Continuous Production of Structured Phospholipids in a Packed Bed Reactor with Lipase from *Thermomyces lanuginosa*

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ABSTRACT: The possibilities of producing structured phospholipids between soybean phospholipids and caprylic acid by lipase-catalyzed acidolysis were examined in continuous packed-bed enzyme reactors. Acidolysis reactions were performed in both a solvent system and a solvent-free system with the commercially immobilized lipase from *Thermomyces lanuginosa* (Lipozyme TL IM) as catalyst. In the packed bed reactors, different parameters for the lipase-catalyzed acidolysis were elucidated, such as solvent ratio (solvent system), temperature, substrate ratio, residence time, water content, and operation stability. The water content was observed to be very crucial for the acidolysis reaction in packed bed reactors. If no water was added to the substrate during reactions under the solvent-free system, very low incorporation of caprylic acid was observed. In both solvent and solvent-free systems, acyl incorporation was favored by a high substrate ratio between acyl donor and phospholipids, a longer residence time, and a higher reaction temperature. Under certain conditions, the incorporation of around 30% caprylic acid can be obtained in continuous operation with hexane as the solvent.

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KEY WORDS: Enzyme bed reactor, lipase-catalyzed acidolysis, Lipozyme TL IM, structured phospholipids, *Thermomyces lanuginosa*.

Phospholipids (PL) are widely used in the food, pharmaceutical, and cosmetic industries. The uses of PL are mostly based on natural products having a very complex composition, such as lecithins, which usually are by-products from soybean oil production. These products consist of many individual molecules with FA having chain lengths between 16 and 22 carbon atoms. Desired PL with new physical and chemical properties can be produced by exchanging FA in the PL. Modified PL with different FA compositions and distributions may be used in various applications. Incorporation of medium-chain FA into natural PL by interesterification will result in better emulsifying and better heat-stability properties for some emulsion systems such as salad dressings and mayonnaise (1). Besides being food additives, the structured or modified PL, into which different functional FA have been specifically incorporated, also can be used in lipid membrane research or for application as pharmaceuticals and cosmetics (2).

Several studies have been published on the modification of PL with phospholipases or lipases (3–6). Compared with chemical methods, enzymatic modifications of PL have the advantage that enzymes are selective and specific. Phospholipase A_1 (PLA_1) and lipases are specific for the ester bond in the *sn*-1 position of the lipids, whereas phospholipase A_2 (PLA_2) is specific for the ester bond in the *sn*-2 position. So far, little effort has been made to scale up the enzymatic modifications of PL to pilot plant or production scale because of problems such as mass transfer limitations and side reactions, which result in low yields. For industrial applications, enzymes are preferred in an immobilized form because it is possible to reuse the enzymes. However, only lipases can currently be obtained commercially in immobilized form for the PL modification; phospholipases are unavailable.

Most of the investigations reported to date have studied the enzyme-catalyzed reactions for production of structured PL in laboratory vessels. Almost all previous studies on lipase-catalyzed acidolysis of PL used pure PC as the substrate (3–6). For commercial considerations, deoiled soybean lecithin might be favored since the price is considerably lower than purified PC. Natural soybean PL are usually a mixture of several PL species including PC, PE, and PI.

Packed (or fixed) bed bioreactors (PBR) have been investigated and applied in the lipase-catalyzed production of structured lipids (7,8). These studies have demonstrated that PBR have promise in developing lipase-catalyzed lipid modifications. The application of PBR for production of structured PL may also be promising for further scale-up. PBR are used for most large-scale enzymatic processes because of high efficiency, low capital investment, ease of construction, possibility of continuous operation, and easy maintenance. Only a few reports are available on the enzymatic modification of PL using PBR. Härröd and Elfman (9) reported the synthesis of PC from lyso-PC and FA using PLA_2 as catalyst under supercritical conditions.

