Lipase-Mediated Synthesis of Dodecyl Oleate and Oleyl Oleate in Aqueous Foams

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ABSTRACT: Engineering media for optimal product yield in enzyme-catalyzed reactions is an important strategy. We report here synthesis of dodecyl oleate and oleyl oleate by lipase *(Candida rugosa)* in solvent-free substrate foams. Ester formation was characterized with respect to enzyme concentration, pH, temperature, and substrate concentration. The kinetics of ester formation suggest that the formation of ester was 80% complete in 2 h. The pH and temperature optima of lipase suggest that the behavior of lipase in substrate foams was similar to its behavior in water or in organic solvents. The denaturing effect of foams on enzyme was evaluated. Rapid loss in activity (>70% in 1 h) was observed in the presence of oleic acid and dodecanol. The large surface areas generated in aqueous foams offer better accessibility of substrate to lipase for esterification.

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The ability of lipases to catalyze reactions in organic media has been extensively studied (1–3). The mild operating conditions and excellent stereoselectivity of enzyme-catalyzed reactions make them attractive to organic chemists (4). The ease of controlling the direction of lipase reactions by altering the composition of the reactants has led to a variety of reaction media. The essential components of these reactions are enzyme, water, organic solvent, and substrates. The immiscibilities of various components result in multiphasic reaction media with several advantages and disadvantages associated with each medium. A wealth of information is available on the adoption of strategies, such as the use of biphasic, water-in-oil emulsions and solvent-free reaction media, wherein the objectives were to solubilize the substrates and the products, improve the mass action, and keep the enzyme from losing activity (5–7). Several innovative techniques were tested to make enzymes more soluble, stable, or reusable in organic media, by modifying the surface of the enzyme preparations (8,9). Mass action limitations are of primary concern in media design since the contact area of enzyme–substrate will have direct bearing on the reaction yield and kinetics. Further, partitioning coefficients of the substrates and the products between polar and nonpolar phases and interface would influence the reaction rate (10). Minimizing the contact of bulk nonpolar phase with the enzyme is important so as to maintain water associated with the enzyme and also to keep the equilibrium toward esterification (1).

Earlier we reported the utility of aqueous foams of lipase substrates as media for conducting lipase-mediated reactions (11,12). The principal advantage of foam media is that they offer substantial contact area between substrates and enzyme (13). Foams are colloidal dispersions of discontinuous gas phase in a continuous, albeit tiny, water phase. Foam, being highly dynamic, offers thorough mixing of the components, which consequently reduces several mass action limitations (14). Foams or air–water interfaces have been used to conduct lipase reactions (13,15,16). We have demonstrated that the surface activity of the substrates was positively correlated with the reaction rate (11). The higher the surface activity of the substrates, the better was the yield of the esters. Foams were also shown to support esterification of glycerol by fatty acids (13,15,16). The rates and the extents of ester formation in foams were comparable to reported values obtained using other reaction media (11). Oleyl glyceride synthesis was also supported in foam reactions and the reaction went to 90% completion in 15 h (12). Others have reported similar glyceride synthesis in foam reactors, using either glycerol and lauric acid (13) or glycerol and stearic acid (15). Yeh and Gulari (13) used glycerol at a concentration of 5.6 M, whereas we have used 100 mM glycerol (12). In addition, in our foam reactions we have maintained the height of the foam by preventing evaporative loss of water by pumping air, whose relative humidity was adjusted. Pinha-Melo *et al.* (16) demonstrated 50% glyceride synthesis in 7 min in oleic acid monolayers in a Langmuir trough with glycerol (>99.5% pure) as subphase using cutinase enzyme. Monomolecular films formed in a Langmuir trough are similar to substrate foams since the disposition of enzyme and substrate is similar. Glyceride synthesis was also shown to be dependent on surface pressure in such a system. Another important difference between foams and other reaction geometries is the content of water in the system. Based on chemical equilibrium considerations, the presence of water in the reaction correlates negatively with ester formation (1). The role of water in the equilibrium of a reaction occurring in a multiphasic medium is not clear. Using water activity as a descriptor, significant negative correlation was shown between reaction

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rates and water activity with a few enzymes (17). However, several studies used water content or water activity as a variable and demonstrated an absence of such correlations (18,19). Reported high rates of ester formation in foams by ourselves and others are intriguing and our effort is to understand the influence of water on esterification in foam.

Air–water interfaces are discontinuities in phases and have denaturing effects on proteins (19). In foams the lipase is exposed continuously to an air–water interface which may cause denaturation of lipase and thereby decrease enzyme activity. We studied the stability of lipase in foam reactions using *p*-nitrophenyl oleate as substrate (20). In this report we have studied the formation of dodecyl oleate and oleyl oleate formation catalyzed by lipase from *Candida rugosa* in foam reactions.

