Lipase-Catalyzed Alcoholysis with Supercritical Carbon Dioxide Extraction 2: Phase Behavior

Helga Gunnlaugsdottir[a],*, Anders Å. Karlsson[b], and Björn Sivik[a]

[a]Department of Food Technology, Lund University, 221 00 Lund, Sweden, and [b]Chemical Ecology and Ecotoxicology, Department of Ecology, Lund University, 223 62 Lund, Sweden

ABSTRACT: The phase behavior of systems containing ethanol plus lipid samples with different lipid compositions plus carbon dioxide was studied visually at 40°C and pressures of 0.01, 9, 15, and 23 MPa by means of a high-pressure sapphire cell. The systems studied represent the main components present in a lipase-catalyzed alcoholysis reaction of cod liver oil in supercritical carbon dioxide. Two phases, a vapor and a liquid phase, were observed in all systems studied at supercritical conditions. *JAOCS 74,* 1491–1494 (1997).

KEY WORDS: Alcoholysis, enzyme, ethanol, lipase, lipids, phase behavior, supercritical carbon dioxide.

Enzyme catalysis in nonaqueous media (NAM) is currently an active research area (1-3). A common feature for enzyme catalysis in NAM is the presence of at least two phases (3), where one phase, normally the bulk, is the nonaqueous medium and the other contains the biocatalyst. Hence, enzyme catalyses in NAM are always heterogeneous. For biocatalysis, supercritical fluids (SCF) are an especially promising class of NAM. Enzyme catalyses in SCF have been investigated by several research groups, and the status of this field recently has been reviewed (4-7). In the published literature, the phase behavior of the reaction system in SCF normally has not been investigated in detail. Information on the phase behavior of the components is important, as many substrates and products may either not dissolve entirely or precipitate out of solution at the pressures and temperatures used; such behavior may result in several phases and affect the efficiency of the process.

The goal of many researchers has been to carry out the reaction in two-phase systems consisting of an enzyme (solid) and a supercritical or near-critical fluid phase containing the solubilized substrates and products, here termed vapor–solid reaction system. The reason for this trend is that systems of this kind minimize diffusion limitations of substrates, which in turn could enhance the rates of biocatalytic processes. Kamat and coworkers (8) have shown that for a lyophilized enzyme, SCF can be expected to enhance the rate of a diffusion-limited reaction. In order to guarantee that solely a vapor–solid system is present, the concentrations of the substrates and products in the reaction system have to be carefully controlled, as multiphases will occur if such concentrations are above solubility. The most commonly applied methods to ensure this are either to place a saturation vessel, in which substrates in the liquid state or impregnated on, e.g., glass wool are stored, upstream of the enzyme reactor (9–14) or to carry out a continuous process in which the substrates are injected into the SCF flow by means of high-pressure pumps (15–18).

Using a vapor–solid reaction system may not always be advantageous. In certain cases, such as in processes where the products or by-products are selectively extracted from the reaction mixture using the SCF, a three-phase system will be preferred. These three phases are the supercritical or near-critical fluid phase containing the solubilized product; a liquid phase containing the substrates and side products; and, finally, the enzyme (often immobilized), here called vapor–liquid–solid reaction system. In this case, a three-phase system will favor the selective extraction of the product, given that it is more soluble than the other reaction components. In our earlier work, we have studied this kind of extractive reaction (19,20). The enzyme reaction that we have investigated is the lipase (E.C. 3.1.1.3)-catalyzed ethanolysis of cod liver oil. This reaction results in a complex mixture of lipid components consisting of triglycerides (TG), diglycerides (DG), monoglycerides (MG), and ethyl esters (EE). Our goal has been to extract preferentially the product (EE) from the reaction mixture with supercritical carbon dioxide (SC-CO_2). In that work we investigated the effect of pressure on the selective extraction of the EE (19) and the effect of changing the flow rate of the extraction fluid on the extraction rate and purity of the product obtained (20).