The use of an enzyme in an organic media has attracted increased interest in recent years. One reason is that in organic media, hydrophobic compounds such as lipids can be dissolved and thereby made accessible for enzymatic conversion. In this study we looked into the possibilities of producing structured PL in PBR with or without solvent using Lipozyme TL IM as

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TABLE 1
FA Distribution in Soybean Lecithin and Structured Lecithin

FA	Soybean lecithin	Structured lecithin[a]
8:0	0.0	35.4
16:0	19.8	9.7
18:0	4.4	2.4
18:1	10.1	6.3
18:2	59.2	41.9
18:3	6.6	4.6

[a]Reaction conditions: solvent ratio, 7.5 mL/g (hexane/substrate); substrate ratio, 36 mol/mol caprylic acid/phospholipids; reaction temperature, 40°C; flow rate, 0.2 mL/min; water content, 0%.

the catalyst. Effects of the molar ratio of reactants, reaction temperature, residence time, and water content on caprylic acid incorporation into PL were monitored as major variables.

MATERIALS AND METHODS

Materials. Natural PL in the form of soybean PL (Sterninstant PC-30) were donated by Stern Lecithin & Soja GmbH (Hamburg, Germany). The PL profile (area%) was PC 54.8, PE 15.6, PI 15.3, phosphatidic acid (PA) 4.3, lyso-PC 0.4, and others 9.6 (unidentified). The FA composition (mol%) of the soybean lecithin can be seen in Table 1. Caprylic acid (8:0, purity 97%) was purchased form Riedel-de-Haen (Seelze, Germany). Lipozyme TL IM, a silica-granulated *Thermomyces lanuginosa* lipase, was donated by Novozymes A/S (Bagsvaerd, Denmark). All solvents and chemicals used were of analytical grade.

PBR. The lipase-catalyzed acidolysis was performed using caprylic acid and soybean PL as substrates. Acidolysis reaction was conducted in both a solvent system and a solvent-free system. With each new experiment with specific parameters, preheated and conditioned substrates were pumped into the enzyme bed for the acidolysis reaction. Approximately 4–5 enzyme bed void volumes (Vs) were discarded before sampling. When a new enzyme bed was used, short-time equilibration with new substrate was performed to stabilize the bed. The substrate mixture was fed upward into the column, and the column temperature was held constant by a circulating water bath (Fig. 1). The reaction substrates, kept in a circulating water bath, were pumped through the enzyme reactor by a pump from Fluid Metering Inc. (New York, NY). For the acidolysis in the solvent system, the bioreactor was a jacketed stainless steel column (l = 200 mm, i.d. = 21 mm) packed with 36 g of Lipozyme TL IM (l = 196 mm). For the experiments using a solvent system, the enzyme was replaced once with new material during the study (as described in the Results and Discussion section). For acidolysis in the solvent-free system, the bioreactor was a jacketed glass column (l = 230 mm, i.d. = 26 mm) packed with 48 g of Lipozyme TL IM (l = 180 mm). The enzyme was reused throughout the study. For stability studies, substrates were passed through the columns for several days, and samples were collected continually.

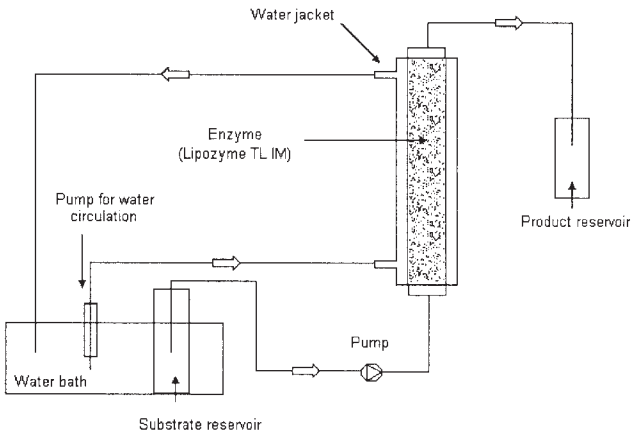

FIG. 1. Process diagram of lipase-catalyzed acidolysis for production of structured phospholipids (PL) in a packed bed reactor.

Determination of bed void fraction (ε). The bed void fraction was determined in the stainless steel column used for the solvent system experiments. Hexane was fed into the newly packed column at room temperature. The column was weighed before and after filling the bed with hexane. The volume of the enzyme bed (V) was calculated from the diameter of the column and length of the bed and the volume of substrate (Vs) was calculated using the density and weight of hexane. The void fraction was then calculated as $\varepsilon = Vs/V$. The same void fraction was used for the calculations in both the solvent-free and solvent-containing systems. The residence time of the substrate in the bed was calculated as $V{\cdot}\varepsilon/V_f$, where V is the enzyme bed volume, ε the void fraction, and V_f the substrate flow rate. The void fraction was calculated to be 0.67. Measurement was conducted at room temperature.

