Immobilization of Lipases on Porous Polypropylene: Reduction in Esterification Efficiency at Low Loading

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ABSTRACT: *Rhizomucor miehei, Humicola* sp., *Rhizopus niveus,* and *Candida antarctica* B lipases were immobilized by physical adsorption onto a macroporous polypropylene support. In an esterification reaction, the enzyme efficiency, and therefore cost-effectiveness, is greatly affected by enzyme loading, with an apparent suppression of efficiency at low lipase loadings for both *R. miehei* and *Humicola* sp. lipases. This results in the appearance of a pronounced maximum in the efficiency-loading relationship at approximately 100,000 lipase units (LU)/g for *R. miehei* lipase (10% of its saturation loading) and at approximately 200,000 LU/g for *Humicola* sp. lipase (50% of its saturation loading). The other lipases studied do not show similar trends. At low loadings, only a small portion of the surface area is occupied and gives the lipase the opportunity to spread; it is hypothesized that the reduction in efficiency at low loadings is due to a distortion of the active molecular conformation caused by the lipase maximizing its contact with the support as a result of its high affinity for the support surface. The relationship between efficiency and loading was different for each of the lipases studied, which may reflect both differences in the strength of the affinity of the lipase for the support and in the ease at which the molecular conformation of the lipase can be distorted.

JAOCS 74, 107–111 (1997).

KEY WORDS: *Candida antarctica* B, *Humicola* sp., immobilization, lipase, *Rhizomucor miehei, Rhizopus niveus*, polypropylene.

The application of lipase biocatalysts in industrial processes (e.g., fat hydrolysis, triacylglycerol modification, and ester synthesis) is now of major commercial importance, and this interest is expected to grow over the coming years as a wider range of lipase catalysts becomes available (1–3). Immobilized lipases are now being used on a commercial scale under essentially nonaqueous conditions for the production of structured triacylglycerols (4) and a range of fatty acid esters, e.g., decyl oleate (5). Immobilization technology offers the benefits of the ability to reuse enzyme, easy separation of product from enzyme, and the potential to run continuous processes *via* packed-bed reactors. In addition, the mild reaction conditions used (in comparison to equivalent chemical processes) lead to high-quality products that require the minimum of refining.

There is now a great deal of literature that describes the immobilization of lipases to various support materials. A wide range of methods has been used, including drying or precipitation onto hydrophilic materials (6,7), adsorption to hydrophobic or ionic surfaces (7–11), covalent attachment to suitable ligands (7,10,12,13), and entrapment in polymer gels (7,9,14).

Work has also been undertaken on establishing the ideal physical and surface chemistry requirements of support materials for the immobilization of lipases *via* hydrophobic interactions (15). However, the high cost of the controlled-pore glasses used in that study precludes them from most direct commercial applications. Supports based on macroporous polypropylene are now becoming widely used for lipase immobilization (16–18) because they largely fulfill the key physical requirements and are relatively inexpensive.

One of the other main factors in the development of highly cost-effective lipase biocatalysts is the selection of an appropriate enzyme loading. While it is undoubtedly correct to assume that the highest activities will be obtained at the highest loadings, these may not necessarily reflect the optimum loading with respect to enzyme efficiency. To determine optimum enzyme loadings, we have studied the efficiency-loading relationship for four lipases of commercial interest immobilized onto macroporous polypropylene.

EXPERIMENTAL PROCEDURES

Materials. Accurel EP100 (macroporous polypropylene, particle size 200–400 μm) was obtained from Akzo Nobel (Obernburg, Germany). *Rhizomucor miehei* lipase (Lipozyme 10,000L; LMN 006), *Humicola* sp. lipase (SP398, Batch PPW 2542), and *Candida antarctica* B lipase (SP434, Batch 28–08) were all gifts from Novo-Nordisk (Copenhagen, Denmark). *Rhizopus niveus* lipase (Lipase N) was obtained from Amano (Nagoya, Japan). All lipases were used as received; no purification was carried out. Oleic acid (92%) was obtained from BDH (Poole, United Kingdom). All other reagents were of standard laboratory grade.

Lipase immobilization. The following general method was used to prepare the catalysts. For the extremes of low and

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high loading, variation in the amount of support and lipase used was necessary.

