Lipase-Catalyzed Synthesis of Lysophospholipids in a Continuous Bioreactor

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A novel enzymatic method of lysolecithin synthesis was developed with immobilized lipase as a catalyst. The enzymatic transesterification was carried out in a number of alcohols, and the reaction was optimized with regard to the water content and temperature of the medium. Similar kinetics of transesterification were observed with several individual phospholipids. The reaction was also performed continuously in a packed-column bioreactor, which was operated for 1180 h. The lipase displayed strict regioselectivity toward *sn*-1 fatty acid in the phospholipid molecule, thus yielding exclusively *sn*-1 lysolecithins as the final product. *sn*-2 Lysophospholipids were subsequently obtained by acyl migration catalyzed by ammonia vapor. Advantages associated with the use of lipases as opposed to conventional, phospholipase-A_2 catalyzed hydrolysis are briefly discussed.

KEY WORDS: Continuous bioreactor, enzyme stability, lecithin, lipase, lysophospholipids, transesterification.

Application of enzymatic methods to the production of surfactants has attracted much attention in recent years. A number of surface active agents have been enzymatically synthesized, including mono- and diglycerides (1–3), sugar fatty acid esters (4–8), fatty acid amides (9–11) and alkylglycosides (12). Lysophospholipids constitute another class of industrially important surfactants that are currently prepared on a large scale by regioselective, phospholipase-mediated hydrolysis. This batch reaction is typically performed in 30% phospholipid emulsion with aqueous buffer in the presence of phospholipase A_2 (13).

However, this process suffers from several complications, one of which is the necessity to inactivate phospholipase A_2 after completion of the reaction because it is practically impossible to recover and reuse the enzyme from the heterogeneous reaction mixture. Irreversible inactivation of phospholipase A_2 is achieved either by a combination of alkalization and heat treatment or by digestion with protease(s), followed by temperature-induced inactivation of the protease (13). Thus, the production of lysophospholipids presents a rather unusual case where enzyme stability is a major drawback in the manufacturing process.

Ideally, one would like to run this process in a homogeneous reaction mixture and, if possible, continuously. This would avoid complications arising from the necessity to inactivate phospholipase A_2 and result in a more cost-effective use of the enzyme. However, phospholipase A_2 displays poor activity in primary alcohols or other organic solvents (14,15), which would be natural choices for phospholipid biotransformations. Another problem associated with the use of phospholipases in such a system would be the enzyme requirement for Ca^{2+}. On the contrary, fungal lipases are known to function perfectly well in nearly anhydrous alcohols with no requirement for cofactors (16), and some of them have been shown to accept phospholipids as substrates (17–20).

This communication describes a novel method of lysophospholipid production in a homogeneous reaction mixture with immobilized lipase as a transesterification catalyst in a range of alcohols. The alcoholysis of phospholipids has also been performed continuously in a packed-column bioreactor operated for 1180 h.

MATERIALS AND METHODS

Chemicals. An immobilized lipase (E.C. 3.1.1.3) from *Mucor miehei* (Lipozyme IM-60) and a crude preparation of lipase from *Humicola lanuginosa* were obtained from Novo-Nordisk A/S (Bagsvaerd, Denmark) and Biocatalysts Ltd. (Pontypridd, Wales), respectively, and were used as acquired. Crude soybean lecithin (containing 90–96% phosphatidylcholine) was supplied by Natterman Chemie (Cologne, Germany), while pure DL-α-phosphatidylcholine dipalmitoyl; DL-α-phosphatidyl-monomethylethanolamine dipalmitoyl; DL-α-phosphatidyl-DL-glycerol dipalmitoyl; and L-α-lysophosphatidylcholine were purchased from Sigma Chemical Co. Ltd. (Poole, United Kingdom). All organic solvents used in this study were supplied by Aldrich Chemical Co. Ltd. (Gillingham, United Kingdom) and were stored over 3Å molecular sieves. High-performance liquid chromatography (HPLC) solvents were purchased from Rathburn (Walkerburn, Scotland).

Incubation conditions. In a typical experiment 100 mg mL^{-1} of immobilized *M. miehei* lipase (Lipozyme) was added to a reaction mixture containing 150 mg mL^{-1} crude lecithin or 50 mg mL^{-1} of individual phospholipids dissolved in the appropriate alcohol. Incubations were carried out at 25, 37 or 55°C in orbital shakers (180 rev min^{-1}) in 2 mL, sealed vials. At the indicated time intervals, aliquots were withdrawn and diluted for HPLC analysis.

