Lipase-Catalyzed Synthesis of Lysophosphatidylcholine Using Organic Cosolvent for *in situ* Water Activity Control

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ABSTRACT: Lysophosphatidylcholines (LPC) were synthesized from L-α-glycerophosphatidylcholine (GPC) by lipase-catalyzed esterification in a solvent-free system. Adding small amounts of a water-mimicking solvent such as dimethylformamide (DMF) to the reaction media significantly enhanced the reaction rate and the product yield. The role of solvent was studied with regard to changes in substrate solubility, the water activity of the reaction system, and the water content of the enzyme. Whereas the solubility of GPC was virtually unaffected by the addition of DMF at controlled water activity, it was considerably affected by water activity. DMF itself lowered the water activity of the system and deprived Lipozyme IM of water. The LPC production was also controlled by varying the initial water content of the enzyme. When two kinds of controls were employed together, a synergistic effect was observed and a 90% conversion was achieved. As a result, an operating window was suggested for LPC production, including water activity of Lipozyme IM and concentration of DMF as two parameters.

Paper no. J9487 in *JAOCS 77,* 791–797 (July 2000).

KEY WORDS: Esterification, lipase, lysophosphatidylcholine, solvent-free system, water activity.

Lysophospholipids are good emulsifying and solubilizing agents as well as synthons for many synthetic phospholipids used in foods, cosmetics, agrochemicals, and pharmaceuticals (1,2). Such diversity in their functional ability is attributed to their unique structural properties. The most widely used lysophospholipid is lysolecithin, which is obtained by the hydrolysis of one fatty acyl residue from naturally abundant sources of lecithin (3–9). Lysophospholipid can be synthesized through enzymatic catalysis by phospholipase A_2 (3–5,9), phospholipase A_1 (6,9), or lipase (7–9) with fairly good yields. But the limitation of such approaches comes from the fact that the fatty acid and head group compositions are determined by the source of phospholipids. Usually the fatty acids on the *sn*-1 position are saturated fatty acids such as palmitic acid and stearic acid, and on the *sn*-2 position are unsaturated ones such as linoleic acid. The most abundant head group on the phosphatidyl residue is choline, while others such as ethanolamine and glycerol are present as minor components. Since the two moieties of the lysophospholipids, i.e., fatty acid and head group, determine their properties, the structures and usage of lysolecithins are somewhat restricted by the sources of lecithin. Therefore, synthetic phospholipids and derivatives with defined structures were investigated to produce phospholipids with unique and uniform properties.

Lipase catalyzes the esterification of glycerophosphatidyl derivatives with free fatty acid to produce lysophospholipids (10–13). Lipase catalyzes the esterification mainly at the *sn*-1 position of a glycerophosphatidyl moiety. This process is interesting because it is a simple one-step esterification, where only two substrates—a free fatty acid and a molecule with a glycerophosphatidyl group—and enzyme are needed for the reaction. However, this process has a few drawbacks, including the use of excess fatty acid and low yield. Compared to the lipase-catalyzed regiospecific transesterifications with fatty acid anhydride (14) or with fatty acid vinyl ester (13), this process is still impractical owing to low yield. The maximal yields of lysophosphatidic acid (LPA) obtained from prior art are below 70% (11), and much less for other lysophospholipids such as lysophosphatidylcholines (LPC) (10,11).

To improve the yield of lysophospholipids, factors affecting the esterification of L-α-glycerophosphatidylcholine (GPC) in a solvent-free system have been examined (10–12). Lipase-catalyzed GPC esterification is a water-producing reaction in nonaqueous media, where free fatty acid condenses with a hydroxyl group of GPC. Water activity control is very important to accomplish nonaqueous enzymatic reactions successfully. The importance of water control was demonstrated in the first report of LPC production by the incorporation of vacuum during the esterification of GPC (10). Typically, 20–30 mm Hg of vacuum was used to remove water from the reaction mixtures. Han and Rhee (11) achieved more sophisticated water activity control with salt hydrate pairs for the production of lysophospholipids in a solvent-free system. All of these approaches are based on water activity control in the reaction mixture during the water-producing esterification. Here, we found that incorporating a small amount of hydrophilic solvent and controlling the water content of the enzyme are effective means to maximize the yield of LPC. Water activity control with solvent engineering is a promis-

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ing technique that does not use additional facilities such as vacuum control or require an additional separation process to isolate salt hydrates from the reaction mixture. Through the determination of the optimal conditions for lysophospholipid synthesis using a cosolvent system for water activity control, we suggest an optimal operating window in terms of cosolvent and water activity of enzyme.