MATERIALS AND METHODS

Chemicals. Oleic acid, oleyl alcohol, dodecanol, *p*-nitrophenol, Triton X-100, *N,N′*-dicyclohexyl carbodiimide (DCC), and lipase (EC 3.1.1.3) from *C. rugosa* (Type VII, 950 units per mg of solid) were purchased from Sigma Chemical Co. (St. Louis, MO).

Foam reaction. Foam reactions were performed as described earlier (11,12). In brief, foam reactions were conducted in double-jacketed glass columns (2.5″ × 10″ column fitted with a sintered glass joint at the bottom) by passing compressed air into a mix of the reaction components at room temperature (25°C). The air was preequilibrated with water to give 90% relative humidity to prevent drying of the foam due to water evaporation. In a typical reaction, lipase (0.4 mg/mL for dodecanol and 1 mg/mL for oleyl alcohol) was mixed with 500 µmol alcohol and 500 µmol oleic acid in 50 µmol Tris-HCl (pH 7.2) in a 5-mL volume. During the reaction the foam was maintained at a constant height (10 ± 2 cm). The reaction was stopped by adding 5 mL of dichloromethane, the aqueous phase was extracted once more with dichloromethane, and the extracts were pooled. The substrates and products were quantitated by separating them on a gas chromatograph (GC) using an HP-1 (methyl silicone gum as immobile phase) column on an HP 5890 Series II Plus GC (Hewlett-Packard, Wilmington, DE) using a 100–200°C temperature ramp. By using known amounts of synthesized dodecyl oleate and oleyl oleate, GC standard graphs (peak area vs. amount) were constructed and used to calculate the extent of conversion. The temperature of the reaction was maintained by connecting the foam column to a water bath. The pH of the reaction was adjusted with buffers: acetate in the range of 4–7 and Tris in the range of 7–9. Lipase-catalyzed reactions in organic solvents were conducted in heptane. Enzyme (0.4 mg/mL) and both the substrates (100 µmol each) were added to 1 mL of heptane in screw-capped tubes and shaken. Aliquots were directly injected into the GC for quantitation. Reactions in buffer were conducted by shaking (but not to the extent that would allow foam formation) enzyme and the substrates in 1 mL of 10 mM Tris-HCl (pH 7.2) buffer. The extraction and analyses were similar to foam reactions.

Synthesis of lipase substrates. *p*-Nitrophenyloleate, oleyl oleate, and dodecyl oleate were synthesized using DCC as a catalyst in dichloromethane, and the products were separated using silica gel column chromatography. Purity of esters was confirmed on thin-layer chromatography and by proton nuclear magnetic resonance.

Lipase activity. Lipase activity was calculated based on hydrolysis of *p*-nitrophenyl oleate/Triton X-100 co-micelles at room temperature (20). The reaction was monitored by following the formation of *p*-nitrophenol, as an increase in absorbance at 410 nm. The reaction consisted of 4 µmol of *p*-nitrophenyl oleate, 0.8% (wt/vol) of Triton X-100, and 50 µmol of phosphate buffer in 1 mL (pH 7.2). Protein content was estimated by the method of Lowry *et al.* (21) using serum albumin as standard.

RESULTS AND DISCUSSION

Figure 1A shows data on the time course of dodecyl oleate and oleyl oleate formation by *C. rugosa* lipase at a concentration of 0.4 mg/mL in foams. The reaction went to >80% conversion with dodecyl alcohol as substrate and to >90% conversion with oleoyl alcohol as substrate in 4 h. Beyond 8 h the height of the foam column was unstable. The kinetics and the extent of ester formation were similar with both oleyl alcohol and dodecanol. In identical substrate and reaction conditions, in heptane and in buffers (stirred but not foamed) dodecyl oleate formation was less than 10% in 6 h. Ester formation was dependent on the lipase concentration in the medium (Fig. 1B). The ester formation in foam reactions was higher compared to similar reactions performed in either solvent-free reactions or in hexane (22,23). A yield of 60% octyl oleate was achieved in solvent-free medium after 4 h of reaction catalyzed by lipase from *Rhizomucor miehei* covalently linked to a graft copolymer (22). In reactions catalyzed by surfactant-coated lipase in hexane, 50% dodecyl laurate was formed in 10 h (23). The influence of water on reaction kinetics was tested in both reactions, and an in-

