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# Enzymatic synthesis of tricaprylin in a solvent-free system: lipase regiospecificity as controlled by glycerol adsorption on silica gel

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Enzymatic synthesis of tricaprylin from caprylic acid (octanoic acid) and glycerol was carried out under solvent-free conditions. Kinetics of mono-, di- and tricaprylin synthesis and caprylic acid esterification in standard conditions (free glycerol) were slightly different from those obtained in the case of silica gel adsorbed glycerol. The critical role of the silica gel which behaves as a "reservoir" is proposed and it was shown that the use of silica gel facilitates reactions and improves the conversion yields at high glycerol concentrations.

## Introduction

Lipase catalysed synthesis of triacylglycerols has been largely studied over the last decade. Most of the published works deal with enzymatic reactions conducted in organic solvents (Klibanov, 1986, 1989). Some authors have also shown that such reactions could be achieved in a medium solely composed of substrates (Goldberg *et al.*, 1988; Ergan *et al.*, 1990). In this latter case, no organic solvent was used to solubilize the substrates, which led to the use of enzymes in a three-phase system: the enzyme preparation in a solid phase and the two non-miscible reactants (glycerol and fatty acid) in two liquid phases. Such a system presents an enormous advantage of avoiding the problems of separation, toxicity and flammability of organic solvents, lowering the cost of the final product and permitting recovery of product without further purification or evaporation step. The esterification of fatty acid with glycerol without adding a solvent also finds applications in the food industries. There are other advantages in carrying out bioconversions under those conditions. In fact, when no solvent is used, it means that the whole reaction medium can be composed of only the necessary substrates and thus, these substrates can be used at very high concentrations. So far, few processes involving lipases have been studied without organic solvent. This approach was used to synthesize triolein (Ergan *et al.*, 1990), medium-chain acylglycerol (Kim and Rhee, 1991) butyl laurate (Gandhi *et al.*, 1995) and sugar acetal fatty acid esters (Fregapane *et al.*, 1991). Among

the immobilized lipases tested for triacylglycerol synthesis from free fatty acids and glycerol, some present a 1,3-specificity (Macrae, 1983; Sonnet, 1988; Bloomer *et al.*, 1990; Ergan and Trani, 1991) allowing, in theory, the synthesis of homogeneous 1,3-diacylglycerols. However, very high yields of triacylglycerols have been obtained (Ergan and Trani, 1991). The explanation for that fact is the spontaneous isomerisation of the enzymatically formed 1-monoacylglycerol or 1,3-diacylglycerol into 2-monoacylglycerol or 1,2-diacylglycerol, respectively, followed by the enzymatic esterification of the free primary hydroxyl (Ergan and Trani, 1991).

In the present paper, we report the effects of silica gel as support for the polar substrate (glycerol) and of the regiospecificity of lipase on the synthesis of tricaprylin in a solvent-free medium.

## Materials and methods

### Materials

Reactions were catalyzed with Lipozyme, a 1,3-specific lipase from *Mucor miebei* immobilized on a macroporous anion exchange resin or with Novozym SP435, a non-specific lipase from *Candida antarctica* immobilized on macroporous acrylic resin, generously provided by Novo Nordisk Bioindustri S. A. (France). Glycerol (G), caprylic acid (CA), dicaprylin (DC), tricaprylin (TC) and butyl laurate were purchased from Sigma. Hexane was obtained from Carlo Erba. Silica gel 100 was purchased from Fluka Chemica.

### Adsorption of glycerol on silica gel

Glycerol was adsorbed as described by Berger *et al.* (1992): 1 g glycerol and 1 g silica gel were gently mixed until an homogeneous powder was obtained.

### GC analysis

Caprylic acid, mono-, di- and tricaprylin were analyzed by a Varian gas chromatograph equipped with a flame-ionization detector, a compact low-thermal mass on-column capillary injector and a Varian 4290 data integrator. The GC was fitted with a 12 m × 0.32 mm × 0.25 μm SGE BPX5 Column. Helium was used as the carrier gas with a flow rate of 30 ml/mn. The detector temperature was 370°C. The column temperature was set to 80°C for 0.5 mn and was then programmed firstly at 50°C/mn to 280°C and secondly at 10°C/mn to 360°C which was maintained constant for 10 mn.

5 μl of the reaction medium (enzyme is previously allowed to settle) were dissolved in 1 ml of hexane that contained lauric acid butyl ester as internal standard (1.72 g/l) and, 0.5 μl were directly injected into the gas chromatograph. The amounts of each component were calculated as percentages of the initial amount of caprylic acid (Charlemagne and Legoy, 1995) and the sum of mole fractions of fatty acid, mono-, di-, and triacylglycerol is 1.

