Synthesis of Oleic Acid Esters Catalyzed by Immobilized Lipase

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Lipase-catalyzed syntheses of oleic acid esters with various primary alcohols have been performed in a batch stirred tank reactor in an almost nonaqueous medium without organic solvents. For all syntheses 50 °C was found to be the optimal temperature. Initial reaction rates were influenced by the alcohol chain length. The study of the pressure stability of the immobilized lipase from *Rhizomucor miehei* (lipozyme IM) showed that the lipase preparation is quite stable; it does not lose its activity when it is exposed to carbon dioxide at 300 bar for 24 h. Esterification rates at high pressure were determined, and it was found that they were higher than at atmospheric pressure. The highest rate and maximal conversion were near the critical point of carbon dioxide.

Keywords: *Esterification; batch reactor; lipase; Mucor miehei; oleic acid esters; high pressure; carbon dioxide; enzyme stability*

INTRODUCTION

In the past few years several authors have reported the use of the lipase from *Rhizomucor miehei* for hydrolysis or ester synthesis (Miller et al., 1988). Many of the enzyme-catalyzed processes were performed in organic solvents (Bloomer et al., 1992; Chulalaksananukul et al., 1992; Knez et al., 1990; Zaks and Klibanov, 1985) or in almost nonaqueous media without any solvent for immobilized *R. miehei* lipase (Ergan et al., 1990; Habulin and Knez, 1993; Pioch et al., 1991), continuous or batchwise or in a membrane reactor (Habulin and Knez, 1991; Luck and Bauer, 1989, 1991). A relatively new technique to obtain pure products and to integrate the separation process is the use of supercritical fluids as solvents. Several authors have shown the advantages of enzymatic catalysis in supercritical carbon dioxide (Adschiri et al., 1992; Cernia et al., 1994; Chi et al., 1988; Dumont et al., 1993; Erickson et al., 1990; Hammond et al., 1985; Knez and Habulin, 1994; Marty et al., 1992; Nakamura et al., 1986).

The study of lipase-catalyzed oleic acid esterification by oleyl alcohol in almost nonaqueous media without a solvent is presented in this work. The product is an analogue of jojoba oil, the use of which has increased in the past few years. Jojoba oil applications include cosmetics, pharmaceuticals, food additives, and high-pressure lubricants. Because of the problem of how to obtain a great amount of jojoba oil and to run at the same time an economical process, our aim was to use cheaper raw materials and develop a simple synthesis process.

The same synthesis has already been done by other authors (Martinez et al., 1988; Sánchez et al., 1992) with zeolite and cobalt chloride as catalysts and by Garcia et al. (1993), who used *R. miehei* lipase as a catalyst. The energy of activation and the constant of the deactivation process of the enzyme at temperatures above 50 °C were calculated. It was reported that alcohol inhibits enzyme-catalyzed esterification by disturbing the water bound to the enzyme (Mukesh et al., 1993). Therefore, special attention was given to esterification of oleic acid with various alcohols in a solvent-free system.

The integration of separation processes and synthesis of the product is the main interest of recent research in chemical and biochemical engineering. Biochemical reactions give the conversion of substrates up to 90%. By using supercritical fluids, it should be possible to integrate synthesis and separation of the products and nonreacted substrates. Therefore, enzyme preparation was tested for stability under high pressure. Because no obvious changes were observed in enzyme activity at supercritical conditions, some experiments in the batch reactor under high pressure were also performed.

MATERIALS AND METHODS

Reagents. *Enzyme Preparation*. The enzyme preparations lipozyme IM and palatase 1000 L were kindly donated from Novo Nordisk AS (Copenhagen, Denmark). Lipozyme IM is a *R. miehei* lipase, immobilized on a macroporous anion exchange resin. Palatase 1000 L is a lipase from *R. miehei* in a water-soluble form.

Lipase Activity. Lipase activity was measured by Novo Nordisk AS. The activity of lipozyme used for our syntheses was 42 batch interesterification units (BIU) g. One BIU corresponds to 1 μmol of palmitic acid incorporated in triolein/min at standard conditions (Preliminary Product Information, 1985).