HPLC analysis. This was performed with Gilson 303 pumps (Middleton, WI) equipped with an ACS (Macclesfield, United Kingdom) 750/14 light-scattering mass detector (evaporation temperature, 70°C; carrier gas, N_2 at 20 psi) and a 5 μm Hichrom (Reading, United Kingdom) H5 (0.46 × 250 mm) normal-phase silica column, maintained at 55°C. Sample injection was performed by a Shimadzu SIL-9A autoinjector (Tyne and Wear, United Kingdom) and elution was carried out with a linear gradient of hexane (5–23%) against 2-propanol/water (3:1) over 5 min at 1.75 mL/min^{-1}.

Synthesis and characterization of sn-*2 lysophospholipids.* Lysophosphatidylcholine-2-palmitoyl was enzymatically prepared from phosphatidylcholine dipalmitoyl (50 mg mL^{-1} substrate in 95:5 ethanol/water; 100 mg mL^{-1} IM-60; 37°C; 72 h) and was separated from fatty acid ester by extraction of the latter with hexane at 55°C. The lysophospholipid was subsequently dried, evacuated and exposed to ammonia vapor at 75°C overnight to catalyze fatty acid migration from *sn*-2 to *sn*-1 position (21). Nuclear magnetic resonance (NMR) spectra were

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recorded on a Jeol EX400 spectrometer (Tokyo, Japan) at 399.65 MHz (^1H) and 100.40 MHz (^{13}C) as solutions in $CDCl_3$. Fast atom bombardment-mass spectrometry (FAB-MS) spectra were determined on a Kratos MS9/50TC spectrometer (Manchester, United Kingdom). Samples were analyzed in a glycerol matrix with a copper probe tip and xenon at a translational energy of 5-8 keV. Because phosphatidylcholines lose CH_3 during FAB (22), the corresponding mass minus 15 results were obtained for the starting material (DL-α-phosphatidylcholine dipalmitoyl), enzymatically prepared lysophosphatidylcholine-2-palmitoyl and the product of acyl migration, lysophosphatidylcholine-1-palmitoyl.

Continuous synthesis of lysophospholipids in a packed-column bioreactor. A continuous-flow, fixed-bed bioreactor was constructed by packing 30 g of Lipozyme into a 250 × 25 mm (i.d.) glass, preparative chromatography column (Omnifit, Cambridge, United Kingdom), equipped with a thermostated water jacket (26°C) and adjustable end filters. Soybean lecithin, at a concentration of 150 mg mL^{-1} in ethanol, was pumped up the column with an LKB (Milton Keynes, United Kingdom) Varioperpex peristaltic pump. Details regarding specific water contents and flow rates are given later in Figure 3.

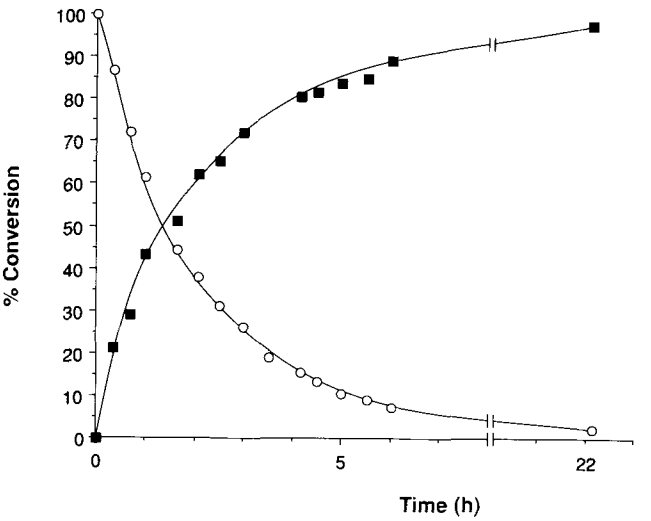

FIG. 1. Kinetics of crude lecithin transesterification catalyzed by Lipozyme IM-60 in ethanol containing 5% water. The consumption of lecithin (○) and accumulation of lysolecithin (■) are shown. For experimental details refer to Materials and Methods section.

RESULTS

Among a number of commercially available lipases, initially tested to determine their transesterification activity toward phospholipids dissolved in primary alcohols, only immobilized *M. miehei* enzyme displayed significant activity in ethanol (Fig. 1) and other short-chain alcohols (Table 1). *Humicola lanuginosa* lipase also catalyzed the transesterification in octanol, but the activity of this lipase dropped significantly as the alcohol chainlength was decreased. In ethanol, for example, its activity was at least 20 times less compared to the *M. miehei* enzyme. Thus, only the latter lipase was selected for further studies.

