Lipase-Catalyzed Hydrolysis of Canola Oil in Supercritical Carbon Dioxide

Karamatollah Rezaei and Feral Temelli*

Department of Agricultural, Food, and Nutritional Science, University of Alberta, Edmonton, Alberta, T6G 2P5 Canada

ABSTRACT: The effect of pressure, temperature, and CO_2 flow rate on the extent of conversion and the product composition in the enzyme-catalyzed hydrolysis of canola oil in supercritical carbon dioxide ($SCCO_2$) was investigated using lipase from *Mucor miehei* immobilized on macroporous anionic resin (Lipozyme IM). Reactions were carried out in a continuous flow reactor at 10, 24, and 38 MPa and 35 and 55°C. Supercritical fluid chromatography was used to analyze the reaction products. A conversion of 63–67% (triglyceride disappearance) was obtained at 24–38 MPa. Mono- and diglyceride production was minimum at 10 MPa and 35°C. Monoglyceride production was favored at 24 MPa. The amount of product obtained was higher at 24–38 MPa due to enhanced solubility in $SCCO_2$. Complete hydrolysis of oil should be possible by increasing the enzyme load and/or decreasing the quantity of the oil substrate. There was a drop in triglyceride conversion over a 24-h reaction time at 38 MPa and 55°C, which may be an indication of loss of enzyme activity. Pressure, temperature, and CO_2 flow rate are important parameters to be optimized in the enzyme-catalyzed hydrolysis of canola oil in $SCCO_2$ to maximize its conversion to high-value products.

Paper no. J9490 in *JAOCS 77*, 903–909 (August 2000).

KEY WORDS: Canola oil, diglycerides, enzyme, glycerol, hydrolysis, monoglycerides, *Mucor miehei*, supercritical.

Supercritical fluids (SCF) have received increasing attention as a medium to conduct enzymatic reactions since high reaction rates can be achieved. Their higher diffusivity and lower viscosity compared to organic solvents make them more attractive as reaction media to transport reactants and products to and from the enzyme. Furthermore, the marked temperature and pressure dependence of their solvent power eases the post-reactional separation of the products. Supercritical CO_2 ($SCCO_2$) with a critical point of 7.4 MPa and 31°C has been used for the extraction and fractionation of lipids (1). Recently, it has been studied as a medium to conduct enzymatic reactions involving lipids. Use of enzymes as catalysts in $SCCO_2$ medium is possible since enzymes have been shown to maintain their activity at pressures as high as 400 MPa (2).

Liu *et al.* (3) synthesized a cocoa butter equivalent using the enzyme-catalyzed interesterification of triglycerides (TG) high in POP (P, palmitate; O, oleate) and POO in $SCCO_2$. Among the five different lipases studied, immobilized lipase from *Mucor miehei* was the most efficient and specific. Erickson *et al.* (4) investigated the effect of pressure-induced changes in the physical properties of $SCCO_2$ on the rate of lipase-catalyzed transesterifications. When studying the lipase-catalyzed hydrolysis of triolein in a batch system, Chi *et al.* (5) reported an increase in the initial rate of the reaction with an increase in water content in both *n*-hexane and $SCCO_2$. They considered water to act as a modifier of the solvent having a 100-fold higher solubility in $SCCO_2$ than in *n*-hexane at 14.7 MPa and 50°C. Glowacz *et al.* (6) studied the hydrolysis of triolein and its partial glycerides in a batch reactor system by porcine pancreas lipase in $SCCO_2$. They showed that the enzyme activity and the enantiomeric ratios of the products were dependent on the water content of the enzyme, substrate, and the reaction time.

High temperatures used in the conventional hydrolysis of fats and oils using the steam-splitting method (>250°C) can lead to oxidation, dehydration, and interesterification of lipids (7). The enzymatic hydrolysis of lipids is performed under much milder conditions with fewer hazards and lower energy consumption. Hydrolysis products of fats and oils can vary depending on the extent of reaction. During a partial hydrolysis, a mixture of mono- and diglycerides (MG and DG, respectively) and free fatty acids (FFA) is obtained, whereas a full hydrolysis results in the production of glycerol and FFA. MG and DG are traditionally produced through glycerolysis of fats and oils and are important ingredients used in numerous food and nonfood products for emulsification purposes. FFA and glycerol are used in the manufacture of many products such as detergents, cosmetics, surfactants, and pharmaceuticals.

The literature lacks information on the enzymatic hydrolysis of vegetable oils in a SCF medium. In addition, studies conducted using a continuous flow reactor packed with an immobilized enzyme bed are quite limited. Therefore, the objective of this study was to investigate the effect of pressure, temperature, and CO_2 flow rate on the overall conversion and product composition in the lipase-catalyzed hydrolysis of canola oil in $SCCO_2$ using a continuous flow reactor.

