Glyceride Synthesis Catalyzed by Cutinase Using the Monomolecular Film Technique†

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ABSTRACT: The monomolecular film technique previously used to study the kinetics of lipase hydrolysis was adapted to synthesizing oleoyl glycerides (monoolein, diolein, and triolein). The water subphase was replaced by glycerol, and a film of oleic acid was initially spread on the glycerol surface. In this system a recombinant cutinase from *Fusarium solani* was able to catalyze oleoyl glyceride synthesis. More than 50% of the oleic acid film was acylated after 7 min of reaction. The surface pressure applied to the monomolecular film acts as a physical selectivity factor since glyceride synthesis can be steered so as to produce either diolein or triolein.

When enzymatic catalysis takes place in systems with a low water content, the resulting thermodynamic equilibrium favors synthesis over hydrolysis. Hydrolytic enzymes can therefore be used to catalyze the formation of ester bonds in these systems. Among the hydrolytic enzymes, lipases have been widely used to perform esterification or transesterification reactions.

Lipid monolayers have been used as lipase substrates with the monomolecular film technique (Verger & de Haas, 1976), offering several advantages: very little lipid is needed to be able to perform kinetic measurements, the variables measured (surface pressure, potential, radioactivity, etc.) often yield unique information, and the "quality of the interface" can be controlled and used to modulate the enzymatic reaction (Verger et al., 1991).

In the present study, the monomolecular film technique was adapted for use in the synthesis of oleoyl glycerides. The water subphase was replaced by glycerol and oleic acid was spread over the glycerol surface to form a monolayer. This method makes it possible to study and perform ester synthesis in a new and original system involving a specific array of self organized lipid substrate molecules. We continuously recorded the surface pressure and furthermore estimated the glyceride synthesis after film recollection and HPLC analysis, at the end of the experiment. A purified recombinant cutinase from *Fusarium solani* (Lauwereys et al., 1991) was used as a biocatalyst in this study.

MATERIALS AND METHODS

Chemicals. Purified recombinant cutinase was a gift from Dr. G. Matthyssens, CORVAS International N.V. (Gent, Belgium). This preparation was in the form of a lyophilized powder at more than 95% purity (w/w).

Oleic acid (OA)[1] (approx. 99% pure), monoolein (MO) (1-monooleoyl-*rac*-glycerol, approx. 99% pure), diolein (DO) (85% 1,3 and 15% 1,2 isomer, approx. 99% pure), triolein (TO) (approx. 99% pure), and glycerol (≥99.5% pure) were obtained from Sigma. Hydrochloric acid (min. 37% pure) and acetic acid (min. 99.8% pure) were from Riedel-de Haën. Acetic acid was distilled before use. Chloroform, tetrahydrofuran, and heptane were purchased from SDS, Peypin (France) (HPLC grade) and were also distilled before use. β-Cyclodextrin (Roquette Frères) was recrystallized twice from a solution in distilled water. The water content of this material was measured by prolonged vacuum drying at 104 °C until constant weight. Salts were from Merck (analytical reagent grade).

Surface Pressure−Molecular Area Curves. Monolayers were obtained by spreading a lipid solution (0.4 mg mL^{-1}) in chloroform over the subphase. Surface pressure−molecular area curves were recorded with either pure glycerol as a subphase or double-distilled water adjusted to pH 2.0 with HCl. The Wilhelmy method was used to measure the surface pressure, with a thin platinum plate attached to a Beckman LM-600 electromicrobalance. This method was also suitable for measuring the air/glycerol interfacial tension, since we obtained a value of 63.4 ± 1.6 mN m^{-1}, which is very similar to the previously reported interfacial tension value (63.4 mN m^{-1}) measured at 20 °C (Weast et al., 1988). Water was used as the reference fluid at 20 °C with a tension value at the air/water interface of 72.8 mN m^{-1} (Weast et al., 1988). With stepwise additions of a lipid solution, successive amounts were spread over the subphase placed in a Teflon trough (circular in shape, 3 cm in radius, and 1 cm deep). Alternatively, continuous surface pressure−molecular area curves could be obtained by spreading a known amount of lipid over the subphase placed in a rectangular Teflon trough (32.5 × 6 × 0.5 cm, l × w × d). The monolayer was then continuously compressed by a

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[1] Abbreviations: OA, oleic acid; MO, monoolein; DO, diolein; TO, triolein.

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motor-driven Teflon barrier at a velocity of 2.46% of its initial area per minute (Verger & de Haas, 1973).

Glyceride Synthesis in Monomolecular Films. Synthesis assays were performed at a constant surface pressure in a "zero-order trough" (Verger & de Haas, 1973) composed of a reaction compartment (circular in shape, 3 cm in radius, and 1 cm deep) and a reservoir compartment (rectangular in shape, 32.5 × 6 × 0.5 cm, l × w × d) communicating by means of a narrow surface canal. The bulk of the reaction compartment was continuously stirred with a magnetic rod at 200 rpm.