EXPERIMENTAL PROCEDURES

Enzymes and chemicals. Lipozyme IM (from *Mucor miehei*) was a generous gift from Novo-Nordisk A/S (Bagsvaerd, Denmark). GPC was donated by Doosan Technical Center (Yongin, Korea). GPC was used after it had been dried at 50°C in a vacuum for 1 h and stored over P_2O_5. All other chemicals were of reagent grade.

Esterification of GPC. Reactions were performed in a 50-mL water-jacketted glass flask equipped with a top-driven Teflon blade. The shaft of the blade was sealed with a Teflon ring; water loss was not observed during the operation, as determined by measuring the humidity of the head space. The reaction temperature was slightly different according to the fatty acids used: 60°C with lauric acid, 65°C with myristic acid, and 70°C with palmitic acid. In general, 0.5 g (8 units) of Lipozyme IM was added to the mixture of 0.5 g of GPC and 5.0 g of fatty acid. After removing suspended particles by filtration, LPC was purified by flash column chromatography. Typically, excess fatty acid was removed with $CHCl_3/CH_3OH$ (95:5 vol/vol), then the column was washed with $CHCl_3/CH_3OH$ (1:1 vol/vol). LPC was finally eluted with $CHCl_3/CH_3OH/H_2O$ (65:25:4 by vol). LPC was identified by nuclear magnetic resonance and fast-atom bombardment mass spectrometry (FAB-MS) with their molecular ions, $[M^+]$. The phosphocholine moiety was also confirmed by the ion appearing at m/z = 184 by FAB-MS.

Analytical methods. Aliquots of samples were applied to silica-coated thin-layer chromatography (TLC) plates and eluted with $CHCl_3/CH_3OH/H_2O$/28% NH_4OH (65:30:4:2, by vol). LPC was detected by iodination or molybdenum blue spray reagent (15,16). Phospholipids were analyzed by high-performance liquid chromatography (HPLC; Young-Lin Instrument Co. Ltd., Ahnyang, Korea) equipped with a Varex MKIII evaporative light-scattering detector (Alltech, Deerfield, IL) using Jordi Gel DVB 500A GBR column (4.6 × 150 mm, Alltech). Five microliter samples were eluted isocratically with $CHCl_3/CH_3OH$/0.2% NH_4OH (25:65:10, by vol) at 0.5 mL/min (drift tube temperature, 75°C; gas flow, 1.7 SLPM).

Measuring water activity and water content. The relative humidity was measured from the headspace of the reactor with a digital thermohygrometer (Oregon Scientific Co., Taiwan). Because the temperature of the headspace is somewhat lower than that of the reaction mixture, the measured humidity in the headspace would not be the value of the reaction mixture. However, we can see the trend of the humidity during the reactions. The temperature of the headspace was kept at 28 ± 3°C. Various saturated salt solutions were selected according to the data of Valivety *et al*. (17) and Svensson *et al.* (18), and their water activities were confirmed by measuring the water activity of saturated salt solution in the gas phase. Water content was measured with a Karl Fisher titrator (Mettler, Greifensee, Switzerland).

RESULTS AND DISCUSSION

Screening of reaction system. For the production of LPC, two reaction systems, i.e., solvent and solvent-free systems, were examined. In solvent systems using hexane and isooctane, which are universal solvents for the esterification by lipase, LPC was not produced at all as a result of the insolubility of GPC in solvents. GPC is soluble in water and lower alcohols, and slightly soluble in fatty acids. When the lower alcohols were used as the solvent, fatty esters of alcohols formed, because the alcohols are competitive hydroxyl donors for the esterification. The formation of LPC was observed only in the solvent-free system, where the substrates and enzyme were well mixed as found by Han and Rhee (11), who synthesized LPA and other lysophospholipids with lipase.