The activity of the palatase preparation was 1000 lipase unit (LU)/g. One LU is the amount of enzyme that liberates 1 μmol of butyric acid/min from a tributyrin substrate at standard conditions.

Chemicals. Alcohols were supplied by Aldrich Chemical Co. (Milwaukee, WI) [1-propanol (99%), 1-butanol (99%), 1-hexanol (98%), 1-octanol (99%), 1-decanol (99%), 1-tetradecanol (97%), 1-hexadecanol (99%), oleyl alcohol (85%)]. Oleic acid (extra pure, BP, Ph Helv, NF) was purchased from Merck (Darmstadt, Germany). All other chemicals were from Kemika (Zagreb, Croatia). Carbon dioxide was 99.97% volume pure and was supplied by Linde Plin (Linde, Celje, Slovenia).

Procedure. *Analytical Method*. For determination of the oleic acid amount in the reaction mixture a volumetric method was used (Leitgeb and Knez, 1990). First, 0.1 g of sample of reaction mixture was diluted in 20 mL of 0.1 wt % phenolphthalein solution in ethanol and titrated with NaOH. Then the amount of free fatty acid was calculated.

Syntheses of Oleic Acid Esters in a Batch Stirred Tank Reactor (BSTR). Solvent-Free System. Each reaction mixture contained 47 mmol of oleic acid, 47 mmol of alcohol, and 1.04 g of enzyme preparation (lipozyme IM). The mixture was

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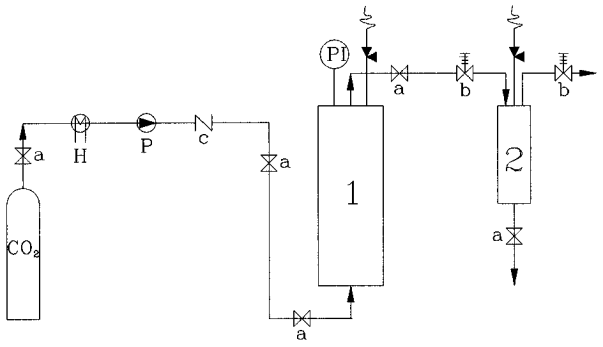

1....reactor
2...separator
P...high pressure pump
PI..pressure indicator
a....valves
b....micrometering valves
c....one way valve
H...heat exchanger

Figure 1. Apparatus for batch esterification at high pressure.

stirred in a 250 mL round-bottom flask with a magnetic stirrer and heated to the desired temperature in a water bath. At defined time periods samples were taken from the reaction mixture and analyzed.

Each result is the average of a minimum of three measurements.

Study of the Enzyme Activity under High Pressure. For this purpose lipozyme IM was placed in an autoclave (Andreas Hofer, Mülheim, Germany; operating pressure, 500 bar; maximum temperature, 100 °C; volume, 500 mL) and kept at supercritical conditions for a defined time. Then batch experiments in a solvent-free system (synthesis of oleyl oleate) were performed with fresh lipozyme and lipozyme that had been exposed to high pressure.

Synthesis of Oleyl Oleate in a BSTR. Supercritical System. The design of the apparatus is shown in Figure 1. The volume of the reactor is 150 mL; the reactor is designed to operate to a pressure of 500 bar. The reactor was shaken via a reciprocal/linear oscillating device, with frequency of 60/min. The whole system was placed in a constant-temperature bath.

First, the reaction mixture, which contained 25 mmol of oleic acid and 25 mmol of oleyl alcohol, was pumped into the reactor. Then, 0.5 g of enzyme preparation (lipozyme IM or palatase 1000 L) was added. Finally, dry CO_2 was pumped into the reactor up to the desired pressure. The solubilities of substrates in carbon dioxide were measured, and the initial concentration never exceeded its solubility limit in gas (Škerget et al., 1995). During the reaction samples were taken out of the reactor and analyzed.

RESULTS AND DISCUSSION

Esterification of Oleic Acid in a BSTR. Solvent-Free System. *Influence of Enzyme/Substrate Mass Ratio on Initial Reaction Rates and Equilibrium Conversion.* For the synthesis of oleyl oleate the influence of different amounts of added immobilized lipase preparation (lipozyme IM) on the reaction rate was studied. From Figure 2 it is obvious that initial velocities increase with greater enzyme amount and at a certain level of enzyme amount they remain constant. The time to reach a certain degree of conversion after 7 h of reaction time decreases with the increased enzyme/substrate mass ratio (Figure 3). Therefore, the decision was made to take 0.05 g of enzyme preparation/g of reaction mixture for further experiments.