The reaction and reservoir compartments were filled with glycerol. A cutinase solution (106−627 μL in 5 mM Tris/HCl, pH 9) was injected into the reaction compartment. The final concentration of cutinase in the reaction compartment was 40 μg mL^{-1}. After a mixing time of 5 min (needed to obtain a homogeneous solution of glycerol and water), the oleic acid solution in chloroform (0.4 mg/mL) was spread in order to reach the desired end-point value of the surface pressure. In the assays with high water contents, the surface of the reservoir compartment was sometimes not large enough to maintain the surface pressure constant throughout the reaction period. Small amounts of the oleic acid solution were respread during the course of the reaction, as required. During the assays the surface pressure varied by 1 mN m^{-1} around the end point value of the surface pressure. The reactions were monitored for 7−10 min. The surface film was then recovered in a vial containing 10 mL of double-distilled water adjusted to pH 2.0 with HCl in order to stop the reaction. The lipids were extracted with 2 mL of distilled chloroform and washed with 5 mL of double-distilled water. After chloroform evaporation the samples were kept under a vacuum for 2 h and then redissolved in 0.3 mL of heptane containing 1% (v/v) acetic acid before HPLC analysis. The final water content of the glycerol subphase present in the reaction compartment was estimated with a Mettler DL 18 Karl Fischer titrator and corresponded to the amount of water injected. Specific activity values were calculated as follows:

$$\text{sp act.} = \frac{(\text{moles of OA converted (HPLC data)})}{(\text{reaction time})} \frac{1}{yS_R M}$$

where y is the overall film recovery and lipid extraction yield, S_R is the surface area (cm^2) of the reaction compartment, and M is the final cutinase molar concentration.

HPLC Analysis. OA, MO, DO, and TO were measured on a Beckmann System Gold high performance liquid chromatograph using a Beckmann Ultrasphere 5-μm silica column (4.6 mm × 25 cm) column and a light scattering detector (Cunow DDL 11). The separation method used was the same as that described previously (Carrière et al., 1991), with some minor modifications as regards the elution steps. Heptane/tetrahydrofuran/acetic acid (94:6:1 v/v/v) was the initial mobile phase. Seven minutes after the sample injection (100 μL), the eluent was switched to heptane/tetrahydrofuran/acetic acid (50:50:1, v/v/v) during a 5-min elution period. Fifteen minutes after the sample injection, the mobile phase was again switched to heptane/tetrahydrofuran/acetic acid (0:100:1, v/v/v) for a further 5 min. A 1 mL min^{-1} flow rate was maintained constant throughout the chromatogaphy.

Hydrolysis Assays in Monolayer. Monolayer hydrolysis of long-chain acyl glycerides cannot be recorded directly because the lipolytic products remain at the surface and induce only slight changes, if any, in the surface pressure with time (Scow et al., 1979). However, when β-cyclodextrin is present in the subphase, the hydrolysis products (OA and MO) of long-chain triglycerides are trapped and further solubilized. The lipase kinetics can therefore be recorded as previously described in the case of medium-chain lipidic substrates (Laurent et al., 1994).

Hydrolysis assays were performed with the previously described zero-order trough, which was also used for glyceride synthesis. The subphase was a 5 mM Tris/HCl (pH 9.0) buffer. Monolayers of 1,3-DO were spread from a chloroform solution (0.4 mg mL^{-1}). A β-cyclodextrin solution was then injected into the reaction compartment to give a final concentration of 0.5 mg mL^{-1}. No changes in the surface pressure were observed. Cutinase was then injected into the reaction compartment to give a final concentration of 0.2 μg mL^{-1}. The enzymatic activity was calculated from the velocity of the movement of the mobile barrier in order to maintain a preset, constant surface pressure, using the following formula:

$$\text{enz. act.} = \frac{\Delta S}{(\text{reaction time})} \frac{\Gamma}{S_R M}$$

where ΔS is the surface area (cm^2) of the reservoir compartment crossed by the mobile barrier, Γ is the surface density of the substrate molecules given by the surface pressure−molecular area curves (Laurent et al., 1994), S_R is the surface area (cm^2) of the reaction compartment, and M is the final molar cutinase concentration.

RESULTS

Surface Pressure−Molecular Area Curves. Two types of surface pressure−molecular area curves for oleic acid spread over either water or glycerol subphases were drawn up. Furthermore, either stepwise additions or a continuous compression method could be used (Figure 1). With the stepwise addition method, known amounts of oleic acid were consecutively spread over the same area, whereas with the continuous compression procedure, a fixed amount of oleic acid was initially spread, and the monolayer was then continuously compressed by a mobile barrier moving at a constant speed. With both methods, the surface pressure was continuously recorded.

