Lipase-Catalyzed Esterification of Stearic Acid with Ethanol, and Hydrolysis of Ethyl Stearate, Near the Critical Point in Supercritical Carbon Dioxide

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ABSTRACT: The effect of pressure on the lipase-catalyzed reaction in supercritical carbon dioxide ($SCCO_2$) was investigated for the esterification of stearic acid (SA) with ethanol and the hydrolysis of ethyl stearate (ES) near the critical point, ranging from 6 to 20 MPa in pressure and 35 to 60°C in temperature. The esterification rate of SA began to increase near the critical point and kept increasing steadily with an increase in pressure, reflecting the increase in SA solubility in $SCCO_2$. The hydrolysis rate of ES showed a maximum at a pressure near the critical point. When the reaction was carried out with an initial overall ES concentration below its solubility limit in $SCCO_2$, the maximum pressure shifted along the extended line of the gas–liquid equilibrium in the supercritical region in the pressure–temperature phase plane. This seems to be related to the singular behavior of some properties in $SCCO_2$ along this line reported in the literature. These results show the importance of pressure, as well as temperature, as a parameter to control enzyme reactions in $SCCO_2$.

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KEY WORDS: Critical point, enzyme-catalyzed, enzyme in supercritical carbon dioxide, esterification, hydrolysis, lipase.

Since the initial finding (1–3) of the applicability of some enzymes in supercritical fluids (SCF), numerous investigations on enzyme catalysis in SCF have been carried out because of their unique solvent properties, such as low viscosity and high diffusivity in comparison with ordinary organic solvents. SCF have two parameters, temperature and pressure, that control their physical properties. Among SCF, supercritical carbon dioxide ($SCCO_2$) has been most frequently used because of its relatively mild critical temperature (31.1°C) and pressure (7.38 MPa) (4). In addition, $SCCO_2$ is nontoxic, nonflammable, and inexpensive, and is therefore expected to be widely applicable as a solvent for extractions and reactions in the food and pharmaceutical industries.

Among enzymes active in $SCCO_2$, lipase (triacylglycerol lipase, EC 3.1.1.3) has been most frequently used because it catalyzes various types of reactions with wide substrate specificity. Thus, lipase-catalyzed reactions in $SCCO_2$ have been carried out for such reactions as hydrolysis of tripalmitin (5) and canola oil (6); alcoholysis of cod liver oil (7) and palm kernel oil (8); glycerolysis of soybean oil (9); synthesis of oleoyloleate from oleic acid (10–12); acylation of glucose with lauric acid (13); thioesterification between oleic acid and butanethiol (14); transesterifications of milk fat with canola oil (15); tristearin with palm oil (16); palm oil with stearic acid (SA) (17); triolein with ethyl behenate and behenic acid (18,19); triolein with SA (20) and propyl acetate with geraniol (21); and randomization of fat and oils (22).

In spite of the many investigations, lipase reactions in $SCCO_2$ are not fully understood and not optimized well because of insufficient accumulation of solubility data and inconclusive characterization of $SCCO_2$ as an enzyme reaction medium compared with ordinary solvents. In a previous paper, we measured solubilities of behenic acid and ethyl behenate in $SCCO_2$, which were described well by a combination of the regular solution model with the Flory–Huggins theory (23,24). The relationship between the substrate solubility and enzyme reaction behavior in $SCCO_2$ has been discussed (19,20).

In a recent paper, we determined log P value for $SCCO_2$ (25), which is an important index for describing the hydrophobicity of solvents. The log P of $SCCO_2$ changed from 0.9 to 2.0 with a change in pressure between 3 and 11.8 MPa at 50°C. Judging from log P, $SCCO_2$ was rather hydrophilic as a solvent. With an increase in pressure, however, the hydrophilicity of $SCCO_2$ decreased even though the water solubility in $SCCO_2$ increased. This extraordinary behavior may characterize a unique property of $SCCO_2$ as a medium for enzyme reactions. $SCCO_2$ showed better performance as a solvent for lipase-catalyzed esterification of SA with ethanol and the hydrolysis of ethyl stearate (ES) as compared to benzene (log P = 2.0) and hexane (log P = 3.5).

