Phospholipase A_2-Catalyzed Hydrolysis of Lecithin in a Continuous Reversed-Micellar Membrane Bioreactor

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ABSTRACT: Lysophospholipids and free fatty acids produced by lecithin hydrolysis are important natural compounds with high potential for application in the food, chemical, and pharmaceutical industries. In this work, the enzymatic hydrolysis of lecithin (essentially phosphatidylcholine) catalyzed by porcine pancreatic phospholipase A_2 (phosphatide 2-acyl-hydrolase, EC 3.1.1.4), encapsulated in mixed reversed micelles of lecithin and *bis*(2-ethylhexyl) sodium sulfosuccinate (AOT) in isooctane, was carried out in a continuous reversed-micellar membrane bioreactor. A tubular ceramic membrane with a 10,000 molecular weight (MW) cutoff was installed in an ultrafiltration module to retain the phospholipase A_2 (MW 14,000) and to continuously separate the products from the reaction media. Water and co-factor (Ca^{++})-containing reversed micelles of lecithin/AOT in isooctane were supplemented to the reactor to compensate for the permeation of reversed micelles and to continuously supply the substrate. The influence of relevant parameters, such as substrate, AOT and enzyme concentrations, water content and fluid hydrodynamics, on the performance of the ultrafiltration membrane bioreactor was investigated. Fluid axial velocity and substrate concentration were the major factors that affected the transport processes through the membrane. Permeate flow rate increased significantly with fluid axial velocity and decreased with substrate concentration; on the other hand, water and enzyme concentrations were identified as critical parameters for the final conversion of lecithin. The relationship between productivity and normalized residence time was analyzed for each set of experimental parameters tested. Operational stability of the bioreactor was tested in a long-term operation to confirm the high stability of this catalytic system.
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KEY WORDS: Fatty acids separation, lecithin hydrolysis, phospholipase A_2, reversed micelles, ultrafiltration membrane bioreactor.

Phospholipase A_2 (phosphatide 2-acyl-hydrolase, EC 3.1.1.4) hydrolyzes the ester bond of 1,2-diacylphospholipids at the *sn*-2 position to produce lysophospholipids and free fatty acids. Lysophospholipids are important emulsifiers in the food industry, especially in the baking industry, and the fatty acids produced by cleavage at position 2 of phospholipids (e.g., lecithins) are mainly unsaturated long-chain fatty acids, which are also useful in the chemical and pharmaceutical industries (1,2).

In recent work, we described the successful use of porcine pancreatic phospholipase A_2 (PLA_2) in mixed reversed micelles of lecithin/*bis*(2-ethylhexyl) sodium sulfosuccinate (AOT)/isooctane for phosphatidylcholine (PC) hydrolysis in a traditional batch reactor (3). Although high conversions were obtained, batch processes are inefficient with respect to enzyme utilization because the enzyme is not usually recovered and reused. Labor requirements are also substantial (repeated time-consuming loading and unloading processes of the reactor are needed), and variations often occur between batches in the quality of the obtained products (4). Some of these disadvantages can be overcome through the use of membrane bioreactors (5). Membrane bioreactors take advantage of the size differences between the enzyme and the reaction products. The enzyme can be trapped on one side of a semipermeable membrane while the substrate is fed directly into the retentate side, where the reaction occurs. The products are removed continuously from the reaction mixture, leaving the enzyme in the reaction zone for further reaction (4). The efficiency of the enzyme (i.e., the amount of products obtained per enzyme loading) can thus be remarkably increased as, for instance, was observed for starch hydrolysis (6).

The present work reports the PLA_2-catalyzed hydrolysis of PC in a continuous reversed-micellar membrane bioreactor. An attempt was made to combine the advantages of biocatalysis in reversed-micellar media (7,8) with the continuous use of PLA_2. The influence of several important process parameters in the reactor performance, such as substrate, AOT and enzyme concentrations, water content and fluid hydrodynamics, was investigated. The operational stability of the membrane bioreactor was also studied in a long-term operation.

EXPERIMENTAL PROCEDURES

Chemicals. Purified phospholipase A_2 (Lecitase™) from porcine pancreas, with a molecular weight around 14,000 Da and an activity of 10,000 IU/mL, was a gift from Novo Nordisk (Bagsvaerd, Denmark).

L-α-Lecithin from fresh egg yolks, consisting of 65% PC (based on choline determination), and surfactant AOT were

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purchased from Sigma (St. Louis, MO) and used without further purification. Isooctane (>99%) was obtained from Riedel-de-Häen (Salze, Germany). Phenol red (phenolsulfonephthalein), oleic acid (*cis*-9-octadecenoic acid, 99%), and Folin-Ciocalteau reagent were from Merck (Darmstadt, Germany). All other reagents were of analytical reagent grade.

Protein concentration. The protein concentration of Lecitase™ aqueous solution was determined by the method of Bradford (9) with phospholipase A_2 from the venom of *Crotalus atrox* cobra (Sigma) as a standard. A protein concentration of 16.8 mg/mL was obtained.

Analytical procedures. In the continuous experiments, the extent of PC hydrolysis in micellar media was estimated by the determination of free fatty acids with a spectrophotometric assay, described by Walde (10), with some modifications. This method enables the determination of free fatty acids released during the hydrolysis of PC in reversed micelles, as previously reported (3). The method uses the fatty acid indicator phenol red (1.25 mM phenol red in 0.1 M Tris–HCl, pH 9.0), solubilized in a solution of 50 mM AOT in isooctane, in such a way that the final solution has a Wo = 11.2 (Wo = $[H_2O]/[AOT]$) and an overall phenol red concentration of 12.5 μM. For free fatty acid determinations, samples of 50 μL were added to test tubes containing 2 mL of the phenol red-containing micellar solution. After vortex mixing, the optical density was measured at 558.5 nm. Standard curves were prepared with oleic acid in isooctane. In the permeation studies, PLA_2 concentration was determined by a modified Lowry assay (11) that can be applicable to organic solutions by the inclusion of a centrifugation/separation step (20 min at 3000 g) (12). Standard curves were prepared with PLA_2 encapsulated in reversed micelles of AOT (40 mM)/isooctane with a Wo = 15, containing 100 mM Tris–HCl buffer and 750 mM calcium in the water pool (pH 8.5). AOT concentrations were determined with the Lowry and Tinsley assay as proposed by Prazeres and co-workers (13); calibration curves were prepared with standard solutions of AOT in isooctane from 0 to 40 mM.