Temperature Influence on Equilibrium Conversion and Initial Reaction Rates. Esterification of oleic acid with various alcohols (1-propanol, 1-butanol, 1-hexanol, 1-octanol, 1-decanol, 1-tetradecanol, 1-hexadecanol, and oleyl alcohol) has been performed at various temperatures. As observed in our previous work on the synthesis of *n*-butyl oleate (Leitgeb and Knez, 1990), the increase of the temperature increases the equilibrium conversion. This effect is greater for the esterification with alcohols of higher molar mass and greatest with the use of oleyl alcohol (Figure 4). The explanation is probably due to the diffusion limitations at low temperatures for larger molecules in the porous enzyme carrier. Initial reaction rates were also determined. The highest value was found to be at a temperature of 50 °C (for all alcohols). With decreasing temperature the reaction rates decrease (Figure 5). They are higher by a factor of 1.3 between 20 and 50 °C with each 10 °C rise in temperature for 1-butanol, 1-hexanol, 1-octanol, and 1-decanol used as substrates and by a factor of 2 for oleyl alcohol.

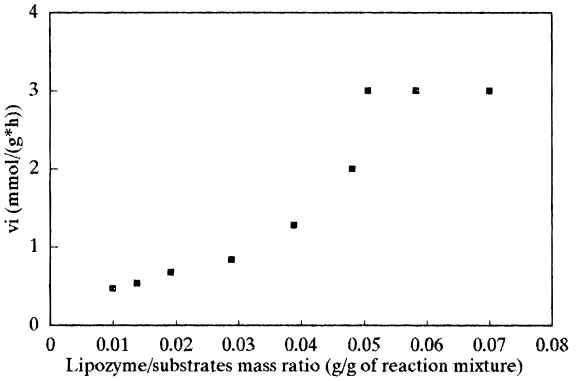

Figure 2. Initial reaction rates for the synthesis of oleyl oleate are influenced by lipase/substrate mass ratio. Reaction mixtures consisted of 47 mmol of oleic acid, 47 mmol of oleyl alcohol, and various amounts of lipozyme. Temperature was kept at 60 °C.

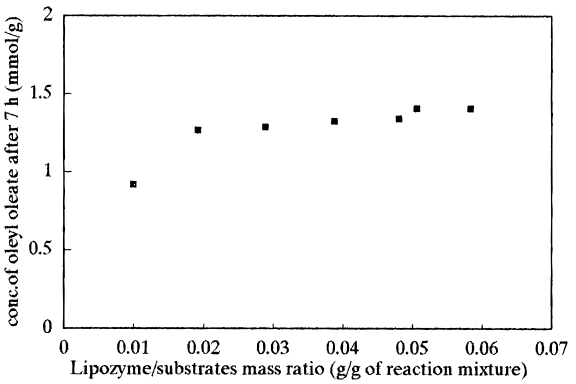

Figure 3. Conversion after 7 h depends on lipase/substrate mass ratio. Reactions were performed at 60 °C. Reaction mixtures consisted of 47 mmol of oleic acid, 47 mmol of oleyl alcohol, and various amounts of lipozyme.

At temperatures higher than 50 °C thermal deactivation of the enzyme occurs, which was not mentioned in the paper by Garcia et al. (1993). Lipozyme IM at 50 °C exists in equilibrium between inactive and active forms. The constant of this process (K_d) was found to be 2.8.