MATERIALS AND METHODS

Materials. *Mucor miehei* lipase immobilized on macroporous anionic exchange resin, Lipozyme IM, was kindly provided by Novo Nordisk (Franklinton, NC). The granular Lipozyme

*To whom correspondence should be addressed.
E-mail: feral.temelli@ualberta.ca.

IM has a particle size of 0.2–0.6 mm and a moisture content of 2–3% (w/w). Lipozyme IM can be used in the temperature range of 30–70°C. It can maintain its declared activity for 1 yr if it is stored at 5°C, but for 3 mon only at 25°C. Enzyme was stored at 5°C until used in the experiments.

Canola oil was obtained from a local grocer. All lipid standards were purchased from Sigma Chemical Co. (Oakville, Ontario, Canada) with purities of ≥99%. Diethyl ether, high-performance liquid chromatography (HPLC)-grade (99.9%), and hexadecane (99%) were obtained from Aldrich Chemical Co. (Milwaukee, WI); petroleum ether, Optima from Fisher Scientific (Fair Lawn, NJ); CO_2 as reaction medium, bone dry (99.8%); hydrogen, nitrogen, and helium, ultra high purity grade (99.999%) and air, United States Pharmacopoeia grade (19.5–23.5% O_2), from Praxair (Mississauga, Ontario, Canada) and CO_2 as mobile phase in SFC, SFC/super critical fluid extraction grade (99.9999%), was from Air Products (Allentown, PA).

Experimental set-up and design. A laboratory-scale supercritical extraction system (Newport Scientific, Inc., Jessup, MD) was modified for use as a continuous flow reactor (Fig. 1). Water was introduced to the CO_2 stream using a piston pump (Gilson 305; Gilson, Inc., Middleton, WI) equipped with a manometric module (Gilson 805; Gilson, Inc.) at a rate of 0.004 mL/min. Oil was introduced by another piston pump (Varian 2010; Varian Instruments, Walnut Creek, CA) at a rate of 0.02 mL/min. A small stainless steel reaction cell (15 cm × 13 mm i.d.) was fabricated and inserted into the original extraction cell. To ensure the uniformity of the feed mixture, water, oil, and CO_2 were combined and passed through a mixer prior to entry at the bottom of the reaction cell. The immobilized enzyme beads (1.0 g) were dispersed in the top portion of the reaction cell using 7.9 g glass beads (3.76 mm average o.d.) and the remaining space was filled with glass wool. A thermocouple held within the enzyme bed was used to measure the reaction temperature, which was maintained within ±2°C of the desired temperature with a controller. Pressure was maintained by a back-pressure regulator. The CO_2 flow rate was adjusted manually with the depressurization valve. Reaction product samples were collected in two successive side-armed test tubes held in a cold bath at −20°C over the collection period (0.5–2 h), weighed, and dissolved in a 1:1 (vol/vol) mixture of diethyl ether and petroleum ether for analysis.

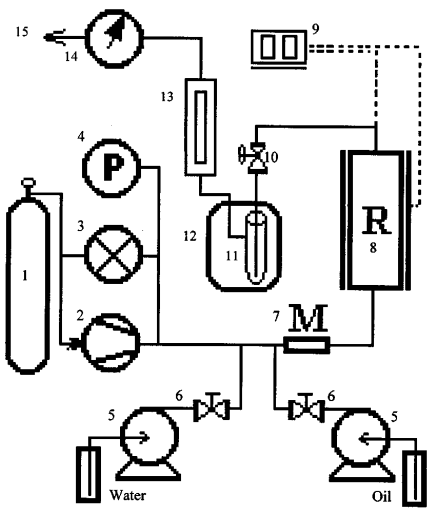

FIG. 1. Schematic of the supercritical reaction system. 1—CO_2 tank, 2—compressor, 3—back-pressure regulator, 4—pressure gauge, 5—high-performance liquid chromatography pumps, 6—on/off valve, 7—mixer, 8—reaction cell, 9—temperature control, 10—depressurization valve, 11—sample collection tube, 12—cold bath, 13—rotameter, 14—gas meter, 15—vent.

CO_2 flow rate was maintained at 3.7 L/min, measured at ambient conditions, except where the flow-rate effect was studied at 1.02 ± 0.02 and 3.71 ± 0.10 L/min, in which case the averages of two runs were reported. Reactions were carried out at 10, 24, and 38 MPa and 35 and 55°C. For each run, a new batch of enzyme was used except where the enzyme stability was studied. In addition, a blank run employing the same water and oil flow rates was conducted with no enzyme at each pressure and temperature condition studied. Reactions were continued for 4–8 h. Although all product samples were collected and analyzed, only samples obtained at ≥4 h were used for comparison of reaction conditions. This was necessary for the product composition to stabilize under steady CO_2, oil, and water flow rates at constant temperature and pressure.

