Immobilization of Porcine Pancreatic Lipase on Celite for Application in the Synthesis of Butyl Butyrate in a Nonaqueous System

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ABSTRACT: For immobilization of lipase, the use of a porous support material is recommended so that suitable amounts of lipase can be spread on a surface area without conformational changes. In this work, porcine pancreatic lipase was deposited on Celite, either by direct binding from aqueous solution or by deposition from aqueous solution by the addition of organic solvent. The influence of the immobilization procedure on the activities of the derivatives has been studied regarding their ability to synthesize butyl butyrate. The reaction rates were compared with the rate of esterification with free lipase. Better properties were displayed when the immobilized lipase form was prepared in an apolar solvent such as hexane. Under suitable reaction conditions, esterification yields as high as 90% were attained. Batch operational stability tests indicated that no enzyme deactivation occurs after 15 consecutive batches of 24 h each.

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KEY WORDS: Butyl butyrate, Celite, enzyme immobilization, enzymatic synthesis, enzyme reutilization, porcine pancreatic lipase.

Until relatively recent times, it was thought that biocatalysts would have little utility for organic synthesis, since a liquid organic reaction medium is usually required for such processes and biocatalysts were thought to be inactive in contact with organics. We know now that many biocatalysts can be effectively used in some types of organic liquids and, in some cases, with the virtual absence of water (1–3). This is important, since the thermodynamic equilibrium of many synthesis processes, such as the production of esters from carboxylic acids and alcohols or peptides from amino acids, is unfavorable in water (3). In organic synthesis, the great advantage in using catalysts that have significant specificity for the product of interest, rather than for byproduct formation, is that costly separation, purification, and waste treatment are reduced (3,4).

Although it has been estimated that over 95% of organic chemical products could be obtained by biotransformations, only a very limited number of biocatalysts have been studied for this purpose (4,5). Furthermore, the frequency of use of biocatalysts is not well distributed among the various types of biotransformations, but follows a pattern in which 75% of the organic syntheses reported in the literature involves the use of hydrolytic enzymes such as proteases, esterases, and lipases (5,6).

The potential application of lipase for the production of several high-value chemicals and the advantages associated with the use of this biocatalyst in nonaqueous media have motivated our group to develop research lines related to the production of flavor esters (6–9). Parallel to this interest, we have also carried out experimental work to establish methodologies for immobilization of pancreatic and microbial lipase on different types of supports (10,11). The advantages of such derivatives include the possibility of recovery and reuse, simplicity in operation, and improved stability (12,13).

The extent of stabilization depends on the enzyme structure, the immobilization method, and the type of support (13). Numerous supports for the immobilization of lipases have been investigated. Comparative studies indicate that dramatic differences in activity are observed among lipases on supports of different materials (14). Therefore, the support must be chosen with great care. The support can affect the partitioning of substrates, products, and water in the reaction mixture and, thereby, indirectly influence the catalytic properties of the enzyme (15,16). The effects of the support on water partitioning in the system can be interpreted by measurements of the aquaphilicity (water-attracting capacity) of the supports (14,15). Normally, high reaction rates are obtained with supports having low aquaphilicity (14–16). Based on this, we chose to carry out a set of experiments with Celite, a natural inorganic material which has a low polarity, that is, an aquaphilicity value lower than 0.2 (16). Despite the number of published works using this support (16–20), the reported results suggested that these derivatives have poor stability under operational conditions. This is a serious limitation, since under industrial scale both higher activity and increased stability offer great economical advantages. Increased stability of the lipase-immobilized forms can be achieved by using

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nonaqueous instead of aqueous media. This procedure worked well in our lab for the immobilization of *Candida rugosa* on controlled-pore silica (CPS) and styrene-divinylbenzene copolymer (10,11). In this paper emphasis was placed on developing a procedure to obtain a lipase immobilized on Celite with high activity and stability for use in the synthesis of flavor esters. Comparison was made between immobilization in aqueous and nonaqueous media (acetone and hexane).

MATERIALS AND METHODS

Materials. Commercial porcine pancreatic lipase (Type II) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). The lipase was a crude preparation with a specific activity of 2484 units/mg protein and was used without further purification. Celite (341) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Olive oil (low acidity) was purchased in a local market. Substrates for esterification reactions were dehydrated, with 0.32 cm molecular sieves (aluminum sodium silicate, type 13 X-BHD Chemicals, Toronto, Canada), previously activated in an oven at 350°C for 6 h. Solvents were standard laboratory grade, and alcohols, organic acids, and other reagents were purchased either from Aldrich Chemical Co. or Sigma Chemical Co.