Analysis of FA composition. Samples were directly methylated by KOH-catalyzed esterification as described elsewhere (10). The FAME were analyzed with an HP6890 series gas–liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a fused-silica capillary column (SUPELCOWAX-10, 60 m × 0.25 mm i.d, 0.20 μm film thickness; Supelco Inc., Bellefonte, PA). The oven temperature was programmed from 70 to 225°C. The initial temperature was held for 0.5 min, increased to 160°C at 15°C/min, then increased to 200°C at a rate of 1.5°C/min, held for 15 min, and finally the temperature was increased to 225°C at a rate of 30°C/min and held for 10 min. An FID was heated at 300°C. The injector was used in split mode with a ratio of 1:20. The carrier gas was helium with a column flow of 1.2 mL/min. The FAME were identified by comparing their retention times with authentic standards (Sigma Chemical Co., St. Louis, MO), and the molar composition was calculated together with response factors and M.W. of the FA.

RESULTS AND DISCUSSION

Different parameters were studied for the acidolysis reaction in PBR using either a solvent-containing or a solvent-free system. The solvent-free system was expected to have higher volumetric productivity than the solvent system if the comparison is based on enzyme load and volume of reaction mixture. However, the reaction time needed was expected to be longer in the

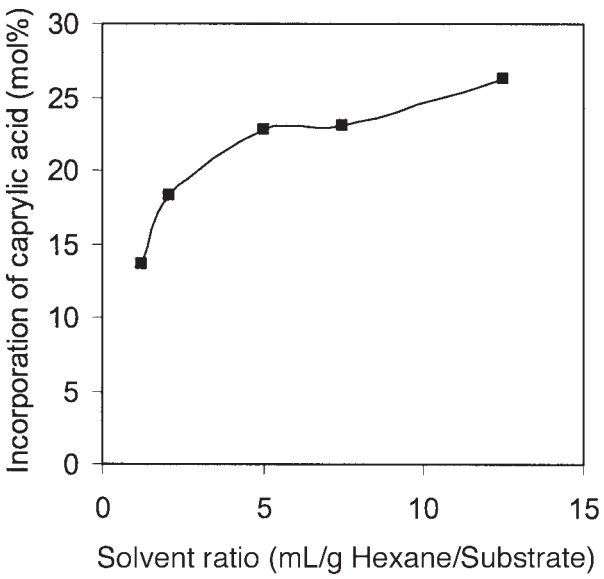

FIG. 2. Effect of solvent ratio (mL/g, hexane/substrate) on the incorporation of caprylic acid (mol%). Reaction conditions: substrate ratio, 3.5 mol/mol caprylic acid/PL; flow rate, 0.2 mL/min; water content, 0%; reaction temperature, 40°C. For abbreviation see Figure 1.

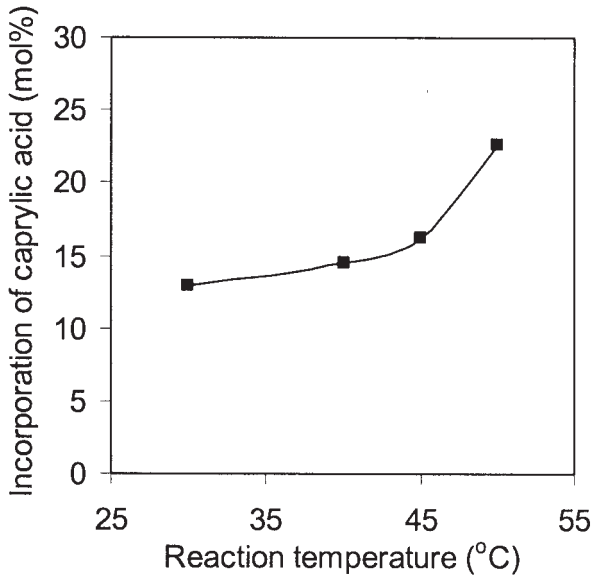

FIG. 3. Effect of temperature on the incorporation of caprylic acid (mol%) in a solvent system. Reaction conditions: solvent ratio, 12.5 mL/g, hexane/substrate; substrate ratio, 3.5 mol/mol caprylic acid/PL; flow rate, 0.2 mL/min; water content, 0%. Immobilized enzyme was reused from previous experiments. For abbreviation see Figure 1.

solvent-free system since the substrate amount was higher. Therefore, it was decided to use a column with a larger volume in the solvent-free system compared with the solvent system.