Lecithin in isooctane was determined by an enzymatic procedure based on the PLA_2-catalyzed hydrolysis of PC in PC/AOT/isooctane mixed reversed micelles. For this analysis, an enzymatic micellar solution was prepared by injecting 340 μL of an aqueous solution (0.159 M Tris–HCl buffer and 1.191M $CaCl_2$, pH 8.5) and 200 μL of Lecitase™ in 25 mL of AOT (30 mM) in isooctane and mixing with strong magnetic stirring. Then, 0.5 mL of this solution was placed in a test tube at 40°C, and 0.5 mL of the sample (PC in isooctane) was added. After strong vortex mixing, the hydrolysis of PC proceeded at 40°C during 30 min (this reaction time was previously shown to be more than enough for the total conversion), after which the free fatty acid content of the reaction medium was analyzed with the phenol red indicator assay (3,10). The PC concentration was then calculated from a calibration curve.

Preparation of feed solutions. Substrate feed solutions were prepared by adding appropriate volumes of an aqueous solution (containing 100 mM Tris–HCl buffer, 750 mM $CaCl_2$, pH 8.5) to a certain amount of an AOT/isooctane solution (with an AOT concentration double the final concentration required). The mixture was then submitted to vigorous magnetic stirring to solubilize the aqueous phase in the organic medium. Finally, an equal volume of a PC/isooctane solution (with a PC concentration double the final level required) was added under magnetic stirring to obtain a micellar medium with the desired Wo [Wo = $[H_2O]/([AOT] + [PC])$] and PC and AOT concentrations (25 and 15 mM, respectively, unless otherwise stated).

Membrane module and experimental set-up. A Carbosep® ultrafiltration tubular ceramic membrane, from Rhone-Poulenc (Miribel, France), was used to retain PLA_2 and to separate the reaction products. The ceramic membrane, which is resistant to organic solvents, was made of a zirconium oxide layer over a porous carbon support with a 10,000 molecular weight cut-off. The inner diameter was 6 mm, wall thickness 2 mm, and effective length 20 cm. A cylindrical stainless-steel bar (with a diameter of 5 mm), accommodated inside the membrane and leading to a decrease of the inner drainage section from 28.3 to 8.6 mm^2, was used in the continuous experiments, after checking its advantageous effect on the reactor productivity. The internal permeation area was around 38 cm^2. The membrane was accommodated in a tubular stainless-steel housing, forming a monochannel ultrafiltration module (14,15).

The bioreactor was operated in a continuous mode as shown in Figure 1. The reaction mixture was recirculated from the thermostated vessel into the membrane by means of a gear pump (Micropump® Cole Parmer, Chicago, IL), flowing tangentially to the membrane. Part of the solution permeated through the wall of the semipermeable membrane due to the hydrostatic pressure difference. The reactor was fed with the substrate solution by a peristaltic pump (Pharmacia-Pl; Pharmacia, Uppsala, Sweden). The connecting pipings in the system were made of isooctane-resistant Viton® (Cole Parmer).

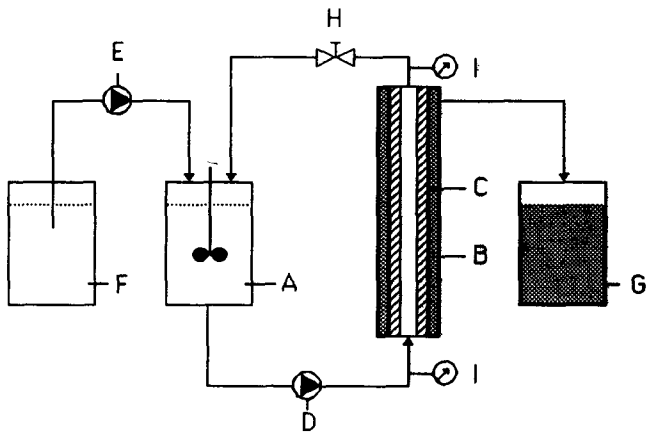

FIG. 1. Experimental apparatus: A, reactor; B, ultrafiltration module; C, ceramic membrane; D, E, pumps; F, substrate reservoir; G, product reservoir; H, back-pressure valve; and I, pressure gauge.

The volume of the concentrate was around 100 mL, and the hold-up of the permeate chamber was approximately 3 mL. To obtain the required residence times, the reaction mixture was recirculated with flow rates (Q_r) in the range of 370–1410 mL/min, corresponding to an average transmembrane pressure (ΔPTM) and permeate (or outlet) flow rate (Q_p) range of around 0.15–1.0 bar and 0.12–1.48 mL/min, respectively.

The membrane was periodically cleaned with 0.1 M NaOH, 0.03 M HNO_3, and water, consecutively. After drying the membrane with hot air, the flux restoration was checked with isooctane. Flow rates across the cleaned membrane were in the range of 1.8–1.9 mL/min for a recirculation rate of about 1320 mL/min and an average transmembrane pressure around 0.8 bar.

Continuous experiments. Reaction experiments were carried out at 40°C, pH 8.5, $[Tris–HCl]_{water\ pool}$ = 100 mM and $[CaCl_2]_{water\ pool}$ = 750 mM, the optimum conditions for activity of this particular reversed micellar system (3). Unless otherwise stated, Wo = 15, [PC] = 25 mM, [AOT] = 15 mM, and $[PLA_2]$ = 16.9 μg/mL were used.

The bioreactor was loaded with the PLA_2 micellar solution in the following way: first, 50 mL of AOT/isooctane solution with a concentration double the final concentration required in reaction was added. Then, appropriate volumes of Tris–HCl buffer, containing $CaCl_2$, and of the aqueous solution of PLA_2 were added to the external vessel, after which the mixture was circulated for 5 min to allow solubilization of the aqueous phase in the reversed-micellar media.

The operation was started in a batch mode (recycling the permeate stream) by adding 50 mL of PC/isooctane solution with the appropriate concentration (double the final concentration required in reaction), to obtain a micellar medium with the desired Wo, PC, and AOT concentrations. Agitation was provided by the recirculation of the reaction mixture. Following a start-up period of 20–30 min of reaction, continuous operation was started by feeding the external vessel with the substrate solution and recovering the permeate stream. Inlet (Q_{in}) and outlet (Q_{out}) flow rates were controlled and kept equal to maintain a constant level in the reactor. Adjustments were made in the inlet flow rate to compensate for the usual initial permeate flux loss.

Permeate and concentrate samples were analyzed for free fatty acids during the course of hydrolysis. Permeate flow rates were measured throughout the operation. Whenever an apparent steady state was achieved (described by the invariability of the permeate flow rate and fatty acid concentration in two consecutive samples within an interval of 15 min), the residence time was changed by varying the inlet and outlet flow rates through the recirculation flow rate and transmembrane pressure. In this system, instead of "steady state," it is more appropriate to use the expression "apparent steady state" because, as will be shown later, the retentate fatty acid concentration never reached a clear plateau but increased during the operation, even after the permeate flow rate and permeate fatty acid concentration had stabilized. After each experiment, the bioreactor was emptied and washed with isooctane until the initial permeate flow rate was reestablished.