Influence of the Alcohol Molar Mass on Initial Velocities and Equilibrium Conversion. As can be seen from Figure 6, reaction rates (at the same temperature) for the esterification of oleic acid with propanol are higher than those for the esterification by alcohols with larger molecules. It is obvious that the molar mass of the alcohol influences the diffusion rates. Reaction rates are almost linearly dependent on the alcohol molar mass with the number of carbon atoms in the alcohol from 3 to 10. Alcohols with higher molar mass do not affect

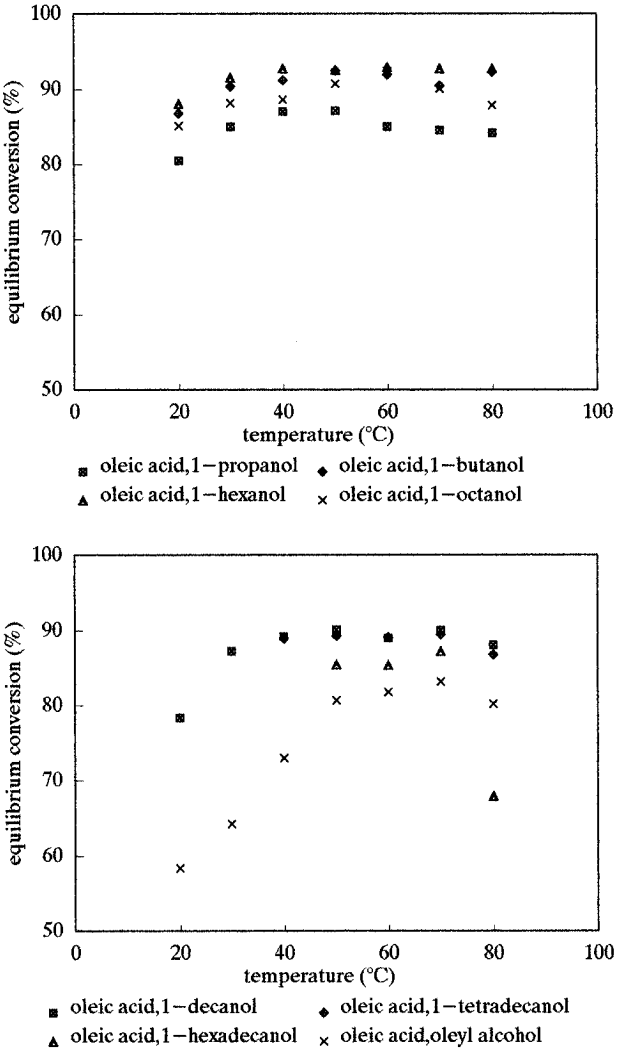

Figure 4. Temperature influences the equilibrium conversion of esterification of oleic acid with various alcohols. Substrates (47 mmol of each) and 1.05 g of lipozyme were stirred with a magnetic stirrer and thermostated at various temperatures.

