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Lipase-catalyzed esterification in monolayers and microemulsions

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Abstract

Studies were carried out to explore the enzymatic synthesis of glycerol and fatty acid in monolayers and microemulsions. Monolayers of stearic acid were taken on subsolutions of glycerol and water, and the enzyme (lipozyme) solution was injected under the compressed monolayers. Monolayers, when analyzed by TLC and HPLC, confirmed the esterification reaction. Interestingly, triglyceride and monoglyceride were the major products even though the lipozyme was 1,3 specific. The same reaction was carried out in water-in-oil microemulsions with sodium di(2-ethylhexyl)sulfosuccinate (AOT) as surfactant and monoglyceride and diglyceride were obtained as the major products. It was also noted that the esterification reaction in the monolayer could take place at a much higher water/glycerol ratio than in the microemulsion. The results are explained on the basis of residence time of the product at the interface, curvature of the interface, orientation of substrate at the interface, and the interfacial activation effect on the lid of the active site of the enzyme.

Key words: Fatty acid; Glycerol; Lipozyme; Microemulsion; Monolayer

Introduction

The development of molecular enzymology has come about mainly through studies on free enzymes. Experiments can be designed to elucidate the structure of the catalytic site and physicochemical conditions for its optimal activity. Lipases are enzymes that catalyze the hydrolysis or synthesis of ester bonds. Maximal activity of lipase is observed in the presence of an interface. The best studied lipases are water soluble and their substrates are water insoluble. Thus, monolayers and microemulsions are suitable media in which to study the effect of curvature of the interface, fluidity of the interface, pH of the aqueous phase and the residence time of reactants and products at the interface in enzymatic reactions.

The hydrolysis of lipid monolayers has been studied extensively for various substrates using different enzymes [1–6]. However, very little work has been done on enzymatic synthesis in monolayers. Synthesis in monolayers is of great interest because of the specific molecular orientation of polar and non-polar groups as well as the high surface concentration of substrate molecules. In addition, factors such as molecular area, surface charge density and surface fluidity can be varied at will in monolayers.

The hydrolysis of lecithin monolayers by phospholipase A has been studied by measuring the decrease in surface potential [1]. Several types of phospholipases that catalyze the hydrolysis of acyl-ester bonds in monolayers of glycerophosphatidyls have also been reported [2,3]. It was observed that the charge on the substrate surface is important in the initiation of the enzymatic reaction. Shah and

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Shulman [4] measured the action of snake venom phospholipase A on lecithin monolayers. They investigated the influence of divalent cations, various buffers and the presence of other lipids on hydrolysis of dipalmitoyl, egg, soybean and dioleoyl lecithin monolayers. It was shown that a lecithin monolayer does not hydrolyze when it is compressed above a critical surface pressure. The critical surface pressure required to block the penetration of the enzyme into the monolayer increased with molecular area (i.e. dioleoyl lecithin > soybean lecithin > egg lecithin > dipalmitoyl lecithin). Colacicco has reported the use of surface potential measurements as an important technique to monitor enzymatic reactions of phospholipid monolayers [5]. Thus monolayers represent a promising system for studying the synthesis reaction of stearic acid (as a monolayer) and glycerol (in the subphase) using *Mucor miehei* lipozyme (10000 L Novo Enzyme).

Enzymatic reactions in microemulsions with low water content have been topics of interest for several groups [7–12]. The anionic double-tailed surfactant AOT (sodium di-(2-ethylhexyl)sulfosuccinate) is most frequently used to form microemulsions. Unlike most surfactants, AOT does not require additional amphiphiles as cosurfactants for the formation of reverse micelles because of its wedge-shaped molecular geometry. The rate of enzymatic reaction in reverse micelles depends on the surfactant concentration, the water-to-surfactant ratio, the temperature, concentration of buffers and pH. The enzymatic synthesis of monoglycerides, diglycerides and triglycerides has been studied to establish the mechanism of reaction between oil-soluble fatty acid and water-soluble glycerol by the enzyme (lipase) in reverse micellar solutions [13,14].

Experimental

Materials

Lipozyme, a product of Novo Laboratory Inc. was made available by Kraft Inc. Hexane, isooctane, isopropanol and buffer solutions were bought from Fisher Scientific Company (USA). Fatty acids, lipids and AOT were bought from Sigma Chemical Company (USA).