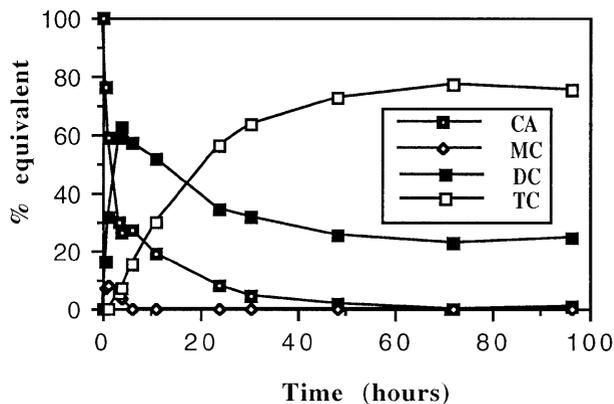
### Tricaprylin synthesis

Given quantities of free glycerol or silica-gel adsorbed glycerol and caprylic acid were mixed together followed by the addition of the immobilized lipase. The three-phase systems were mixed in a 4 ml vial and incubated on a rotatory shaker at 250 rev/min placed in an incubation chamber at 60°C. The progress of the reaction was followed by withdrawal of 5 μl aliquots at various time intervals, further analyzed by the GC method described above. Water produced by the reaction is removed by free evaporation from the reaction medium as described by Ergan *et al.* (1990)

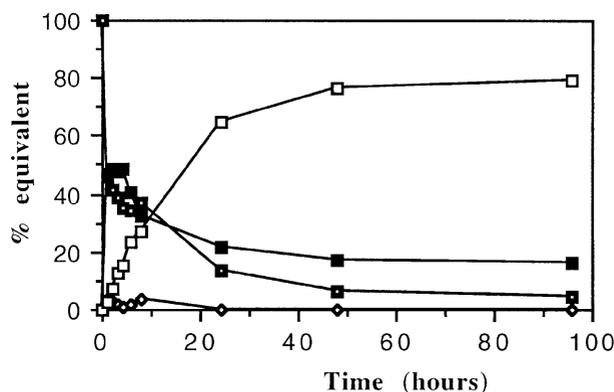
## Results and discussion

### Effect of silica gel-adsorbed glycerol on tricaprylin synthesis

The syntheses of tricaprylin in standard conditions (free glycerol) and with silica gel adsorbed glycerol were compared (Figure 1 and Figure 2). Under standard conditions, equilibrium is reached at 72 hours and the final tricaprylin yield is 78% of the initial free fatty acid (FFA). At 4 hours, the dicaprylin yield reaches a maximum corresponding to 63% of the initial FFA. Monocaprylin never exceeds 8.5% which is reached at



**Figure 1** Progress curve of intermediates (CA: caprylic acid, MC: monocaprylin, DC: dicaprylin, TC: tricaprylin) of tricaprylin synthesis at 60°C, reaction medium kept uncovered: 1.032 g of caprylic acid, 0.218 g of glycerol and 0.1 g of Lipozyme.



**Figure 2** Progress curve of intermediates of tricaprylin synthesis at 60°C, reaction medium kept uncovered: 1.032 g of caprylic acid, 0.436 of glycerol immobilized on silica gel 100 and 0.1 g of Lipozyme. Symbols: see Figure 1.

1 hour, and is no longer detected after 3 hours. These results suggest that using a kinetic control would not be efficient for synthesizing monocaprylin, but could be valuable to synthesize dicaprylin. This dicaprylin would have to be purified later on by crystallization if necessary.

When glycerol is adsorbed on silica gel, the same kinetic profile is observed. Nevertheless, under these conditions, the maximum dicaprylin yield occurs earlier (2 h) and its value is lower than that measured in the first case (49% instead of 63%). The maximum monocaprylin synthesis is also lower when glycerol is adsorbed on silica gel but some traces can be detected during a longer period (20 h).