Upon comparing the surface pressure−molecular area curves obtained over water and glycerol, three major differences were noted. First, the surface pressure at which the film of oleic acid collapsed was lower when the film was spread over glycerol (25.8 mN m^{-1}) rather than a water subphase (30.5 mN m^{-1}). Second, at surface pressures of more than 5 mN m^{-1}, the surface densities of oleic acid over glycerol were higher than those over water. Third, both stepwise and continuous surface pressure−molecular area curves over water were superimposable, which was not the case with the curves obtained with glycerol as a subphase. Over the latter subphase, continuous compression gave higher surface densities than when stepwise additions were performed. After a continuous compression over 4 min (at a rate of 14% min^{-1}) corresponding to a reduction of 50% of

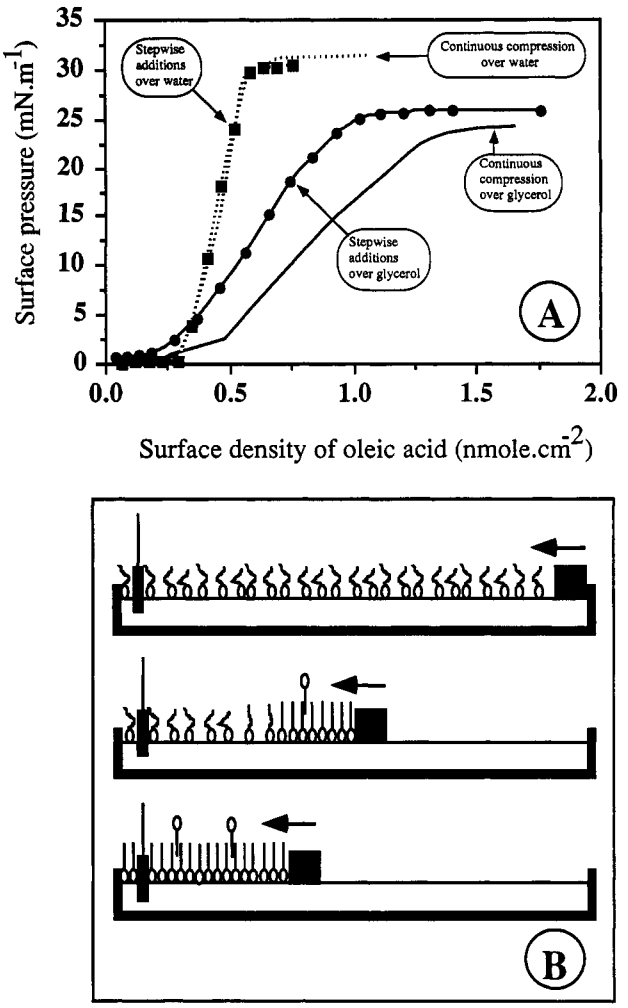

FIGURE 1: (A) Oleic acid surface pressure–molecular area curves at the air/glycerol interface (solid lines) and at the air/water interface (dashed lines): (■) stepwise additions over a water subphase; (– –) continuous compression over a water subphase; (●) stepwise additions over a glycerol subphase; (—) continuous compression over a glycerol subphase. (B) Diagram of an oleic acid monolayer over a glycerol subphase under continuous compression. During compression, some oleic acid molecules collapse and leave the monolayer. Rigid and flexible tails correspond to condensed and expanded conformations of oleic acid, respectively. The platinum plate is depicted as a thick vertical bar.

the initial area of the film, the beginning of the surface pressure increase was detected only 15 min after the end of the compression. This is indicative of a very slow relaxation process, illustrated schematically in Figure 1B.

The surface pressure–molecular area curves obtained by stepwise additions of oleic acid and oleoyl glycerides over glycerol are given in Figure 2. At high surface pressures (above 15 mN m^{-1}) we observed a small decrease in surface pressure (±0.1 mN m^{-1} min^{-1}) probably indicative of a slow desorption process. The plot of the surface pressure versus the surface density of the oleoyl moiety clearly shows that the number of oleoyl chains per unit surface does not depend upon the compound used; i.e., the acyl chains occupy around the same area whether they are in the free fatty acid or the mono-, di-, or triglyceride form. This clearly indicates that the desorption of oleic acid into the glycerol subphase is negligible during the time course of a kinetic experiment.