With $SCCO_2$ as a solvent for enzyme reactions, pressure, as well as temperature, is an important process parameter. In the present paper, the effect of pressure is investigated for the lipase-catalyzed esterification of SA with ethanol and hydrolysis of ES in $SCCO_2$, especially near the critical point.

MATERIALS AND METHODS

Materials. Immobilized lipase (Lipozyme IM from *Rhizomucor miehei*; 1,3-specific) with the enzyme adsorbed on a macroporous anion exchange resin was purchased from Novo Nordisk (Tokyo, Japan). SA and ES were purchased from

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Sigma (St. Louis, MO). Ethanol (99.5%), acetonitrile, tetrahydrofuran, dichloromethane of high-performance liquid chromatography (HPLC) grade, and acetic acid (99.7%) were purchased from Kanto Chemical Co. (Tokyo, Japan). Liquid CO_2 (99.99%) was from Suzuki Shokan Co. (Tokyo, Japan).

Lipase-catalyzed reactions in pressurized CO_2. The enzyme reaction was carried out in pressurized CO_2 by batchwise operation. Immobilized lipase (0.045 g) with water content adjusted by addition of water, a magnetic stirring bar, and substrates for the enzyme reaction were placed separately at the bottom of a high-pressure reaction vessel (40 mL in volume), which was submerged in a water bath (8,9). Prior to starting the reaction, pressurized CO_2 was passed through a molecular sieve column and then flushed into the reactor. The reaction was started by mixing the enzyme and the substrates in the reactor with the magnetic stirring bar. The reaction was stopped after 30 min by rapid cooling of the reactor with a dry ice–acetone mixture to solidify the reaction mixture inside. The apparent reaction rate was calculated from the increase in product concentration during the reaction.

Analytical procedure. Concentrations of substrates and water were calculated from the initial loading and the reactor volume. When the initial molar ratio of ES and CO_2 was kept constant ([ES]/[CO_2] = 1:1835.3, mol/mol) in the process with pressure varied, the initial loading of ES was adjusted depending on the density of CO_2, which was obtained from the web site of the National Institute of Standards and Technology (http://webbook.nist.gov/chemistry/fluid/) and based on Reference 26. The solidified reaction mixture, after sublimation of CO_2, was extracted with 10 mL of diethylether. The extract was filtered through a disc membrane filter (5.0 μm filter unit; Millipore Co., Tokyo, Japan) resistant to organic solvent, and then diethylether was evaporated in a rotary evaporator. The residue was dissolved in HPLC eluent and subjected to HPLC analysis. The HPLC system was composed of a pump (PU980; Jasco Co., Tokyo, Japan), a refractive index detector (Shodex SE-51; Showa Denko Co., Tokyo, Japan), and a column (Inertsil ODS 2 column, 4.6 × 250 mm; GL Science, Tokyo, Japan) held at 37°C. A mixed solution of acetonitrile, tetrahydrofuran, dichloromethane, and acetic acid (70:20:20:0.8, by vol) was used as HPLC eluent at a flow rate of 0.9 mL/min.

Calculation of solubilities. The solubilities of SA, water, and ES in $SCCO_2$ were calculated from our previous data (24), data by Wiebe and Gaddy (27), and data by Bharath *et al.* (28), respectively.

RESULTS AND DISCUSSION

Effect of pressure on the esterification rate. The effect of pressure on the lipase-catalyzed esterification rate of SA with ethanol in $SCCO_2$ was measured between 35 and 60°C (Fig. 1). At all the temperatures tested, the esterification rate steadily increased with an increase in pressure.