Effect of solvent ratio. The use of solvents in the reaction system can dramatically reduce the viscosity of the substrates and thus increase the reaction rate by increasing the mass transfer of the substrates. Solvents with too high a polarity are, however, not suitable in enzymatic esterification reactions because they are strong water distorters and thereby inactivate the enzyme (11). The best measure of polarity is the logarithm of the partition coefficient (log *P*) of the organic liquid between *n*-octanol and water; the higher the log *P*, the less polar the solvent is. Solvents with log $P < 2$ are not considered suitable for enzyme reactions. Commonly used solvents for the acidolysis of PL are toluene and hexane, both having log $P > 2$ (12–15). It was reported that reactions conducted in hexane were faster than reactions conducted in toluene (6). Hexane was selected for further studies since it is generally accepted in the fat and oil industry. The immobilized enzyme is compatible with this solvent. The solvent ratio (mL/g, hexane/total substrate) was varied to determine how it influences the incorporation of caprylic acid into PL in the PBR. Five different solvent ratios were tested with the same substrate ratio. The substrates were run through the column with the highest solvent ratio first and then in the order of decreasing solvent ratios. With the decrease of the solvent ratio, the incorporation of caprylic acid decreased (Fig. 2) because the substrate/enzyme ratio was increased as well. Therefore, a longer residence time will be needed to reach the same incorporation of the novel FA. From the lowest to the highest solvent ratio, there was a 10-fold increase in the PL concentration; however, the incorporation was less than twofold. The highest rate of production would therefore be found for samples having the lowest solvent ratio. However, if the conversion is low, additional separation steps of the product and original PL substrate are needed if the goal is to obtain PL with a high incorporation of caprylic acid. For practical applications, the hexane amount should be kept as low as possible, because of downstream processing and environmental considerations. Therefore, a compromise has to be made concerning the amount of solvent.

Effect of temperature. According to the supplier of Lipozyme TL IM, the enzyme is most active in the temperature range of 55–70°C. Usually, an increase of reaction temperature results in an increased reaction rate, according to the Arrhenius law, during enzyme-catalyzed reactions. A high temperature favors higher yields for endothermic reactions owing to the shift of thermodynamic equilibrium. When lipase activity decreases, it is possible to compensate by increasing the operating temperature at a rate that permits the system to maintain a constant conversion rate. For the solvent system, the temperature was varied in the range of 30–50°C to minimize hexane evaporation. A higher temperature gave higher incorporation, and the highest incorporation of caprylic acid was seen at 50°C (Fig. 3). A further increase in temperature could be interesting but is limited by the b.p. of the solvent (b.p. for hexane: 66°C). In the absence of solvent, increasing the column temperature can control the viscosity of the substrates. At elevated temperatures, operation is easier, since higher temperatures increase the solubility of reagents and decrease the viscosity of solutions. This is useful only within the optimal temperature range of the enzyme involved, because higher temperatures will deactivate the enzyme. For the solvent-free system, the temperature was varied from 50 to 70°C with the following reaction conditions: substrate ratio, 6 mol/mol caprylic acid/PL; water content, 0.5%; flow rate, 0.3 mL/min. The incorporation of caprylic acid into