Table 1 shows the surface pressure and the surface densities at the collapse points of oleic acid and oleoyl

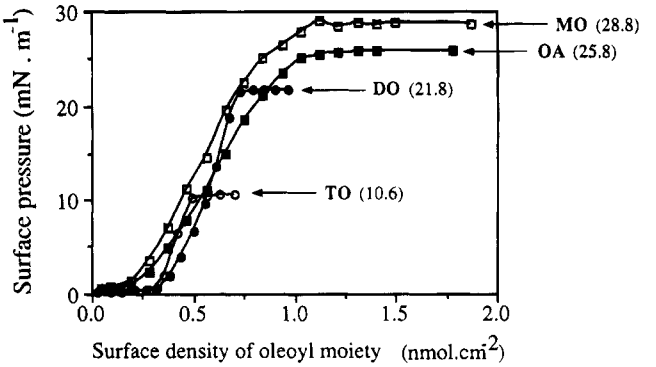

FIGURE 2: Stepwise additions to obtain surface pressure–molecular area curves of (■) OA, oleic acid; (□) MO, monoolein; (●) DO, diolein; and (○) TO, triolein at the air/glycerol interface. Numbers in parentheses are the values of the corresponding collapse pressures. The surface densities are expressed in terms of the number of oleoyl moieties per unit area.

Table 1: Pressure and Surface Density (at the Collapse Point) of Oleic Acid and Oleoyl Glyceride Monolayers Spread over a Water or Glycerol Subphase

	OA	MO[b]	DO[c]	TO
collapse pressure, glycerol/H_2O (mN m^{-1})	25.8/30.5	28.8/31.2[a]	21.8/31.0[a]	10.6/12.3[a]
surface density, glycerol/H_2O (nmol cm^{-2})	1.13/0.64	0.87/0.52[a]	0.33/0.33[a]	0.17/0.17[a]

[a] Values based on stepwise compression surface pressure–molecular area curves over double-distilled water adjusted to pH 2.0 with HCl (Laurent et al., 1994). [b] MO, 1-monoolein (Laurent et al., 1994). [c] DO, 1,3-diolein (Laurent et al., 1994).

glyceride monolayers spread over glycerol and water subphases, respectively. The surface pressures at the collapse point were always lower when the monolayers were spread over glycerol. With OA and MO, the surface densities were higher when measured over glycerol than over a water subphase, whereas similar surface densities were obtained with DO and TO with both types of subphase.

Continuous Recording of the Synthetic Activity of Cutinase. After injection of the enzyme, the adsorption of cutinase at the interface leads to a small increase ($\Delta\pi_c$ around 2 mN m^{-1}) in the initial air/glycerol surface pressure (Figure 3A). It is possible that this adsorption causes a partial denaturation of the enzyme at the air/glycerol interface. Spreading an oleic acid monolayer over a glycerol subphase containing cutinase resulted in a rapid increase followed by a slow decrease in the surface pressure (Figure 3A). A similar but slower decrease in the surface pressure was observed after injecting the same amount of buffer (pH 9.0) without any cutinase, as illustrated in Figure 3B. The latter decrease, taken as a control experiment, was due to the solubilization of ionized oleic acid since it was not observable at pH 5 (Figure 3C). In order to be able to monitor the synthetic activity of a lipolytic enzyme from the decrease in the surface pressure, it is therefore preferable to use acidic subphase pH values. The value of pH 9 was used because the synthetic activity of cutinase was found to be maximal in the pH range 9 to 11. Negligible activity was found under acidic conditions (Sebastião et al., 1993). Due to the desorption process of the ionized form of oleic acid at pH 9.0, the enzymatic synthesis rates calculated from the decrease in the surface