As shown in Figure 1, the initial amounts of SA and ethanol were 22.6 and 415 mM, respectively. In $SCCO_2$, the solubility of ethanol is high but that of SA is very limited so

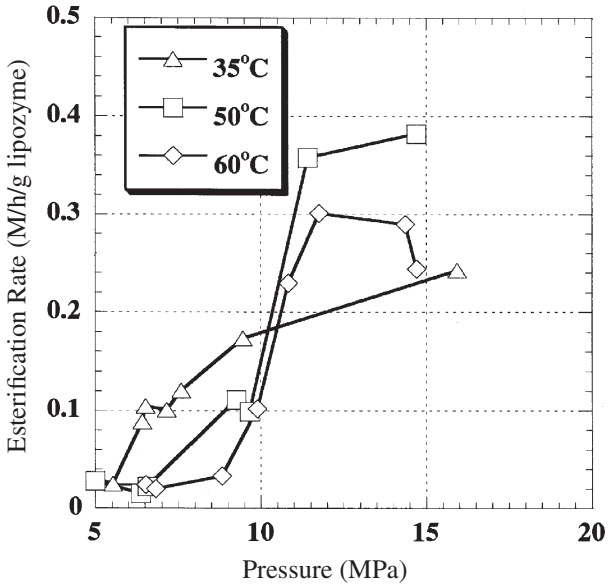

FIG. 1. Effects of pressure and temperature on the reaction rate of lipase-catalyzed esterification of stearic acid (SA) with ethanol (EtOH) in $SCCO_2$ at 50°C. Initial overall concentrations of SA, EtOH, and water were 22.6, 415, and 9.17 mM, respectively. The reaction time was 30 min.

that the overall SA concentration in the present experiment was much higher than its solubility limit. In Figure 2, effects of pressure and temperature on the calculated solubility of SA are shown. The calculated solubility of SA began to increase near the critical point with an increase in pressure. This increase in the solubility of SA corresponded well to the increase in the esterification rate of SA shown in Figure 1. The $SCCO_2$ phase was the only reaction phase because the undissolved SA phase was solid (melting point = 69.6°C). Therefore, the esterification of SA seemed to have been limited by the solubility of SA.

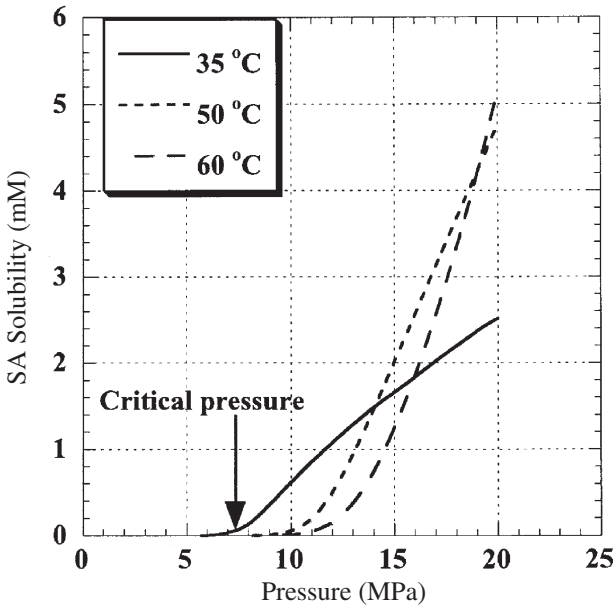

FIG. 2. Solubility of SA as a function of pressure at various temperatures. See Figure 1 for abbreviations.

Effect of pressure on the hydrolysis rate. On the contrary, the hydrolysis rate of ES was affected by solubilities and concentrations of water and ES and also by the dilution effect of water and ES on the mole fraction basis with an increase in pressure by addition of CO_2. In addition, the reaction might have occurred not only in the $SCCO_2$ phase but also in the undissolved liquid ES phase depending on the solubility and the initial overall concentration of ES.

Figure 3 shows the effects of initial overall concentrations of water, varied from 9.17 to 31 mM, and ES, varied from 2.4 to 22.6 mM, on the hydrolysis rate of ES at 50°C. With increases in initial concentrations of water and ES, the reaction rate increased. Maxima in the hydrolysis rate, indicated by the marks A1, A2, B1, etc., were observed in all the experimental conditions tested. With an increase in ES concentration, the maximal pressure shifted low and the shape of the maximum became sharper.