Hydrophilic water-mimicking solvents such as DMF are protein-denaturing solvents that rapidly inactivate enzyme (19). However, when a small amount of hydrophilic solvent is used in an organic solvent, it often enhances the enzyme reaction rate and yield by increasing enzyme flexibility (18,20). The production of LPC in the solvent-free system is such a case. By using water-mimicking solvents in the solvent-free system, the esterification of GPC with oleic acid was enhanced. Therefore, the effect of water-mimicking solvent concentrations on yield were compared with water.

An optimal concentration of each solvent gives maximal enhancment of GPC esterification, whereas the reactions are stopped by adding more than 2% (vol/wt) water (Table 1). DMF exhibited the best enhancing effect at 4% (vol/wt), while 20% (vol/wt) of formamide completely inhibited the formation of LPC. Methanol enhanced the reaction rate up to a 2% (vol/wt) blend level, but the formation of methyloleate was observed at all methanol concentrations examined. LPC was not produced in the presence of >2% (vol/wt) methanol. Further optimization revealed that the optimal concentrations of DMF and formamide for LPC production were around 5–10% (vol/wt).

Time courses of the reactions were obtained with three saturated fatty acids. Reaction temperatures were set slightly over the melting temperatures of the fatty acids to maintain a fluid state. Inclusion of 5% (vol/wt) DMF increased both reaction rates and final yields (Fig. 1). When the initial rates and yields were investigated by varying the DMF concentrations, an optimal condition for the initial rate was observed at 7.5% (vol/wt) DMF, and the yield reached the maximum at that concentration (Fig. 2). Next, the effect of enzyme loading on the production of LPC was measured in 7.5% (vol/wt) DMF. Lipozyme IM, at a concentration of 10% based on the weight of fatty acid, was sufficient to give a higher initial rate and optimal yield at that condition (Fig. 3).

TABLE 1
Effect of Solvents on Production of LPC by Lipozyme IM with Oleic Acid

Solvent	Concentration (% vol/wt)	Relative LPC production compared to solvent-free system[a]
Formamide	4	0.9
	10	1.6
	20	0.0
Dimethylformamide	2	2.1
	4	2.5
	10	1.6
Methanol	2	1.7
	4	0.0
	10	0.0
Water	2	0.0
	4	0.0
	10	0.0
None	—	1.0

[a]Reactions were performed with 0.1 g of L-α-glycerophosphatidylcholine, 0.5 g of oleic acid, 0.2 g of Lipozyme IM, and specified amounts of solvent, based on the weight of oleic acid, in a shaking incubator at 37°C, 250 rpm for 1 d. The conversion without any solvent was 25.1%. Lipozyme IM from Novo Nordisk, Bagsvaerd, Denmark. LPC, lysophosphatidylcholine.

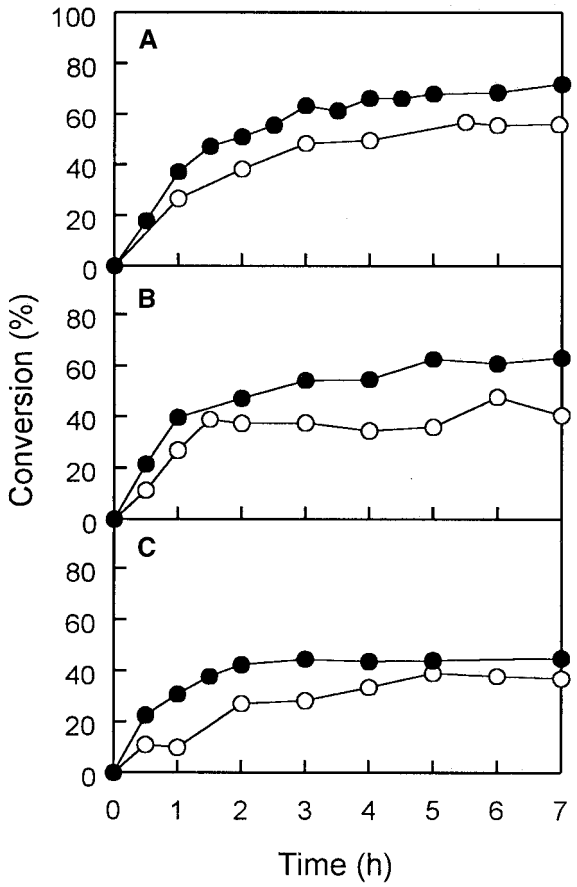

FIG. 1. Production of lysophosphatidylcholines (LPC) by Lipozyme IM-catalyzed esterification of L-α-glycerophosphatidylcholine (GPC). (A) Lauric acid, (B) myristic acid, and (C) palmitic acid were used as acyl donors. A solvent-free system (○) was compared with a system containing dimethylformamide [DMF; 5% (vol/wt), based on the weight of fatty acid (●)]. The reaction temperatures were (A) 60, (B) 65, and (C) 70°C, respectively. Lipase IM was supplied by Novo Nordisk, Bagsvaerd, Denmark.