Transmission studies. Studies on the permeation of PLA_2, AOT, and PC through the membrane were carried out at 40°C to determine the rejection coefficients of these three important components of the reaction mixture. The rejection coefficient of PLA_2 was assayed in reversed micelles of AOT (40 mM)/isooctane with Wo = 15 containing 100 mM Tris–HCl buffer and 750 mM calcium in the water pool (pH 8.5). The rejection of AOT was assayed in isooctane at two different concentrations of surfactant (15 mM and 40 mM). The rejection coefficient of PC was determined either when pure in isooctane (Wo = 0) or when in a mixed reversed-micellar solution of PC/AOT/isooctane (Wo = 15), containing Tris–HCl buffer and calcium (100 and 750 mM, respectively) in the water pool (pH 8.5) of the hydrated micelles.

In these permeation studies, the bioreactor was operated in a batch mode with recycling of the permeate stream (Q_r = 750 mL/min). The bioreactor was loaded in the following way: first, the module and the pipings were filled with pure isooctane (40 mL); then, a solution of isooctane (60 mL) with appropriate concentrations of the components in study was added to the external vessel in such a way that, after dilution with the isooctane present within the module and pipings, the required concentrations were reached. Samples of concentrate and permeate were taken for analysis at specific time intervals to follow permeation. Rejection coefficients [$\sigma = 1 - (C_p/C_c)$] were calculated when concentrate (C_c) and permeate (C_p) concentrations reached equilibrium.

RESULTS AND DISCUSSION

Membrane permeation studies. The behavior of the membrane toward three important system components (PLA_2, AOT, and PC) was evaluated in a series of transmission experiments. The ability to retain the enzyme is one of the most important membrane requisites for successful continuous operation of an enzymatic membrane reactor (14,16).

The permeation study of PLA_2 was carried out with the enzyme encapsulated in reversed micelles of AOT/isooctane (Fig. 2). The experiment was performed at Wo = 15, 40 mM AOT, and 67.4 μg/mL of PLA_2. The chosen AOT concentration was 40 mM to equal the sum of AOT plus PC concentrations used in most of the continuous experiments (15 and 25 mM, respectively). Protein concentrations in the concentrate and permeate streams were determined during the time course of the experiment. The initial decrease of concentration on the retentate observed in transmission studies (Figs. 2–4) is due to a dilution effect of the component under study (PLA_2, AOT, or PC) added to the external vessel, when mixing with the isooctane present within the module and pipings takes place. The rejection coefficient of PLA_2 in reversed micelles (but not under reaction conditions) was around 70% after 2.5 of recycling. No adsorption of the enzyme to the membrane was detected because the enzyme concentration in the retentate remained essentially constant during the opera-

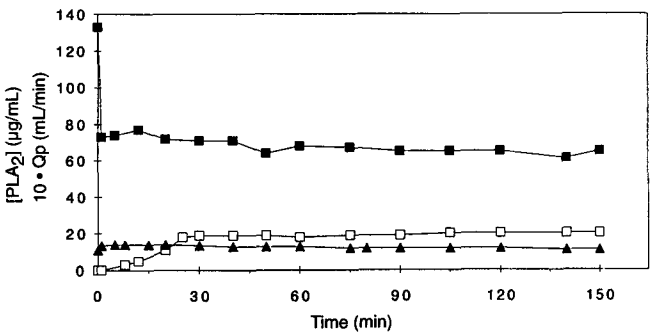

FIG. 2. Protein concentration in the concentrate (■) and in the permeate (□) streams and permeate flow rate (▲) during the transmission experiment of porcine pancreatic phospholipase A_2 (PLA_2) in reversed micelles of *bis*(2-ethylhexyl) sodium sulfosuccinate (AOT)/isooctane (Wo = 15, [AOT] = 40 mM; [PLA_2] = 67.4 µg/mL, 40°C, pH 8.5; $[Tris–HCl]_{water\ pool}$ = 100 mM; $[CaCl_2]_{water\ pool}$ = 750 mM; and $Q_r \approx$ 750 mL/min).

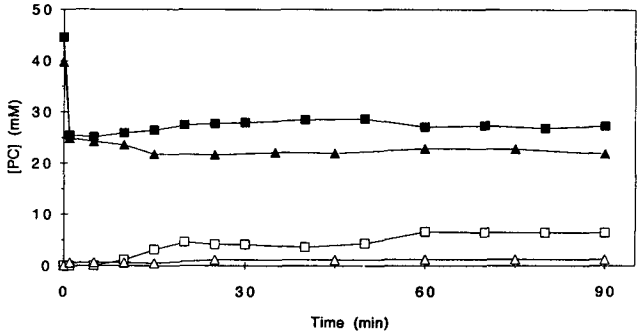

FIG. 4. Lecithin concentration in the concentrate (■,▲) and permeate (□,△) streams during the permeation experiments of phosphatidylcholine (PC), either pure in isooctane (■,□) or in reversed micelles of PC/AOT/isooctane (▲,△) (in both experiments: [PC] = 25 mM, 40°C; $Q_r \approx$ 750 mL/min; in the experiment with the mixed reversed-micellar media: Wo = 15; [AOT] = 15 mM, pH 8.5; $[Tris–HCl]_{water\ pool}$ = 100 mM; and $[CaCl_2]_{water\ pool}$ = 750 mM). Abbreviations as in Figure 2.

tion time (Fig. 2). Furthermore, no decrease on the permeate flow rate was observed during the experiment, which seems to be in accordance with the absence of protein adsorption. The commercial PLA_2 preparation was obtained after tryptic activation of prephospholipase A_2 and, although a further purification to remove the proteolytic activity was performed, small contaminant peptides are likely to be present in Lecitase™ (17). These peptides with a small molecular weight (lower than the cut-off of the membrane) are probably responsible for the observed rejection coefficient of PLA_2, taking into account that protein content is being measured (and not the enzyme specifically). So, it is possible that the intrinsic rejection coefficient of PLA_2 is higher than 70%, as would be expected from the enzyme molecular weight (≈14,000 Da) and the membrane cut-off (10,000 Da).

The rejection of AOT was determined in isooctane (Wo = 0) at two different concentrations of this surfactant (15 and 40 mM). Figure 3 shows the evolution of AOT concentration in the concentrate and in the permeate streams obtained in both experiments. The rejection coefficient of AOT was around 11% in both experiments, meaning that the surfactant is not retained by the membrane. As with PLA_2, no decrease on the permeate flow rate was detected during the experiment.

These results are similar to those obtained by Prazeres *et al.* (14) who observed that reversed micelles of AOT in isooctane were not retained by an identical membrane, independent of their size (Wo). Permeation of AOT might be a drawback for operation of the reactor, if the main purpose is development of a continuous reversed-micellar membrane bioreactor—first of all, it means that AOT must be continuously supplied to the reactor to compensate for its leakage from the system; on the other hand, the presence of AOT in the permeate stream is undesirable because it implies a further purification of the reaction products.

