celite, Montmorillonite K10 and Silica gel). Amberlite MB-1 was found to be the best support, with a conversion of 38.2%, enantiomeric excess of 50.7% and enantiomeric ratio (E value) of 19 in 72 h of reaction. After a thorough optimization of several parameters, the E value of the immobilized *Aspergillus niger* lipase was increased ($E = 23$) in a shorter reaction period (48 h) at 35°C. Moreover, the immobilized *Aspergillus niger* lipase maintained an esterification activity of at least 80% after 8 months of storage at 4°C and could be reused at least six times.

Keywords Immobilization · Lipase · Ibuprofen · *Aspergillus niger*

V. C. F. da Silva · P. de Oliveira Carvalho (✉)
Curso de Farmácia, Universidade São Francisco,
Av. São Francisco de Assis, 218, Bragança Paulista,
São Paulo CEP 12916-900, Brazil
e-mail: patcarvalho@saofrancisco.edu.br

F. J. Contesini
Laboratório de Bioquímica de Alimentos,
Faculdade de Engenharia de Alimentos,
Universidade Estadual de Campinas (UNICAMP),
C.P. 6154, Rua Monteiro Lobato, 80, Campinas,
São Paulo CEP 13083-970, Brazil

Introduction

Lipase (triacylglycerol hydrolases, EC 3.1.1.3) catalyzes the hydrolysis of triacylglycerols in water, and, in non-aqueous medium, is the enzyme most widely used in organic synthesis, mainly because of its property of catalyzing the resolution of racemic drugs such as (*R,S*)-ibuprofen [2-(4-isobutylphenyl) propionic acid]. It has been reported that (*S*)-ibuprofen is 160-fold more active than its antipode in the in vitro synthesis of prostaglandin [1]. The present authors previously reported the production of a lipase from *Aspergillus niger* AC-54 that was able to preferentially esterify (*R*)-ibuprofen, and provided the best results for enantioselectivity and thermostability when compared with other native lipases [3, 4]. However, use of the crude freeze-dried powder of the free enzyme requires the production of large quantities of enzyme for each set of assays, making the process lengthy and expensive.

The main advantage of using immobilized enzymes as biocatalysts is that it is possible to reuse them since they can be easily recovered, thereby making the process economically feasible. Various techniques, and even more support materials have been studied, and consequently many immobilized preparations with a wide range of efficiency, stability and activity are available. With respect to immobilized enzymes, several parameters are important when considering industrial applications: mechanical strength, chemical and physical stability, hydrophobic/hydrophilic character, enzyme loading capacity and cost, to cite some. On account of the relatively high surface hydrophobicity of lipases, the simple adsorption of lipases onto suitably hydrophobic supports has been the more popular strategy. In addition, immobilization by adsorption is economically feasible and attractive [10, 14]. Industrially

successful immobilized lipases are based on rigid, stable supports (e.g., Novozyme 435, immobilized *Candida antarctica* lipase B and RM-IM, immobilized *Rhizomucor miehei* lipase).

Despite the large amount of work reported on the use of lipases in enantioselective reactions, there are far fewer reports on how best to immobilize a given lipase without affecting its enantioselective properties. In the search for suitable and low cost materials, the present study examined and compared five inexpensive materials (Accurel EP-100, Amberlite MB-1, Celite, Montmorillonite K10 and Silica gel) as supports for the immobilization of a native *Aspergillus niger* lipase. The esterification activity and enantioselectivity of the free and immobilized enzymes were assessed in the resolution of (*R*,*S*)-Ibuprofen in isooctane. In addition, important variables (amount of enzyme adsorbed onto the support and the reaction temperature) were established in order to optimize the process. The reusability and storage stability of the immobilizate selected were also determined.

Materials and methods

Chemicals

Isooctane, 1-propanol, (*R*,*S*)-ibuprofen and the pure enantiomers were purchased from Sigma-Aldrich Chemical (St. Louis, MO). Yeast extract and Bacto peptone were purchased from Difco Laboratories, (Detroit, MI). Culture media components, Celite, Silica-gel 60 Amberlite MB-1, Montmorillonite K-10, chemical reagents and the other solvents were obtained from Merck (Darmstadt, Germany) and from Sigma-Aldrich with the highest purity available. Low acidity olive oil (Carbonel, Spain) was purchased at a local market. Accurel was donated by Membrana Underlining Performance (Germany).