**Table 1** Effect of the glycerol adsorption on silica gel on the initial rates of dicaprylin and tricaprylin synthesis

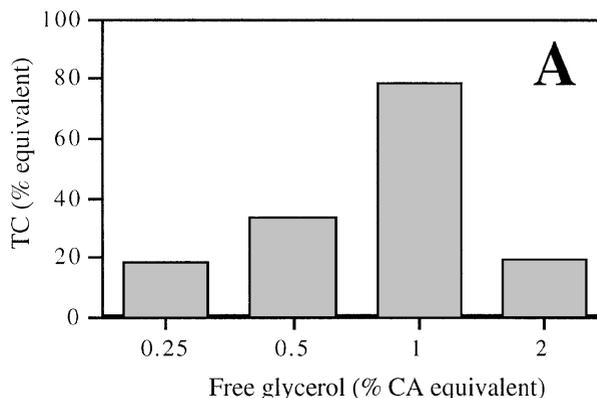
	Initial rate in $\mu\text{M}/\text{mn}/\text{g}$ Lipozyme	
	Dicaprylin	Tricaprylin
Free glycerol	120	7.8
Silica gel adsorbed glycerol	200	8.95

At equilibrium, 80% of the initial FFA are under the form of tricaprylin. The other products are FFA and dicaprylin that represent respectively 4% and 16% of the initial FFA. The tricaprylin yield is the same in both cases and is obtained in the same time. This production profile of tricaprylin strongly agrees with the results published by Ergan *et al.* (1990) and the kinetic model proposed by Lortie *et al.* (1993) for the enzymatic synthesis of triolein in a solvent-free medium.

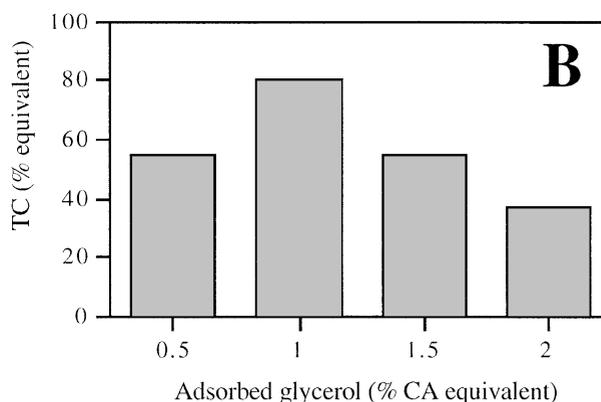
A comparison of the initial rates of di- and tricaprylin formation under the two sets of conditions is presented in Table 1. It can be observed that glycerol adsorption on silica gel induces an increase in the initial rate of di- and tricaprylin synthesis. This increase of the initial rates is the most favourable effect that could be detected.

In order to get a better view about the effect of glycerol adsorption, we measured the total tricaprylin synthesis at equilibrium for different ratios of glycerol-caprylic acid with free and silica gel adsorbed glycerol (Figure 3A and 3B). For the standard conditions (Figure 3A) the best result was obtained with stoichiometric amounts (molar ratio = 1). When glycerol/caprylic acid ratio equals 2, tricaprylin yield is less than 20%. In the case of silica gel adsorbed glycerol (Figure 3B) the molar ratio of 1 is also the best ratio for tricaprylin synthesis. As opposed to standard conditions, higher or lower ratios do not induce a drastic decrease of the level of synthesized tricaprylin. For example a molar ratio of 2 leads to the synthesis of tricaprylin two times higher when glycerol is adsorbed on silica gel.

The study of tricaprylin synthesis from caprylic acid and silica gel-adsorbed glycerol in a medium solely composed of substrates shows that the presence of silica gel-adsorbed glycerol improves significantly the initial rate of di- and tricaprylin syntheses and the conversion yield of tricaprylin at equilibrium for the different ratios of glycerol/caprylic acid (except for ratio 1). The following explanation for the critical role of silica gel in this type of reactions is proposed: the silica gel having



**Figure 3a** Total tricaprylin synthesis at equilibrium after uncovered batch experiments using free glycerol: 1.032 g of caprylic acid, 0.1 g of Lipozyme and different ratios of glycerol/caprylic acid.



**Figure 3b** Total tricaprylin synthesis at equilibrium after uncovered batch experiments using adsorbed glycerol: 1.032 g of caprylic acid, 0.1 g of Lipozyme and different ratios of glycerol/caprylic acid.