The initial concentration of water was lower than its solubility limit at all the maxima in Figure 3. The initial overall concentration of ES, however, was higher than its solubility limit at some of the maxima in Figure 3, which were distinguished by parentheses at the mark of maxima. In these cases with ES over its solubility limit, sharp maxima were observed, and the reaction rate increased with an increase in ES concentration. This strongly suggests the possibility of the reaction in the undissolved ES phase because had the reaction occurred only in the $SCCO_2$ phase, the reaction rate would have been limited only by the solubility of ES in $SCCO_2$ and the excessive ES might have no relation to the reaction rate. In Figure 3, however, the reaction rate increased with an increase in overall ES concentration even when the ES concentration was higher than its solubility limit. In a separate experiment, the occurrence of the lipase-catalyzed hydrolysis of ES was confirmed to take place in the ES phase without $SCCO_2$. Therefore, the increase in the hydrolysis rate of ES in Figure 3 with an increase in the ES concentration must have been affected by the reaction in the ES phase. Interestingly, a sharp maximum was observed at A1 in Figure 3 (A), at which the pressure was below the critical point of CO_2. This would be a mixed result of reactions in the $SCCO_2$ phase and the ES phase. A similar maximum in the reaction rate below the critical pressure was also reported in the lipase-catalyzed transesterification of triolein with SA in $SCCO_2$ (20). When the reaction occurred only in the $SCCO_2$ phase (B2, B3, C2, C3 in Fig. 3), the maximal pressure converged to almost the same pressure at about 10 MPa, which is higher than the critical pressure (7.38 MPa).

Effect of temperature on the pressure at maximum in hydrolysis rate. It has been shown that the pressures yielding maxima in the hydrolysis rate of ES were about 10 MPa at 50°C when the ES concentration was below its solubility limit. The effect of variations in temperature on the pressure at maximal hydrolysis was investigated with ES and water concentrations fixed at 8.0 and 24 mM, respectively. As shown in Figure 4, sharp and temperature-dependent maxima were observed in the reaction rate near the critical point. The pressure at maximal activity increased with an increase in temperature from 40 to 60°C. At these peaks, concentrations of ES and water were under their solubility limit.