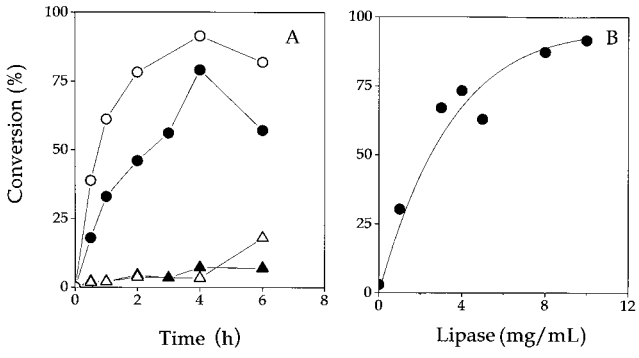

FIG. 1. Formation of dodecyl oleate and oleyl oleate by lipase in foams. (A) Time course of formation of oleyl oleate (○) and dodecyl oleate (●) by lipase at 1 and 0.4 mg/mL concentration, respectively. Dodecyl formation was also followed, under similar conditions of concentration and temperature, in heptane (▲) and in buffer (△). In both cases the reaction was stirred and not foamed. (B) Esterification of dodecylalcohol with oleic acid as a function of lipase concentration.

crease in water content in the reaction was found to decrease the rate of ester formation. However, the effects were less compared to expectations based on reaction equilibrium considerations. Recently, a mixture of cholesterol/docosahexaenoic acid (3:1 mol%), 30% water, and 3,000 units/g of lipase was stirred at 40°C for 24 h, and the esterification extent attained was 89.5% (24). In foam, the esterification occurs at the air/water interface. The lipase substrates and products occupy the air–water interface. Lipases are interfacially active, and the reaction rate primarily would depend on the interaction between the lipase and the substrate in the monolayer (4). Foam being a biphasic medium, the partitioning coefficients and surface activities of the substrates and the products would play an important role. Pinho-Melo *et al.* (16) have shown that the surface pressure applied to monomolecular films acts as a physical selectivity factor in glyceride synthesis catalyzed by cutinase.

The apparent K_m of dodecanol for ester formation was estimated by conducting foam reactions at various dodecanol concentrations and a fixed oleic acid concentration (100 mM). Apparent K_m for dodecanol was calculated to be 50 mM based on Lineweaver-Burk transformation of the data (Fig. 2). At dodecanol concentrations more than 100 mM, ester formation was inhibited. This may be partly due to the surface denaturation of lipase by dodecanol (see below). In hexane, the K_m for lauric acid in dodecyl ester formation was 10 mM (23). Glycerol showed an apparent K_m of approximately 5 M in a foam reaction, which may be due to the low surface activity of glycerol compared to fatty acids (12). The observed ester yield would be a sum of ester formation and ester hydrolysis in foams, since foam reaction would allow ester hydrolysis to proceed simultaneously with ester formation.

pH and temperature dependence of dodecyl oleate formation by lipase. To understand whether dodecyl oleate formation in foams by lipase is similar to other reactions catalyzed by lipase, we studied its formation at various pH values and temperatures. Between pH 6–9 lipase-mediated formation of both dodecyl oleate and oleyl oleate was insensitive to pH changes. Below pH 6 and above pH 9 the activity decreased sharply (Fig. 3A). We did not observe any obvious changes in foamability of the reactions at various pH values. Lipase from *R. miehei* showed a pH optimum between 5 and 8 in catalyzing glyceride formation in foams (12). Temperature dependence of dodecyl oleate was studied in the range of 15 to 50°C. Beyond 40°C the activity fell very sharply. We plotted the data in the range of 15–35°C using the van't Hoff relation and estimated the activation energy (E_a) to be 24.4 kJ/mol (Fig. 3B). This value of E_a is in the range of other E_a values determined for other esterification reactions catalyzed by lipase (25). The loss in enzyme activity beyond 40°C may be due to a combination of such features as enzyme inactivation and changes in the partitioning terms of the substrates to the interface. At higher temperatures it was difficult to maintain the foam height, probably because the stability of the foam may depend sharply on temperature (14).

Activity of lipase in foams. Air–water interfaces are known to have denaturing effects on proteins (19). The structure of soluble proteins is primarily due to isotropic interactions of protein surface groups with the surrounding water molecules. However, when proteins are exposed to phase discontinuities such as air–water interfaces, the structure, and consequently the function, suffers. In foams, lipase is continuously exposed to the interface throughout the reaction period. We tested the activity of lipase, after exposure to foams for various times. At each time point an aliquot of the sample was briefly spun and the water phase, containing the lipase, was kept in the cold. The enzyme activity and the protein content were then determined as given in the Materials and Methods section. The loss in lipase activity was very sharp (nearly 80%) even after 30 min of exposure to foam (Fig. 4A). After 30 min of exposure to heptane the enzyme lost only 20% of its activity. This suggests that the loss of activity was rapid and that inactivation may partly be responsible for the decrease in esterification seen in Figure 1A. To verify this we have compared percent conversion in two reactions after 1 h: in one reaction (5 mL), 4 mg of lipase was used and in another reaction (5