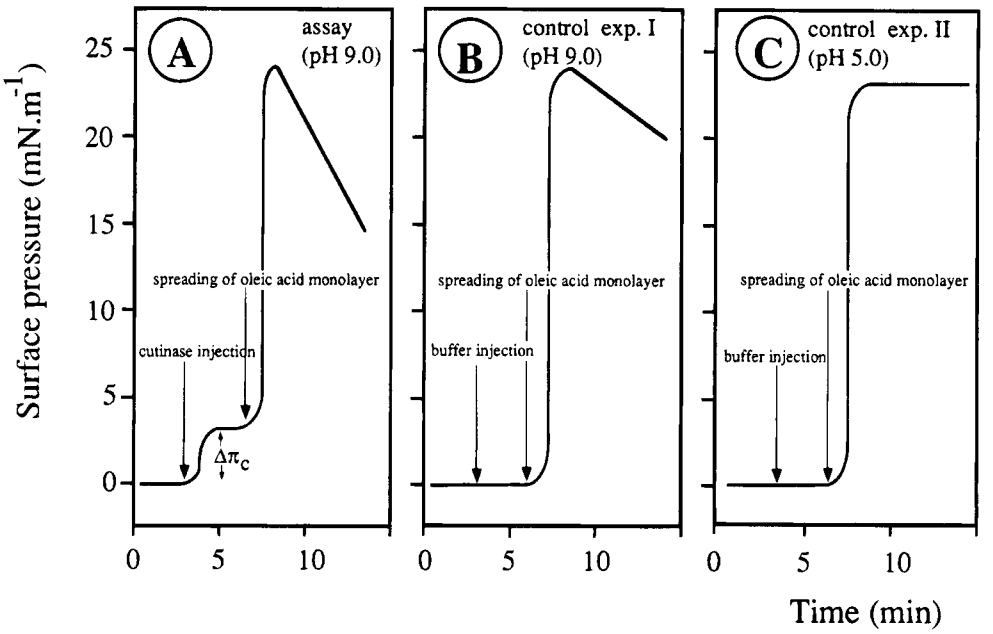

FIGURE 3: Recordings of surface pressure changes at the glycerol/air interface under the following conditions: (A) During an enzymatic assay. $\Delta\pi_c$ denotes the increase in surface pressure due to cutinase adsorption at the interface. After the spreading of an OA film (second arrow), the surface pressure rise is followed by a decrease. (B) During a control experiment without any cutinase (control I—pH 9.0). (C) During a control experiment without any cutinase (control II—pH 5.0).

pressure are always higher than those calculated from HPLC analysis of the reaction products.

Degree of Conversion and Synthetic Activity of Cutinase. The degree of conversion of oleoyl chains after 7 min of reaction with cutinase were determined after film recovery, lipid extraction, and HPLC analysis (Figure 4). High conversion levels were obtained. For instance, at a pressure of 15.4 mN m^{-1} with low water contents (0.33−0.98% H_2O), around 50% of the oleic acid was converted into products (Figure 4). At higher surface pressures, lower degrees of conversion were observed. At 24.5 mN m^{-1} and a low water content, only around 35% of the oleic acid initially present at the interface was converted within 7 min. Figure 4 also shows that the amounts of MO formed were higher at 24.5 mN m^{-1}, while TO was almost exclusively detected at a surface pressure of 15.4 mN m^{-1}. No TO was produced at 24.5 mN m^{-1} except at low water levels (0.33 and 0.65% H_2O).

Since part of the initial oleic acid spread at the interface was solubilized under alkaline conditions (Figure 3B), the question arises as to whether the synthesis really occurs at the interface and not in the bulk phase. To check this point, we performed a lipid extraction on a sample of the bulk phase collected at the end of the synthesis reaction catalyzed by cutinase. The fact that no glyceride products were detected (data not shown) indicates that the synthesis reaction actually takes place at the air/glycerol interface. The amount of oleic acid detected in the bulk phase after 8 min of reaction, at a working surface pressure of 24.5 mN m^{-1}, was about 8% of the oleic acid spread initially at the air/glycerol interface. Thus, the rate of desorption of oleic acid (1% min^{-1}) does not greatly affect the values of the synthetic activity catalyzed by cutinase. Furthermore, the total number of oleoyl chains converted into products (MO, DO, and TO) is comparable at low (15.4 mN m^{-1}) and high (24.5 mN m^{-1}) surface pressures.

The values of the synthesis levels were based on the film recovery, lipid extraction, and HPLC data. Table 2 compares the synthesis activity with the hydrolysis rates of 1,3-DO monolayers (measured with β-cyclodextrin in the aqueous phase). The rates of hydrolysis were 900 and 400 times higher than the synthesis activities measured at 15.4 and 24.5 mN m^{-1}, respectively.

DISCUSSION

Water is a highly polar solvent having a high surface tension value at the air/water interface. Stable monomolecular layers of amphipatic molecules can therefore be formed at air/water interfaces. In order to adapt the monomolecular film technique to monitoring lipase-catalyzed synthetic reactions, the choice of the alcohol forming the subphase depends on the physical requirements cited above. A high initial interfacial tension value is needed to obtain a wide range of interfacial tension changes caused by modifications in the chemical composition of the monolayer. During preliminary assays, two alcohols, glycerol and ethylene glycol, were selected, giving air/liquid surface tensions at 20 °C of 63.4 and 47.7 mN m^{-1}, respectively (Weast et al., 1988). Furthermore, these alcohols have a low enzymatic denaturation capacity (Khmelnitsky et al., 1991), which is also an important feature to be considered when working with enzymes in organic solvents. An extensive amount of monolayer work on alcohol/water systems with fatty acids and esters was reviewed by Lim and Berg (1975).

On the other hand, oleic acid was a good candidate for obtaining stable liquid-expended monolayers at the air/alcohol interface. Since oleic acid is fairly soluble in glycol, glycerol was finally selected to perform glyceride synthesis using the monomolecular film technique.