Lipase

The lipase was produced in a basal medium with an initial pH value of 6.0 and consisting of 2% (m/v) peptone, 0.5% (m/v) yeast extract, 0.1% (m/v) $NaNO_3$, 0.1% (m/v) KH_2PO_4, 0.05% (m/v) $MgSO_4{\cdot}7H_2O$ and 2% (m/v) olive oil. The cultures were grown in Erlenmeyer flasks (500 mL) containing 120 mL growth medium. The cultures were inoculated with 1 mL spore suspension (10^5–10^6 spores/mL) and the flasks shaken on a rotary shaker (130 rpm) at 35°C for 72 h. The cultures were filtered and the supernatants treated with ammonium sulphate (80% saturation). The precipitates were dialyzed against water and freeze-dried for use as an extracellular crude lipase preparation in powder form with a residual water content of 0.2% (m/m).

Assay of lipase activity

The hydrolytic activities of free and immobilized lipase were measured by a titrimetric method previously described [4] using olive oil as the substrate. Activities are expressed in international units, where 1 U lipase is the amount of enzyme able to catalyze the release of 1 μmol oleic acid per minute at pH 7.0 and at 37°C. The amount of protein was determined by the method of Lowry [13] using egg albumin as a standard and measuring absorbance at 280 nm.

Immobilization by simple adsorption

The lipase obtained from *A. niger*, which had a specific lipolytic activity of 0.56 U/mg protein, was immobilized by adsorption onto Accurel EP-100, Amberlite MB-1, Celite, Montmorillonite K10 and Silica gel supports. For immobilization, crude lipase preparation (0.1 g) was dissolved in 5 mL sodium phosphate buffer (50 mM pH 7.0), mixed thoroughly with 500 mg dried support and shaken at 200 rpm for 12 h. After immobilization, the preparation was vacuum filtered, washed thoroughly with deionized water and rinsed with a sodium phosphate buffer solution of the same pH. The final preparation was then freeze dried and stored at 4°C for further use.

Immobilization yield

Immobilization yield was defined as follows:

$$\text{Immobilization yield } (\%) = (a_{imm}/a_{free}) \times 100$$

where a_{imm} is the total activity of immobilized enzyme (U/mg), and a_{free} is the total activity of initial enzyme preparation (U/mg).

Esterification reaction

The standard reaction mixture was composed of (*R*,*S*)-ibuprofen (66 mM), 1-propanol (66 mM) and isooctane (10 mL), without the addition of water, contained in stoppered conical flasks. The reaction was started by the addition of 40 mg free lipase (or 240 mg immobilized lipase, which equates to 40 mg lipase and 200 mg support) and incubated in an orbital magnetic shaker at 35°C with stirring at 180 rpm. Experiments without the addition of the enzyme were carried out to evaluate the percentage of spontaneous esterification in the system. The above experiments were repeated varying the enzyme

concentration from 2.4 to 4.8% and the temperature from 25 to 50°C. Samples of 100 µL of each solution were withdrawn at different times (12–96 h) and diluted in 1.4 mL isooctane. The amount of ester (conversion degree) formed during the reaction and the enantiomeric excess of (*S*)-ibuprofen were determined by gas chromatography (GC) and high performance liquid chromatography (HPLC), respectively.

Chromatography analysis

Gas chromatography was performed using a Chrompack CP 9001 gas chromatograph equipped with a flame ionization detector (FID) and a CP-Sil 5 CB column (10 m × 0.25 mm × 0.12 µm). The injector temperature was 300°C and that of the detector 350°C; the oven temperature was maintained at 180°C. The carrier gas was hydrogen with a flow rate of 1.0 mL/min. An external standard method was employed to quantify the ester formed and the acid remaining. The enantiomers of the unreacted ibuprofen were separated by HPLC using a chiral column (Chiralcel OD, Daicel Chemical Industries, Japan). The mobile phase was a mixture of n-hexane/Isopropanol/trifluoracetic acid (HPLC grade) (100/1/0.1 v/v/v) at a flow rate of 1.0 mL/min, and detection was by UV at 254 nm. The retention time of each compound was 7.835 min for (*S*)-ibuprofen and 7.061 min for (*R*)-ibuprofen.