Methods

Monolayer formation

Stearic acid dissolved in a solvent (hexane: methanol:chloroform, 3:1:1 v/v/v) was spread on a subsolution of water and glycerol using an Agla micrometer syringe. After the evaporation of the solvent, the monolayer was compressed with a waxed glass bar up to a desired initial surface pressure. When the surface pressure reached a steady value, lipozyme was injected under the monolayer and stirred gently by a rolling magnet. This assured the uniform distribution of the enzyme in the subsolution. The surface pressure and surface potential were measured with time. After 3 h, the monolayer was removed by suction and the product was dissolved in methanol:chloroform (2:1 v/v) solvent. The sample for HPLC was taken from the methanol and chloroform solvent phase.

Microemulsion

A typical microemulsion consisted of 14 ml of alkane and 0.004761 mol of fatty acid in the oil phase, 3.3 g of AOT as the surfactant, and 0.010043 mol of glycerol, 0.1 ml of lipozyme and water in the aqueous phase. The microemulsion was prepared by mixing the above components under constant stirring. The reaction was monitored by NaOH titration using phenolphthalein as the indicator (for extent of reaction) or by HPLC (for quantitative analysis of products formed).

Reaction

The synthesis reaction taking place in the microemulsion and monolayer is

$$\text{glycerol} + \text{fatty acid} \xrightarrow{\text{lipase}} \begin{matrix}\text{monoglyceride}\\ \text{diglyceride}\\ \text{triglyceride}\end{matrix} + \text{water}$$

Monitoring the reaction using HPLC and TLC

Normal phase HPLC on a silica column (10 mm × 2.4 mm) fitted with a UV absorbance detector of 213 nm cut-off wavelength and 0.05 absorbance units full-scale (a.u.f.s.) was used to separate monoglyceride, diglyceride, triglyceride and fatty acid in order to monitor the reaction. A mixture of iso-octane and isopropanol (94:6 v/v) was used as the mobile phase. The flow rate of the mobile phase was kept at 1 ml min^{-1}. All analyses were done at room temperature. The absorbance detector of the HPLC is from Spectra Physics (Model SP8450). The integrator (Model SP8880) plots raw signals from the detector and determines the presence of peaks. This signal is analyzed by Spectra Physics software LNET2 and SPMENU.

TLC plates were run twice in diethyl ether up to 2 cm, and then in hexane–diethyl ether–acetic acid mixture (70:30:1 v/v/v).

Results and discussion

Reactions in monolayers

A monolayer of stearic acid was spread on a subsolution of glycerol and water (1:1 v/v; total volume, 85 ml at pH 6.8). The monolayer was compressed to the desired surface pressure (22 dyn cm^{-1}) and lipozyme (*Mucor miehei*) was injected under the monolayer. Lipozyme was gently mixed under the monolayer. The surface pressure and surface potential were measured with time as described earlier [15]. It is evident from Figs. 1 and 2 that surface pressure remained constant whereas surface potential increased by about 90 mV. The synthesis reaction was carried out at three concentrations of lipozyme (144, 216 and 288 LU per milliliter of subsolution). No change in the surface potential of the stearic acid monolayer was observed without lipozyme. In order to establish that the change in the surface potential is indeed due to the formation of ester bonds, we carried out additional experiments using a stearyl alcohol monolayer (instead of stearic acid) under the same conditions. There was no change in the surface potential after injecting the lipozyme under the stearyl alcohol monolayer. However, cleavage of the ester bond in lecithin monolayers decreases the surface potential [4,5]. The change in surface potential of the monolayer was measured at various initial surface pressures in order to elucidate the effect of surface pressure on the synthesis reaction (Fig. 3). It is evident from Fig. 3 that an initial surface pressure of 20 dyn cm^{-1} was optimum for maximum change in the surface potential (i.e. maximum conversion). Initially the change in surface potential increased with the increase in the surface pressure. This was mainly due to the increase in the two-dimensional concentration of reactant at the air/water interface. After the opti-

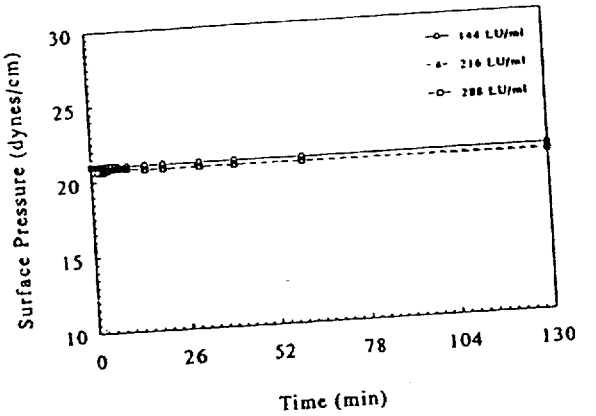


Fig. 1. Surface pressure vs. time curves for enzymatic reactions in monolayers at various lipozyme concentrations.