Surface Pressure−Molecular Area Curves. As shown in Figure 1, the differences between the surface pressure−molecular area curves obtained with either the continuous compression technique or the stepwise addition method were probably due to the viscosity of glycerol, which is about 1500 times higher than that of water (Weast et al., 1988). The

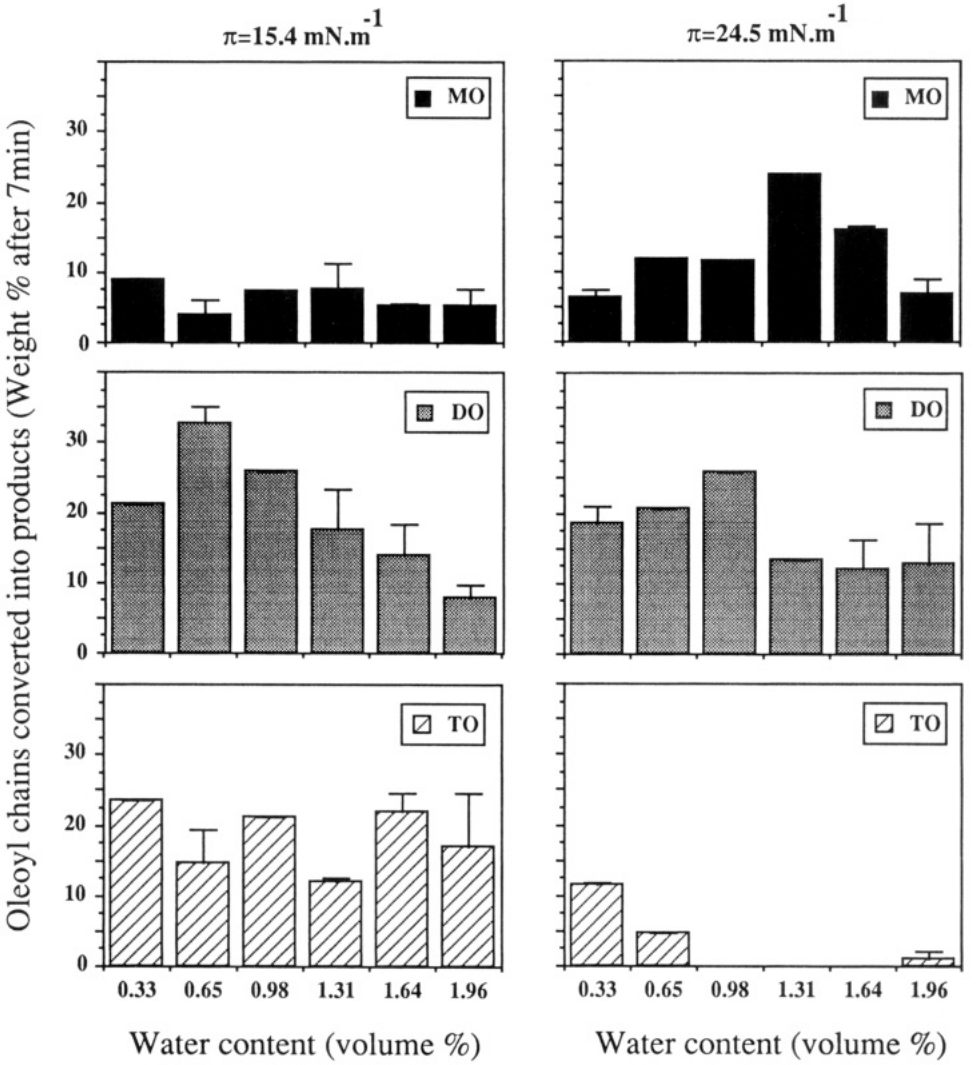

FIGURE 4: Percentage (wt %) of oleoyl chains converted into glyceride products (MO, DO, and TO) after 7 min of reaction as a function of the water content (vol %) of the glycerol subphase. Two surface pressure values (15.4 ± 0.9 and 24.5 ± 1.1 mN m^{-1}) were selected in a zero-order trough.

Table 2: Synthesis and Hydrolysis Rates of Glycerides Catalyzed by Cutinase, Using the Monomolecular Film Technique and Expressed as mol cm^{-2} min^{-1} M^{-1} [a]

	surface pressure (mN m^{-1})	
	15.4	24.5
synthesis/ hydrolysis	$4.7 \times 10^{-5}/4.2 \times 10^{-2}$	$7.8 \times 10^{-5}/3.2 \times 10^{-2}$

[a] Synthesis activities were measured at 0.33% water content (vol/vol). Hydrolysis rates were measured with monolayers of 1,3-DO and β-cyclodextrin present in the subphase, as described under Materials and Methods.

