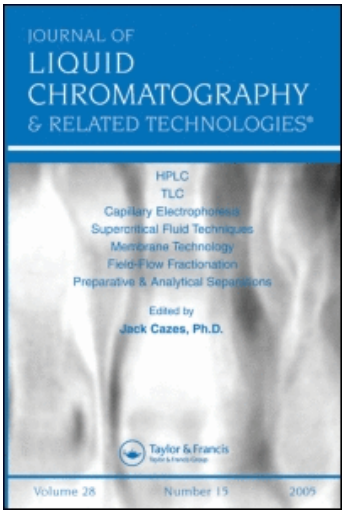

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DIRECT HPLC MONITORING OF LIPASE ACTIVITY IN REVERSE MICELLAR MEDIA

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ABSTRACT

Given the profusion of biotechnological applications of the nonaqueous use of lipases, we have evaluated the possibilities of exploiting the inherent advantages of high performance liquid chromatography (HPLC) for a simple, rapid assay of lipase activity in reverse micellar media, as a convenient alternative to previously reported spectroscopic methods, using both a model system and esterification reaction, and different commercial lipases. The results obtained after a screening for optimized chromatographic conditions in the reverse-phase mode indicate that a satisfactory resolution of the reaction components can be obtained following a straightforward protocol, which permits an accurate, reliable quantitation of the reaction progress, regardless of the enzyme used.

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INTRODUCTION

Nonaqueous enzymology has consolidated during the last decade as a promising, exciting area of research (1-3) with important biotechnological implications, particularly those concerning organic syntheses and bioconversions in food, pharmaceutical, agricultural, petroleochemical or even military industries. Among the different experimental approaches proposed to place a functional enzyme in a water-restricted environment (each having its inherent advantages), one of the most popular is the solubilization of the protein in the so-called reverse micelles (4,5), where the enzyme is physically confined in an aqueous "micropool" separated from the bulk organic phase ("oil") by a monolayer of surfactant. This distribution contributes to a quite efficient biocatalysis by enabling a maximization of the water-oil interfacial area and a ready solubilization of many nonpolar substrates (and products) in the organic phase.

Although reverse micelles have been also employed as membrane mimetic systems for the study of membrane-bound or membrane-associated proteins (6,7) or hydrophobic peptides (8), or as microemulsion preparations for topic drug delivery (9), the attention in these systems has been mainly polarized towards applied enzymology facets, and in particular quite profusely towards the use of lipases in varied transformations of evident industrial interest, such as ester synthesis, triglyceride hydrolysis or interesterification, etc.

An overview of the literature during the last years evidences that whereas the monitoring of lipase activity in organic solvents using insoluble enzyme (powdered, entrapped or adsorbed to a support) has made use in some instances of liquid chromatographic procedures, in the case of reverse micellar solutions most of the work has been based on spectroscopic determinations. Thus, among the methods most often employed are that of Lowry & Tinsley (10) or modifications of it (11), that of phenol red cosolubilized in the micelle as an acid-base indicator (12), one based on Fourier-transfrom Infrared (FTIR) spectroscopy (13, 14) or the use

of chromogenic lipase substrates, such as p-nitrophenyl palmitate (15) or more sophisticated ones (16,17). To a lesser extent, gas chromatography (18), thin-layer chromatography (19) or alkalimetric titrations (20,21) have been also used occasionally.

Paradoxically, however, in spite of all the inherent advantages of high-performance liquid chromatography (HPLC), the use of this technique is scarcely found in the direct monitoring of lipase-mediated conversions occurring in reverse micellar media, which is somewhat understandable taking into account the number of components to be separated *a priori*: organic solvent, surfactant (plus sometimes cosurfactant), enzyme, water, substrate(s) and product(s).

In this preliminary work, we have made an initial evaluation of the possibilities of HPLC in its reverse-phase (RP) mode, as a versatile tool for the simple, rapid assay of lipase activity in reverse micelles, by selecting a reverse micellar model system [Aerosol OT (AOT) in isooctane], model lipases (from *Candida rugosa*, *Rhizopus delemar* and *Pseudomonas sp.*) and a model reaction (the esterification between n-decanoic acid and 1-butanol) (Fig. 1). A screening for chromatographic conditions has been carried out to obtain a satisfactory resolution of most of the reaction components and a fast, straightforward sample preparation procedure is proposed, valid for other lipases and model reactions assayed. It is shown that the lipase activity can be accurately and reliably followed by RP-HPLC, the results being comparable to those obtained by other techniques.

EXPERIMENTAL PROCEDURES

Materials.