Enantioselectivity-value measurements

The enantioselectivity value (*E* value) was calculated from the enantiomeric excess of the remaining (*S*)-Ibuprofen (ee), and the conversion degree (*c*) according to the method described by Chen et al. [5] (Eq. 1).

$$E = \frac{\ln[(1-c)](1-\mathrm{ee}S)}{\ln[(1-c)(1+\mathrm{ee}S)]} \quad (1)$$

Results and discussion

Enantioselective esterification for the optical resolution of racemic ibuprofen

The results obtained in the immobilization studies of *A. niger* lipase in the resolution of (*R*,*S*)-ibuprofen are shown in Table 1. As described earlier, a molar ratio of 1:1 (1-propanol:ibuprofen) with 40 mg (0.4% m/v) free enzyme was able to catalyze the kinetic resolution of ibuprofen by esterification [4]. When 2.4% (m/v) of immobilized lipase (corresponding to 0.4% m/v free enzyme) was used, the conversion was between 7.2 and 38.2%. This is less than the expected theoretical value that could be achieved in kinetic resolutions (50%). Although the desired result was not obtained in the kinetic resolution catalyzed with the lipase immobilized on the different supports, it was evident that the catalytic conversion, enantiomeric excess, and consequently the *E* value, shown by enzyme immobilized on Amberlite MB-1 ($E = 19$) in the resolution of (*R*,*S*)-ibuprofen were much better than enzyme immobilized on the other support materials used here. As a rule of thumb, enantiomeric ratios below 15 are unacceptable for practical purposes, those from 15 to 30 are considered as moderate to good and above this value they are considered to be excellent [8]. Under the test conditions used, the free lipase had an *E* value of 6.9, and when the conversion reached 14.6%, the enantiomeric excess of (*S*)-ibuprofen showed a maximal value of 12% after 72 h. In addition, as can be seen from the data in Table 1, lipase adsorbed on Amberlite MB-1 gave an

Table 1 Kinetic resolution of (*R*,*S*)-ibuprofen catalyzed by free and immobilized *Aspergillus niger* lipase immobilized on different supports. Reaction conditions: (*R*,*S*)-ibuprofen (66 mM), 1-propanol (66 mM), isooctane (10 mL), and 22.4 units of free and immobilized lipases at 35°C for 72 h

Type of support	c[a] (%)	ee[b] (%)	*E*[c]	Immobilization yield (%)
No support (free lipase)	14.6 ± 1.3	12.0	6.9	–
Accurel EP-100	30.6 ± 2.5	31.8	9.5	36.8 ± 3.8
Amberlite MB-1	38.2 ± 1.8	50.7	19	61.7 ± 5.4
Celite	26.0 ± 0.8	6.0	1.5	32.4 ± 6.8
Montmorillonite K10	32.0 ± 0.8	34.8	9.8	53.5 ± 5.4
Silica gel	7.2 ± 1.3	ND[d]	ND	15.8 ± 4.2

[a] Conversion (c) is given as the percentage of initial racemic ibuprofen esterified after the reaction time

[b] Enantiomeric excess (ee) of the (*S*)-ibuprofen active (enantiomer not esterified)

[c] Enantiomeric ratio

[d] Not detected

immobilization yield of approximately 62%. Based on these results, *A. niger* lipase immobilized in Amberlite MB-1 was selected and used in all subsequent experiments.

Amberlite MB-1 consists of hydrophobic beads of a strongly basic and acidic resin [12]. On the other hand, Montmorillonite K10 is the commercially available acid activated form of smectite (2:1 dioctahedral). Other common hydrophobic supports include polyethylene, polypropylene, styrene and acrylic polymers. Celite and Silica gel belong to the hydrophilic group of supports, which also include Duolite, activated carbon, clay and Sepharose. This might explain why lipase immobilized on Celite and Silica gel showed the lowest enantioselectivity, because hydrophilic supports generally result in high losses of lipase activity upon immobilization. These losses in activity have been attributed to the following possibilities: a situation in which only small quantities of lipase are immobilized; a change in the conformation of the lipase on adsorption, into a form with reduced activity; a decrease in the ability of hydrophobic substrates to reach the active site of the enzyme; or the existence of steric hindrance imposed by the carrier matrix, which constrains the flexibility of the lipase molecule [9]. These results are in agreement with data reported in the literature. Serri et al. [15] reported that Amberlite was much better than Celite as a support material for the immobilization of *C. rugosa* lipase in the synthesis of citronellyl laurate. Chen and Tsai [6] reported that the immobilization of *Candida rugosa* lipase on Accurel suppressed the enantioselectivity in the synthesis of the (*S*)-ibuprofen ester in cyclohexane.