Enzyme stability. Enzyme stability was examined by using the same enzyme batch for three consecutive runs of 8 h each at 38 MPa and 55°C. The system was depressurized after each run, but the enzyme was left in the reaction cell at room temperature. A 5- and a 2-d time lapse were between the runs. Leakage of the enzyme from the reactor was not expected throughout the runs and depressurization steps since the enzyme is specified by the manufacturer to be strongly adsorbed to the anionic exchange resin.

Analysis of reaction products. A supercritical fluid chromatography unit (SFC/GC Series 600; Dionex, Mississauga, Ontario, Canada) equipped with a fused-silica column (10 m × 50 μm i.d.) with 0.25 μm film (SB-100-methyl silicone) and a timed-split injector was used to analyze the reaction products. The rotor capacity in the injector was 500 nL. Injection time was varied between 0.5–4.0 s to obtain a consistent response since the amount of product collected at the various conditions of the study was different. The flame-ionization detector (FID) was maintained at 350°C. $SCCO_2$ was the mobile phase. The column temperature and pressure programming were modified from that of Temelli *et al.* (8). Column temperature was held at 100°C for 2 min and then increased to 190°C at a rate of 40°C/min. Column pressure was programmed as follows: held at 12.2 MPa for 2 min, ramped (I) at 0.5 MPa/min to 15.2 MPa and held for 5 min, ramped (II) at 1.5 MPa/min to 17.2 MPa and held for 3 min, ramped (III) at 1.5 MPa/min to 22.8 MPa and held for 1 min, ramped (IV) at 45.1 MPa/min to 27.4 MPa and held for 3 min, and ramped (V) at 4.1 MPa/min to 31.5 MPa and held for 10 min. The Dionex Al-450 Chromatography Automation Software Release 3.32 was used to collect and analyze the data. Successive runs were ≥1 h apart.

Oleic acid, mono-, di-, and triolein were used as standards for FFA, MG, DG, and TG, respectively. Hexadecane was used as internal standard (IS). Standard solutions containing 5, 10, and 20 mg/mL of standards and IS were prepared. Triplicate injections of each standard solution were made, and the detector response of each component in the standards was normalized to its equivalent concentration in the original solution. Relative standard deviation (RSD) in a standard mixture of FFA, MG, DG, and TG was determined as 4.0, 4.5, 2.9, and 2.5%, respectively. Relative response factors of FFA, MG, DG, and TG were determined by dividing the normalized responses by that of IS in each chromatogram. Since relative response factors should be independent of injection volume and solute concentrations (9), a mean relative response factor for the triplicate injections of all concentrations of each species was used in calculations, which was 0.80 ± 0.02, 0.81 ± 0.02, 0.73 ± 0.02, and 0.96 ± 0.02, for FFA, MG, DG, and TG, respectively. For the quantification of reaction product samples, peak area counts of FFA, MG, DG, and TG were divided by their respective relative response factors and normalized to 100% (w/w). To determine the molar ratios of FFA, MG, DG, and TG, the average molecular weights of 280, 356, 617, and 880 a.m.u., respectively, based on canola oil fatty acid composition data provided by Canola Council of Canada (10), were used.

Terminology. The terms conversion and production, defined as follows were used to characterize the reaction: Conversion was used to indicate the fraction of original TG consumed in the reaction. To determine the conversion, moles of each of MG, DG, TG, and glycerol in the product at any given time were measured and normalized to 100%, since based on the reaction stoichiometry (Eqs. 1–4) each molecule of TG can only be converted to any one of DG, MG, or glycerol and any further breakdown of the products is not anticipated in the CO_2 environment.

$$\text{Step 1:} \quad TG + H_2O \rightarrow DG + FFA \qquad [1]$$

$$\text{Step 2:} \quad DG + H_2O \rightarrow MG + FFA \qquad [2]$$

$$\text{Step 3:} \quad MG + H_2O \rightarrow \text{glycerol} + FFA \qquad [3]$$

Overall reaction:

$$TG + 3\ H_2O \rightarrow \text{glycerol} + 3\ FFA \qquad [4]$$

Then, the mole fraction of TG (f_{TG}) was subtracted from unity.

$$\text{conversion} = 1\text{-}f_{TG} \qquad [5]$$

The term Production was used to quantify the formation of individual products, i.e., MG, DG, glycerol, or FFA at a given time and a specific set of conditions. The relative weight of each component found in both collection tubes of each run was reported as production.