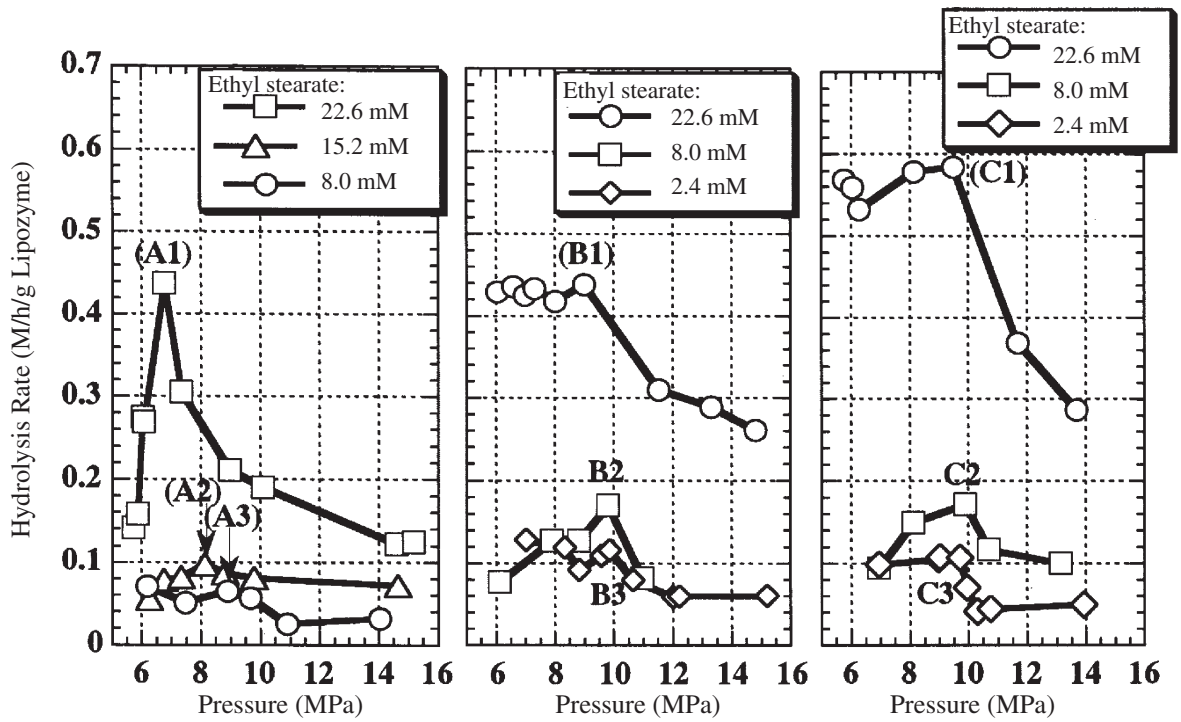

FIG. 3. Effect of pressure on the apparent reaction rate of lipase-catalyzed hydrolysis of ethyl stearate (ES) in 30 min at various initial overall concentrations of ES and water at 50°C: (A), [water] = 9.17 mM; (B), [water] = 24 mM; (C), [water] = 31 mM. The reaction time was 30 min. Maxima in the reaction rate are marked by A1, A2, etc. Parentheses at the mark of maxima show that the initial ES overall concentration was higher than its solubility limit.

At pressures higher than the maxima, reaction rates steadily decreased with an increase in pressure (Fig. 4). This decrease in the reaction rate might be affected by the dilution of ES on a molar fraction basis by the addition of CO_2 to increase pressure (29). Therefore, in a further set of studies the initial molar ratio of ES and CO_2 was kept constant ([ES]/[CO_2] = 1:1835.3, mol/mol) across the pressure range. As shown in Figure 5, sharp maxima in the hydrolysis rate of ES were also observed, and pressures at maxima shifted upward with an increase in reaction temperature. After reaching maxima, the reaction rate once decreased down to a minimum, then increased again.

Although the reaction behaviors in the higher pressure range are different between Figures 4 and 5, pressures at maxima were not different. Those pressures at maxima are plotted in the pressure–temperature diagram of CO_2 in Figure 6. It turned out that pressures at maxima shifted along the extended line of the gas–liquid equilibrium of CO_2. In the literature (30), it has been reported that correlation lengths and density fluctuations in $SCCO_2$ form a ridge along this extended line, and some properties in $SCCO_2$, such as solubility rate and rate constants of some reactions, showed singular behavior on this extended line. This suggests the mechanism of the unusual reaction behavior near the critical point in $SCCO_2$ in the present case. With singularities described above, the intermolecular interaction between substrates and the enzyme would have increased to maximize the reaction rate.

In the near-critical region, the existence of solute–solute aggregation was theoretically proved in extremely dilute supercritical mixtures (31). Ellington and Brennecke (32) observed that the rate constant for the uncatalyzed esterification of phthalic anhydride with methanol at 97.5 bar was 30 times higher than that at 166.5 bar in $SCCO_2$, suggesting the increased local concentration of the methanol around the phthalic anhydride near the critical point. Ikushima *et al.* (33) also observed a sharp maximum near the critical point in the lipase-catalyzed esterification rate of *n*-valeric acid and citronellol when pressure was varied. They suggested that the formation of the lipase–substrate complex would be facilitated by an increase in the electron-accepting power of $SCCO_2$ at the maximum. They also reported the conformational change of lipase to trigger the enzymatic activity in the pressure range near the critical point (34).