The lipase-catalyzed reaction investigated results in a constantly changing reaction mixture. In the beginning of the experiment, the oil is present in the form of TG. During the reaction, the lipase splits the individual fatty acids from the glycerol backbone to form EE, a nonpolar lipid component, as well as DG, MG, and glycerol which are more polar. Polar lipids, such as DG and MG, are amphiphilic; i.e., they have a hydrophilic part (a polar headgroup) and a hydrophobic part (a nonpolar lipid tail). Amphiphilic components are usually

*To whom correspondence should be addressed at Department of Food Technology, Lund University, P.O. Box 124, 221 00 Lund, Sweden.
E-mail: Helga.Gunnlaugsdottir@livsteki.lth.se.

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surface-active and, hence, good emulsifiers. It was thus considered possible that the presence of these lipid classes might affect the phase behavior of the reaction system, e.g., result in an isotropic microemulsion composed of carbon dioxide, TG, MG, and glycerol. It has, for example, been reported that a stable microemulsion is formed when fish oil/MG/water or glycerol are mixed in the proportions of 77:20:3 (21). In an extractive reaction process, the formation of a microemulsion during the alcoholysis reaction would result not only in quite different conditions for the enzyme reaction but also in the entrainment of the reactants with the outgoing CO_2 flow and, consequently, much less selective extraction of products. The objective of this work was to investigate the phase behavior of the reaction system by simulating the conditions used in our earlier work on extractive reaction (19,20).

MATERIALS AND METHODS

The cod liver oil used was of commercial quality and obtained from Lysi h.f. (Reykjavik, Iceland). Lipid residues were obtained after lipase-catalyzed ethanolysis of cod liver oil in SC-CO_2 (20). Dimodan LS, a commercial MG containing mainly monooleoyl and monolinoleoyl glycerols, was obtained from Grindsted (Aarhus, Denmark). CO_2 (purity 99.99%) was supplied by Air Liquide Gas AB (Malmö, Sweden). High-performance liquid chromatography (HPLC)-grade hexane, 1-propanol, and water were from Fisher Scientific (Loughbarough, United Kingdom), and acetic acid was p.a. grade from Prolabo (Paris, France).

Analysis of products. The overall content of TG, DG, and MG, as well as free fatty acids and fatty acid ethyl esters of the lipid samples, was determined by liquid chromatography. Separations were performed at ambient temperature by use of a column (250 × 3 mm) packed in the laboratory with diol-modified silica (5 μm, LiChrospher 100 DIOL) from Merck (Darmstadt, Germany). The mobile phase consisted of (i) hexane/acetic acid (99:0.5, vol/vol) and (ii) hexane/1-propanol/acetic acid/water (85:15:0.5:0.1, by vol). The linear gradient timetable was: at 0 min, 100:0; at 6 min, 50:50; at 18 min, 50:50; at 24 min, 100:0; at 45 min, 100:0, (%A/%B, respectively) at a solvent flow rate of 0.4 mL/min between 0 to 24 min, 0.8 mL/min between 24 to 35 min, and 0.4 mL/min between 35 to 45 min. A Waters (Milford, MA) model 600E HPLC instrument with a Rheodyne 7125 injector (20 μL loop) was used. Detection was accomplished with an evaporative light-scattering detector (SEDEX 55; Sedere, Alfortville, France) set at an air inlet pressure of 2.0 bar and a temperature of 45°C.

Calibration. A stock solution of a five-component composite standard, representing the lipid classes detected in the lipid samples, was made up in hexane. All standards were from Sigma (St. Louis, MO). The lipid classes and standards were as follows: EE, palmitic acid EE; TG, tripalmitin; free fatty acid, palmitic acid; DG, dipalmitin (mixed isomers); and MG, 1-monopalmitoyl-*rac*-glycerol. Several different dilutions of the stock solution were made, and a dose–response curve of the standards (light-scattering detection) was constructed by injecting 20 μL of the composite standard (0.002 to 0.5 μg/μL). The correlation between dose and response of each standard was then fitted to an equation.