The effect of temperature on the catalytic activity of the immobilized enzyme

The effect of temperature on the resolution of (*R*,*S*)-ibuprofen using *A. niger* lipase immobilized on the Amberlite MB-1 support was examined in the range of 25–50°C after 72 h of reaction (Fig. 1). The results showed that the conversion degree and enantiomeric excess increased with increase in temperature from 25°C to 45°C, increasing from 15 to 40% and 8 to 50%, respectively. The values were quite similar in the range from 35 to 45°C, and the *E* values remained constant throughout the 72 h of reaction, being approximately 20. However, at 50°C the selectivity (*E* value) decreased from 20 to 7.1. This was probably due to some denaturation of the protein structure, resulting from the breakdown of the weak ionic and hydrogen bonding that stabilized the three dimensional structure [7]. Taking into account the above results, a temperature of 35°C was selected for use in the study of the influence of enzyme loading on the resolution of (*R*,*S*)-ibuprofen.

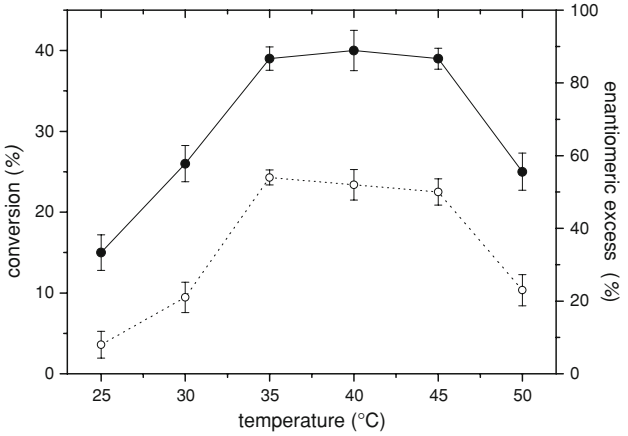

Fig. 1 Effect of temperature on the conversion (*filled circle*) and enantiomeric excess (*open circle*) obtained with *Aspergillus niger* lipase immobilized on Amberlite MB-1 for the resolution of (*R*,*S*)-ibuprofen (72 h). Each *data point* represents the average of three experiments; *error bars* standard deviation

The effect of enzyme loading on the catalytic activity of the immobilized enzyme

The effect of lipase loading on the support was studied using different enzyme concentrations (Fig. 2). The conversion and enantiomeric excess improved as the amount of enzyme adsorbed increased. When the reaction was carried out with 3.6% (w/v) of enzyme, excellent conversions (45.6%) and enantiomeric excess (69%) were obtained after 48 h, this shorter time being very important for the cost of the process. More importantly, the enantioselectivity was significantly improved ($E = 23$). However, larger amounts

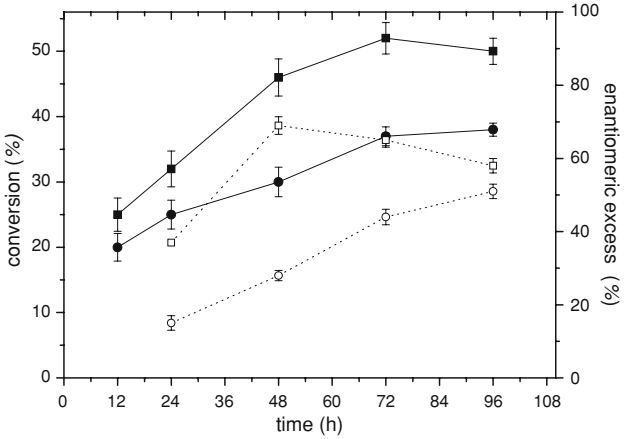

Fig. 2 Conversion (*c*) and enantiomeric excess (*ee*) obtained with *A. niger* lipase immobilized on Amberlite MB-1 for the resolution of (*R*,*S*)-ibuprofen at different enzyme concentrations as a function of time at 35°C. *filled square* c for 3.6% m/v, *filled circle* c for 2.4% m/v, *open square* ee for 3.6% m/v, *open circle* ee for 2.4% m/v. Each *data point* represents the average of three experiments; *error bars* standard deviation

of enzyme (4.8%) did not further improve the esterification reaction (data not shown), probably due to saturation of the reaction rate as a result of the increase in substrate diffusion inhibition caused by enzyme aggregation. With higher enzyme loading, the onset of mass transfer limitations poses another difficulty that prevents the enzyme from exerting its activity at a higher rate.