Glycerol quantification. Since the amount of glycerol produced in the reaction could not be measured directly with SFC analysis, its relative quantity was calculated based on overall moles of FFA formed along with the production of each of DG, MG, and glycerol. The equivalent moles of FFA produced for Equation 1 ($n_{FFA,\,DG}$) is equal to the number of moles of DG present in each product mixture (n_{DG}). Therefore, $n_{FFA,DG} = n_{DG}$. Similarly, based on reaction stoichiometry, the following expressions can be obtained: $n_{FFA,\,MG} = 2\ n_{MG}$ and $n_{FFA,\,Glycerol} = 3\ n_{Glycerol}$, where $n_{FFA,\,MG}$ and $n_{FFA,\,Glycerol}$ are the number of moles of FFA equivalent to the moles of MG (n_{MG}) and glycerol ($n_{Glycerol}$), respectively. Therefore, the total FFA present in the product mixture ($n_{FFA,\,total}$) can be expressed as

$$n_{FFA,\,total} = n_{DG} + 2\ n_{MG} + 3\ n_{Glycerol} \qquad [6]$$

from which the relative moles of glycerol can be calculated.

RESULTS AND DISCUSSION

Analysis of the canola oil used as the substrate for the hydrolysis reaction showed only TG peaks. A maximum of 4.8% (mol%) hydrolysis was observed in the blank runs carried out at different pressure and temperature conditions with no enzyme, which was negligible compared to the enzymatic runs. A typical SFC chromatogram of the reaction products is given in Figure 2. The retention times of FFA, MG, DG, and TG were 10–16, 17–19, 23–27, and 27–31 min, respectively. The wider peaks of FFA and TG indicate that these lipid classes consist of a mixture of different components. On the other hand, the sharper peaks of MG and DG represent the narrower range of product spread within MG and DG. According to the fatty acid composition of canola oil reported by the Canola Council of Canada (10), C_{18} fatty acids make up >90% of the total fatty acids in canola oil. It is possible that the MG and DG fractions contain C_{18} unsaturated fatty acids only. However, this needs to be confirmed by determining the fatty acid composition of each of the FFA, MG, DG, and TG fractions.

Enzyme stability in $SCCO_2$. Changes in product composition over a 24-h hydrolysis period at 38 MPa and 55°C are given in Figure 3. The high DG content in the product is an indication of a low extent of hydrolysis since DG is the first intermediate product of the TG hydrolysis. A higher concentration of FFA at the start of each 8-h run is due to the start-up

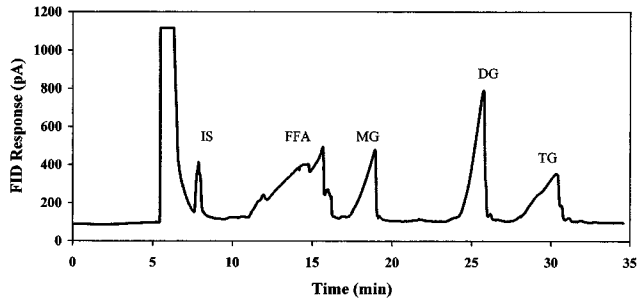

FIG. 2. Typical supercritical fluid chromatogram of a product mixture from the enzymatic hydrolysis of canola oil in supercritical carbon dioxide ($SCCO_2$); IS, internal standard; FFA, free fatty acids, MG, monoglycerides; DG, diglycerides; TG, triglycerides.

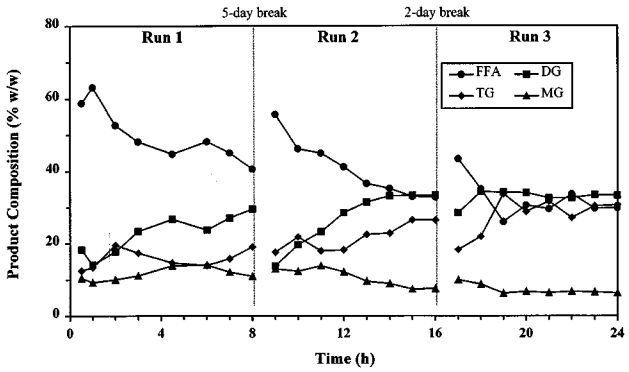

FIG. 3. Change in the product composition in the lipase-catalyzed hydrolysis of canola oil in $SCCO_2$ at 38 MPa and 55°C over the course of a 24-h interrupted hydrolysis. See Figure 2 for abbreviation.

