# Effect of Pressure on an Enzymatic Reaction in a Supercritical Fluid

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Three different authors have reported on the use of four different enzymes in supercritical fluids. Nakamura *et al.* (1986) have demonstrated that lipase carries out transesterification reactions in the presence of supercritical carbon dioxide. Hammond *et al.* (1985) have shown that polyphenyl oxidase is active in supercritical $CO_2$ and fluoroform. Randolph *et al.* (1985) have shown that alkaline phosphatase and cholesterol oxidase are active in supercritical $CO_2$. More recently, Randolph *et al.* (1988) have examined the effect of aggregation of cholesterol on cholesterol oxidase activity in $CO_2$ using electron paramagnetic resonance (EPR). They found that when cosolvents which promoted aggregation were added, the reaction rate increased in proportion to the amount of aggregation. To date, no data on the effect of pressure on reaction rate have been presented.

The objective of this work is to determine whether pressure-induced changes in the physical properties of a supercritical fluid solvent affect the rate of an enzymatic reaction and if so, which properties are responsible for the change.

The enzyme used in this work is lipase from *Rhizopus arrhizius,* which catalyzes the transesterification of triglycerides in the presence of free fatty acids and low water activity. It is not specific for any particular triglyceride, but only acts on the fatty acid residues at the 1 and 3 position of the glycerol backbone. Thus, the 2-substituted product is not formed. This leaves only two products, the 1-(or 3-) and 1,3-substituted triglycerides. A microbial lipase was chosen because it does not require cofactors and because different batches have a more consistent activity than a tissue-derived enzyme such as porcine pancreatic lipase.

The reactants are trilaurin (LLL) and palmitic acid and the products are 1,2-dilauryl-3-palmitoyl-rac-glycerol (PLL) and 1,3-dipalmitoyl-2-lauryl-rac-glycerol (PLP). Since a small amount of water is present, the diglycerides 1,2-dilauryl-glycerol (LL) and 1-palmitoyl-2-lauryl-glycerol (PL) are formed as well. Carbon dioxide was chosen as the supercritical fluid because it is nontoxic, nonflammable, and inexpensive. It has a critical temperature of 31°C and a critical pressure of 7.28 MPa.

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## Apparatus and Procedure

The enzyme was immobilized on Hyflo Supercel by dissolving 100,000 units (Calbiochem units) of purified lipase (specific activity: 4,509 U/mg) from *Rhizopus arrhizius* (Calbiochem) in 11 mL of reagent grade water and then adding 5 g diatomaceous earth (Hyflo Supercel from Manville) to the enzyme solution to make a wet paste. The paste was placed in a vacuum chamber at room temperature overnight to dry, and stored at 4°C until used.

The reaction experiments were carried out batchwise in the 50-mL autoclave shown in Figure 1. The experiment proceeded as follows: first, 100 mg of the enzyme-containing Hyflo Supercel was hydrated with reagent grade water (10% v/w) and allowed to stand in a closed vial in the refrigerator for 2 to 3 hours so that the water was distributed evenly. Then, 100 mg of the hydrated catalyst, 25 mg of Hyflo Supercel hydrated with 75% (v/w) water, and substrate (LLL and palmitic acid) were charged to the reactor. The wet Hyflo Supercel was added to saturate the $CO_2$ with water, so that the enzyme would not be dehydrated. The initial concentration of a reactant never exceeded its solubility limit in $CO_2$ based on our previous solubility studies (Bamberger *et al.*, 1988). Next, $CO_2$ that had been dried over a molecular sieve was introduced to the reactor, temperature and stirring control activated, and more $CO_2$ pumped into the reactor until the desired pressure was reached.

After one hour, the reaction was terminated by depressurizing the reactor, causing reactants and products to precipitate. A U-tube fitted with glass wool in the downstream end was connected to the depressurization valve to collect any material soluble in the $CO_2$. The reactor and U-tube were rinsed with chloroform to collect all reaction products.