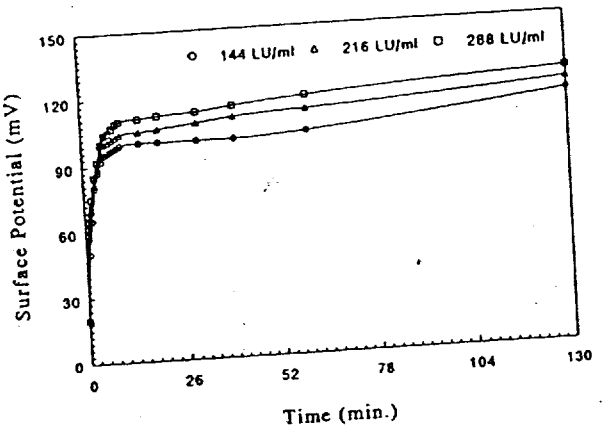


Fig. 2. Surface potential vs. time curves for enzymatic reactions in monolayers at various lipozyme concentrations.

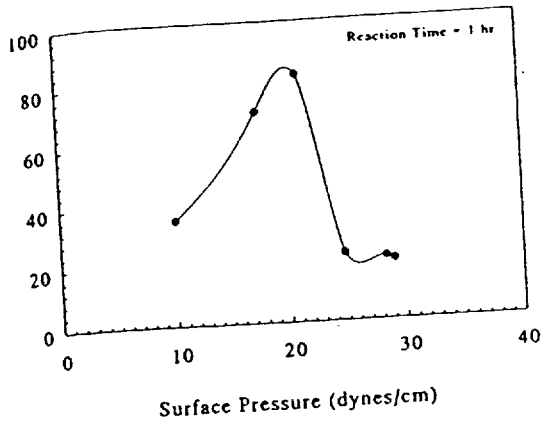


Fig. 3. Effect of initial surface pressure on the change in surface potential in 1 h.

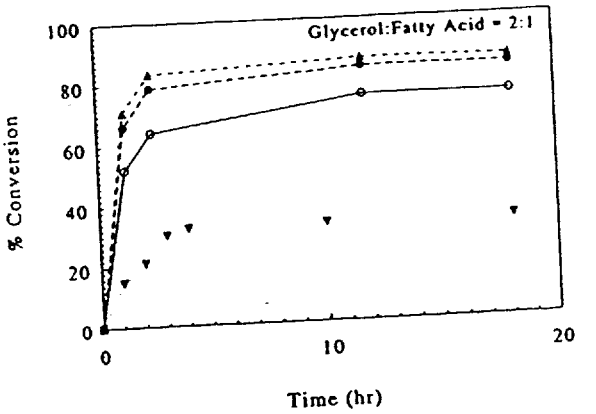


Fig. 4. Effect of unsaturation in fatty acid on the percentage conversion of fatty acid into glycerides in microemulsion mediated reactions: O, oleic acid; ▲, linoleic acid; ●, linolenic acid; ▼, stearic acid.

mum surface pressure (20 dyn cm^{-1}), the change in the surface potential decreased with increase in initial surface pressure because stearic acid molecules were so tightly packed that the packing reduced the reaction.

The product from the monolayer was analyzed to confirm the synthesis reaction. After the completion of the reaction, the monolayer was removed and mixed with chloroform and methanol (1:2 v/v) solvent to dissolve possible monoglycerides, diglycerides and triglycerides. HPLC of this sample indicated the presence of tristearin (32.6%), monostearin (55.7%), and stearic acid (11.7%) in the monolayer. TLC of the extracted monolayer also confirmed the presence of these products.