The conversions obtained for Lipozyme-catalyzed transesterification of lecithin in various alcohols are summarized in Table 1. It should be noted that the optimal reaction temperature was markedly dependent on the alcohol used, being significantly lower in ethanol than in 1-butanol. No appreciable activity was detected in methanol at room temperature. Individual phospholipids were transesterified at similar rates. For example, the conversion of DL-α-phosphatidyl-DL-glycerol dipalmitoyl was 55% after 8 h of incubation, 21% for DL-α-phosphatidylmonomethylethanolamine dipalmitoyl and 47% for DL-α-phosphatidylcholine dipalmitoyl under the same incubation conditions (50 mg mL^{-1} phospholipid in 95:5, vol/vol 2-propanol/water; 100 mg mL^{-1} IM-60; 55°C).

Generally, the reaction rate could be significantly enhanced by the addition of a small amount of water to the reaction mixture (Fig. 2). Predictably, more water was required to reach the maximum activity in water-miscible alcohols than in more hydrophobic homologues. No significant hydrolysis was observed under the above conditions even at water content of 10% (vol/vol) in ethanol.

Having established the optimal reaction conditions and that individual phospholipids are converted by the enzyme at comparable rates, we attempted to perform the reaction continuously in a packed-column bioreactor operated in ethanol at different water contents (Fig. 3). It is evident from the results that 95 + % conversion can be obtained by optimizing the flow rate and the water content of the solvent. When operated at a steady state, the bioreactor was capable of producing 0.5 g of product per gram of immobilized lipase per day. Practically no inactivation of the enzyme was observed over 1180 h of continuous operation, as evident from comparison of the

TABLE 1

Effect of Temperature and Solvent on Lipozyme-Catalyzed Transesterification of Lecithin[a]

Time (h)	Yields obtained at 15% wt/vol lecithin								
	Ethanol (°C)			2-Propanol (°C)			1-Butanol (°C)		
	22	37	55	22	37	55	22	37	55
2	38	28	12	32	31	20	37	56	41
5	68	55	26	62	60	42	67	87	73
8	84	72	38	79	77	59	85	96	87
24	>98	83	74	>98	96	87	>98	>98	>98

[a]The conversion of lecithin was determined under the experimental conditions described in Materials and Methods and in the legend to Figure 1.

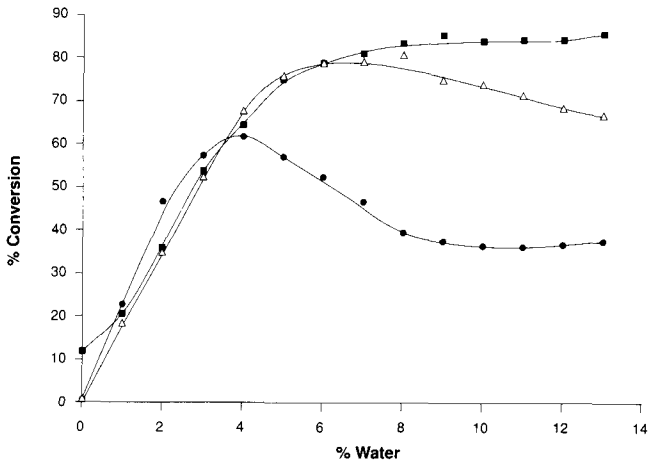

FIG. 2. Dependence of Lipozyme activity on water content of ethanol (■), 2-propanol (△) and 1-butanol (●). Transesterification of crude lecithin was carried out as described in the Materials and Methods section.

productivities achieved at the beginning and the end of the experiment. In fact, the conversion appeared to be slightly higher when the bioreactor was returned to the initial conditions of flow rate and water content (670 h) than during the first 50 h of operation (Fig. 3). This was probably due to the tight retaining of residual water by the immobilized enzyme particles. The substrate conversion did not quite decline to the original level, even after an additional 100 h of operation (not shown).