Apparatus and ester synthesis. The experimental device used to study the phase behavior of the lipids in SC-CO_2 has been described in detail by Hammam and Sivik (22). The experiments were carried out at 40°C and pressures of 0.01, 9, 15, and 23 MPa. In this work, the sapphire cell (1.62 mL) was charged with 0.22 g lipid sample and 0.14 g ethanol (99.5%), and the phase behavior of this mixture was observed visually. The amounts of ethanol and lipid samples used were chosen so that they would correspond to the proportions of ethanol, lipids, and SC-CO_2 present at the beginning of an actual enzyme reaction experiment. The experimental apparatus and the reaction conditions used for the lipase-catalyzed alcoholysis of cod liver oil have been described (20).

RESULTS AND DISCUSSION

In this work, the system consists of three main components, i.e., lipids, ethanol, and CO_2. In the lipase-catalyzed reactions, the immobilized enzyme is also present. The phase behavior of mixtures containing ethanol + lipid samples + CO_2 at different pressures was studied visually using a high-pressure view cell. The results from these investigations are compiled in Table 1. The main differences between the lipid samples under investigation are their lipid class compositions. The unreacted cod liver oil contains nearly exclusively TG whereas the main difference between Residues A and B was the amount of TG and EE present in the sample. As there was no significant difference in the amounts of amphiphilic components (DG and MG) present in the two residues studied, additional MG (Dimodan LS) was added to one of the residues to investigate the effect of such variation in component ratio. Two phases, a vapor phase and a yellow liquid phase, were observed in all systems and at all pressures studied except at the 0.01 MPa pressure for the unreacted cod liver oil. At the 23 MPa pressure, the phase border tended to become slightly more diffuse, possibly because the densities of the two phases become very similar at this pressure and temperature (40°C). At 0.01 MPa, three phases were observed for the unreacted cod liver oil sample: $liquid_1$ (ethanol)–$liquid_2$ (cod liver oil)–vapor (CO_2 gas). This occurred because the amount of ethanol used did not dissolve in the unreacted cod liver oil, and thus two liquid phases were present. When these two liquids were mixed, they formed an unstable emulsion that disappeared as soon as the sapphire cell was charged with the CO_2. Consequently, only two phases (vapor–liquid) were noted at higher pressures. For all other lipid samples under investigation, the ethanol used dissolved in the samples at 0.01 MPa, and hence only two phases (vapor–liquid) were observed. In addition, we studied whether intensive mixing would affect the phase behavior of the systems studied. This was done by turning the sapphire cell up and down a couple of times. At supercritical pressures, this resulted in a hazy

TABLE 1
Phase Behavior of the Ethanol + Lipid + Carbon Dioxide Mixtures[a] at 40°C and Different Pressures

Lipid sample	Lipid class composition	Phases observed without mixing			
		0.01 MPa	9 MPa	15 MPa	23 MPa
Unreacted cod liver oil	TG 98%	Vapor $Liquid_1$ $Liquid_2$	Vapor Liquid	Vapor Liquid	Vapor Liquid Diffuse phase border
Residue A	TG 11%, EE 78% DG12%, MG 0.5%	Vapor Liquid	Vapor Liquid	Vapor Liquid	Vapor Liquid Diffuse phase border
Residue B	TG 7%, EE 84% DG 9%, MG 1%	Vapor Liquid	Vapor Liquid	Vapor Liquid	Vapor Liquid Diffuse phase border
Residue A/Dimodan LS (50:50 wt%)	TG 6%, EE 39% DG 6%, MG 51%	Vapor Liquid	Vapor Liquid	Vapor Liquid	Vapor Liquid

[a]0.22 g lipid sample and 0.14 g ethanol (99.5%) charged to cell (1.62 mL). Abbreviations: EE, ethyl esters, TG, triglycerides; DG, diglycerides; MG, monoglycerides.