The role of water-mimicking solvent. The effects of water-mimicking solvent on the reaction rate and yield can be explained in several ways. Its hydrophilic nature enables it to change the water activity of the reaction medium. In water-producing reactions such as esterification, water activity control is critical to maintain the reaction rate and to improve yield. Since the water activity of the reaction media determines the thermodynamic equilibrium state, the final yield of the esterified product (LPC) would be expected to increase on addition of DMF, due to its ability to keep the water activity low. Furthermore, the partition of water molecules around the enzyme must be changed by DMF. The accumulation of water during esterification severely decreases the activity of Lipozyme IM (20). Hence, making the reaction medium so that it is more hydrophilic or removing enzyme-bound water intermittently with hydrophilic solvents is required to remove excess water molecules. Hydrophilic solvents deprive enzyme particles of water (21). Therefore, fast partitioning of water using DMF would increase the esterification rate by lowering the surrounding water concentration of enzyme (18).

The effect of DMF on the partitioning of water between Lipozyme IM and the fatty acid was measured using caprylic acid as a reference. Water content of Lipozyme IM stored on the shelf was 64 mg/g. When this enzyme was added to caprylic acid containing 0.7% (vol/vol) water, the water content of the enzyme increased to 74 mg/g within 1 h. In this case the amount of water contained in the caprylic acid corresponds to the amount of water generated from 10% (w/w) of GPC during the esterification, when 100% conversion of GPC to LPC is assumed. However, the situation was reversed when DMF was added to the mixture. When the enzyme was added to the caprylic acid containing 10% (vol/vol) of DMF and 0.7% (vol/vol) of water, Lipozyme IM lost its water and the water content even decreased to 50 mg/g within 1 h. This result suggests that the enzyme activity in the fatty acid can be changed by differences in water activity caused by the addition of DMF.

Another possible role of DMF is the alteration of substrate solubility. In organic media the substrate solubility is mainly affected by solvent polarity (22,23). Because of the low solubility of GPC in fatty acids, the reactions usually occur while some solid GPC particles are suspended in the fatty acid melt, especially for long-chain saturated fatty acids. The remaining GPC particles are slowly solubilized as the reaction proceeds. The hydrophilic nature of DMF may help dissolve the GPC and elevate the reaction rate. Taking these considerations into account, we examined the role of DMF in LPC synthesis.

First, we measured the solubility of GPC in caprylic acid. GPC is insoluble in most solvents and slightly soluble in fatty acids. We chose caprylic acid as a reference because GPC solubility in long-chain fatty acids is difficult to measure owing to their high melting temperatures. GPC was added to the

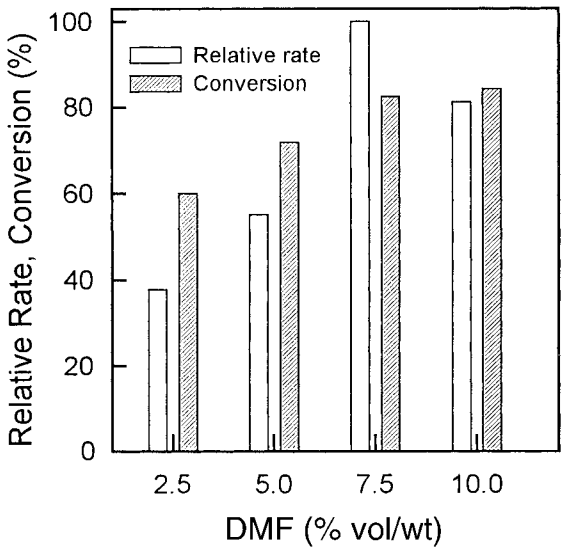

FIG. 2. The effect of DMF concentration on LPC production. DMF was added to mixtures of 5 g lauric acid + 0.5 g GPC; the reactions then were initiated by adding 0.5 g Lipozyme IM at 60°C. The conversion was calculated as the ratio of the final concentration of LPC after 10 h of reaction to the initial GPC concentration. Relative rate was obtained from the initial LPC production rate. For abbreviations see Figure 1.