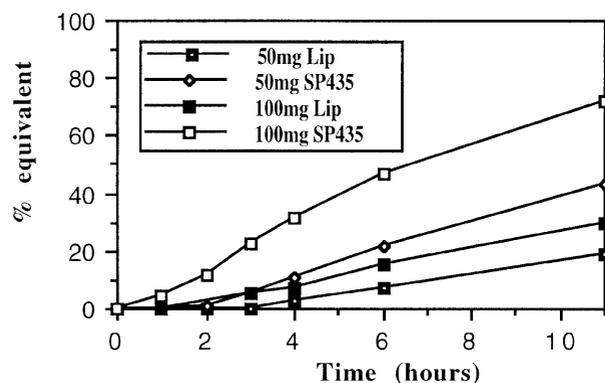
a strong affinity for the glycerol (Stevenson *et al.*, 1994), behaves as a glycerol “reservoir” and plays a protective role for the immobilized enzyme by avoiding polar substrate (glycerol) adsorption on the Lipozyme support. Similar results have been obtained when working with organic solvents and a reaction mechanism has been proposed (Castillo *et al.*, 1997). Secondly, the adsorption of glycerol onto a silica-gel support facilitates the contact between Lipozyme, the hydrophilic glycerol and hydrophobic caprylic acid.

### Effect of lipase regiospecificity on tricaprylin synthesis

Novozyme SP435 is prepared with *Candida antarctica* lipase, which is non-specific. Lipozyme is prepared with *Mucor meihei* lipase, which is 1,3-specific. The synthesis

**Table 2** Effect of enzyme quantity (mg) on the initial rate of tricaprylin synthesis

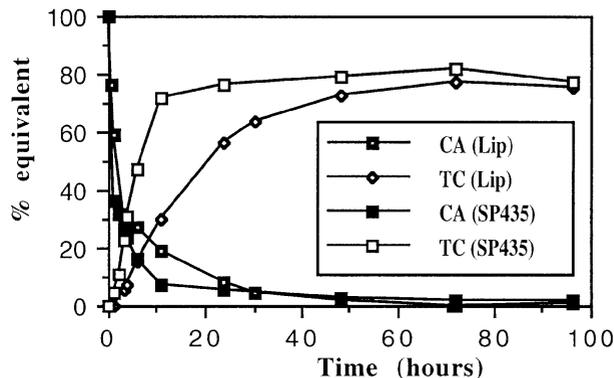
Enzyme quantity (mg)	Initial rate of tricaprylin synthesis in $\mu\text{M}/\text{mn}$
<b>Lipozyme</b>	
50	0.44
100	0.78
<b>SP435</b>	
100	1.85

**Figure 4** Effects of lipase specificity and quantity on the rate of tricaprylin synthesis at 60°C, reaction medium kept uncovered: 1.032 g of caprylic acid and 0.218 g of glycerol. Lip: Lipozyme; SP435: SP435 lipase.

of tricaprylin was carried out in standard conditions (see Materials and methods). We investigated the same reaction with the two types of lipase and looked at the effect of enzyme concentration on the rate of tricaprylin synthesis (Figure 4).

Clearly, increasing the amount of the non-specific or 1,3-specific lipase increases the rate of tricaprylin synthesis. The initial rates of tricaprylin formation are calculated (Table 2). The rate of the reaction with the two kinds of lipase is directly proportional to the enzyme quantity, which is expected if no limiting step other than enzymatic occurs in the reaction.

In order to quantify the extent of tricaprylin synthesis with the two types of immobilized lipase, the composition of the reaction medium as a function of time has been analyzed. As shown in Figure 5, we can clearly distinguish that SP435 is faster than the same amount of Lipozyme. This comparison is valid only in terms of amounts of enzyme preparation, since the amounts of protein are not known.

**Figure 5** Progress curve of caprylic acid (CA) consumption and tricaprylin (TC) synthesis at 60°C, reaction medium kept uncovered: 1.032 g of caprylic acid, 0.218 g of glycerol and 0.1 g of Lipozyme or 0.1 g of SP435 lipase.

At equilibrium, similar medium compositions are obtained in both cases: 80% TC and 4% CA left. A higher conversion yield after 24 hours is obtained with SP435 (76%) as compared to that obtained with the Lipozyme (57%). These results are in agreement with previous works (Ergan and Trani, 1991) with oleic acid and glycerol.

## Conclusion

A good yield of tricaprylin synthesis can be achieved with a 1,3-specific immobilized lipase (Lipozyme) and a non-specific immobilized lipase (SP435) without any organic solvent. As a result of this study, we can present the following observations:

- The silica gel-adsorbed glycerol improves significantly the initial rate of di- and tricaprylin synthesis and the conversion yield of tricaprylin at equilibrium for the different ratios of glycerol/caprylic acid (except for ratio 1).
- Stoichiometric amounts of substrates are necessary and sufficient to achieve the best production of tricaprylin with free or silica gel adsorbed glycerol.
- For the two immobilized lipase preparations, the yield of tricaprylin synthesis in a medium solely composed of substrates is directly proportional to the quantity of immobilized enzyme preparation.

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