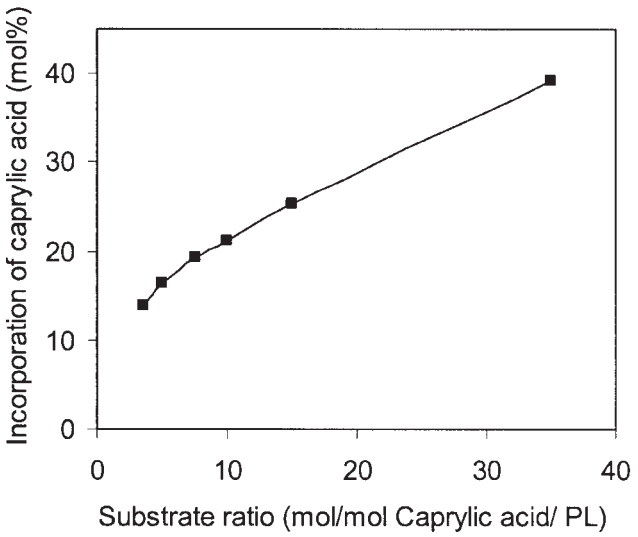

FIG. 4. Effect of substrate ratio on the incorporation of caprylic acid (mol%). Reaction conditions: solvent ratio, 7.5 mL/g, hexane/substrate; reaction temperature, 40°C; flow rate, 0.2 mL/min; water content, 0%. Immobilized enzyme was reused from previous experiments. For abbreviation see Figure 1.

PL was 14.6, 16.7, and 21.1% at 50, 60, and 70°C, respectively. The data show that in the 50–70°C range, the incorporation of caprylic acid increased with increasing temperature, with the highest incorporation seen at 70°C. Lipase stability is influenced by temperature; a high temperature will greatly reduce the enzyme stability and its half-life. A higher temperature will also increase the lipid oxidation rate, especially if PUFA are used as acyl donors. In batch reactions for the same enzyme and substrate, the maximum incorporation was observed at 57.5°C (10). This was an optimized temperature, which is believed to be associated with other parameters. However, results presented in this study show that it is possible to increase the temperature to 70°C and still get an increase in the incorporation of caprylic acid in the solvent-free system.

Effect of substrate ratio. The PL composition of the product in the enzymatic acidolysis reaction depends on the substrate ratios (mol acyl donor/mol PL). The effect of the PL to caprylic acid mole ratio in the reaction mixtures on lipase-catalyzed acidolysis when using a solvent system is shown in Figure 4. The incorporation of caprylic acid increased with increasing substrate mole ratio. For the solvent-free system, two substrate ratios were tested, 6 and 8 mol/mol caprylic acid/PL with the following reaction conditions in the PBR: water content, 0.5%; flow rate, 0.3 mL/min; and temperature, 60°C. The incorporation of caprylic acid into PL was 13.1 and 24.4% with substrate ratios of 6 and 8, respectively. In batch reactions, substrate ratios above 5.5 gave rise to substrate inhibition when the same enzyme and substrate were used (10). These results indicate that an even higher molar ratio can increase the incorporation of caprylic acid into PL in PBR using a solvent-free system. The results from the solvent-containing and the solvent-free systems show that the substrate ratio certainly moves the reaction equilibrium to the product side and improves acyl incorporation. The choice of substrate mole ratio also relates to the downstream processing cost and difficulties in separating the FA (acyl donor and exchanged FA) from the products. Therefore, a compromise has to be made. By varying the substrate ratio, the PL substrate applied to the enzyme per unit is also changed; therefore, it should be noted that with high substrate ratios the overall productivity will decrease.

Effect of residence time. Residence time can be increased or reduced by varying the volume flow rates. The flow rate and residence time have a reciprocal relationship described by the following equation:

$$\text{residence time} = \frac{\pi \cdot r^2 \cdot l \cdot \varepsilon}{V_f} \quad [1]$$

where r = inner radius of the column, l = column length, ε = the bed void fraction, and V_f = flow rate of substrates. For the solvent system, the flow rate was varied from 0.1 to 1.0 mL/min, giving a residence time from 0.8 to 8 h. For this study, a new enzyme column was prepared. For the solvent-free system, the residence time was varied from 1.5 to 13 h, corresponding to flow rates between 0.1 and 1.1 mL/min. In Figures 5A and 5B, the incorporation of caprylic acid as a function of the residence time is depicted.

The results indicate that a low flow rate is required for a high incorporation. Having low flow rates gives rise to the problem of external mass transfer limitations. This indicates that for an efficient operation, several steps should be used since a long reaction time is needed. Further study on this issue is necessary to optimize the system.