open bottles containing caprylic acid with different amounts of DMF, then the bottles were placed in the closed bottles containing saturated salt solution. The GPC solutions were mixed with magnetic stirring for a month, and the GPC was added until no further solubilization was observed. After removing the solid from the mixture by centrifugation, the samples were filtered through a Millex-SR filter (0.5 μm; Millipore, Bedford, MA). Then the GPC concentration was measured by HPLC. As a result of fixing the water activity at 0.22 with saturated aqueous potassium acetate at 30°C, DMF virtually had no effect on the solubility of GPC (189.3 ± 5.5 mg/mL) up to 10% (vol/vol). At a water activity controlled to 0.08 with saturated aqueous potassium hydroxide, the solubility of GPC decreased from 148.0 to 124.8 mg/mL as DMF content increased up to 10% (vol/vol). The effect of DMF on the solubility of GPC was rather small compared to the significant change caused by the water activity. Furthermore, as DMF gave a somewhat negative effect on the solubility of GPC at the controlled water activity, we investigated the effect of DMF on water activity in detail.

Relative humidity of the reactor headspace was measured as a function of the DMF concentration. As expected, the relative humidity decreased as the concentration of DMF increased (Fig. 4). Generally, relative humidity reached equilibrium in about 15 to 30 min after adding DMF to lauric acid at 60°C. Relative humidity dropped dramatically from 19 to 11% following the addition of 20% (vol/wt) DMF, then no further effect on the relative humidity was observed (Fig. 4). Because the lower relative humidity of the headspace directly indicates the lower water activity in the reaction media, we see that the addition of DMF can result in yield improvement by shifting the thermodynamic equilibrium.

The roles of DMF can be summarized. DMF not only decreases the water activity of the reaction system but also deprives the enzyme of water. These effects shift the thermodynamic equilibrium to produce more LPC and to increase the enzyme activity in the esterification of GPC with fatty acid. Although the addition of DMF changes the solubility of GPC slightly, this effect is a minor one and is mediated by the DMF-induced reduction in water activity.

Effect of the hydration state of Lipozyme IM. One interesting feature of this reaction is that the reaction components are solid at room temperature. The water contents of the fatty acid [<0.2% (w/w)] and GPC [<0.1% (w/w)] are very low. Most

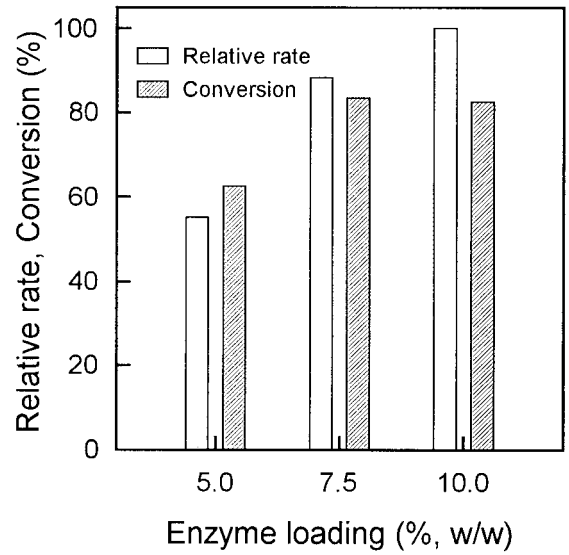

FIG. 3. The effect of enzyme loading on the production of LPC by Lipozyme IM. Lipozyme IM was added to mixtures of 0.5 g GPC, 5 g lauric acid, and 7.5% (vol/wt) DMF. Reactions were incubated at 60°C for 10 h. For abbreviations see Figure 1.