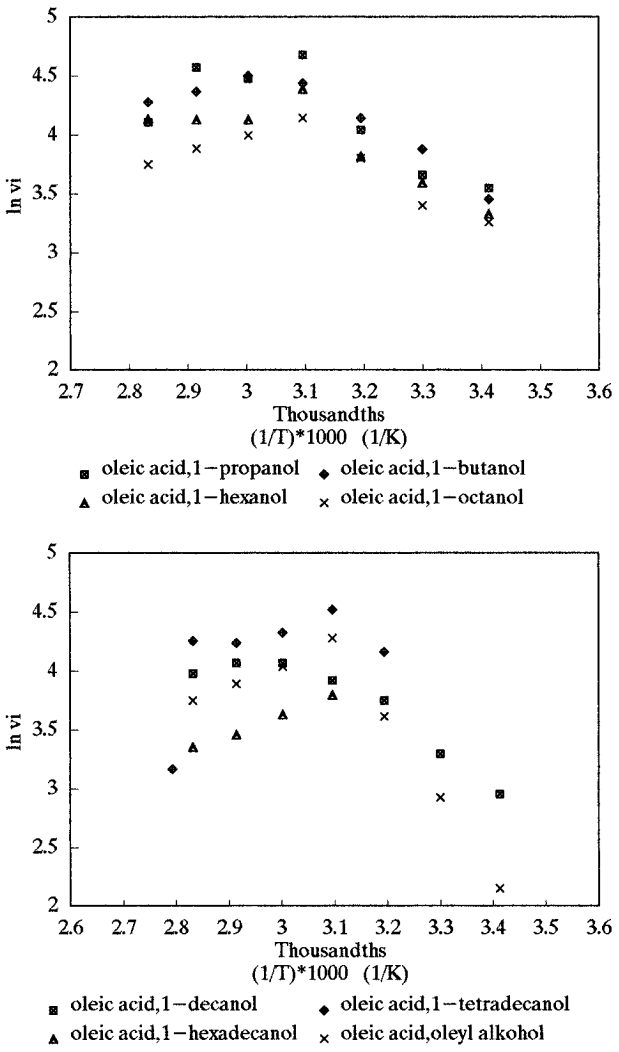

Figure 5. Initial reaction rates of esterification of oleic acid with various alcohols are temperature dependent. Reactions were performed in a batch stirred reactor. Reaction mixture consisted of 47 mmol of oleic acid, 47 mmol of alcohol, and 1.05 g of lipozyme.

the rates of reaction much more than decanol. Synthesis of oleyl oleate is somewhat faster, probably due to the favorable transport properties (viscosity, diffusivity, surface tension) of oleyl alcohol compared to those of the other applied alcohols.

Energies of activation increase with higher alcohol molar mass. As shown in Figure 7, they are linearly dependent on alcohol molar mass. This dependence is valid for butanol, hexanol, octanol, and decanol. For tetradecanol and hexadecanol, energies of activation were not determined. These two alcohols are solid; therefore, without a solvent, reaction could not proceed between 20 and 40 °C.

As can be seen from Figure 8, equilibrium conversion for propanol as the substrate is lower than for butanol at the same temperature. With further increase of the alcohol molar mass final conversion slightly decreases. Besides the influence on the diffusion rates, alcohol chain length (molar mass) also influences the enzyme activity.

Enzyme Deactivation under High Pressure. Many authors have reported testing enzyme stability at high pressure (Erickson et al., 1990; Marty et al., 1992; Miller et al., 1991; Nakamura, 1990; Van Eijs et al., 1988). From these studies it is obvious that lipases are the most stable enzymes. For lipase activity determination experiments were made only below 300 bar. Therefore, lipozyme was exposed to carbon dioxide at 300 bar and 40 °C for 4 and 24 h. As can be seen from Figure 9, batch experiments with fresh enzyme and enzyme that was kept at supercritical conditions showed that there is almost no loss of lipase activity in the supercritical media.

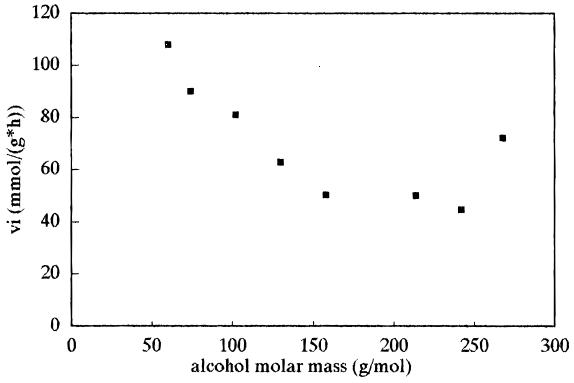

Figure 6. Initial reaction rates at 50 °C are influenced by the alcohol molar mass.

Synthesis of Oleyl Oleate in a BSTR. Supercritical System. Esterification of oleic acid with oleyl

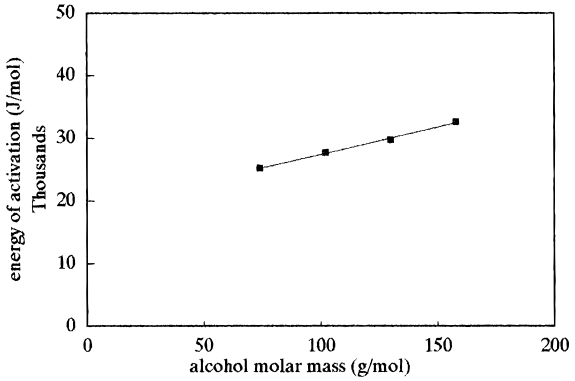

Figure 7. Energies of activation are linear dependent from alcohol molar mass.