Water content and operative stability. The operative stability of the enzyme in a solvent system over a week (168 h) is shown in Figure 6A. The enzyme reached equilibrium within 48 h and thereafter was stable with only a slight decline in the incorporation of caprylic acid (enzyme activity). The influence of water in the solvent system was tested during the operative stability study by adding 0.25% water to the substrate after 168 h of running. A slight decrease could be observed for the incorporation of caprylic acid. Addition of excessive amounts of water should also be avoided since it would result in emulsion formation and complicate the product recovery (16). Operative stability of the solvent-free system was tested for several days with two different water contents (Fig. 6B). With both substrates, the incorporation was highest in the beginning and decreased until 30 h, where it stabilized. The incorporation of caprylic acid was slightly higher when the water content was 0.25%. For the solvent-free system, it seems that small amounts of water are beneficial for the incorporation. The results indicate that the PL amount in a substrate mixture has a great influence on the catalytic activity of the enzyme. Increasing PL in the substrate will probably remove more water from the enzyme, thus reducing the catalytic activity. Therefore, it is necessary to add water to the solvent-free system to increase the transacylation rate, and apparently the addition of water to the solvent system did not increase the incorporation at all.

Table 1 contains the FA composition for structured PL having a high incorporation of caprylic acid. From the present and

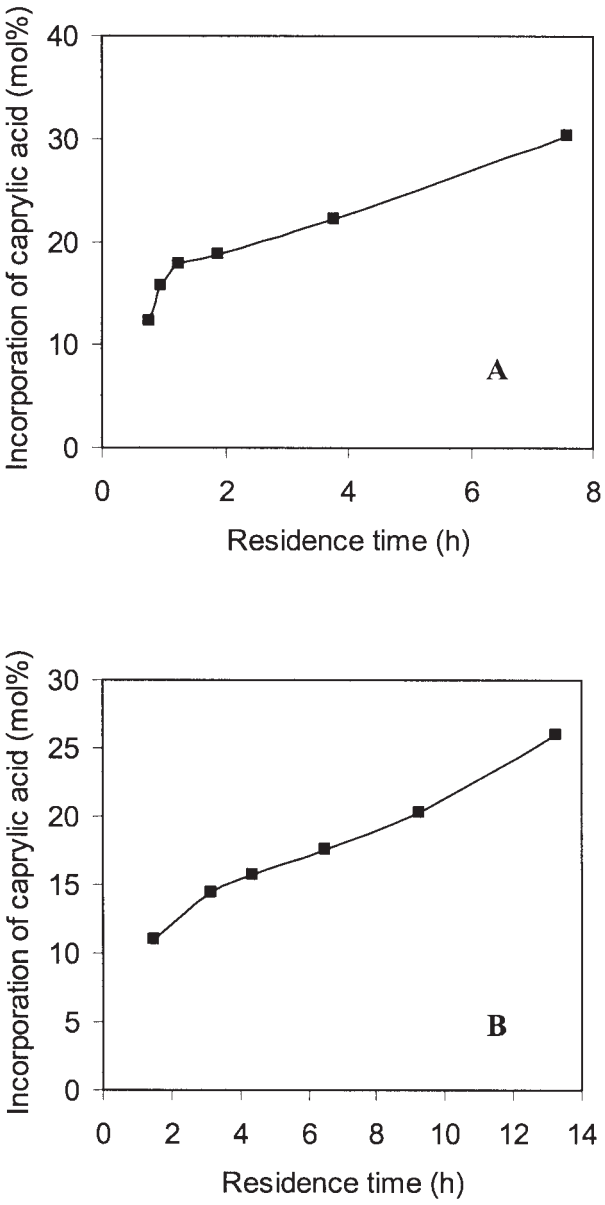

FIG. 5. Effect of residence time on the incorporation of caprylic acid (mol%). (A) Solvent system. Reaction conditions: solvent ratio, 7.5 mL/g, hexane/substrate; substrate ratio, 3.5 mol/mol caprylic acid/PL; reaction temperature, 40°C; flow rate, 0.2 mL/min; water content, 0%. (B) Solvent-free system. Reaction conditions: substrate ratio, 6 mol/mol caprylic acid/PL; reaction temperature, 60°C; water content, 0.50%. For abbreviation see Figure 1.