Lipase (EC 3.1.1.3) from *Candida rugosa* (formerly named *cylindracea*), type VII, was purchased from Sigma (St. Louis, MO.) and used without further purification. Lipases from *Rhizopus delemar* and from *Pseudomonas sp.* were generously provided by Amano Intl. Enzyme Co. (Nagoya, Japan) and also used without additional purification. AOT was

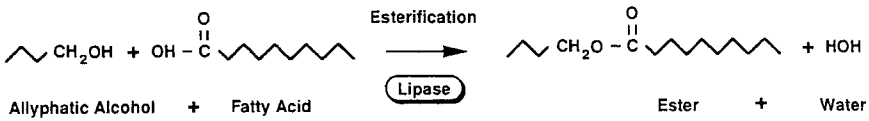

FIGURE 1. Lipase-catalyzed model esterification reaction in reverse micelles.

obtained from Serva (Heidelberg, Germany), purified by preparative RP-HPLC, and vacuum-dried overnight prior to use to ensure a minimal water content. Organic solvents were of analytical or HPLC grade, from Merck (Darmstadt, Germany). Bidistilled water was purified through a Millipore Milli-Q system (Millipore, Milford, MA). Fatty acids, alcohols and salts were obtained from commercial suppliers with a >99% purity.

Sample Preparation.

The reverse micellar solutions were prepared by adding appropriate quantities of alcohol (typically 250 mM) and fatty acid (typically 100 mM) to a solution of 100 mM AOT in isooctane. To start the reaction, a few microliters of lipase solution in 10 mM Tris-HCl (pH 7.5) were injected to the substrate(s)-containing reaction medium, and after a few seconds of gentle shaking a clear, transparent, enzyme-containing reverse micellar solution was obtained (zero time). Aqueous lipase concentrations and injected volumes were adjusted to obtain in all cases an overall enzyme concentration in the organic medium of 0.4 mg of lipase sample per mL. The water content of the micelles is defined by the parameter w_0 (4), which denotes the quotient of molar concentrations of water and surfactant.

Activity Measurements.

The reactions were carried out in screw-capped vials at 25 °C. Periodically, aliquots of 20 μL of the reaction medium were withdrawn and placed in an eppendorf and the solvent was rapidly evaporated under a N_2 stream. The residue was redissolved in 40 μL of chromatographic mobile phase, and after centrifugation at 13,000*g* for 3 min to separate the nonsolubilized enyme, 10 μL of the supernatant were injected onto a

LiChroCART 125-4 (LiChrospher 100 RP-18) cartridge, from Merck. The liquid chromatograph consisted of M-510 solvent-delivery systems, an automated gradient controller, a U6K universal injector and a 410 differential refractometry detector, all from Waters (Milford, MA).

Other details of experimental conditions can be found in the corresponding legends to Figures.

RESULTS AND DISCUSSION

Although *gradient conditions* for elution are not unusual in RP-HPLC separations and can undoubtedly afford both higher versatility and resolution, our purpose throughout this work was to design and optimize a simple, reliable protocol for a rapid, 'comfortable' monitoring of lipase activity using *isocratic elution* (preferable for differential refractometry detection), to make the chromatographic technique attractive when compared to the reported spectroscopic methods.

Our first attempts were aimed at the direct injection onto the reverse-phase column of the enzyme-containing reverse micellar solution and subsequent chromatographic separation of lipase, surfactant, isooctane, n-decanoic acid, 1-butanol and butyl decanoate. As a first trial, methanol-water and acetonitrile-water (containing 0.1% TFA) mixtures around 50 % (by volume) of organic component were tested as mobile phase as a compromise both to have a reasonable resolution of substrates and products and to dissolve (although denatured) the lipase in the eluent. Unfortunately, under these conditions the reaction product could not be eluted from the column (not shown), a requisite that we considered mandatory for reliable quantitation of enzyme activity (based on simultaneous *appearance of the ester* and *disappearance of the substrates*). Since even at 70% (by volume) of acetonitrile in the mobile phase, the reaction product was not eluted, we made an estimation of the approximate composition of organic component in the eluent necessary for