time for the system to reach the operating conditions during which residual oil in the lines was in contact with the enzyme. Therefore, composition at ≥4 h was considered. The 5- and 2-d delays in between the runs did not seem to affect the enzyme since there was a smooth transition in product compositions from one run to the next excluding the 4-h start-up delay. The decline in TG consumption and the decrease in FFA and MG production throughout the 24-h run indicated a decrease in the conversion as the reaction proceeded. This is consistent with the results of Marty *et al.* (11), who used *M. miehei* lipase immobilized on a macroporous anionic resin and reported a 10% loss in the enzyme activity in both $SCCO_2$ and *n*-hexane after 6 d of exposure to 13 MPa and 40°C. Although the extent of inactivation increased with the number of pressurization–depressurization steps (2), its effect on enzyme activity in this experiment is not anticipated to be significant. Giessauf *et al.* (12) reported that lipase from *Pseudomonas* sp. and lipase from *Candida cylindracea* lost only 36.1% of its activity after 30 pressurization–depressurization steps in 24 h in $SCCO_2$ at 15 MPa and 75°C. In this study, only three pressurization–depressurization steps were involved, and the reaction temperature (55°C) was milder, whereas the pressure was higher (38 MPa) than those applied by Giessauf *et al.* (12). It has been reported that such moderate pressures do not cause enzyme inactivation (13).

Different enzymes exhibit different activity patterns in supercritical media, possibly because of different immobilization supports (14) and different structures. For example, *C. cylindracea* lipase supported on Celite 545 was inactivated at a higher rate than *M. miehei* lipase supported on macroporous anionic resin beads (14). At 13.6 MPa and 40°C, the residual activity of the former was 75% and that of the latter was 85–90% after 7 and 6 d, respectively (14). Chymotrypsin and trypsin can be mentioned to illustrate the effect of structure on enzyme stability. These enzymes contain disulfide bridges and undergo partial inactivation during slow depressurization, ~5–10 min, from 10 MPa after exposure to $SCCO_2$ (2). However, penicillin amidase, which does not have cysteine, appears to be the least stable during depressurization (2). Giessauf *et al.* (12) reported that hydrolases (lipases and esterases, crude preparations) with disulfide bridges had a lower degree of inactivation compared to an enzyme without cystine after several pressurization–depressurization steps at 15 MPa and 75°C for 24 h. However, thermal stability was not any better. Zagrobelny and Bright (15) reported inactivation during pressurization as well. How Lipozyme IM was affected in this study needs further detailed evaluation.

Effect of CO_2 flow rate on product composition. Effect of CO_2 flow rate on the hydrolysis of canola oil in $SCCO_2$ was studied at 38 MPa and 55°C at CO_2 flow rates of 1.02 ± 0.02 and 3.71 ± 0.10 L/min over 8 h. Figure 4 depicts the changes in glycerol content of the products at the two different flow conditions. The fluctuations in the data within the first 2 h demonstrate the instability in the product composition early in the reaction. The mole fractions of MG and DG were lower at the lower flow rate, indicating further conversion to glycerol. This is evident in the higher fraction of glycerol in the final product at this condition. Thus, the enzyme is capable of fully hydrolyzing TG molecules if enough residence time is provided. TG content of the product was higher at the lower flow rate. This result along with those obtained for MG and DG demonstrated that there was a parallel conversion of MG and DG to glycerol after a certain amount of MG and DG was produced. This was verified by plotting the total FFA production at the two different flow rates for the last 4 h of the reaction where product composition was stabilized (Fig. 5). There was no flow rate effect on the FFA production.

The solubility of canola oil in $SCCO_2$ at different conditions was calculated from the slope of the linear portion of extraction curves by Fattori *et al.* (16). The solubility of canola oil in $SCCO_2$ at 36 MPa and 55°C is 11 mg oil/g CO_2 (16) and is expected to be slightly higher at 38 MPa and 55°C, i.e., conditions of this study. Also, the solubility of water in $SCCO_2$ at 55°C and 40.5 MPa is ~7 mg water/g CO_2 (17), which is close to the condition of this study. Therefore, up to a total of ~40 g oil and ~25 g water at 3.71 L/min CO_2 flow rate and ~11 g oil and ~7 g water at 1.02 L/min CO_2 flow rate would have been dissolved over 8 h if they were supplied in

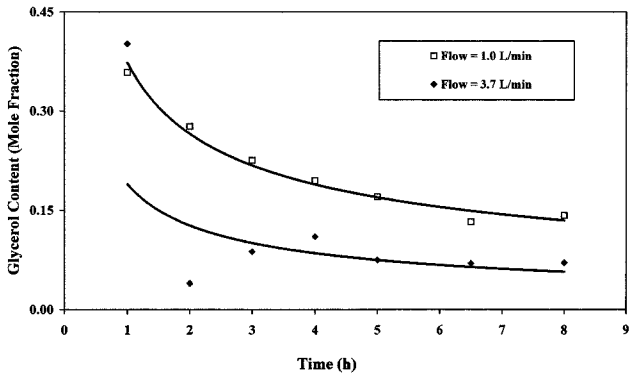

FIG. 4. Flow effect on glycerol content in the continuous hydrolysis of canola oil in $SCCO_2$ at 38 MPa and 55°C. See Figure 2 for abbreviation.