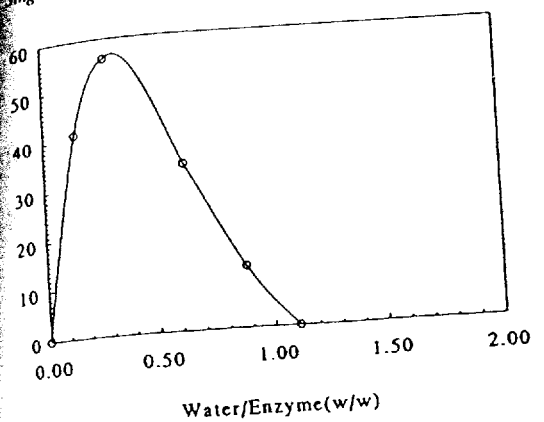
Reactions in microemulsions

Effect of unsaturation of fatty acid

The structure of the fatty acid had a profound effect on the percentage conversion of fatty acid to monoglycerides, diglycerides or triglycerides in microemulsions (Fig. 4). A maximum conversion of 84% was found in the case of linoleic acid, 80% for linolenic acid, 71% for oleic acid and 33.4% for stearic acid. This occurs due to the change in partitioning of fatty acid between the interface and the oil caused by unsaturation of the fatty acid. As unsaturation in the fatty acid increases, its solubility in hexane increases. This increase in solubility from stearic acid to linoleic acid gives a driving force to the products at the interface to move into the oil phase quickly. This results in an increase in conversion. There is a very small difference between linoleic acid and linolenic acid because both of these fatty acids are unsaturated, and although unsaturation is greater for linolenic acid, the difference in solubility of the products in hexane is very low. HPLC of a sample from a microemulsion containing stearic acid indicated the presence of monostearin (12.4%), distearin (21.0%) and stearic acid (66.6%). TLC of this microemulsion sample also confirmed the presence of these products.

Effect of water/enzyme ratio on the equilibrium conversion

Lipozyme was freeze-dried to remove all the associated water and then increasing amounts of water were added to it to study the effect of water content on the equilibrium conversion (originally, before freeze-drying, the enzyme contained 30 wt% water). A maximum in the equilibrium conversion was found near 30% water (Fig. 5). This 30% water is bound to enzyme molecules and AOT molecules in the microemulsion. A further increase in water induces accumulation of free water inside the aqueous core and this free water causes hydrolysis of ester bonds. So the equilibrium percentage conver-



ig. 5. Effect of water/lipozyme ratio on the percentage conver-ion of oleic acid in microemulsion mediated reactions.

ion decreased at higher concentrations of water n microemulsions, reaching zero conversion at 1.10 (w/w) with respect to the enzyme.

Discussion

Although the enzyme is 1,3 specific, the presence of triglyceride was observed in the monolayer products but not in the microemulsions. This is mainly due to the long residence time of the products and substrate at the air/water interface in monolayers. It is likely that the products and substrates are relatively more stationary at the air/water interface of monolayers than at the oil/water interface of microemulsions. The same enzyme maintains its specificity in the reverse micelle or microemulsion systems.

It is well known [16] that lipases are able to catalyze synthesis reactions under conditions of "water starvation", and this was confirmed using suspensions of the enzymes in organic solvents [17]. Synthesis of glycerides from glycerol and fatty acids has been attempted in microemulsions of very low water content [14,18]. No synthesis reaction was observed in microemulsions with a water/glycerol molar ratio above 1:1 [19], whereas we have shown that the same synthesis reaction is possible in monolayers with a water/glycerol molar ratio of 4:1 (1:1 v/v). In fact a significant increase in surface potential (approximately 60 mV) was observed with a water/glycerol molar ratio of 12:1, indicating the synthesis in monolayers. This is due to the orientation of the polar group of the fatty acid toward the active site of the enzyme and to the high concentration of fatty acid molecules near the active site. The concentration of fatty acid per unit interfacial area in monolayers is much higher than the surface concentration of fatty acids in microemulsions. Tristearin and monostearin were the main products from the reaction in monolayers whereas distearin and monostearin were the main products from the reaction in microemulsions. The result obtained from the reaction in the microemulsion was consistent with the results obtained by Hayes and Gulari [19]. They used tetradecanoic acid as fatty acid and lipase from *Rhizopus delemar*. The presence of triglyceride in reactions in monolayers is mainly due to the residence time of reactants and/or products at the interface (which is much higher in the case of monolayers than microemulsions) and to the favorable orientation of stearic acid molecules. The most interesting part of the synthesis in monolayers is the absence of distearin. This may be due to some conformational changes in the enzyme when it is exposed to a planar surface with high concentration of stearic acid molecules. In the case of microemulsions, as distearin is formed at the interface, it diffuses into the oil phase owing to its solubility (i.e. partitioning in the oil phase).

Conclusions

Our results indicate that enzymatic synthesis in monolayers is indeed possible at higher water content than in microemulsions. Monolayers provide higher conversions of fatty acid to glycerides than do other systems such as microemulsions or aqueous solutions. Moreover, the concentration of the substrate can be varied with surface pressure in monolayers. We also observed that monoglycerides and triglycerides were the major products in monolayers, whereas monoglycerides and diglycerides were the predominant products in microemulsions.

Acknowledgement

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