Accurel EP100 (2.0 g) was wetted with a minimum volume of absolute ethanol (typically 12.0 mL). After standing for a few minutes, 65 mL of 0.01 M Na_2HPO_4 buffer (pH 7) was added, followed by the required amount of lipase dissolved in 175 mL of the same buffer. The mixture was then left mixing on an orbital shaker (Model R100, Luckham Ltd., Burgess Hill, Sussex, United Kingdom) at room temperature (approximately 20°C). The adsorption of lipase onto the support was monitored by loss of enzyme activity from the solution by using the tributyrin hydrolysis assay described below. After the adsorption process was complete (4–16 h, depending on loading), the catalyst was collected by filtration, washed twice with 0.01 M Na_2HPO_4 pH 7 buffer (100 mL), then with distilled water, and dried under vacuum at room temperature (approximately 20°C) until no further weight loss was recorded. The lipase theoretical loading is defined as the amount of lipase activity lost from the solution per unit weight of support.

Hydrolysis assay. The activity of lipase solutions was determined by a standard Novo assay (19). A sample of enzyme solution is added to a gum arabic-stabilized suspension of tributyrin in distilled water (20 mL) at 30°C. The suspension is prepared by homogenizing a mixture of tributyrin (3 mL), distilled water (47 mL), and emulsification reagent (10 mL) at 24,000 rev • min^{-1} for 15 s (Ultra-Turrax Model T25, Janke & Kunkel GmbH, Staufen, Germany). The emulsification reagent is prepared by dissolving gum arabic (6.0 g), glycerol (540 mL), NaCl (17.9 g), and KH_2PO_4 (0.41 g) in distilled water (400 mL) and then making up the total volume to 1000 mL with distilled water. After addition of the lipase, the pH is maintained at pH 7 in an Autotitrator (VIT 90; Radiometer, Copenhagen, Denmark) by titration with 100 mM sodium hydroxide solution. One lipase unit (LU) is defined as the amount of lipase that liberates 1 µmol butyric acid per minute under these conditions.

Esterification assay. The immobilized lipase (typically 5–20 mg of the vacuum-dried material, depending on lipase loading) was placed in a vial, and a mixture of oleic acid (5.88 g), octan-1-ol (2.7 g) and distilled water (0.175 g) was added. The vial was sealed, placed in a waterbath at 50°C for 30 m, and shaken at 200 strokes • min^{-1}. A sample (100 µL) was then removed and immediately eluted down a small alumina column (2 mL, basic, activity II) with diethyl ether, together with a solution of methyl stearate (2.5 mg) dissolved in petroleum ether (b.p. 100–120°C) as an internal standard. The ether was then removed by evaporation and replaced with petroleum ether (4.0 mL, b.p. 60–80°C). The ratio of octyl oleate to methyl stearate was then determined by gas–liquid chromatography (GC) (column: 1 µm BP20 phase, 25 m × 0.53 mm). The activity was expressed as µmol ester formed per minute per mg catalyst. The lipase efficiency is obtained by dividing lipase activity by lipase loading.

Porosity and surface area. Mean pore diameter and surface area of the Accurel EP100 were determined by mercury intrusion porosimetry and BET (Brunauer-Emmett-Teller) adsorption isotherms (N_2), respectively. These were carried out by MCA Services (Melbourn, Cambridgeshire, United Kingdom).

RESULTS AND DISCUSSION

Physical properties of Accurel EP100. Particles of Accurel EP100 typically have a wide distribution of pore diameters: from 10 nm up to macroporous channels of 5 µm or more. The pore size distribution of a typical batch of Accurel EP100 is shown in Figure 1. The surface area of this material = 85 $m^2 • g^{-1}$, and its total pore volume = 2.95 mL • g^{-1}. Using a cylindrical pore model, its mean pore diameter is calculated to be approximately 140 nm (4 × total pore volume from porosimetry ÷ surface area from BET).

Catalyst efficiency: Rhizomucor miehei. A series of catalysts was prepared by adsorbing *R. miehei* lipase onto Accurel EP100 at various loadings. The saturation loading was found to be 1190 KLU • g^{-1} (1 KLU = 1000 LU) for this lipase, which, by using a figure of 15 KLU • m^2 for surface coverage on a hydrophobic support (15), gives a surface area of approximately 80 $m^2 • g^{-1}$. This represents 95% of the BET surface area and shows that, despite having a wide pore size distribution, most of the surface of this carrier is available for lipase immobilization.