The results in Figure 6 and discussions above show that the singular point in the hydrolysis rate exists along the extended line of the gas–liquid equilibrium in the pressure–tem-

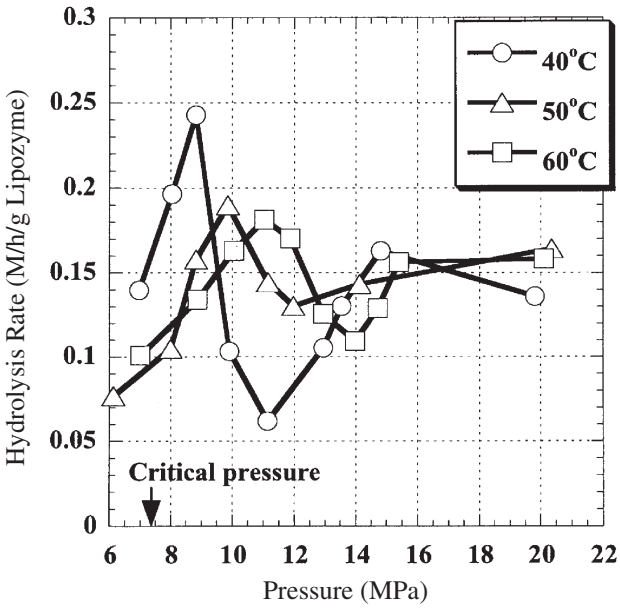

FIG. 5. Effect of pressure on the apparent rate of lipase-catalyzed hydrolysis of ES in pressurized CO_2 at various temperatures. Initial molar ratio of ES and CO_2 was kept constant at 1:1835.3. The reaction time was 30 min. See Figure 3 for abbreviation.

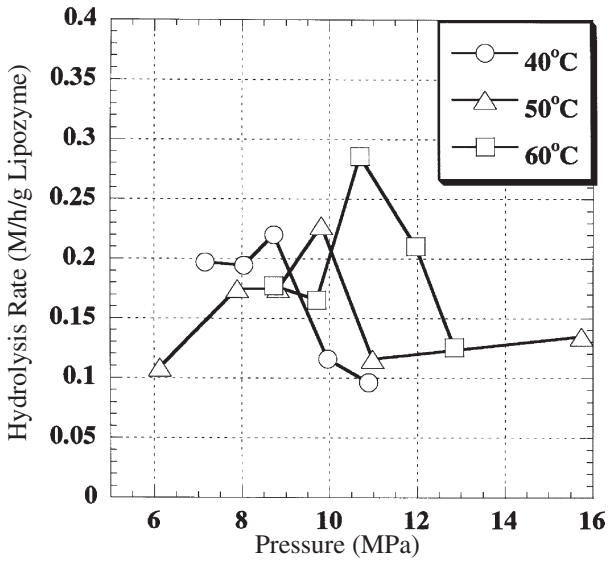

FIG. 4. Effect of pressure on lipase-catalyzed hydrolysis rate of ES in pressurized CO_2 at various temperatures. Initial concentrations of ES and water were fixed at 8.0 and 24 mM, respectively. The reaction time was 30 min. See Figure 3 for abbreviation.

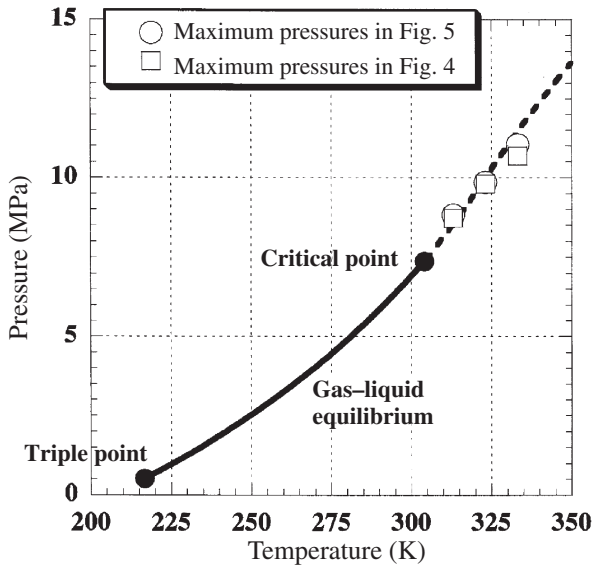

FIG. 6. Pressures at maxima in ES hydrolysis rate in Figures 4 and 5 plotted in pressure–temperature phase diagram of CO_2. See Figure 3 for abbreviation.