(slight opalescence) vapor phase, while the yellow liquid phase remained transparent. When the cell was merely swirled around once or twice, however, exclusively the vapor phase in the immediate vicinity of the phase border became hazy. These results suggest that in the actual enzyme reaction experiments the continuous mixing of the substrates in the liquid phase may result in a hazy vapor phase close to the phase border. The opalescence of the vapor phase may indicate that the mixture of components present in that phase is close to its critical point. This kind of critical opalescence has been described by Stanley (23). However, it is also possible that the mixing of the two phases results in micellar changes of the components present in the vapor phase.

In conclusion, these results show that in the actual lipase-catalyzed alcoholysis experiments (19,20) which were carried out at 9 to 24 MPa and 40°C, the reaction system comprises three phases, i.e., vapor–liquid–solid; the solid phase is here the immobilization support containing the enzyme. Furthermore, this vapor–liquid–solid system probably prevails during the entire reaction. This information is of vital importance for the extractive reaction process under investigation in our earlier work (19,20) since it indicates that the possibility of vapor–solid systems that severely would diminish the selectivity, owing to coextraction of substrates and side-products, does not exist in this process.

Notwithstanding the differences between vapor–solid and vapor–liquid–solid reaction systems, it was considered interesting to compare the conversion obtained in our earlier work to some of the published work on lipid modification using lipases carried out using vapor–solid systems in SC-CO_2. Information on the reactions, lipases, etc. used by various researchers is compiled in Table 2. Dumont and coworkers (16)

TABLE 2
Various Reactions, Lipases, and Reaction Conditions Used for Lipid Modification in Supercritical Carbon Dioxide

Reaction	Lipase	Reaction conditions	System studied	Reference
Esterification of myristic acid by ethanol	*Mucor miehei*[a]	12.5 MPa 50°C	Vapor–solid	16
Transesterification of myristic acid by trilaurin	*Rhizopus arrizus*[b]	8.3–11.1 MPa 35°C	Vapor–solid	12
Alcoholysis of ethyl acetate by isoamyl alcohol	*M. miehei*[a]	10 MPa 60°C	Vapor–solid	15
Alcoholysis of cod liver oil by ethanol	*Candida antarctica*[c]	9 MPa 40°C	Vapor–liquid solid	20

[a]Lipozyme from Novo Nordisk A/S (Bagsvaerd, Denmark).
[b]Immobilized on porous aminopropyl glass beads.
[c]Novozym from Novo Nordisk A/S.

reported that at constant reactant flow rate the optimal conversion obtained was 60% when an SC-CO_2 flow rate of 340 g/h was used. Miller and coworkers (12) demonstrated that the conversion of trilaurin was dependent on residence time. It was at best approximately 86% when the residence time was 125 s. van Eijs *et al.* (15) found that the productivity of the immobilized lipase was 120 g isoamylacetate/kg enzyme/h at optimal moisture content. In our work on the effect of changing the flow rate of the extraction fluid (20), a conversion of 86%, considering only EE extracted from the reaction mixture, was observed for a flow rate of 0.3 NL CO_2/min (liter CO_2 at atmospheric pressure and room temperature/min). This conversion corresponds to a production rate of approximately 440 g EE/kg immobilized enzyme/h. The rather small amount (0.0007 kg) of immobilized lipase used in this work gives rise to this fairly high production rate. The perception from this comparison is that the production rate of the vapor–liquid–solid system studied seems to be of the same order or possibly even higher than that observed for vapor–solid systems. Further support for this conclusion is the report of Jackson and King (24) that, when a glycerolysis reaction of soybean oil and 1,2-propanediol was carried out in SC-CO_2, the conversion rate to MG increased when the concentrations of the soybean oil and 1,2-propanediol exceeded their solubilities in SC-CO_2.

In summary, the conclusion from this work is that a multiphase system is not inhibitory for carrying out lipase-catalyzed lipid modifications in SC-CO_2. Further, information on the phase behavior of the system can be important for the efficiency of the process as well as for the purity of the product.

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