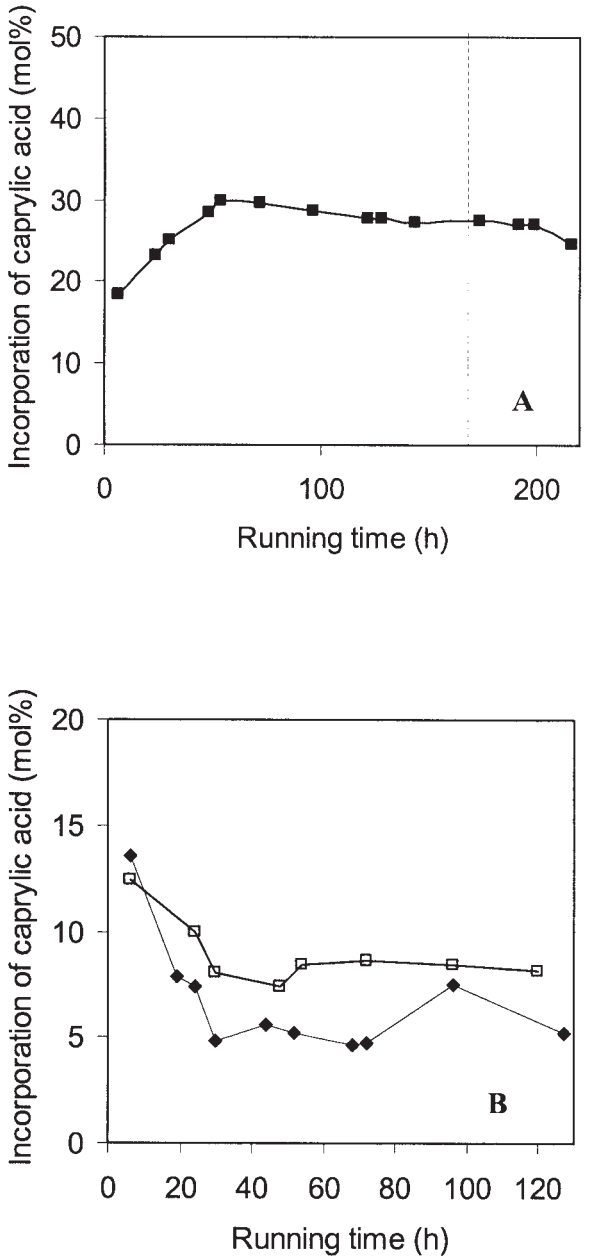

FIG. 6. Operative stability of Lipozyme TL IM. (A) Solvent system. Reaction conditions: solvent ratio, 7.5 mL/g, hexane/substrate; substrate ratio, 9.5 mol/mol caprylic acid/PL; reaction temperature, 40°C; flow rate, 0.2 mL/min; water content, 0 (0–168 h) and 0.25% (168–216 h). (B) Solvent-free system. Reaction conditions: substrate ratio, 6 mol/mol caprylic acid/PL; reaction temperature, 70°C; flow rate, 0.3 mL/min; (◆) water content: 0%, (□) water content: 0.25%. For abbreviation see Figure 1.

also previous publications, it has been reported that the maximum incorporation of caprylic acid into the deoiled soybean lecithin is 38–40% (10). The nature of the PL affects the incorporation rates of caprylic acid catalyzed by Lipozyme TL IM in hexane. The following order of reactivity was observed: PC > PE > PI > PS (10). Incorporation of caprylic acid into PC was high within 48 h, whereas the incorporation into other PL species was low. Since soybean lecithin actually is a mixture of PL species, this is probably the reason for the lower incorporation into this substrate compared with purified PC. Previously, we showed that the water content had no effect on the incorporation of caprylic acid under a solvent-free batch system (10). However, in this study we found that water has some influence on the incorporation using a PBR. Before these types of reactions can be implemented industrially, further work will need to be done to increase efficiency. However, this study provides a few clues for the importance of the system, e.g., that water addition is necessary for the solvent-free system, that the reaction needs a long residence time, and that an increase of temperature

within the thermostability of the enzyme gives higher incorporation. Furthermore, a high amount of solvent and substrate ratio will increase incorporation of novel FA as well.

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