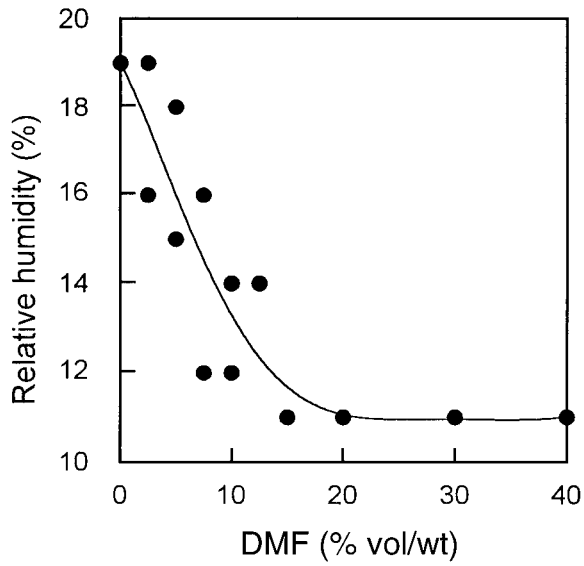

FIG. 4. Changes in relative humidity according to the amount of DMF added to lauric acid at 60°C. For abbreviation see Figure 1.

of water in the beginning of the reaction comes from the enzyme particles [typically, about 7% (w/w) of water is present in commercial enzyme powder]. Because the main role of DMF in this reaction system was identified as water activity control, reducing the water content of the enzyme appears to be very important to control the initial water content in the reaction mixture.

When the relative humidity from the reactor headspace was measured during the reactions, it was greatly affected by the water content of the enzyme. Humidity data collections were started when enzyme particles were added to the mixture of fatty acid and GPC. When enzyme equilibrated over saturated aqueous potassium hydroxide (a_w = 0.08) was used for the reaction, the relative humidity started at about 30% and gradually increased to 60% by the end of the reaction at 10 h. On the other hand, when enzyme equilibrated over saturated aqueous magnesium chloride (a_w = 0.33) was used, where the highest conversion was obtained, the final relative humidity rose to 90% in 10 h. By using enzyme preincubated at a higher water activity such as against potassium chloride (a_w = 0.84), relative humidity rapidly increased to 98%, i.e., the upper limit of the humidity sensor, within 1 h. This result suggested that the water content in the reaction system is very sensitive to the initial water content of the enzyme, as expected.

The water content of the enzyme was controlled by placing the enzyme particles over saturated salt solutions for 1 d at 30°C. The water content of the enzyme varied from 43 to 145 mg/g according to the water activity of the salt solution (Fig. 5). One day's incubation of the enzyme was sufficient to reach equilibrium, as confirmed by comparing with the water content of the enzyme after 6 d. Lipozyme IM was stable under the incubation conditions for at least 1 d. Optimum enzyme activity occurred at water activities between 0.33 and 0.53 (Fig. 6). Beyond this region, both the reaction rates of the enzyme and the yields of LPC decreased. The highest conversion was about 80%, which approximately corresponds to the highest conversion obtained by controlling the reaction with DMF concentration.

Production of LPC with the two modes of water activity control. In this paper, two methods to control the water activity during the esterification of GPC have been described. In a sense, the two methods are similar in that they control the water content of the enzyme and the water activity of the reaction system. Since the control of the water activity is a kind of kinetic process, the outcome would be very different depending upon the method employed. We combined the two modes of water activity control described above. Lipozyme IM was equilibrated over saturated magnesium chloride for 1 d, and 7.5% (vol/wt) of DMF was added from the start of the reaction. The conversion of GPC to LPC reached a maximum of 90% (Fig. 7), which is about 10% higher than that using any single control method.

An optimal water content of the enzyme is usually needed to give the highest initial activity. However, as shown previously the water content of the enzyme increases owing to the water generated during the esterification reaction. A hydrophilic solvent such as DMF can decrease the water activity of the reaction system, suggesting that DMF performs like a buffer for the water molecules produced through the esteri-