two-dimensional diffusion coefficient of a molecule present in a monolayer is inversely related to the viscosity of the subphase (Dimitrov et al., 1978). The diffusion process over a glycerol subphase is therefore much slower than over a water subphase. The continuous compression mode probably results in the situation schematically depicted in Figure 1B, where a lateral heterogeneity of the film is expected to occur because oleic acid molecules diffuse more slowly than the barrier moves. During the course of a continuous compression, some molecules probably collapsed since the average surface densities obtained at all the surface pressures used were always higher when this compression mode was used (see Figure 1A). The slow diffusion rates of the lipid molecules over the glycerol subphase may delay the quantitative supply of oleic acid molecules from the reservoir to the reaction compartment when working with a zero-order trough (Verger & de Haas, 1973). It is therefore difficult to experimentally regulate the surface pressure at a constant end point value during the enzymatic synthesis reactions.

Substituting water for glycerol led to higher surface densities only in the case of OA and MO monolayers (Table 1). A stronger interaction of a single acyl chain with the glycerol subphase may have been responsible for these higher molecular surface densities. The relatively lower surface pressure values at the collapse points of oleoyl glycerides and oleic acid monolayers spread over a glycerol subphase may have been due to the fact that the absolute air/glycerol surface tension (63.4 mN m^{-1}) was lower than the absolute air/water surface tension (72.8 mN m^{-1}).

Recording the Synthetic Activity of Cutinase. Ideally, continuous recordings of glyceride synthesis using the monomolecular film technique with fatty acid monolayers spread over a glycerol subphase can be obtained based on the decrease in the surface pressure due to the collapse of the formed reaction products. In general, however, some of these products can either remain (MO) at the interface,