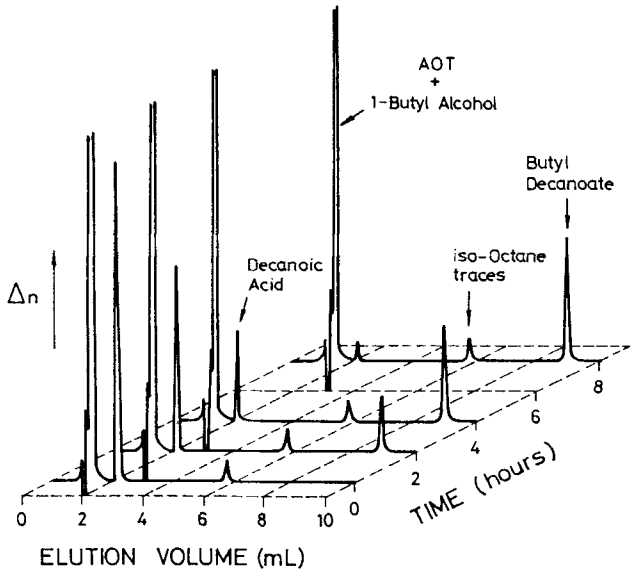

FIGURE 2. RP-HPLC monitoring of the *Rhizopus delemar* lipase-mediated esterification of n-decanoic acid and 1-butanol in an AOT/isooctane reverse micellar system (w_0=8.9). The elution profiles correspond to aliquots from the reaction medium taken at different incubation times and treated as described in the Experimental Procedures. The column was isocratically eluted with 0.1% TFA-containing acetonitrile-water (85:15, v/v) at a flow rate of 1.0 mL/min. Samples were monitored using differential refractometry detection.

isocratic elution of the ester, by performing a screening experiment under gradient conditions (using UV detection) from 50% to 90% acetonitrile (not shown). It was decided that an isocratic mobile phase (containing 0.1% TFA) composed of acetonitrile-water (85:15, v/v) should be adequate for the separation and visualization of both substrates and products. Since in this case the direct injection of the reaction mixture was not possible due to the high content or organic component of the eluent (in which the lipase was not soluble), a simple, rapid protocol to remove the enzyme before sample injection was therefore developed, which is described in the Experimental Procedures.

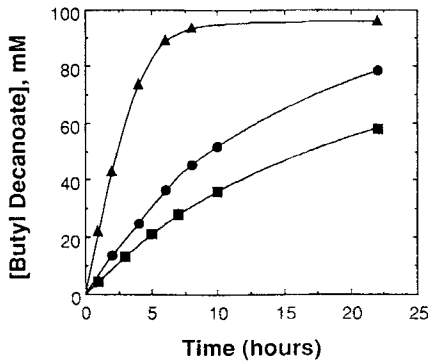

FIGURE 3. Time course of lipase-catalyzed formation of butyl decanoate in reverse micelles (w_0=8.9), as monitored by RP-HPLC, for different lipases assayed: (▲) *Rhizopus delemar*; (●) *Pseudomonas sp.*; and (■) *Candida rugosa*.

Fig. 2 depicts the gratifying results obtained upon implementation of the above conditions and illustrates, as an example, how the time course of *Rhizopus delemar* lipase-catalyzed ester formation in reverse micelles can be easily followed by RP-HPLC using a differential refractometry detector. Notice that elution of all the components in the reaction mixture (except the lipase) takes less than 10 min and that both one of the substrates (n-decanoic acid) and the ester product (butyl decanoate) are completely resolved from the other components.

Fig. 3 depicts, as an example, kinetics of the production of butyl decanoate in the reverse micellar medium by the different lipases assayed, as monitored by RP-HPLC. These results show that the method proposed can be conveniently used not only to monitor the progress of synthetic reactions and obtain accurate kinetic data, but to establish a comparison of the relative activities of enzymes from different sources.

On the other hand, when the influence of the water content of the reverse micelles (w_0) on the activity of the *Candida rugosa* lipase was analyzed, the profile obtained from HPLC data (Fig. 4) was found to be similar to that previously reported for the same enzyme and micellar system using spectroscopic determination of the reaction progress (22).

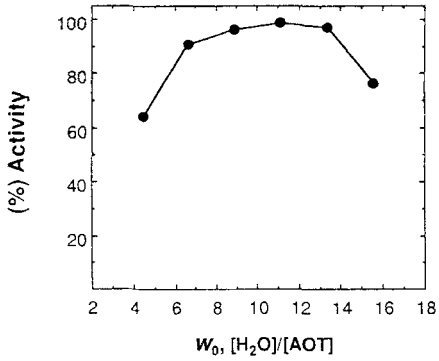

FIGURE 4. Profile of the dependence of the HPLC-determined activity of *Candida rugosa* lipase on the water content of the reverse micelles (w_0).

CONCLUSION

We provide here preliminary data which show that the widely acknowledged advantages of HPLC can be exploited as an alternative methodology for a rapid, direct monitoring of lipase activity in reverse micellar media, especially for comparative purposes which only require simple model reactions. In this case, the chromatographic approach can afford a higher reliability for quantitative determinations than some conventional spectroscopic assays and the possibility of automation and easy manipulation of a large number of samples.

On the other hand, optimization of conditions for the HPLC assay of model reactions involving longer fatty acids will require to explore other mobile phases to attain satisfactory separations in reasonable times. As an example, we have verified that isocratic elution on the above chromatographic system with a mobile phase as simple as pure methanol yields elution volumes for hexyl myristate, palmitate, stearate and oleate of 3.6, 4.9, 6.8 and 5.3 mL, and permits an easy monitoring of ester formation catalyzed by different lipases. Work is in progress in this direction.

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