Finally, we investigated the regioselectivity of lipase-catalyzed transesterification by structural analysis of the lysophospholipid product. To this end, DL-α-phosphatidylcholine dipalmitoyl was treated with Lipozyme in ethanol, and lysophosphatidylcholine was isolated as described under Materials and Methods. NMR analysis of the product confirmed that, in accordance with earlier reports (17–20), the lipase is highly specific to the *sn*-1 position. Lysophosphatidylcholine-2-palmitoyl was further converted to *sn*-1-palmitoyl allowing access to both *sn*-1 and *sn*-2 lysophospholipids. This was achieved by the exposure of lysophosphatidylcholine-2-palmitoyl to ammonia vapor under conditions previously described for partial acylglycerols (21). The acyl migration was proved by assigning the chemical shift values of the *sn*-2-CH absorption of all three compounds in the ^{1}H NMR spectra using a combination of ^{13}C DEPT and ^{1}H-^{13}C heteronuclear COSY NMR experiments. Lysophosphatidylcholine-2-palmitoyl showed a small change to high-field in chemical shift value (δ 4.9) relative to the starting material—phosphatidylcholine dipalmitoyl (δ 5.2). Lysophosphatidylcholine-1-palmitoyl (the product of acyl migration), however, displayed a large shift to high-field (δ 3.9) relative to the starting material. Mass determination by −ve FAB-MS gave the expected molecular ions of $[M - 15]^- = 718$ for phosphatidylcholine dipalmitoyl and $[M-15]^- = 480$ for both *sn*-1 and *sn*-2 lysophosphatidylcholines.

DISCUSSION

Lysophospholipids are an important class of natural emulsifiers that are widely used in the food industry to improve the emulsification and digestibility of fats (23). The present communication describes a novel, facile method of their preparation with immobilized lipase. The developed methodology has a number of distinct advantages over the conventional process where hydrolysis of lecithin is catalyzed by phospholipases: (i) The reaction is performed in a homogeneous reaction mixture, thus simplifying overall process control and allowing continuous operation; (ii) dispensation of co-factor (Ca^{2+})

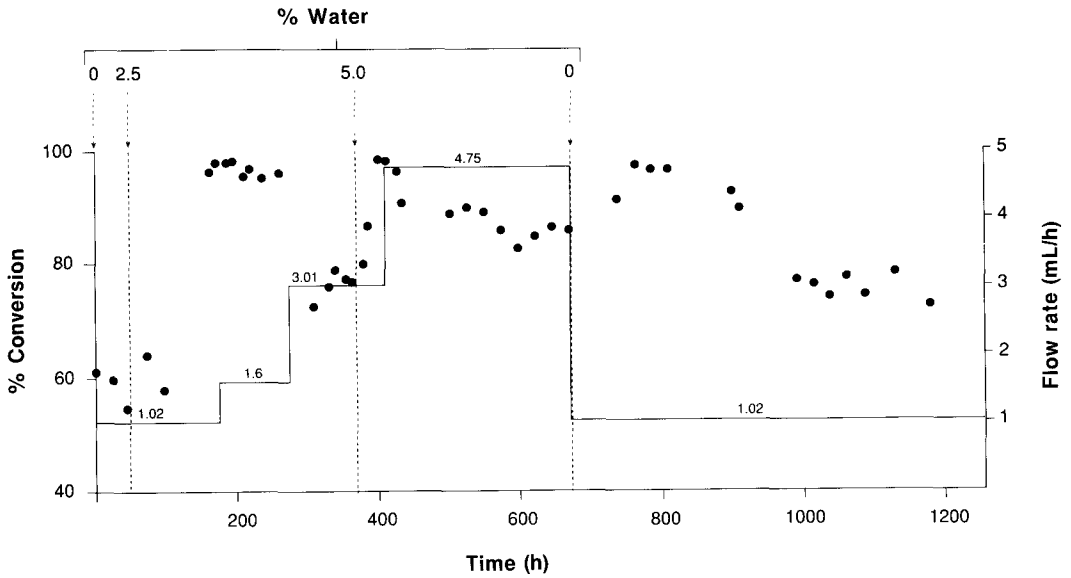

FIG. 3. Continuous synthesis of lysolecithin in a packed-bed bioreactor. Closed circles represent substrate conversion at the given time intervals. Changes in the flow rate, expressed in mL min^{-1}, are shown as an unbroken line.

requirement and the performance of the reaction in predominantly organic solvents significantly facilitate product recovery; (iii) the enzyme can be easily recovered by filtration and reused; and (iv) both *sn*-1 and *sn*-2 lysophospholipids can be produced with the same enzyme through ammonia-mediated migration of the acyl group. The latter and the easy control of lecithin/lysolecithin ratio in the final preparation is especially important for producing a preparation with the desired emulsifying properties (24).

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