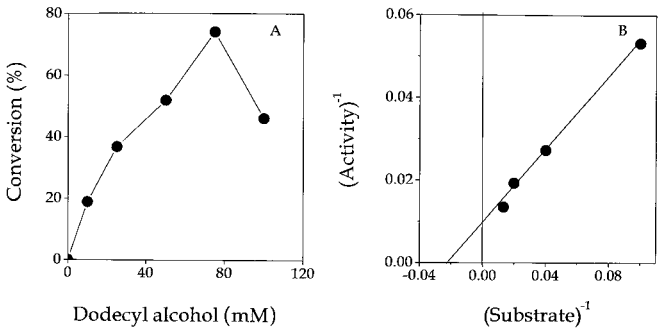

FIG. 2. Dependence of esterification on concentration of dodecyl alcohol in foams. (A) The oleic acid concentration was fixed at 100 mM in all the reactions. (B) Lineweaver-Burk transformation of the data. Calculated apparent K_m is 50 mM.

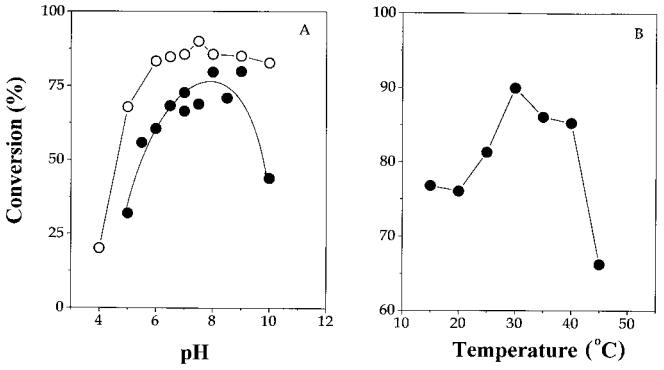

FIG. 3. pH and temperature dependence of esterification catalyzed by lipase in foams. (A) Oleyl oleate (○) and dodecyl oleate (●) formation were studied at various pH values at room temperature. Between pH 4–7 acetate buffer was used, and Tris-HCl buffer was used between pH 7–10. Comparison of esterification at overlapping pH values in the two buffers suggested that there were no buffer-specific effects. (B) Temperature dependence of dodecyl oleate formation in foams by lipase studied at pH 7.2. The activation energy of dodecyl oleate formation was calculated to be 24.4 kJ/mol.

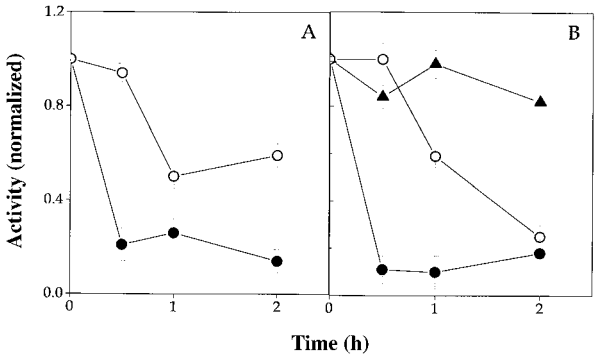

FIG. 4. Stability of lipase in foams. (A) Remaining activity of lipase was measured at various times in foams (●) and in heptane (○). *p*-Nitrophenyloleate was used as a substrate to quantitate the enzyme activity. The activities were normalized by quantitating the protein in each sample. The activity at time zero was taken as 1. (B) Lipase activity was measured at various times in foams containing oleic acid (○), dodecylalcohol (▲), and oleic acid and dodecylalcohol together (●).

mL), 2 mg of lipase was added at the beginning of the reactions and another 2 mg after 30 min. The percent conversion was 10% higher than the control in the second reaction, indicating that the loss of activity was very rapid (more than 50%) and could be compensated by adding fresh lipase (data not shown). Furthermore, this demonstrates that the loss of enzyme activity was not due to product inhibition. We further studied the kinetics of inactivation in the presence of either of the substrates and also in the absence of the substrates (Fig. 4B). Denaturation in the absence of substrates was similar to the denaturation observed with dodecanol alone (data not shown).The rate of inactivation was higher in foams containing oleic acid compared to dodecanol. Interfacial inactivation may be the common mechanism for loss of activity of lipases in multiphasic reaction media. Various derivatization strategies such as adding polyethylene glycol, surfactants, lipids, and the like may help in increasing the solubility of lipase in organic media and also might lessen the interfacial denaturing effects of organic phases on the proteins (8,9).

Structurally, and in terms of substrate disposition, foams may be best suited to conduct reactions occurring at interfaces, such as lipase-mediated reactions. Since the bulk of the phase in foams is air, foam reactors will have a large volume. Though the reactions occur at a faster rate in foams compared to biphasic media, space–time yield in foams may be comparable to other media. Foams offer certain advantages over other reaction media used for lipases, *viz*. large interfacial area, absence of organic solvents, and simplicity.

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