Immobilization of lipase on Celite. Lipase was immobilized by adsorption on Celite using buffer (0.1 M sodium phosphate buffer, pH 7.00) or organic solvent (acetone or hexane) as an immobilization medium. To this end, 500 mg of lipase was dissolved in 10 mL of 0.1 M sodium phosphate buffer (pH 7.0) and mixed with 5 g Celite under low stirring for 2 h at room temperature. After this period, 10 mL of 0.1 M sodium phosphate buffer or organic solvent was added to the enzyme-support mixture and the adsorption was allowed for a further 30 min. The lipase adsorbed on the support was filtered and washed three times with 10 mL of 0.1 M sodium phosphate buffer (pH 7.0) or organic solvent. Further experiments were carried out to select appropriate enzyme loading by using a fixed amount of lipase for different amounts of support.

Butyl butyrate synthesis. Ester synthesis was carried out in 20 mL of dry *n*-heptane containing 300 mM *n*-butanol, 300 mM butyric acid, and 12.5 mg/mL immobilized lipase, except where noted. The mixture was incubated at 37°C with reciprocating agitation (150 rpm). The inhibitory effect of butyric acid concentration on the esterification rate was studied by varying the initial acid concentration between 120 mM and 900 mM while keeping the initial butanol concentration at 300 mM. The majority of the esterification reactions were run in parallel with blank experiments. These were conducted under similar conditions by using the same mass of support without enzyme, in order to assess the extent of substrate and/or solvent evaporation.

Batch operational stability tests. The operational stability of the immobilized enzyme was assayed by using 25 mg/mL of immobilized lipase in successive batches carried out under the same conditions as described for butyl butyrate synthesis. Twenty-four hours after starting each batch, the immobilized lipase was removed from the reaction medium and rinsed with hexane in order to extract any substrates or product retained in the matrix. One hour later (length of time required for the solvent to evaporate) the immobilized derivative was introduced into a fresh medium.

Analysis. The activities of free and immobilized lipase were assayed by the olive oil emulsion method according to the modification proposed by Mustranta *et al.* (21). The substrate was prepared by mixing 30 mL of olive oil with 70 mL of emulsification reagent. The emulsification reagent (1 L) contained NaCl (17.9 g), KH_2PO_4 (0.41 g), glycerol (540 mL), gum arabic (10 g), and distilled water. The reaction mixture, consisting of 5 mL of the emulsion, 4 mL of sodium phosphate buffer (pH 7.00), and either free (1 mL, 0.1 mg/mL) or immobilized (100–250 mg) lipase, was incubated for 30 min at 37°C. The reaction was stopped by addition of 10 mL of acetone/ethanol solution (1:1). The liberated fatty acids were titrated with 25 mM potassium hydroxide solution in the presence of phenolphthalein as an indicator. One unit (U) of enzyme activity was defined as the amount of enzyme which liberated 1 μmol of free fatty acid per min under the assay conditions. Protein was determined according to Lowry *et al.* (22) using BSA as standard. The amount of bound protein was determined from the difference between the amount of protein introduced into the coupling reaction mixture and the amount of protein in the filtrate and in the washing solutions. Analyses of the lipolytic activities carried out on initial and spent lipase solutions and immobilized preparations were also used to determine the coupling yield (η%) according to the following expression:

$$\eta\% = \frac{\text{(overall activity of the immobilized enzyme)}}{\text{(overall activity of the initial enzyme solution)}} \times 100 \quad [1]$$

The esterification reactions were monitored by measuring butanol and butyl butyrate concentrations by gas chromatography using a 6 ft 5% DEGS on Chromosorb WHP, 80/10 mesh column (Hewlett-Packard, Palo Alto, CA), and hexanol as internal standard. Water concentrations in the liquid and solid phases were measured by Karl Fischer potentiometric titration using a DL 18 Moisture Apparatus (Mettler, Zurich, Switzerland).

The partition coefficients (support/external organic solvent) of butanol, butyric acid, and butyl butyrate were estimated according to the following equation (9):

$$P = \left(\frac{C_0 - C}{C}\right) \times \left(\frac{V_0}{V - V_0}\right) \quad [2]$$

where P = partition coefficient, C_0 = initial concentration of compound, C = equilibrium concentration of compound, V_0 =

total volume of system (organic phase + support), and V = volume of organic phase. Partition experiments were conducted under the same conditions as their reaction counterparts. In order to estimate the support volume ($V - V_0$), a calibration curve relating volume of Celite vs. mass of the Celite was established [volume of matrix (cm^3) = 0.28 × mass of Celite (g) + 0.02; correlation coefficient = 0.991].