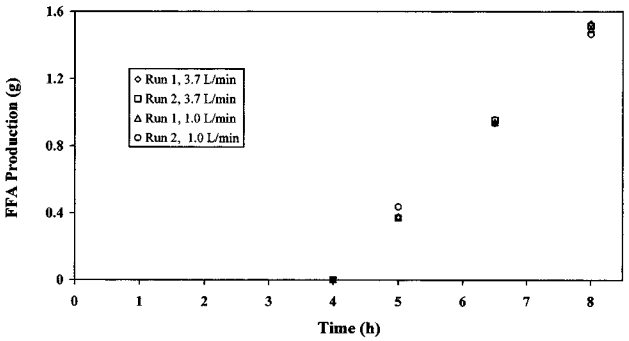

FIG. 5. Effect of CO_2 flow rate on the FFA production after 4 h of reaction at 38 MPa and 55°C. See Figure 2 for abbreviation.

sufficient quantities to the CO_2 stream. The flow rates of oil and water (0.02 and 0.004 mL/min, respectively) were kept constant throughout this study, providing 9.89 g oil and 1.92 g water over 8 h, respectively.

At a higher CO_2 flow rate, the concentration of the substrates in the CO_2 phase is below saturation since the same amount of oil and water was supplied to a larger CO_2 quantity (~440 g) compared to that of the lower flow rate (~120 g) over 8 h. Whether the supercritical medium is saturated or not has an effect on the hydrolysis reaction. The lower concentration of substrates in the reactor as a result of a higher CO_2 flow rate may cause the reaction rate to drop accordingly. Thus, the condition is not appropriate for complete hydrolysis of TG molecules to glycerol, and instead partial conversion to the intermediate products is favored. Glowacz *et al.* (6) showed that the extent of hydrolysis of triolein and its partial glycerides increased with reaction time, and as a result a higher amount of oleic acid was released.

Another effect may be related to the possible loss of enzyme activity as a result of a lack of water around the enzyme at the higher CO_2 flow rate. The layer of water on the enzyme has more of a tendency to leave the enzyme site at the higher CO_2 flow rate since water concentration in the SCF is lower. Hampson and Foglia (18) reported that the immobilized lipase from *C. antarctica* lost 2–6% of its water content per hour in $SCCO_2$ at 27 MPa and 60°C with 0.5 or 1 L/min CO_2 flow rate. They also reported that in the hydrolysis of tripalmitin, the enzyme with 1.5% moisture content gave little evidence of hydrolysis. However, the enzyme with 5.4–23.5% initial moisture content led to products with palmitic acid and unreacted tripalmitin only, which is in agreement with the results of the lower flow rate in this study.

Since the amounts of oil and water introduced to the reaction system were below their saturation levels in $SCCO_2$, the results obtained can be attributed to the net flow rate effect on the hydrolysis reaction. It seems that product composition can be controlled by changing flow rates of substrates and CO_2. Furthermore, one can predict that full hydrolysis of oil is possible if a lower CO_2 flow rate, a higher enzyme load, and/or a lower oil flow rate is selected.

Composition of product mixtures. A typical trend for the change in product composition over a 4-h period is shown in Figure 6, for a reaction at 24 MPa and 35°C. It is apparent that the reaction system requires about 3 h to reach steady state. After 4 h, DG concentration (~20 mol%) was twice that of MG (~10 mol%), indicating that DG production was favored over MG at this condition. Similar results were obtained for all other conditions of this study except for the reactions conducted at 10 MPa and 35°C, in which case the production of both MG and DG was less pronounced. At 10 MPa and 35°C, due to the poor solubility of canola oil in $SCCO_2$ (0.05 mg oil/g CO_2) (16), only a small fraction (0.5%) of the pumped oil was dissolved while water solubility was relatively high (~3 mg water/g CO_2) (17), and as a result direct hydrolysis of TG to glycerol and FFA was favored and MG and DG accumulation was less than that at other conditions. When Glowacz *et al.* (6) studied the hydrolysis of triolein and its partial glycerides, they reported that the (re)activity of triolein was higher than dioleins and within the dioleins; 1,2-diolein was more (re)active than 1,3-diolein; and finally dioleins were more (re)active than 1-monoolein indicating compound specificity of the immobilized porcine pancreatic lipase.