perature diagram of CO_2, and the reaction rate is maximized at that point. This will be of practical importance in the selection of operating conditions for enzyme reactions in $SCCO_2$ such as for the lipase-catalyzed transesterification and hydrolysis of triglycerides.

REFERENCES

1. Hammond, D.A., M. Karel, A.M., Klibanov, and V.J. Krukonis, Enzymatic Reactions in Supercritical Gases, *Appl. Biochem. Biotechnol. 11*:393–400 (1985).
2. Randolph, T.W., H.W. Blanch, J.M. Plausnitz, and C.R., Wilke, Enzymatic Catalysis in Supercritical Fluid, *Biotechnol. Lett. 7*:325–328 (1985).
3. Nakamura, K., Y.M. Chi, Y., Yamada, and T. Yano, Lipase Activity and Stability in Supercritical Carbon Dioxide, *Chem. Eng. Commun. 45*:207–212 (1986).
4. Kamat, S.V., E.J. Beckman, and A.J. Russell, Enzyme Activity in Supercritical Fluids, *Crit. Rev. Biotechnol. 15*:41–71 (1995).
5. 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).
6. Rezaei, K., and F. Temelli, Lipase-Catalyzed Hydrolysis of Canola Oil in Supercritical Carbon Dioxide, *Ibid. 77*:903–909 (2000).
7. Gunnlaugsdottir, H., and B. Sivik, Process Parameters Influencing Ethnolysis of Cod Liver Oil in Supercritical Carbon Dioxide, *J. Supercrit. Fluid 12*:85–93 (1998).
8. Oiveira, J.V., and D. Oiveira, Kinetics of the Enzymatic Alcoholysis of Palm Kernel Oil in Supercritical CO_2, *Ind. Eng. Chem. Res. 39*:4450–4454 (2000).
9. Jackson, M.A., and J.W. King, Lipase-Catalyzed Glycerolysis of Soybean Oil in Supercritical Carbon Dioxide, *J. Am. Oil Chem. Soc. 74*:97–106 (1997).
10. Goddard, R., J. Bosley, and B. Al-Duri, Esterification of Oleic Acid and Ethanol in Plug Flow (Packed-Bed) Reactor Under Supercritical Conditions, Investigation of Kinetics, *J. Supercrit. Fluid 72*: 85–93 (1995).
11. Hablin, M., V. Krmelj, and Z. Knez, Synthesis of Oleic Acid Esters Catalyzed by Immobilized Lipase, *J. Agric. Food Chem. 44*: 338–342 (1996).
12. Knez, Z., M. Habulin, and V. Krmelj, Enzyme-Catalyzed Reactions in Dense Gasses, *J. Supercrit. Fluid 14*:17–29 (1998).
13. Tsitsimkou, C., H. Stamatis, V. Sereti, H. Daflos, and F.N. Kolisis, Acylation of Glucose Catalysed by Lipase in Supercritical Carbon Dioxide, *J. Chem. Technol. Biotechnol. 71*:309–314 (1998).
14. Caussette, M., A. Marty, and D. Combes, Enzymatic Synthesis of Thioesters in Non-conventional Solvents, *Ibid. 68*:257–262 (1997).
15. Yu, Z.-R., S.S.H. Rizvi, and J.A. Zolloweg, Enzymatic Esterification of Fatty Acid Mixtures from Milk Fat and Anhydrous Milk Fat with Canola Oil in Supercritical Carbon Dioxide, *Biotechnol. Prog. 8*:508–513 (1992).
16. 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).
17. Liang, M.-T., C.-H. Chen, and R.-C. Liang, The Interesterification of Edible Palm Oil by Stearic Acid in Supercritical Carbon Dioxide, *J. Supercrit. Fluid 13*:211–216 (1998).