The hydrolysis activity of the catalysts was measured with the tributyrin assay. The results are shown in Figure 2 in the form of an efficiency plot (activity/loading), which shows that efficiency falls as loading is increased. This is typical of immobilized enzyme systems where mass transfer limitations hinder the diffusion of substrate through the pores, leading to an effectively decreased substrate concentration toward the center of the particles. This is particularly important at high enzyme loadings where the enzyme will be distributed throughout the particles; at low loadings, most of the enzyme will be located at or near the surface of the particles, and mass

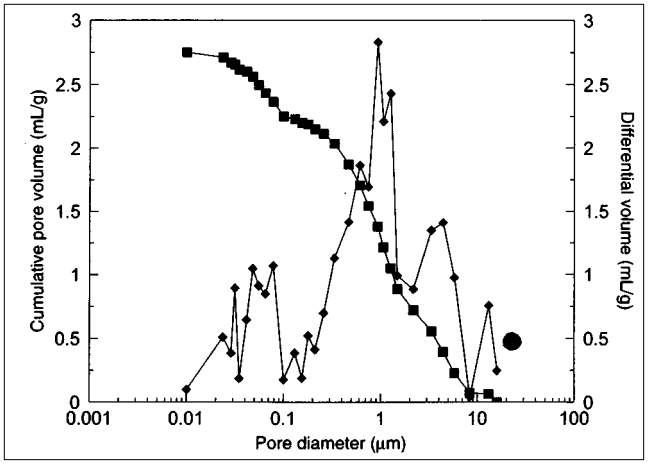

FIG. 1. Pore size distribution of Accurel EP100 polypropylene support (Akzo Nobel, Obernburg, Germany) by mercury intrusion porosimetry. Both cumulative pore volume (■) and differential pore volume (◆) are shown. Differential pore volume is Δ pore volume/Δ log pore diameter.

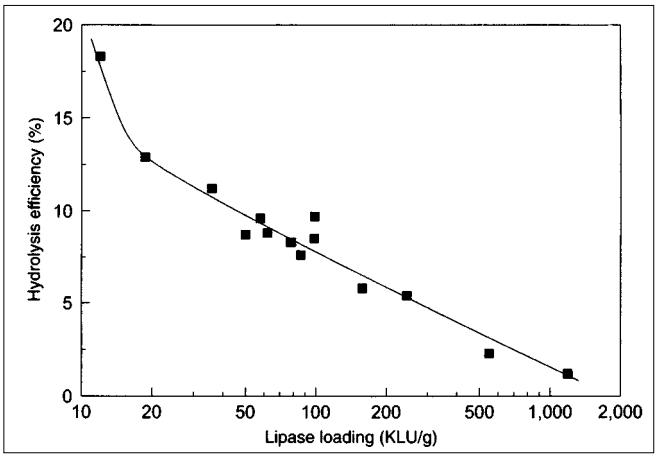

FIG. 2. Effect of lipase loading on hydrolysis efficiency for *Rhizomucor miehei* lipase immobilized on Accurel EP100 (KLU = thousands of lipase units). See Figure 1 for company source.

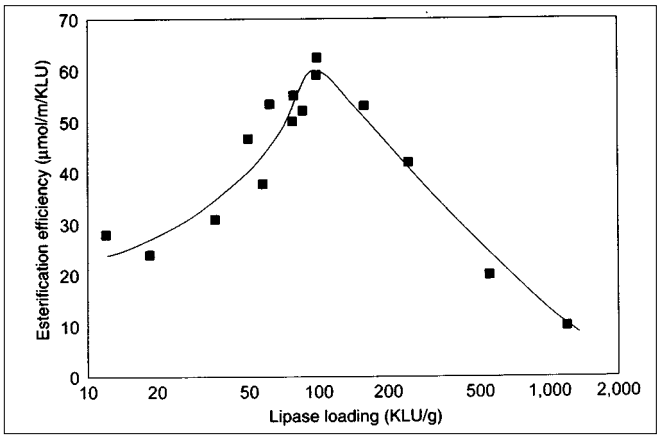

FIG. 4. Effect of lipase loading on esterification efficiency for *Rhizomucor miehei* lipase immobilized on Accurel EP100. See Figure 1 for company source. See Figure 2 for abbreviation.

transfer limitations will be less pronounced. These mass transfer problems can be particularly severe in this system because the substrate is in the form of a suspension of small droplets in water, most of which are larger than the mean pore diameter of the support. Heterogeneous substrate mixtures are generally unsuitable for the assay of enzymes immobilized in porous supports, and this might account for the relatively low (<20%) recovered activity.

The catalysts were assessed for their esterification (octyl oleate) activity, and plots of activity (for all lipases studied) and efficiency (activity/loading for *R. miehei*) vs. loading are shown in Figures 3 and 4, respectively. These plots show that, as expected, the activity of the catalyst increases as more lipase is loaded onto the support, but when converted to an efficiency plot, a maximum at a loading of approximately 100 KLU • g^{-1} is seen.