Effect of pressure and temperature on product composition. Relative concentrations of glycerol, MG, DG, and TG in the product mixture after 4 h of reaction are listed in Table 1 at the different conditions studied. The concentration of each component was mainly dependent on pressure, and small differences were observed with a change in temperature at each pressure. Glycerol concentration was highest (~35 mol%) at 10 MPa and 35°C. This was related to the lower solubility of oil in $SCCO_2$ at this condition, resulting in further hydrolysis. At 38 MPa, glycerol content increased with an increase in temperature from 35 to 55°C. The highest level of MG was obtained at 24 MPa and 35–55°C, which declined with an increase in pressure to 38 MPa. At 10 MPa and 35°C, concentrations of both MG and DG were lower, indicating their immediate conversion. DG content increased dramatically with a change in pressure to 24 or 38 MPa at 35–55°C. The highest fraction of TG was observed at 10 MPa and 35°C, indicating the lowest conversion, ~50%. At 24–38 MPa and 35–55°C and when the CO_2 flow

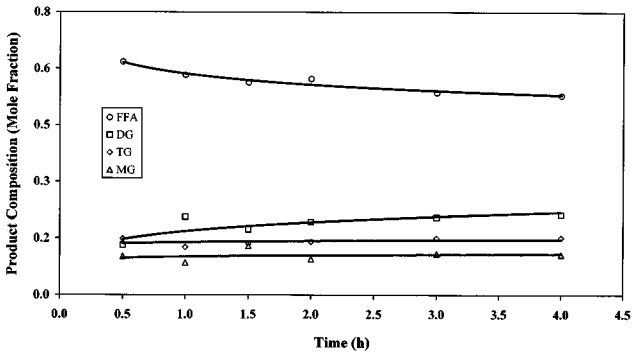

FIG. 6. Change in the product composition in the hydrolysis of canola oil in $SCCO_2$ at 24 MPa and 35°C (CO_2 flow rate: 3.7 L/min, measured at ambient conditions). See Figure 2 for abbreviations.

TABLE 1
Effect of Pressure and Temperature on Product Composition (mol%, normalized to 100%) After 4 h of Continuous Reaction[a]

Compound	10 MPa/35°C	24 MPa/35°C	24 MPa/55°C	38 MPa/35°C	38 MPa/55°C
Glycerol	34.5	5.8	3.5	6.9	9.7
MG	1.3	21.4	20.9	14.4	13.4
DG	15.1	39.5	41.4	42.8	39.7
TG	49.1	33.3	34.1	35.9	37.3

[a]MG, monoglycerides; DG, diglycerides; TG, triglycerides.

TABLE 2
Effect of Pressure and Temperature on the Production of MG, DG, and FFA After 4 h of Continuous Reaction[a]

	Production (g)				
Compound	10 MPa/35°C	24 MPa/35°C	24 MPa/55°C	38 MPa/35°C	38 MPa/55°C
MG	0.01	0.41	0.31	0.25	0.20
DG	0.07	1.32	1.09	1.14	1.02
FFA	0.47	1.72	1.73	1.45	1.29

[a]Flow rates: CO_2 at 3.7 L/min, measured at ambient conditions; water at 0.004 mL/min and oil at 0.02 mL/min. FFA, free fatty acids. See Table 1 for other abbbreviations.

rate was 3.7 L/min, a conversion of 63–67% was obtained after 4 h of reaction, which was not affected by a change in pressure and/or temperature in that range. These results demonstrate that product composition can be altered to achieve a desired mixture by changing reaction conditions. Although a very small amount of oil was soluble at 10 MPa and it was expected that the enzyme would hydrolyze all of the oil, a conversion of only ~50% was achieved. A change in the properties of the microaqueous layer of the enzyme or a reduction in the mass transport properties of the solvent can also be a factor. When studying the acyltransfer activity, Briand *et al.* (19) showed that the acyltransfer (including hydrolysis) activity of *C. parapsilosis* lipase was very sensitive to the experimental temperature, and an optimal temperature of 40–50°C was obtained in an aqueous treatment of rapeseed oil. They further demonstrated that the enzyme was able to maintain its activity at temperatures up to 50°C but was substantially inactivated at temperatures >50°C in less than an hour in aqueous solutions.

Effect of pressure and temperature on the production. The cumulative amount of MG, DG, and FFA in the product mixture obtained after 4 h was highest at 24 MPa (Table 2). The highest production of both MG and DG was obtained at 24 MPa and 35°C, after which an increase in both pressure and temperature or a decrease in pressure resulted in an adverse effect. Temperature effect on FFA production at 35–55°C and 24–38 MPa was not pronounced, but pressure was an important factor. At this range of pressure and temperature, the total oil load of CO_2 did not change since the total oil introduced to the system was kept constant and it was lower than the saturation level of CO_2. FFA production was higher at 24 MPa than at 38 and 10 MPa. At 10 MPa, the lowest production of MG, DG, and FFA was obtained, which is due to the lower solubility of canola oil in $SCCO_2$.