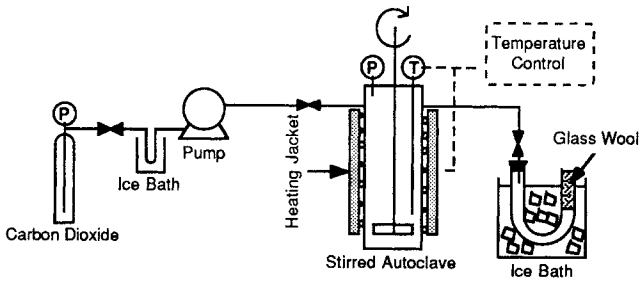


**Figure 1. High-pressure reaction apparatus.**

Reaction products were analyzed by capillary gas chromatography after silation with (N,O)-bis(trimethyl silyl) trifluoroacetamide (BSFTA). The conversion of LLL to PLL was usually 10% ± 5%.

## Control Experiments

Before the effect of physical properties of the SCF on reaction rate could be studied, several control experiments were performed. First, the hydrated immobilized enzyme was exposed to $CO_2$ at 15 MPa and 40°C in the presence of LLL and palmitic acid for 18 h. The enzymatic activity was measured by the rate of transesterification of LLL by palmitic acid in hexane. The activity of the $CO_2$-exposed enzyme was the same as that of the unexposed enzyme.

In order to prove that the reaction was catalyzed by the enzyme, the reactor was loaded with LLL, palmitic acid and wet celite, and pressurized to 15 MPa. After two days, there was no detectable conversion.

Using a typical diffusion constant for solutes in supercritical $CO_2$ ($10^{-8}$ $m^2/s$ from Debenedetti, 1984) the Thiele modulus was $10^{-2}$ and the Damkohler number, which is the ratio of the characteristic reaction rate to the characteristic external diffusion rate, was $10^{-5}$. These results give external and internal effectiveness factors of unity, which means that mass-transfer effects can be neglected.

## Effect of Reactant Concentration on Rates at 15 MPa

Initial transesterification rates were measured in $CO_2$ at 15 MPa and 40°C. Data for rate of PLL appearance ($v_p$) as a function of [LLL] at constant [P] are plotted as the points in double reciprocal form in Figure 2. Since straight lines are obtained, the data fit Michaelis-Menten kinetics at constant [P]. The slopes of the lines in Figure 2 varied linearly with [P], so they were fit to a straight line. The intercepts were not easily fit to a function, so they were interpolated. The resulting model is presented as the lines in Figure 2. This model is strictly empirical but will be useful in explaining the effect of pressure on the reaction rate.

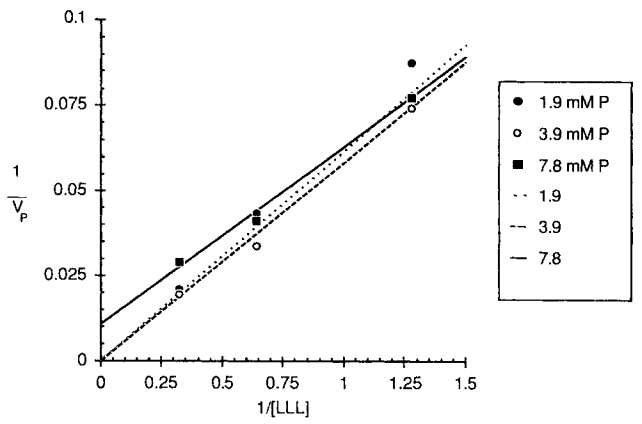


**Figure 2. Double reciprocal plot of reaction rate in $CO_2$ at 40°C and 15 MPa vs. [LLL]**

Points = experimental data; lines = empirical rate law.

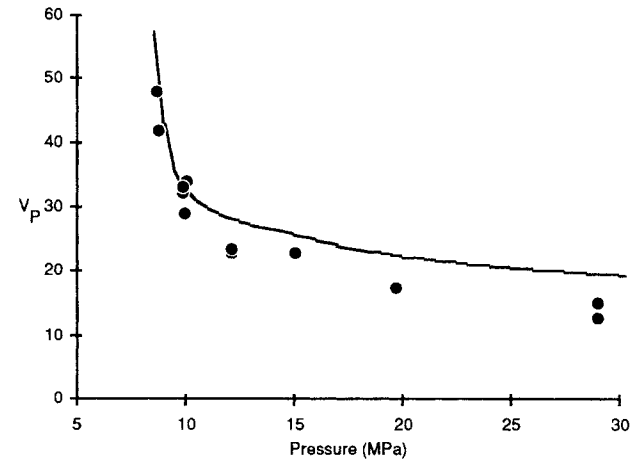


**Figure 3. Effect of pressure on reaction rate in supercritical $CO_2$ at 40°C**

Points = experimental data; line = model based on mole fraction of reactants.