An important parameter that controls the activity of immobilized enzymes is enzyme loading, particularly with lipases, which have strong affinity for surfaces. According to Bosley and Peilow [2], at low enzyme loadings, the lipase attempts to maximize its contact with the surface, which results in a loss of conformation and, consequently, in a reduction of activity. As loading is increased, less area is available for the lipase to spread itself, and therefore more of its active conformation is retained, and the loss in activity is reduced. Lee et al. [11] reported on the immobilization of *Candida rugosa* lipase for the resolution of (*R*,*S*)-ibuprofen, and observed that when the amount of lipase was higher than 10 mg, no further increase in activity was apparent.

Reuse and storage stability of free and immobilized lipase in the resolution of (*R*,*S*)-ibuprofen

The residual esterification activity of the free and immobilized lipase with repeated use is shown in Fig. 3. These data indicate that lipase immobilized on Amberlite MB-1 was stable after repeated operations. Immobilized lipase retained its activity with little loss after six reaction cycles. For the first two reuses, no obvious loss of immobilized lipase activity was observed. After four reuses, the activity

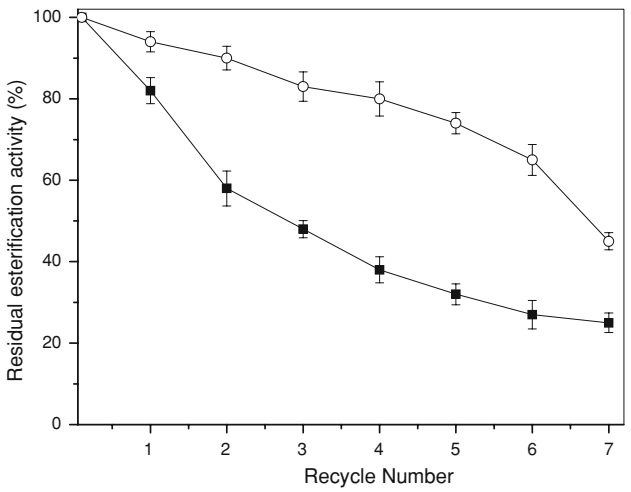

Fig. 3 Residual esterification activity versus recycle number during the reuse study for the resolution of (*R*,*S*)-ibuprofen. *filled square* Free *A. niger* lipase, *open circle* immobilized *A. niger* lipase. Each *data point* represents the average of three experiments; *error bars* standard deviation

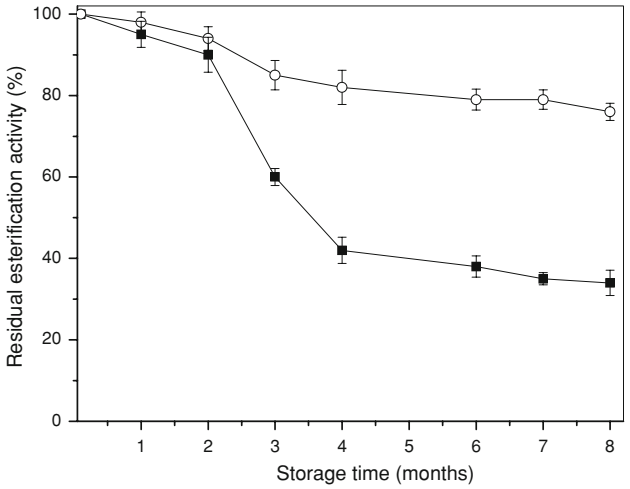

Fig. 4 Stability of free and immobilized *A. niger* lipase preparations during 8 months of storage at 4°C. *filled square* Free *A. niger* lipase, *filled circle* immobilized *A. niger* lipase. Each *data point* represents the average of three experiments; *error bars* standard deviation

loss was only 10%. After six reuses, the immobilized lipase retained 70% of residual esterification. However, almost 20% of the free lipase was lost in the second batch, and its activity was decreased gradually in subsequent cycles, resulting in 23% activity.

The storage stability of the immobilized lipase compared with that of free lipase is shown in Fig. 4. After 8 months of storage at 4°C, the residual esterification activity of the immobilized lipase was 80%, while that of the free lipase was 35%, indicating that the immobilization procedure considerably increased the storage stability.

Although throughout this study the *E* values were not high enough for industrial applications, the significant increase in enantioselectivity of the enzyme immobilized on Amberlite was promising for a biocatalytic reaction, especially when one considers the fact that this enzyme can be recycled and reused at least six times, and retained at least 80% of its esterification activity after 8 months of storage at 4°C.

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