The membrane rejection toward PC was evaluated either when pure in isooctane or in a mixed reversed-micellar solution of PC/AOT/isooctane (Fig. 4). PC concentration was 25 mM in both experiments. The rejection coefficient of PC was around 73% in isooctane and about 94% in the reversed micellar media. A strong decay of the permeate flow rate was observed in both experiments (Fig. 5); the steady-state permeate flow rate was only 23% of the initial permeate flow rate

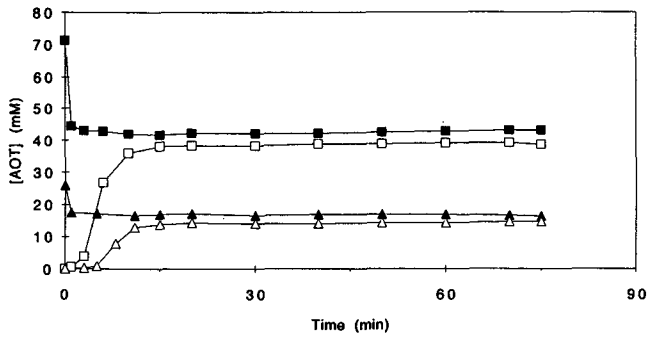

FIG. 3. AOT concentration in the concentrate (■,▲) and permeate (□,△) streams during the transmission experiments: [AOT] = 40 mM (■,□) and [AOT] = 15 mM (▲,△) (in both experiments: Wo = 0, 40°C and $Q_r \approx$ 750 mL/min). Abbreviations as in Figure 2.

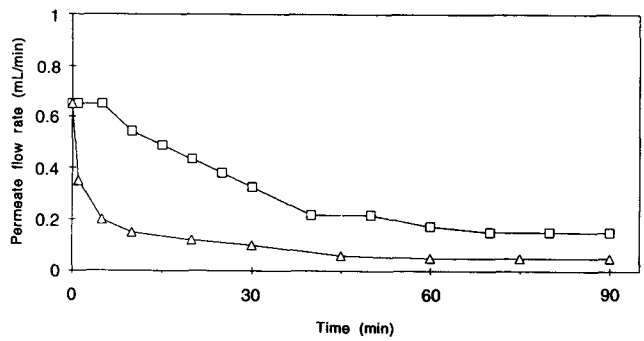

FIG. 5. Permeate flow rate evolution in experiments described in Figure 4 [PC/isooctane (□) and H_2O/PC/AOT/isooctane (△)]. Abbreviations as in Figures 2 and 4.

when using PC in pure isooctane, and around 8% when using the mixed micellar solution. Although high rejection coefficients were obtained for substrate in both cases, which looks promising for the operation of the reactor, the strong decay in the permeate flow rate may limit reactor productivity. On the other hand, changes in the obtained rejection coefficients might be expected for other concentration values and reaction conditions due to the presence of more complex interactions between the different components and the appearance of new species (reaction intermediates) (14). The results obtained in these permeation studies allow us to get a first idea of the membrane separation behavior in regard to the enzymatic system components.

Analysis of continuous performance. The influence of some process parameters (fluid axial velocity, enzyme concentration, water content, AOT, and substrate concentration in feed) in the performance of the system was studied in a series of continuous experiments. Other parameters were held constant (40°C, pH 8.5 $[Tris–HCl]_{water\ pool}$ = 100 mM, and $[CaCl_2]_{water\ pool}$ = 750 mM).

The analysis of data obtained was, in some instances, made in terms of two relevant performance functions: the conversion degree X (%), defined as $X = 100 \cdot$ [fatty acid]/[PC], and the fatty acid productivity P [μmol/(min mg)], defined as $P =$ [fatty acid] $\cdot\ Q_{in}/E_t$, where [PC] is the inlet lecithin concentration (mM), [fatty acid] is the outlet free fatty acid concentration (mM), Q_{in} is the inlet flow rate (mL/min), and E_t is the total amount of PLA_2 in the system (mg).

Effect of fluid axial velocity. The effect of the fluid axial velocity on fatty acid concentration in the outlet stream and in the permeate flow rate is shown in Figures 6 and 7, respectively. This study was carried out by performing two continuous experiments, one using the simple module and the other in the presence of a cylindrical bar of stainless-steel accommodated inside the tubular membrane. The presence of the cylindrical bar inside the tubular membrane led to a decrease of the inner drainage section from 28.3 to 8.6 mm^2 and, consequently, to an increase of the fluid axial velocity by a factor of about 3 (using the same recirculation flow rate, Q_r). In both experiments, similar ranges of recirculation flow rates were tested, 520–1410 mL/min in the absence of the bar and 500–1370 mL/min with the bar (this small difference is due to an enhancement of the attrition in the flow of the fluid inside the tubular membrane when the bar is present). As shown in Figure 6, no significant differences were detected on conversion degrees, which were about 100% in both experiments. Also, the changes in the residence time (indicated in all graphs by the arrows) did not lead to significant variations in the outlet stream fatty acid concentration, which can be attributed to the presence of an excess of enzyme (16.9 μg PLA_2/mL micellar solution).

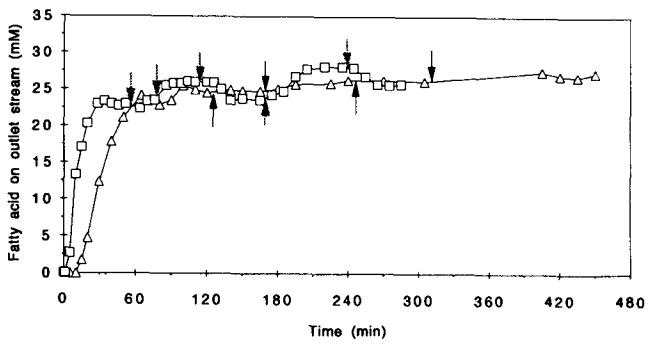

FIG. 6. Effect of fluid axial velocity on the outlet fatty acid concentration: crossflow section = 8.6 mm^2 (i.e., higher axial velocities) (□) and 28.3 mm^2 (i.e., lower axial velocities) (△) (in both experiments: Wo = 15; [AOT] = 15 mM; [PC] = 25 mM, $[PLA_2]$ = 16.9 μg/mL, 40°C, pH 8.5; $[Tris–HCl]_{water\ pool}$ = 100 mM; and $[CaCl_2]_{water\ pool}$ = 750 mM, 500 mL/min ≤ Q_r ≤ 1410 mL/min; the arrows indicate changes on residence time). Abbreviations as in Figures 2 and 4.