At high loadings, the expected diffusion-controlled limitations are seen, but the observation of a maximum suggests that another mechanism must be operating by which enzyme efficiency is being suppressed at lower loadings.

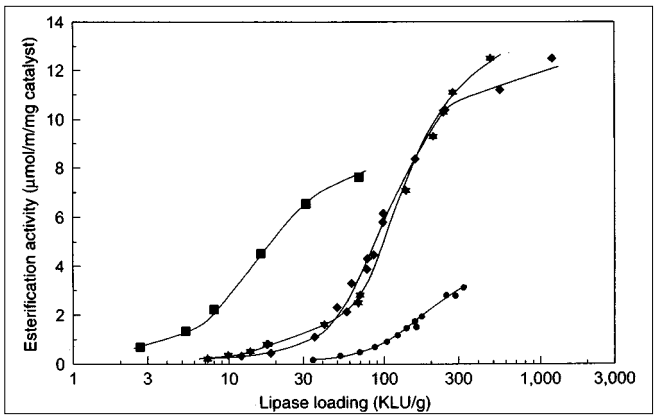

FIG. 3. Effect of lipase loading on esterification activity for *Rhizomucor miehei* lipase (◆), *Humicola* sp. lipase (●), *Rhizopus niveus* lipase (★), and *Candida antarctica* B lipase (■), immobilized on Accurel EP100. See Figure 1 for company source. See Figure 2 for abbreviation.

We believe that this suppression of activity is a result of conformational changes in the enzyme molecule. At low loadings, there is a large excess of surface area that the enzyme can occupy. It is also known that the lipase has a strong affinity for the surface (this is the basis of the immobilization method). At low 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 and efficiency. As loading is increased, less area is available for the lipase to spread itself, more of its active conformation is retained, and the loss in activity is reduced.

This maximum is not observed in the hydrolysis data (Fig. 2), which may be due to the hydrolysis activity measurement being completely dominated by the diffusion of the heterogeneous substrate solution into the pores of the catalyst. If this is not so, it would imply that the changes undergone by the enzyme at low loading suppress the esterification activity more than hydrolysis activity. This seems unlikely.

Humicola sp. catalysts were prepared with *Humicola* sp. lipase from low loadings to near saturation coverage. The esterification activity of these catalysts was measured, and their efficiencies were calculated. The data are shown in Figures 3 and 5.

The efficiency of esterification vs. loading relationship (Fig. 5) for *Humicola* sp. catalysts is broadly similar to the profile found for *R. miehei* catalysts, except that the efficiency maximum (approximately 250 KLU • g^{-1}) is much nearer to the saturation loading. However, in terms of absolute activity, *Humicola* catalysts were much less active than *R. miehei* catalysts at equivalent loadings (Fig. 3). This is due to differences in activity between the lipases toward the tributyrin substrate used to define the enzyme loading and the oleic acid substrate used for measuring the activity of the final catalyst. This is a reflection of enzyme specificity, leading to a preference for particular fatty acids. Because efficiency (as defined by activity/loading) contains these differences in activity toward substrates, it is not correct to use efficiency to compare different lipases that are immobilized on the same support.

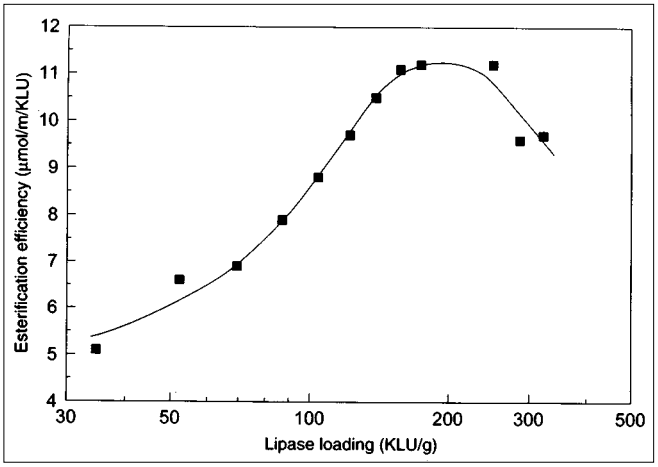

FIG. 5. Effect of lipase loading on esterification efficiency for *Humicola* sp. lipase immobilized on Accurel EP100. See Figure 1 for company source. See Figure 2 for abbreviation.

However, efficiencies can be used to compare the same lipase on different supports.

The explanation for the shape of the efficiency curve for the *Humicola* lipase is broadly the same as for *R. miehei* lipase. Because the absolute activity of immobilized *Humicola* is much lower than the other systems studied, it is possible that diffusional effects are less important in this system, allowing conformational effects to dominate. Alternatively, there may be a much stronger interaction between the *Humicola* lipase and the support, which leads to greater activity loss at lower loadings due to lipase "spreading."