RESULTS AND DISCUSSION

Initial experiments involved adsorption of lipase on Celite at a fixed relationship between support (1000 mg) and enzyme (100 mg), using buffer or acetone as an immobilization medium. Both procedures showed similar performance in terms of protein coupling (Table 1), however, much lower lipolytic activity was attained for the immobilization in buffer medium. In addition, the high concentration of water which surrounds the catalytic surface of this immobilized preparation (48.80%) may raise additional difficulties for its use in the esterification reaction, since for this type of reaction the water content should be low. To verify this, the specific activities of both immobilized forms were calculated and compared with that attained for free lipase (Table 1). Lipase adsorbed on Celite from buffer exhibited a very modest activity compared with lipase deposited from buffer by acetone addition. This behavior was also observed by Norin *et al.* (15) for a similar reaction system (esterification of heptanol with butyric acid). Therefore, buffer was not used as a dispersion medium for lipase immobilization in the following tests.

Another 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 (23), 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, more of its active conformation is retained, and loss in activity is reduced. To determine an efficient relationship between porcine pancreatic lipase and Celite, we studied the influence of the amount of support in the range of 0.5–5.0 g (dry weight) for a fixed amount of enzyme (500 mg) using acetone as an immobilization medium. As expected, when the amount of support decreased for a fixed amount of lipase, there was improved retention of the protein deposited on the solid support (Table 2). Concerning the recovered enzyme activities, no significant differences were found under all conditions used. Although activity retention is an important parameter for the evaluation of an immobilization procedure, the final selection should be based on analysis of the biocatalyst performance under operational conditions. The retention of the biocatalyst activity after repeated use was assessed in terms of butanol conversion rate at the end of each cycle. When the three lipase preparations adsorbed on Celite by acetone precipitation were used for butyl butyrate synthesis, the enzyme preparations were initially active but gradually lost their activity during repeated reactions (Fig. 1), following a trend similar to that reported by several researchers (16–18).

To overcome this constraint, the immobilization was performed by replacing acetone with hexane as dispersion medium, using the intermediate ratio between lipase and Celite (0.25 g protein/g support). This methodology has been successfully applied previously by Mustranta *et al.* (21) using Celite and Duolite as support materials, although no reutilization tests were carried out. According to our results, it appears that the immobilized lipase prepared by using hexane is highly superior to the corresponding preparation using acetone, not only in regard to the coupling yield (Table 2) and the synthesis activity but also in relation to its stability under operational conditions (Fig. 1 vs. Fig. 3).

The actual mechanism of the enhanced properties of immobilized lipase on Celite from hexane is not well understood, although it appears that the use of a solvent which has lower polarity (higher log P value) is capable of creating a specific microenvironment around the enzyme that may enhance its stability and activity (24). This would not be surprising, since it has been shown that water-immiscible hydrophobic solvents such as alkanes (hexane, log P = 3.50) retain an enzyme's high catalytic activity whereas water-miscible hydrophilic solvents such as dimethyl-formamide, dimethyl sulfoxide, lower alcohols, and acetone (log P = –0.23) are usually incompatible with enzyme activity (25).

A typical esterification reaction illustrating the performance of both free lipase and lipase immobilized from hexane is shown in Figure 2. A comparison between the two is made at similar units of activity (0.4×10^3 units/mL). The reactions have been carried out as described in the Materials and Methods section by using equimolar amounts (300 mM) of butanol and butyric acid in heptane.

TABLE 1
Comparison of the Lipase Adsorption Yield on Celite Using Buffer and Acetone as Immobilization Media

Immobilization medium	Protein bound (%)[a]	Coupling yield (η%)[b]	Immobilized water content (%)	Specific activity (μmol • g^{-1} • min^{-1})[c]	Relative activity (%)[d]
Buffer	32.80	6.70	48.80	0.03	4.8
Acetone	42.35	26.70	1.30	0.22	35.5

[a]Total protein offered to the immobilization: 124 mg.
[b]Calculated according to Equation 1 (overall activity offered to the immobilization: 298.14 U).
[c]Specific activities are expressed as μmol butanol reacted per min per g solid by following the rate of disappearance of butanol from the reaction mixture containing butanol and butyric acid.
[d]Relative activity was calculated by dividing the specific activity of the immobilized lipase by the value attained with free lipase (0.62 μmol • g^{-1} • min^{-1}).