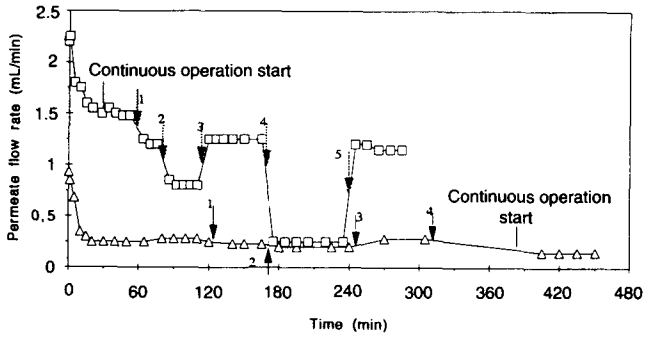

FIG. 7. Effect of fluid axial velocity on the permeate flow rate in the membrane bioreactor: (□) crossflow section = 8.6 mm^2 [continuous operation start: $Q_r \approx$ 1370 mL/min and (ΔPTM) = 1.0 bar; 1, $Q_r \approx$ 1030 mL/min and ΔPTM = 0.8 bar; 2, $Q_r \approx$ 800 mL/min and ΔPTM = 0.6 bar; 3, $Q_r \approx$ 1370 mL/min and ΔPTM = 1.0 bar; 4, $Q_r \approx$ 500 mL/min and ΔPTM = 0.3 bar; 5, $Q_r \approx$ 1370 mL/min and ΔPTM = 1.0 bar] and (△) 28.3 mm^2 [continuous operation start: $Q_r \approx$ 830 mL/min and ΔPTM = 0.6 bar; 1, $Q_r \approx$ 780 mL/min and ΔPTM = 0.5 bar; 2, $Q_r \approx$ 540 mL/min and ΔPTM = 1.0 bar (valv.); 3, $Q_r \approx$ 1410 mL/min and ΔPTM = 1.0 bar; 4, $Q_r \approx$ 520 mL/min and ΔPTM = 0.3 bar]. The notation "valv." means that the back-pressure valve was significantly closed to obtain the indicated ΔPTM value (same conditions as Fig. 6).

Figure 7 shows the permeate flow rates obtained in the presence and absence of the bar. Higher permeate flow rates were obtained in the presence of higher fluid axial velocities (i.e., in the presence of the bar), as expected. Indeed, as the axial velocity increases, the diffusion of solutes away from the membrane into the bulk solution is enhanced and accumulation of solutes on the membrane surface decreases, counteracting concentration polarization (18,19). This results in an increase of the permeate flow rate. The initial flux loss, caused by the build-up of a gel layer in the membrane surface, is clearly visible in the first hours of operation in both experiments.

Figure 7 shows a situation where Q_p was practically identical in both assays. In that situation, Q_r was almost identical in both experiments (≈500–540 mL/min); however, the ΔPTM needed in the presence of the bar was lower (0.30 bar vs. 1.0 bar). The ΔPTM required to obtain that Q_p value in the absence of the bar was achieved with the help of the back pressure valve (Fig. 1). This situation clearly illustrates that

TABLE 1
Apparent Steady-State Productivities (*P*) and Conversion Degrees in the Permeate Stream (*X*) as a Function of Fluid Axial Velocity[a]

Cylindrical bar	Axial velocity (m/s)	Specific productivity (*P*) [μmol/(min • mg)]	*X* (%)
Absent	0.31	2.4	108
Absent	0.49	3.9	99
Absent	0.83	4.3	105
Present	0.97	4.1	112
Present	1.55	12.3	104
Present	2.66	18.4	96

[a]Same conditions as in Figure 6.

TABLE 2
Apparent Steady-State Productivities as a Function of Enzyme Concentration, for the Same Residence Time (90 min)[a]

$[PLA_2]^b$ (μg/mL)	Specific productivity (*P*) [μmol/(min • mg)]	Productivity (μmol/min)
1.7	81.7	13.9
8.4	25.0	21.0
16.9	17.5	29.6

[a]Same conditions as in Figure 8.
[b]Porcine pancreatic phospholipase A_2.

the ΔPTM required to obtain a certain value of Q_p is generally lower when higher tangential velocities are used (19,20).

Table 1 shows some steady-state productivities and conversion degrees obtained in both experiments. Clearly, higher axial velocities led to similar conversions (as already seen in Fig. 6), but to much higher productivities, as a direct consequence of the higher permeate flow rates obtained. Further work was performed in the presence of the cylindrical bar to optimize the performance of the bioreactor.

Effect of enzyme concentration. The effect of enzyme concentration (1.7, 8.4, and 16.9 μg PLA_2/mL) on the performance of the bioreactor was also studied. The experiment with 16.9 μg/mL is the one described in the previous section in the presence of the cylindrical bar. In the assay with 8.4 μg/mL, only one Q_r value (≈1370 mL/min) was tested that corresponded to a ΔPTM of 1.0 bar and a steady-state residence time (= total volume of the reaction mixture/Q_p) of 90 min. In the assay with 1.7 μg/mL, Q_r ranged from 650 to 1370 mL/min, which corresponded to ΔPTM in the range of 0.5–1.0 bar; the steady-state residence time varied from approximately 90 min to 4 h.

Figure 8 shows that the time course of PC hydrolysis in the membrane bioreactor was dependent on the enzyme concentration. Higher PLA_2 concentrations led to higher conversion degrees on the outlet stream and, hence, to higher productivities (in terms of μmol/min). No significant effect was detected on the permeation fluxes (which differed by no more than 10% when using the same Q_r and ΔPTM). The steady-state conversion degrees for 1.7, 8.4, and 16.9 μg/mL of PLA_2 were about 48, 73, and 100%, respectively, when using a residence time of 90 min. The increase of the conversion degree when increasing PLA_2 concentration may be related to inhibition of the enzyme by the reaction products (lysophospholipids and free fatty acids).

Figure 8 also shows a control assay performed in the absence of enzyme. The free fatty acid concentration in the outlet stream was practically zero, indicating that chemical hydrolysis of lecithin is nonexistent.

Table 2 shows that the specific productivity [μmol/(min • mg)] decreased with enzyme concentration, although productivity in terms of μmol/min increased. A similar trend had already been observed with this enzymatic system in a batch stirred-tank reactor. This was attributed to a possible formation of less active enzyme aggregates with increased PLA_2 concentration (3). Further work was performed with 16.9 μg PLA_2/mL. With this concentration, total conversion of 25 mM lecithin was obtained with a residence time of 90 min.