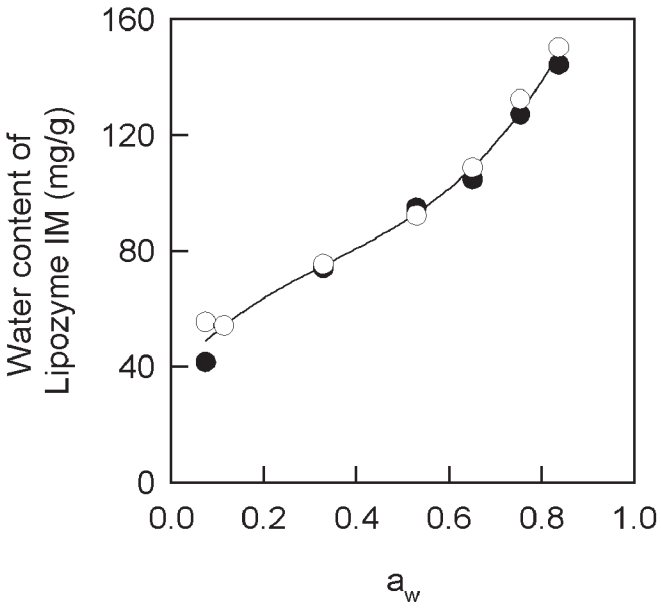

FIG. 5. Changes in water content of Lipozyme IM with different water activity. To control the water activities of the enzymes, they were incubated over following saturated aqueous salt solutions for 1 d (○) and 6 d (●). Equilibrium water activities (a_w) are shown in parentheses: KOH (0.08), LiCl (0.11), $MgCl_2$ (0.33), $Mg(NO_3)_2$ (0.53), $CoCl_2$ (0.65), NaCl (0.75), and KCl (0.84). For enzyme see Figure 1.

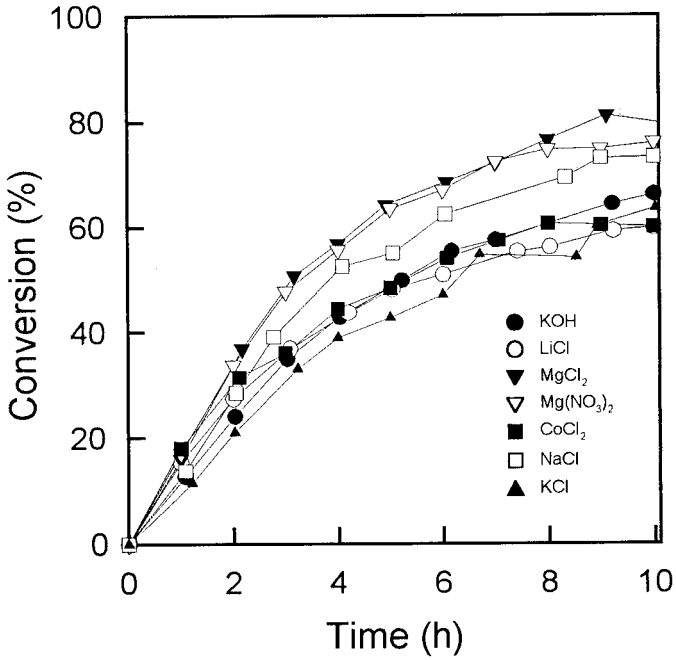

FIG. 6. Production of LPC by Lipozyme IM equilibrated at various water activities. Lipozyme IM was preincubated over various saturated aqueous salt solutions for 1 d before use. Equilibrium water activities of the various salts are listed in the caption for Figure 5. For abbreviations see Figure 1.

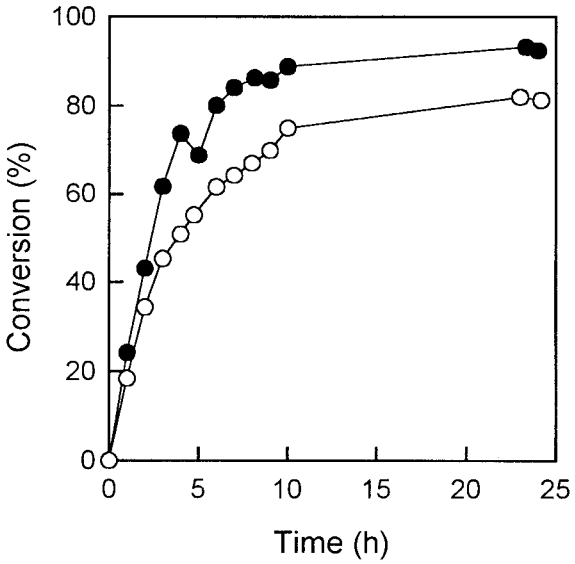

FIG. 7. Production of LPC under the optimized condition using the two methods of water activity control at the same time (●). The reaction was started by adding 0.5 g Lipozyme IM (equilibrated with saturated magnesium chloride solution) to mixtures of 5 g lauric acid, 375 μL DMF, and 0.5 g GPC at 60°C. The enzyme reaction using only one method of water activity control, i.e., controlling water activity of the enzyme without DMF addition, was compared as a control (○). For abbreviations see Figure 1.