TABLE 2
Adsorption of Porcine Pancreatic Lipase on Celite by Using Immobilization Organic Media (acetone or hexane)

Ratio lipase/Celite	Activity (U/mg)	Overall activity (U)	Coupling yield (η) (%)[a]	Protein bound (%)[b]
1:1[c]	75.64	73.37	24.62	61.57
1:4[c]	33.90	84.07	28.20	56.32
1:10[c]	9.97	79.60	26.70	42.35
1:4[d]	62.28	237.30	79.59	79.03

[a]Calculated according to Equation 1 (overall activity offered to the immobilization: 298.14 U).
[b]Total protein offered to the immobilization: 124 mg.
[c]Acetone precipitation.
[d]Hexane precipitation.

The stability of the hexane-immobilized system was also assessed by reusing the immobilized lipase 15 times in the synthesis of butyl butyrate. Lipase adsorbed onto Celite from hexane gave a stable enzyme preparation, and conversions in excess of 90% were maintained for 15 sequential batch reactions over a period of about 2 wk (each batch under standard conditions, 24 h long). The data in Figure 3 indicate that the kinetics of the batch reactions (in terms of the rate of butanol utilization and butyl butyrate formation) remained relatively stable during the repeated tests.

Having established an improved methodology for adsorption of lipase on Celite, further experiments were carried out to determine the influence of butyric acid and the amount of the lipase immobilized on butyl butyrate synthesis. Those parameters were selected on the basis of our previous results by using a commercial immobilized lipase preparation (Lipozyme) as a mean of comparative performance (26). Therefore, no attempt was made to investigate other parameters that may interfere in the esterification performance, such as water concentration and solvent polarity.

The effect of initial butyric acid concentration on the esterification rate with butanol by lipase immobilized on Celite is shown in Figure 4. In the presence of nearly equimolar amounts of *n*-butanol and butyric acid the formation of butyl butyrate was highest. The rate of product formation declined sharply with additional increases of butyric acid.

This behavior is quite different from that attained with a commercial lipase preparation (Lipozyme), in which an excess of butyric acid was found to ensure an optimal esterification performance (8,26). Estimation of the partition coefficients of reactants and product for both enzyme preparations (Table 3) is used here to show the dependence of lipase activity on the medium composition, which, in its turn, is affected by the nature of the support material.

With the lipase adsorbed on Celite (low polar support), similar partition coefficient values were attained for butanol and butyric acid, suggesting that a molar ratio of 1:1 provides a suitable migration balance of both reactants into the aqueous layer around the catalyst, where the enzymatic reaction occurs. The use of Lipozyme (when a lipase is adsorbed on an ion exchange resin, a highly polar support) as a catalyst produced a very different set of results (26): the partition coefficient for butanol was almost two times greater than for butyric acid (26). This has led to claims that the reaction rate might be limited by the concentration of the acid (8,26). As far as the partition coefficient of product is concerned, both enzyme preparations favor the migration of the butyl butyrate

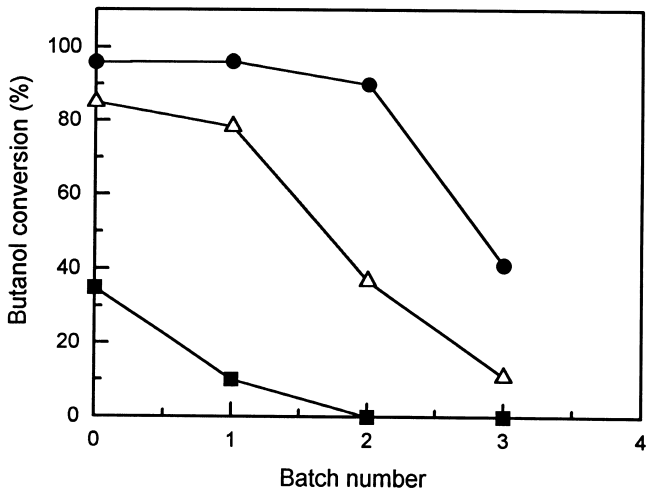

FIG. 1. Repeated use of lipase adsorbed on Celite at different loading levels using acetone in the esterification reaction of *n*-butanol (300 mM) with butyric acid (300 mM) in heptane under shaking (150 rpm) at 37°C. Each reaction was carried out for 24 h. (●) Celite/lipase (1:1); (▽) Celite/lipase (4:1); (■) Celite/lipase (10:1).