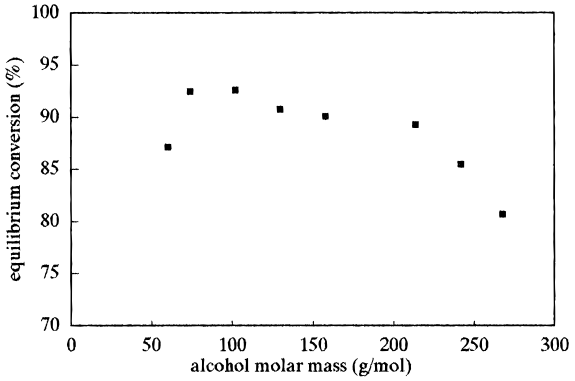

Figure 8. Alcohol molar mass influences equilibrium conversion. Reactions were performed at 50 °C in a batch stirred tank reactor.

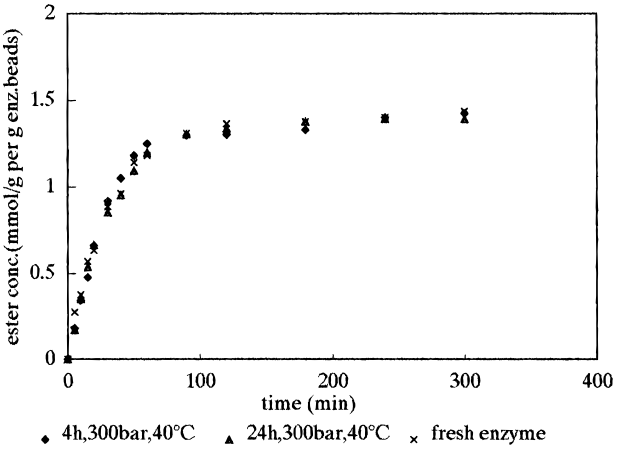

Figure 9. Synthesis of oleyl oleate in a solvent-free system with fresh lipase preparation, and lipase preparation previously exposed to high pressure. Reactions were performed at 60 °C and atmospheric pressure.

alcohol at high pressure was catalyzed with lipozyme and with palatase (Knez and Habulin, 1994). The reaction rates for the synthesis catalyzed with lipozyme were higher than for the synthesis in the solvent-free system (Table 1). The highest value was found for the performance near the critical point of carbon dioxide (31 °C and 84.5 bar). At higher pressure at the same temperature reaction rates decrease.

Water solubility in supercritical carbon dioxide increases with higher pressure above the critical point of CO_2, so supercritical carbon dioxide tends to strip away the layer of essential water from the enzyme (Dumont et al., 1992), leading to lower yields of reaction at high pressure.

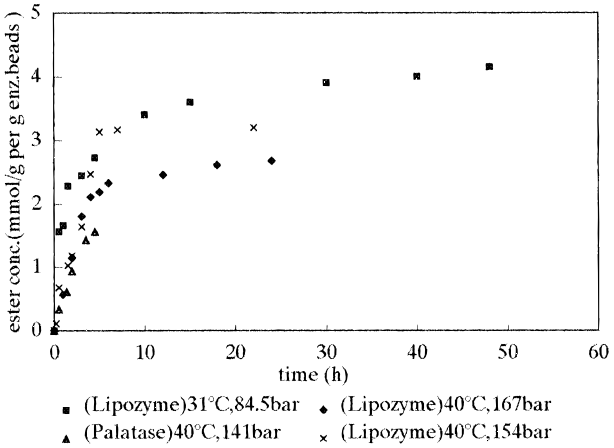

Figure 10. Oleyl oleate concentration rise with time for the syntheses catalyzed with lipozyme IM and palatase 1000L at different reaction conditions.

Table 1. Initial Reaction Rates in a Batch Stirred Tank Reactor for the Synthesis of Oleyl Oleate

synthesis of oleyl oleate	reaction conditions	initial reaction rate [mmol/(g·h)]
solvent-free system	30 °C, 1 bar	0.465
	40 °C, 1 bar	0.625
high-pressure system	31 °C, 84.5 bar	1.428
	40 °C, 154 bar	1.025

For the reaction catalyzed with palatase the initial velocity was the lowest.

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Received for review March 23, 1995. Revised manuscript received August 2, 1995. Accepted September 29, 1995.[⊗]

JF950163K

[⊗] Abstract published in *Advance ACS Abstracts,* November 15, 1995.