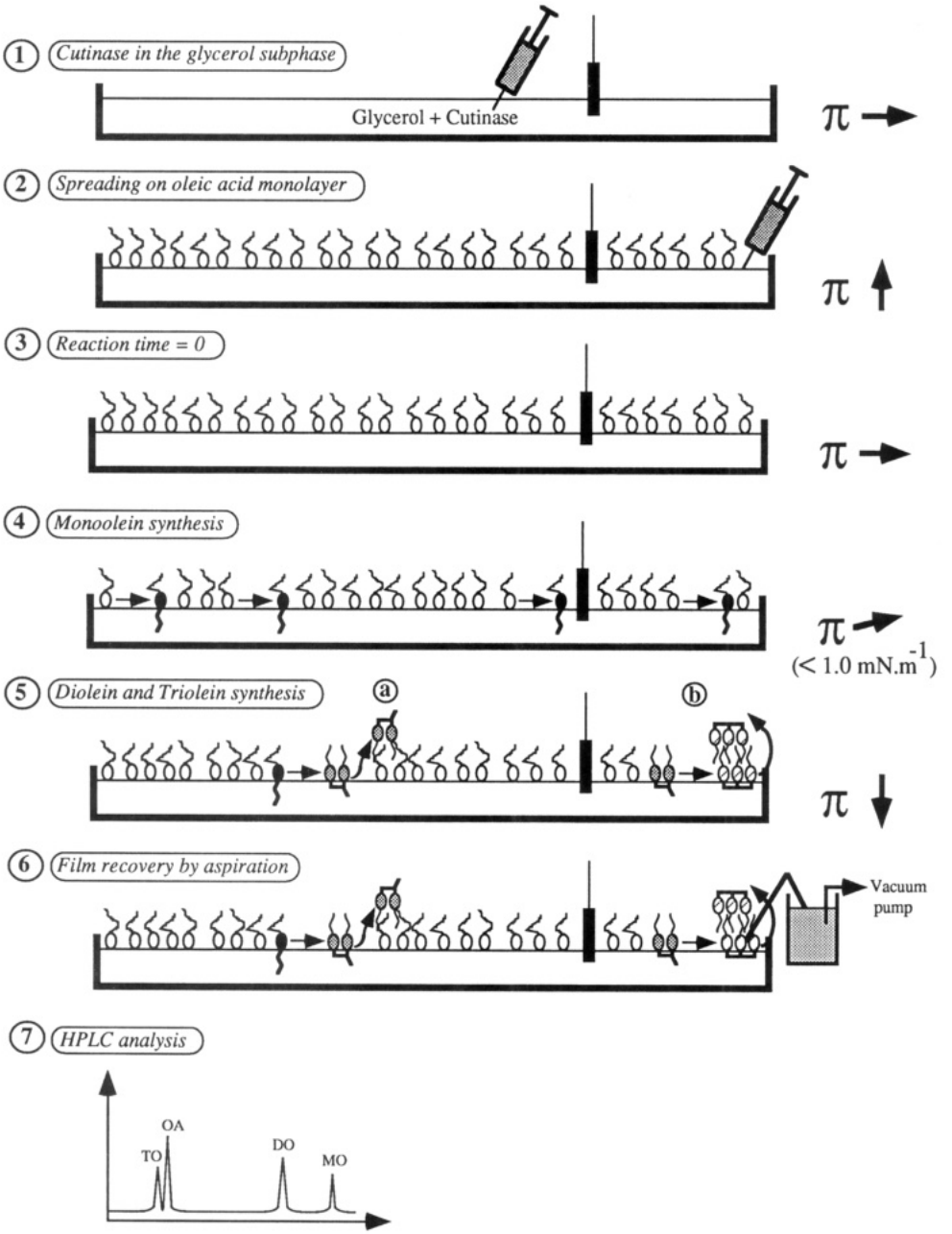

FIGURE 5: Steps occurring in a first-order trough after the spreading of an oleic acid monolayer (2) over a glycerol subphase containing cutinase (1). Monoolein synthesis (4). Diolein and triolein synthesis (5). Film recovery (6) and HPLC analysis (7). Symbols: OA, open ovals; MO, solid ovals; DO, stippled ovals; TO, diagonally striped ovals. Surface pressure changes are indicated by the slopes of the arrows on the right-hand side. The platinum plate is depicted as a thick vertical bar.

contributing to the surface pressure increase, or leave it (DO and TO) after collapsing, resulting in a concomitant surface pressure decrease. Three surface pressure ranges can thus be selected from the surface pressure−molecular area curves shown in Figure 2. First, the selected end-point values of the surface pressure were between the collapse points of OA (25.8 mN m^{-1}) and DO (21.8 mN m^{-1}). The synthesis of MO molecules slightly affects the surface pressure because an expected maximal surface pressure increase of about 4 mN m^{-1} should result from the complete synthesis of MO from OA and glycerol (Figure 2). Experimentally the maximum fraction of MO formed was found to be around 25% (see Figure 4), which means that an increase of 1 mN m^{-1} in the initial surface pressure could be expected to occur. This situation is schematically illustrated in Figure 5 (step 4). Within this surface pressure range, however, the DO molecules synthesized have probably collapsed (Figure 5, step 5a) since the working pressure is above the collapse point of pure DO. Two acyl chains leave the surface each time one molecule of DO is formed and then collapses, resulting in a concomitant decrease in the surface pressure. This decrease reflects only the enzymatic synthesis of DO and not that of MO.

Second, the selected surface pressure range is between the collapse pressures of DO (21.8 mN m^{-1}) and TO (10.6 mN m^{-1}). The synthesized DO molecules therefore remain, forming a stable monolayer at the interface, and are further squeezed out only when TO molecules are subsequently formed and collapse (Figure 5, step 5b). This explains why only TO synthesis activity could be observed under these conditions. Third, at values below 10.6 mN m^{-1}, all the reaction products stay at the interface and no significant

surface pressure change could be observed. To investigate the above situations, two surface pressures, namely 24.5 and 15.4 mN m^{-1}, were selected as end-point values (see figure 4).

Degree of Conversion and Synthetic Activity of Cutinase. Cutinase was able to form oleoyl glycerides from an OA monolayer spread at the air/glycerol interface. When this interfacial technique was applied to glyceride synthesis, more than 50% of oleic acid was converted into glycerides within 7 min of reaction (see Figure 4).

Looking more closely at the chemical nature of the glycerides formed, some pressure selectivity seems to have prevented TO formation. When the selected surface pressure was 24.5 mN m^{-1}, the DO molecules formed were expelled from the interface by collapsing, and practically no TO was detected. This would suggest that collapsed DO cannot be used further as substrate by cutinase and that the enzyme penetration is probably restricted to the first lipid monolayer, whereas when DO molecules stay at the interface (at a selected working surface pressure of 15.4 mN m^{-1}), around 20% of the oleoyl chains are converted into TO within a reaction time of 7 min. This conversion level is independent of the water content of the glycerol subphase.

In spite of the high rate of conversion that we observed (about 8% of oleic acid was converted per minute), the synthesis activity was very low in comparison with the rates of hydrolysis recorded using monolayers of 1,3-DO as substrate (Table 2). It has been observed by using the monomolecular film technique that only the lipase molecules adsorbed at the interface are really enzymatically active (Verger & de Haas, 1976). Given the negligible denaturing effects of glycerol as a bulk solvent, the large differences between synthesis and hydrolysis activities may possibly be attributable to the fact that lower amounts of cutinase were adsorbed to a glycerol/lipid interface than to a water/lipid interface. Two nonmutually exclusive possibilities can be envisaged: the adsorption of cutinase at the air/glycerol interface can cause a partial denaturation of the enzyme; alternatively, cutinase may adopt different conformations in glycerol or in water. Studies are in progress with a view to checking these hypotheses.

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