Rhizopus niveus. A series of catalysts was prepared with *R. niveus* lipase and Accurel EP100 to cover a range of loadings, and their esterification efficiency vs. loading is shown in Figure 6.

The experimental data for this system show much more scatter, and it is difficult to identify trends in the efficiency plot. There are indications of slightly reduced activity at the lowest loadings and some evidence of diffusion-controlled limitations at the highest loadings, but there is no clear evidence of a maximum as seen in the previous systems. This lipase is more difficult to work with than the others because the crude commercial product contains significant protease activity and makes it difficult to measure accurate lipase loadings due to proteolysis of the lipase in the supernatant solution. Differing degrees of proteolysis of the lipase during the relatively long immobilization times may explain the variable activities obtained with this system.

Candida antarctica *B.* The B lipase from *C. antarctica* is of particular interest due to its high thermal stability and ability to catalyze reactions with secondary alcohols. This lipase is now being used for the commercial production of esters, such as decyl oleate and isopropyl myristate, for use as emollients in the cosmetics industry (5).

A series of catalysts was prepared with recombinant *C. antarctica* B lipase in a range of loadings up to near saturation. Esterification activities were determined, and a plot of esterification efficiency vs. loading is shown in Figure 7.

In contrast to the other lipases studied, the catalysts show no depression of efficiency at low enzyme loadings. The maximum efficiencies are obtained at the lowest loadings, and it appears that the expected diffusion-controlled limitations dominate the efficiency relationship over most of the loading range. This suggests that this lipase has a structure that is much more difficult to distort. This may be due to the enzyme having either a more rigid structure or a smaller number of potential binding sites on its surface, making it less likely to "spread" on the support surface.

No attempt has been made to purify any of the lipases studied. It has been shown that the adsorption of nonlipase proteins to the support can affect the activity and efficiency of the catalyst (20). It could therefore be expected that the shape of the efficiency-loading relationship will, to some extent, depend on the level and type of other proteins present in the commercial lipase preparations used.

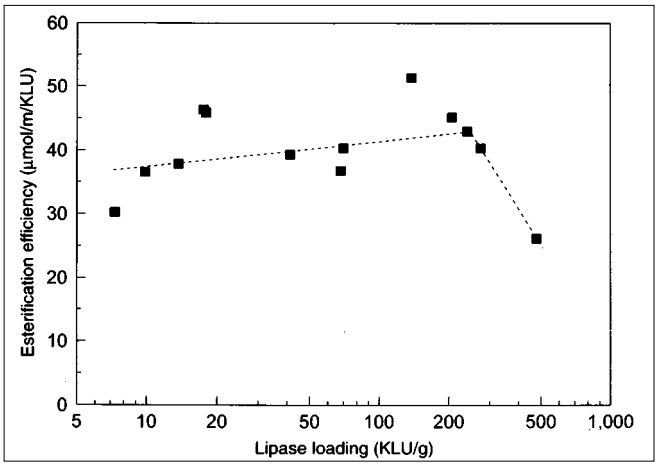

FIG. 6. Effect of lipase loading on esterification efficiency for *Rhizopus niveus* lipase immobilized on Accurel EP100. See Figure 1 for company source. See Figure 2 for abbreviation.

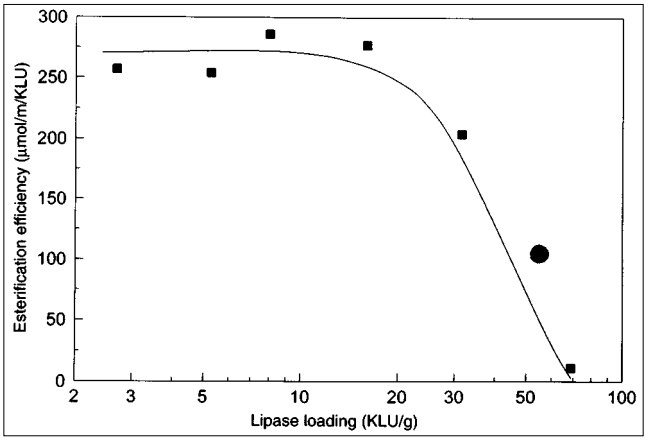

FIG. 7. Effect of lipase loading on esterification efficiency for *Candida antarctica* B lipase immobilized on Accurel EP100. See Figure 1 for company source. See Figure 2 for abbreviation.

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[Received May 20, 1996; accepted November 13, 1996]