## Effect of Pressure on Reaction Rate

In all the experiments described in this section, a single concentration of substrates (1.6 mM LLL and 2 mM palmitic acid) was used. The effect of pressure on $v_p$ is shown by the points in Figure 3. There is a strong effect of pressure on $v_p$, especially as the critical pressure of 7.28 MPa is approached.

Reactions were carried out in ethane as well as $CO_2$. Ethane is not acidic and would therefore not change the reaction rate by changing the pH of the active site. Ethane has a critical temperature (32°C), close to that of $CO_2$ (31°C). The critical pressures are different, so the data are compared on the basis of density in Figure 4. The $v_p$ points for ethane and $CO_2$ all fall along the same line, except at the lowest densities which are near the criti-

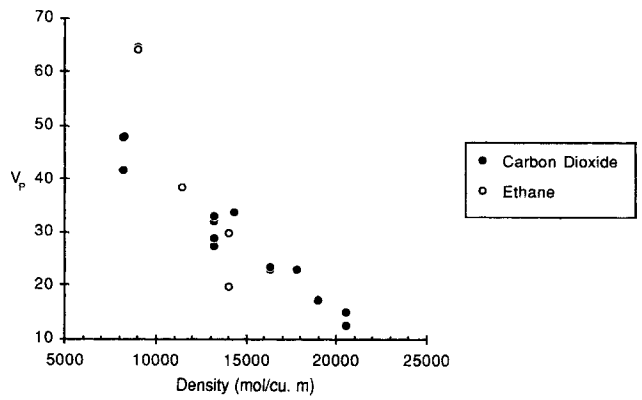


**Figure 4. Reaction rate vs. density for both $CO_2$ and ethane at 40°C.**

cal points of the solvents. Since the same trend is observed with ethane and $CO_2$, the decrease of rate with pressure does not appear to be caused by a pH effect. The effect of pressure on condensed phases is slight (Prausnitz, 1965). Therefore, the changes in reaction rate are probably due to changes in the SCF phase rather than to changes in the immobilized enzyme phase.

The reaction rate is obviously not proportional to the reactant concentration because all the reactions reported in Figure 3 were at the same concentration, but the reaction rate was different. Another explanation of the pressure effect is that pressure affects the way the reactants partition between the SCF phase and the enzyme. In order to quantify this hypothesis, fugacity coefficients for the reactants were estimated from the Peng-Robinson equation of state (EOS). The parameters in the EOS were determined from vapor pressure and liquid density data for the pure components, and from solubility measurements of fatty acids and triglycerides in $CO_2$ (Bamberger *et al.*). The fugacity coefficients change by orders of magnitude over the range of pressures discussed here, so one might expect very large changes in the calculated reaction rates. This is indeed the case and when fugacities were used in the rate expression, no fit of the data could be obtained.

It was found that the effect of pressure on the reaction rate could be modeled by using the mole fractions of the reactants in the rate law. In order to convert molar concentrations (on which the rate law is based) to mole fractions, it is necessary to multiply by the molar volume of the SCF. The rate law for 15 MPa is of the form:

$$v_p = f([\mathrm{LLL}],[\mathrm{P}]) \tag{1}$$

to extend this formula to other pressures, multiply the concentrations by the molar volume, $V$, at the appropriate pressure, and divide by the molar volume at 15 MPa ($V_{15}$).

$$v_p = f\left(\frac{[\mathrm{LLL}] \cdot V}{V_{15}}, \frac{[\mathrm{P}] \cdot V}{V_{15}}\right) = f\left(\frac{x_{\mathrm{LLL}}}{V_{15}}, \frac{x_{\mathrm{P}}}{V_{15}}\right) \tag{2}$$

This rate law is plotted as the line in Figure 3 and uses parameters determined at one pressure to predict rates at other pressures using no additional parameters.


## Acknowledgment

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## Notation

[LLL] = trilaurin concentration
[P] = palmitic acid concentration
$v_p$ = rate of palmitic acid incorporation into triglyceride
$V$ = molar volume
$x$ = mole fraction

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