fication. This result shows that, although the two modes of water control have almost the same effect on the reaction system, they can act synergistically when used together. On the basis of these concepts, an operating window can be drawn for the controlling parameters in the lipase-catalyzed esterification during LPC production (Fig. 8). The operating window illustrates schematically the effects of reaction parameters on the reaction, and can be used for rapid identification of the process (24).

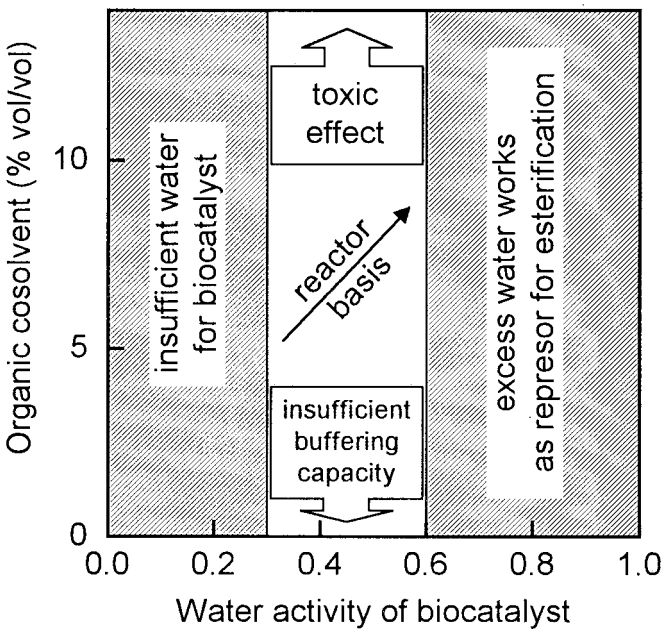

FIG. 8. Operating window for production of LPC by lipase-catalyzed esterification in solvent-free media. Organic cosolvent (DMF) concentration and water activity of biocatalyst (Lipozyme IM) were used to establish a regime for the optimal reaction. For abbreviations see Figure 1.

Water activity is important in nonaqueous enzymology, especially for water-involving reactions such as the esterification in this work. Too low water activity means insufficient water for the proper action of enzyme. On the other hand, excess water suppresses the progress of esterification thermodynamically by shifting the equilibrium to the acids and alcohols from esters. The effect of water activity of the biocatalyst on the reaction has been shown using the water activity-controlled biocatalyst (Fig. 6). Complexity may result from generation of water in nonaqueous media, especially in hydrophobic solvent. In such a system, even a small variation in water concentration has a significant effect on the water activity of the system. Because the water content of the system rapidly affects the water activity of the biocatalyst, the reaction regime can be changed by the small amount of water generated during esterification. For LPC production, water activity was controlled with organic cosolvent (DMF) to lessen the effect of generated water and to enhance reaction performance. The recommended operational conditions are suggested by the upper-right-trending arrow in Figure 8. Organic cosolvent should have a toxic effect owing to its ability to denature protein. However, within proper concentrations, the cosolvent works as a successful ingredient for esterification by acting as water buffer, as shown above.

One interesting feature of using DMF is that it also reduces by-product formation. The formation of diacylphosphatidylcholine was observed with prolonged reaction time—generally after 24 h (11,12)—owing to further acylation of LPC after acyl migration, as described by Virto *et al*. (13). With palmitic acid, about 10% phosphatidylcholine was generated among the total products because of further acylation of lysophosphatidylcholine after 100 h of incubation. However, similar conversions were obtained within 10 h by using the cosolvent, where phosphatidylcholine was not observed at all. As a consequence, the LPC production system is a good and unique model system in that adding hydrophilic solvents such as DMF becomes an effective means to control the water activity in a solvent-free system.

ACKNOWLEDGMENT

This work was partly supported by the Korean Ministry of Science and Technology.

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[Received December 13, 1999; accepted May 19, 2000]