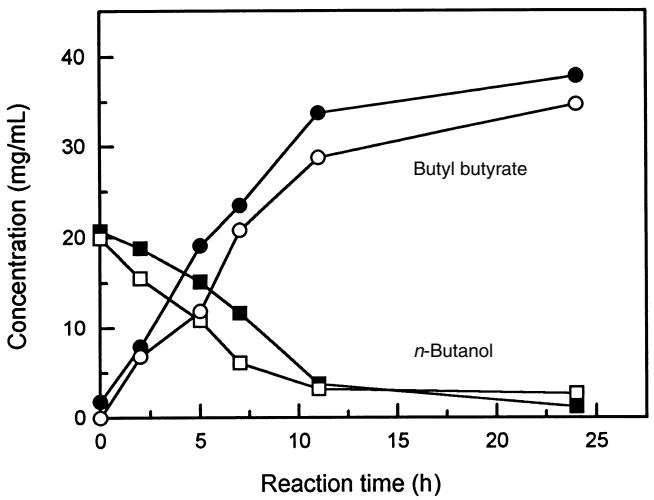

FIG. 2. Concentration profiles during the synthesis of butyl butyrate in batch reaction by free lipase (solid symbols) and immobilized lipase from hexane (open symbols). Reaction conditions were as described for data in Figure 1.

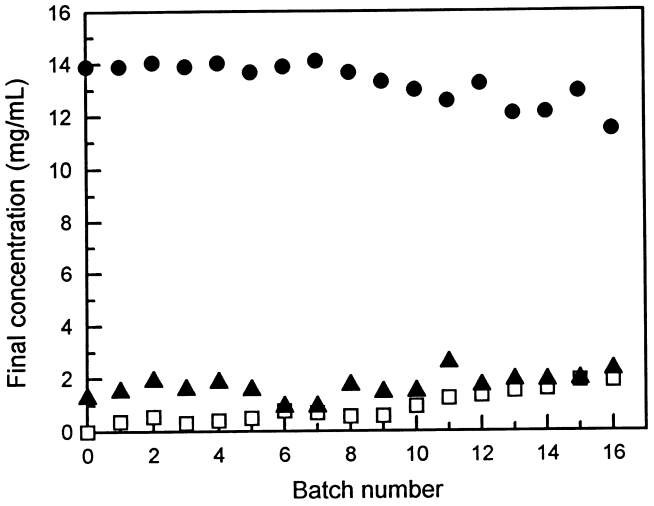

FIG. 3. Batch operation stability test of the Celite-immobilized lipase from hexane. Initial medium composition, 100 mM butanol and 100 mM butyric acid in heptane. Duration of each cycle: 24 h. (□) *n*-Butanol; (▲) butyric acid; (●) butyl butyrate. Reaction conditions were as described for data in Figure 1.

toward the organic solvent. This is helpful in terms of keeping the reaction equilibrium in the desired direction and for product recovery. From this, and in agreement with Frense *et al.* (27) and Castillo *et al.* (28), it is clear that the support polarity and the molar ratio of reactants play an important role in this esterification reaction. Therefore, the partition coefficient estimation can provide a good guide not only to formulate a medium design for biocatalyst purposes but also to evaluate the suitability of a particular carrier for enzyme immobilization. Further investigations related to the optimization of both parameters for biocatalysis in nonconventional media are in progress.

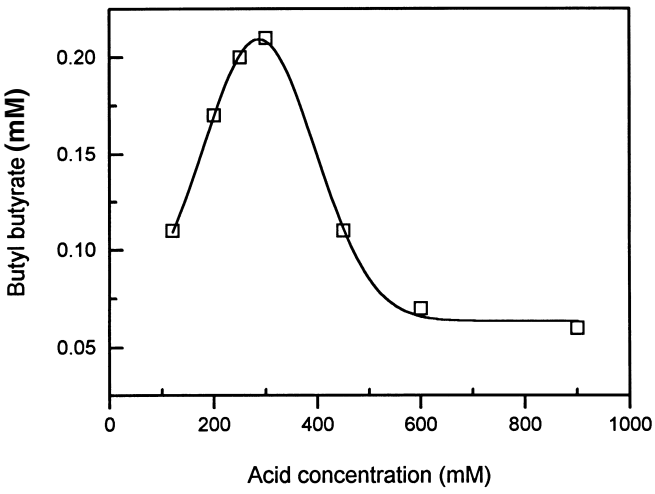

FIG. 4. Effect of initial butyric acid concentration on the esterification rate. Reactions were carried out in heptane containing a fixed amount of butanol (300 mM) under shaking (150 rpm) at 37°C.

TABLE 3
Partition Coefficients onto Lipase Immobilized on Celite and Lipozyme/Heptane of n-Butanol, Butyric Acid and Butyl Butyrate[a]

	Partition coefficient	
Compound	Lipozyme[a]	Lipase-Celite
n-Butanol	2.43	0.23
Butyric acid	1.53	0.24
Butyl butyrate	0.63	0

[a]Estimated according to Equation 2, at concentrations of 300 Mm at 37°C under shaking.
[b]Data from our previous work (26).

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