Effect of water content. The effect of water content, described by the parameter Wo {$Wo = [H_2O]/([AOT] + [PC])$}, was studied by maintaining constant AOT and PC concentrations (15 and 25 mM, respectively) throughout the whole range of Wo (Fig. 9). The recirculation flow rate was varied from 500 to 1370 mL/min to which ΔPTM corresponded in the range of 0.3–1.0 bar; steady-state residence times ranged from 70 min to approximately 6 h 40 min. Although no significant differences were observed for Wo 15 and 10, which

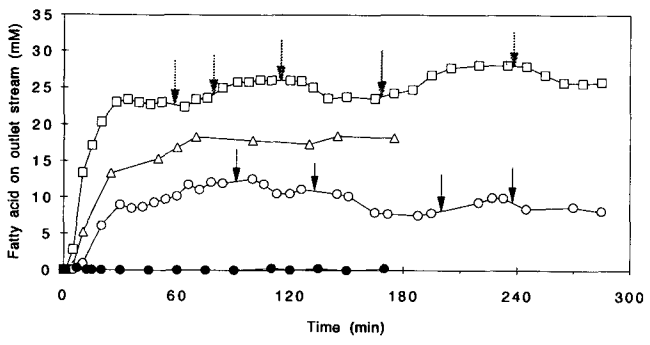

FIG. 8. Effect of enzyme concentration on the outlet fatty acid concentration: $[PLA_2]$ = 16.9 (□), 8.4 (△), 1.7 (○), and 0 (●) μg/mL. In experiments (△) and (●), $Q_r \approx 1370$ mL/min; in experiments (□) and (○), $500 \leq Q_r \leq 1370$ mL/min. Arrows indicate changes on residence time. Abbreviations as in Figure 2.

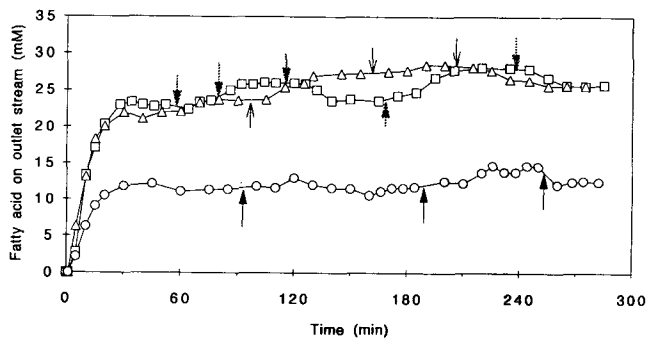

FIG. 9. Effect of water content on the outlet fatty acid concentration: Wo = 15 (□), 10 (△), and 5 (○). Arrows indicate changes on residence time ($500 \leq Q_r \leq 1370$ mL/min). Abbreviations as in Figure 2.

led to 100% conversion, a lower value was obtained (50%) for Wo = 5. This is related to water limitation, referred to in a previous work with this reversed-micellar medium (3). No significant differences were detected in the steady-state permeation fluxes in the range of Wo values tested.

Effect of AOT concentration. The influence of AOT concentration on the continuous hydrolysis of PC in the membrane bioreactor was also studied. Wo and lecithin concentration were maintained constant by controlling the water content throughout the range of AOT concentrations. As shown in Figure 10, lower AOT concentrations led to increasing delays on the set-up of an initial steady state (residence time in the range of 70–100 min, obtained with $Q_r \approx 1370$ mL/min and $\Delta PTM \approx 1.0$ bar). This may be related to the use of rate-limiting amounts of AOT (<15 mM). Indeed, an optimum AOT concentration around 10–15 mM was reported for the initial rate of lecithin hydrolysis, as well as a decrease on PLA_2 activity when AOT concentration was lower (3). That kinetic limitation initially present (when using lower AOT concentrations) is overcome as the reaction proceeds, due to the formation of lysophospholipids, which also exerts a cosurfactant effect. Therefore, no significant differences were detected on the equilibrium conversion degrees.

Although higher initial rates of lecithin hydrolysis in the membrane bioreactor were obtained in the presence of 15 mM AOT, Figure 10 indicates that it is possible to decrease the surfactant concentration in the feed, at least down to 5 mM, without a decrease of steady-state conversion and productivity, which is advantageous in terms of outstream processing because purification of the final products becomes easier.

Effect of substrate concentration. The effect of substrate concentration (25 and 50 mM PC) on continuous hydrolysis in the membrane bioreactor is shown in Figure 11. The water content increased with substrate concentration to keep Wo constant throughout the range of lecithin concentration used. The time course of hydrolysis was very dependent on the substrate concentration. Higher PC concentrations led to higher outlet fatty acid concentrations but generally to lower permeate flow rates. This decrease in the permeation fluxes is probably related to an increase in the reaction mixture viscosity (6) and to a higher accumulation of rejected solutes on the surface

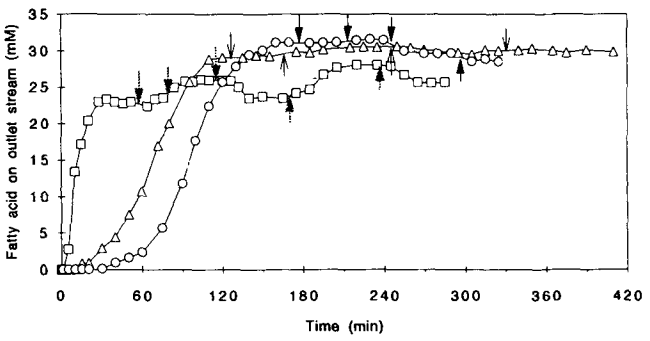

FIG. 10. Effect of AOT concentration on the outlet fatty acid concentration: [AOT] = 15 (□), 10 (△), and 5 (○) mM. Same Q_r range as in Figure 9 (arrows: changes on residence time). Abbreviations as in Figure 2.

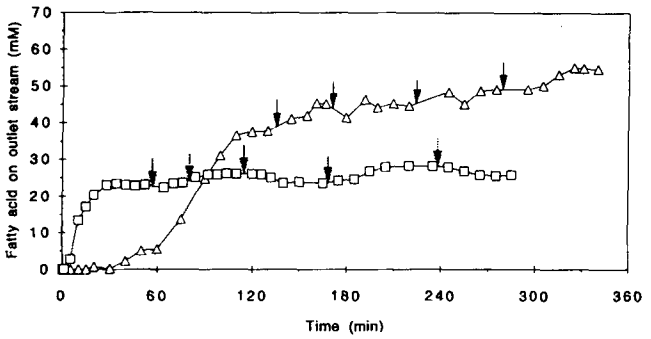

FIG. 11. Effect of substrate concentration on the outlet fatty acid concentration: [PC] = 50 (△), and 25 (□) mM. Same Q_r range as in Figure 9 (arrows: changes on residence time). Abbreviations as in Figure 2.

and within the pores of the membrane (19) (leading to an increase of concentration polarization and membrane fouling). At similar permeate flow rates, productivities were evidently higher for 50 mM lecithin. However, although the productivities were initially (i.e., between 90 and 270 min of the continuous operation, approximately) higher for a 50 mM substrate concentration, lower productivities were obtained toward the end of operation due to a more pronounced loss of permeation ability. This situation is better illustrated in Table 3, which shows five apparent steady-state permeate flow rates (Q_p) and productivities (P) as a function of substrate concentration, obtained by the indicated order during the time course of hydrolysis, and for similar Q_r and ΔPTM values. As shown in Table 3, in the last apparent steady state, obtained at approximately 270 and 330 min when using 25 and 50 mM lecithin, respectively (see Fig. 11), the productivity was higher for the lower substrate concentration used (for the same values of Q_r and ΔPTM). These results indicate that reactor productivity in a long-term continuous operation will probably be higher for the lower PC concentration tested (25 mM).