18. Yoon, S.-H., O. Miyawaki, K.-H. Park, and K. Nakamura, Transesterification Between Triolein and Ethylbehenate by Immobilized Lipase in Supercritical Carbon Dioxide, *J. Ferment. Bioeng. 82*: 334–340 (1996).
19. Yoon, S.-H., H. Nakya, O. Ito, O. Miyawaki, K.-H. Park, and K. Nakamura, Effects of Substrate Solubility in Interesterification with Triolein by Immobilized Lipase in Supercritical Carbon Dioxide, *Biosci. Biotechnol. Biochem. 62*:170–172 (1998).
20. Nakaya, H., O. Miyawaki, and K. Nakamura, Transesterification Between Triolein and Stearic Acid by Lipase in CO_2 at Various Pressures, *Biotechnol. Tech. 12*:881–884 (1998).
21. Chulalaksananukul, W., J.-S. Condoret, and D. Combes, Geranyl Acetate Synthesis by Lipase-Catalyzed Transesterification in Supercritical Carbon Dioxide, *Enzyme Microb. Technol. 15*:691–698 (1993).
22. Jackson, M.A., J.W. King, C.R. List, and W.E. Neff, Lipase-Catalyzed Randomization of Fats and Oils in Flowing Supercritical Carbon Dioxide, *J. Am. Oil Chem. Soc. 74*:635–639 (1997).
23. Iwai, Y., Y. Koga, H. Maruyama, and Y. Arai, Solubility of Stearic Acid, Stearyl Alcohol in Supercritical Carbon Dioxide in 35°C, *J. Chem. Eng. Data 38*:506–508 (1993).
24. Nakaya, H., O. Miyawaki, and K. Nakamura, Solubilities of Behenic Acid and Ethylbehenate in Supercritical Carbon Dioxide, *Kagaku Kogaku Ronbunshu 25*:237–239 (1999).
25. Nakaya, H., O. Miyawaki, and K. Nakamura, Determination of Log *P* for Pressurized Carbon Dioxide and Its Characterization as a Medium for Enzyme Reaction, *Enzyme Microb. Technol. 28*: 176–182 (2001).
26. Ely, J.F., J.W. Magee, and W.M. Haynes, Research Report RR110, Gas Processors Association, Tulsa, OK, 1987.
27. 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).
28. Bharath, R., H. Inomata, K. Arai, K. Shoji, and Y. Noguchi, Vapor–Liquid Equilibria for Binary Mixtures of Carbon Dioxide and Fatty Acid Ethyl Ethers, *Fluid-Phase Equilib. 50*:315–327 (1989).
29. 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).
30. Nishikawa, K., I. Tanaka, and Y. Amemiya, Small-Angle X-ray Scattering Study of Supercritical Carbon Dioxide, *J. Phys. Chem. 100*:418–421 (1996).
31. Wu, R.S., L.L. Lee, and H.D. Cochran, Structure of Dilute Supercritical Solutions: Clustering of Solvent and Solute Molecules and the Thermodynamic Effects, *Ind. Eng. Chem. Res. 29*:977–988 (1990).
32. Ellington, J.B., and J.F. Brennecke, Pressure Effect on the Esterification of Phthalic Anhydride in Supercritical CO_2, *J. Chem. Soc. Chem. Commun.*:1094–1095 (1993).
33. Ikushima, Y., N. Saito, and M. Arai, Promotion of Lipase-Catalyzed Esterification of *n*-Valeric Acid and Citronellol in Supercritical Carbon Dioxide in the Near-Critical Region, *J. Chem. Eng. Jpn. 29*:551–553 (1996).
34. Ikushima, Y., N. Saito, M. Arai, and H.W. Blanch, Activation of Lipase Triggered by Interactions with Supercritical Carbon Dioxide in the Near-Critical Region, *J. Phys. Chem. 99*:8941–8944 (1995).

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