ACKNOWLEDGMENTS

Gratitude is expressed to the Natural Sciences and Engineering Research Council of Canada for financial support and Novo Nordisk for providing enzyme. Karamatollah Rezaei thanks the Ministry of Culture and Higher Education of Iran for scholarship support.

REFERENCES

1. King, J.W., and G.R. List, *Supercritical Fluid Technology in Oil and Lipid Chemistry*, AOCS Press, Champaign, 1993.
2. Randolph, T.W., H.W. Blanch, and D.S. Clark, Biocatalysis in Supercritical Fluids, in *Biocatalysis for Industry*, edited by J.S. Dordick, Plenum Press, New York, 1991, pp. 219–237.
3. Liu, K.J., H.M. Cheng, R.C. Chang, and J.F. Shaw, Synthesis of Cocoa Butter Equivalent by Lipase-Catalyzed Interesterification in Supercritical Carbon Dioxide, *J. Am. Oil Chem. Soc. 74*: 1477–1482 (1997).
4. Erickson, J.C., P. Schyns, and C.L. Cooney, Effect of Pressure on an Enzymatic Reaction in a Supercritical Fluid, *AIChE J. 36*:299–301 (1990).
5. Chi, Y.M., K. Nakamura, and T. Yano, Enzymatic Interesterification in Supercritical Carbon Dioxide, *Agric. Biol. Chem. 52*: 1541–1550 (1988).
6. Glowacz, G., M. Bariszlovich, M. Linke, P. Richter, C. Fuchs, and J.T. Mörsel, Stereoselectivity of Lipases in Supercritical Carbon Dioxide. I. Dependence of Regio- and Enantioselectivity of Porcine Pancreas Lipase on the Water Content During the Hydrolysis of Triolein and Its Partial Glycerides, *Chem. Phys. Lipids 79*:101–106 (1996).
7. Gandhi, N.N., Applications of Lipase, *J. Am. Oil Chem. Soc. 74*: 621–634 (1997).
8. Temelli, F., J.W. King, and G.R. List, Conversion of Oils to Monoglycerides by Glycerolysis in Supercritical Carbon Dioxide Media, *Ibid. 73*:699–706 (1996).
9. Annino, R., and R. Villalobos, *Process Gas Chromatography, Fundamentals and Applications*, Instrument Society of America, Research Triangle Park, 1992.
10. Canola Council of Canada, *Canola Oil Chemical & Physical Properties*, Winnipeg, 1994.

11. Marty, A., W. Chulalaksananukul, R.M. Willemot, and J.S. Condoret, Kinetics of Lipase-Catalyzed Esterification in Supercritical CO_2, *Biotech. Bioeng. 39*:273–80 (1992).
12. Giessauf, A., W. Magor, D.J. Steinberger, and R. Marr, A Study of Hydrolases Stability in Supercritical Carbon Dioxide (SC-CO_2), *Enz. Microb. Technol. 24*:577–583 (1999).
13. Lozano, P., A. Avellaneda, R. Pascual, and J.L. Iborra, Stability of Immobilized α-Chymotrypsin in Supercritical Carbon Dioxide, *Biotech. Lett. 18*:1345–1350 (1996).
14. Yu, Z.R., S.S.H. Rizvi, and J.A. Zollweg, Enzymatic Esterification of Fatty Acid Mixtures from Milk Fat and Anhydrous Milk Fat with Canola Oil in Supercritical Carbon Dioxide, *Biotech. Prog. 8*:508–513 (1992).
15. Zagrobelny, J., and F.V. Bright, *In Situ* Studies of Protein Conformation in Supercritical Fluids: Trypsin in Carbon Dioxide, *Biotech. Prog. 8*:421–423 (1992).
16. Fattori, M., N.R. Bulley, and A. Meisen, Carbon Dioxide Extraction of Canola Seed: Oil Solubility and Effect of Seed Treatment, *J. Am. Oil Chem. Soc. 65*:968–974 (1988).
17. Wiebe, R., and V.L. Gaddy, Vapor Phase Composition of Carbon Dioxide–Water Mixtures at Various Temperatures and at Pressures to 700 Atmospheres, *J. Am. Chem. Soc. 63*:475–477 (1941).
18. Hampson, J.W., and T.A. Foglia, Effect of Moisture Content on Immobilized Lipase-Catalyzed Triacylglycerol Hydrolysis Under Supercritical Carbon Dioxide Flow in a Tubular Fixed-Bed Reactor, *J. Am. Oil Chem. Soc. 76*:777–781 (1999).
19. Briand, D., E. Dubreucq, and P. Galzy, Factors Affecting the Acyltransfer Activity of the Lipase from *Candida parapsilosis* in Aqueous Media, *Ibid. 72*:1367–1373 (1995).

[Received December 16, 1999; accepted May 1, 2000]