Effect of normalized residence time on productivities and conversion degrees. To obtain a clearer interpretation of the performance of the bioreactor, the experimental results were analyzed in terms of productivities and conversion degrees as a function of a normalized residence time (Figs. 12 and 13).

TABLE 3
Apparent Steady-State Permeate Flow Rates (Q_p) and Productivities (P) as a Function of Substrate Concentration[a]

Apparent steady state[c]	[PC][b] = 25 mM		[PC] = 50 mM	
	Q_p (mL/min)	P [μmol/(min • mg)]	Q_p (mL/min)	P [μmol/(min • mg)]
First	1.48	20.1	0.99	22.1
Second	1.20	16.7	0.90	24.0
Third	0.80	12.3	0.63	16.7
Fourth	0.25	4.2	0.34	9.8
Fifth	1.15	17.5	0.40	13.0

[a]Same conditions as in Figure 11.
[b]PC, phosphatidylcholine.
[c]The steady states were obtained by the indicated order during the time course of the operation.

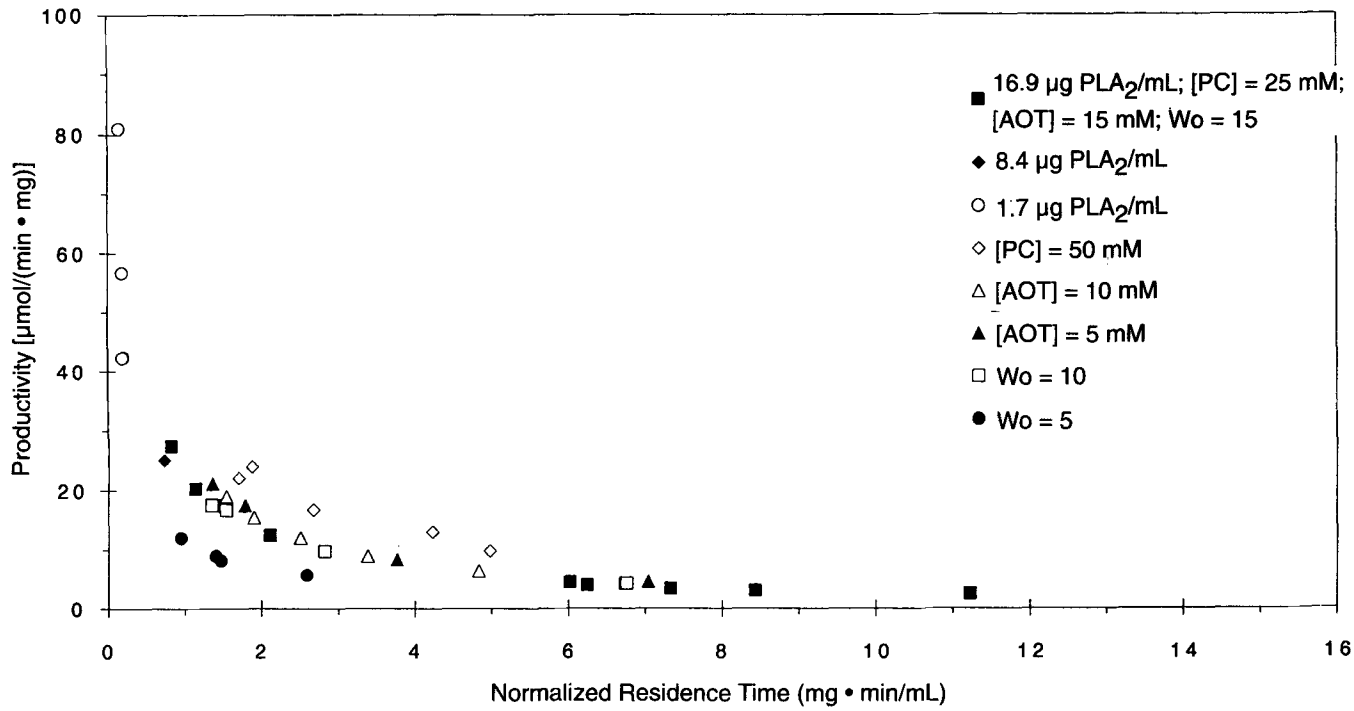

FIG. 12. Effect of normalized residence time on free fatty acid productivity (unless otherwise stated, Wo = 15; [AOT] = 15 mM; [PC] = 25 mM; $[PLA_2]$ = 16.9 µg/mL, 40°C, pH 8.5; $[Tris–HCl]_{water\ pool}$ = 100 mM; and $[CaCl_2]_{water\ pool}$ = 750 mM). Abbreviations as in Figure 2.

The normalized residence time (τ) was defined as: $\tau = E_t/Q_{in}$, where E_t is the total amount of PLA_2 in the system (mg) and Q_{in} ($= Q_{out}$) is the inlet flow rate (mL/min). Figures 12 and 13 were constructed by taking into account all the apparent steady states previously obtained in the continuous experiments, as well as the one obtained in the operational stability experiment (see next section).

Figure 12 shows that the productivity declined sharply as the normalized residence time increased. Moreover, for each normalized residence time, there is a tendency for an increase

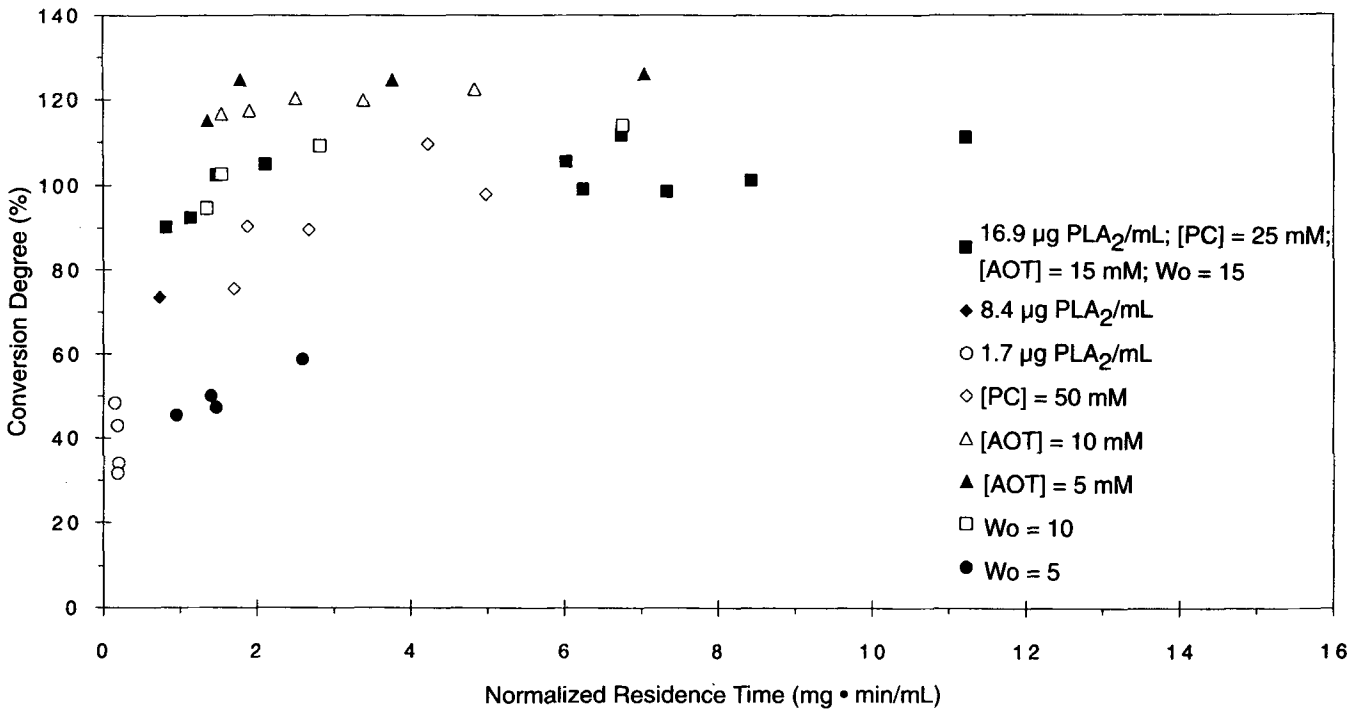

FIG. 13. Effect of normalized residence time on PC conversion. Same conditions as in Figure 12. Abbreviations as in Figures 2 and 4.

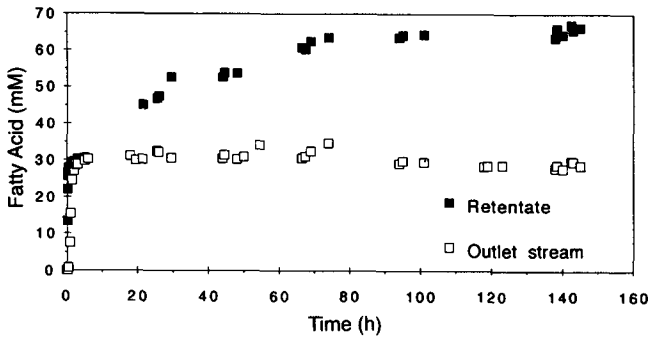

FIG. 14. Operational stability of the bioreactor (Wo = 15; [AOT] = 15 mM; [PC] = 25 mM; [PLA_2] = 16.9 μg/mL, 40°C, pH 8.5; $[Tris\text{–}HCl]_{water\ pool}$ = 100 mM; $[CaCl_2]_{water\ pool}$ = 750 mM, and Q_{in} = Q_{out} = 0.12 mL/min). Abbreviations as in Figures 2 and 4.

of productivity as the substrate concentration rises from 25 to 50 mM; on the contrary, the productivity decreases when water content is lowered to a Wo value of 5. Figure 13 depicts the conversion degree as a function of normalized residence time. A sharp increase in the conversion degree can be observed as normalized residence time increases. In addition, lower conversions were obtained in the presence of lower water content (Wo = 5) and higher substrate concentration (50 mM lecithin); this is related to water limitation and enzyme inhibition by the reaction products (lysophospholipids and free fatty acids), respectively.

Conversion degrees higher than 100% were often obtained. This is probably related to the low and uncertain purity (about 65%) of PC in the commercial lecithin used. Contaminants, such as phosphatidylethanolamine and phosphatidylserine, generally present in lower amounts in egg yolk lecithins, are certainly present in this lecithin (21); the phosphatidylethanolamine content in these lecithins can, in some instances, reach about 24%, depending on the extraction and isolation methods used in PC preparation (21). The great heterogeneity of the commercial preparation of lecithin used, supplied in the form of nonuniform granules, may have also contributed to the dispersion on the obtained conversion degrees.

Operational stability. A long-term operation was carried out to test the operational stability of the reactor ([PC] = 25 mM, Q_{in} = Q_{out} = 0.12 mL/min). The recirculation flow rate was initially around 370 mL/min, but was adjusted during the operation to maintain a constant permeate flow rate. The reactor was operated for 145 h without a significant decrease of the outlet stream conversion degree (≈100%) (Fig. 14), confirming the high stability of PLA_2 in this reaction system and the complete rejection of the enzyme by the ceramic membrane. The total production of the reactor (loaded with 1.69 mg PLA_2), was approximately 27.8 mmoles of free fatty acid, corresponding to an average productivity of 32.0 g oleic acid/(g enzyme • h). This fatty acid productivity is comparable with the one obtained with a similar membrane reactor for hydrolysis of triglycerides catalyzed by a lipase in AOT/isooctane reversed micelles (22).

As shown in Figure 14, the fatty acid concentration in the retentate increased, and thus, the fatty acid rejection coefficient did not remain constant but increased during the operation. This is probably caused by the accumulation of lecithin impurities and lysophospholipids, which are probably highly rejected by the membrane. A slight increase of the reaction mixture viscosity was noted during the operation, which might be related to an accumulation of solutes in the retentate. As a consequence, a gel layer builds up on the membrane surface, superimposing its own rejection characteristics on those of the membrane. These events (concentration polarization and also probably membrane fouling) tend to increase the retention of lower-molecular weight species (18,23).

In our experiment, a low flow rate was selected to facilitate the operation of the reactor during that long period. Nevertheless, the results presented in Figure 12 indicate that higher productivities might be obtained if the reactor is operated with lower residence time.

In summary, the proposed membrane bioreactor for the hydrolysis of PC with PLA_2, encapsulated in mixed reversed micelles of PC/AOT/isooctane, was operated in a continuous mode (i) with retention of the enzyme, (ii) high hydrolysis conversions (≈100%, easily achieved due to the short reaction times needed to accomplish the complete hydrolysis), and (iii) simultaneous separation of fatty acids. The high operational stability of the system, coupled with the good specific productivities and conversion degrees, indicates that the investigated PLA_2/reversed-micellar membrane bioreactor has a high potential for application in biocatalysis.

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