Screening Catalytic Lipase Activities with an Analytical Supercritical Fluid Extractor

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ABSTRACT: Two different screenings of several commercial lipases were performed to find a lipase with superior performance for the conversion of lipid moieties to their fatty acid methyl ester (FAME) derivatives under supercritical conditions. The first screening was done under hydrolytic conditions in a buffer. The second screening was done under supercritical conditions with CO_2, utilizing some of the same lipases for the methanolysis of different lipids. For the substrates studied, there was a significant difference in lipase activity under the two above conditions. Significant hydrolytic activity was demonstrated for three different lipid types (triglycerides, sterols, and phospholipids) with Lipase PS30, but when the same lipase was immobilized on an Accurel carrier (polypropylene), the activity decreased considerably. The opposite was found for Lipase G, which showed strong activity when immobilized and under supercritical conditions. Furthermore, Chirozyme L-1 was superior under supercritical conditions. The altered substrate specificity that some of these lipases show in supercritical CO_2 suggests several interesting synthetic options and applications under these conditions.

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The analysis of lipids in biological samples and food products by chromatography often requires solvent extraction, followed by methylation of the fatty acids involved. However, the rather cumbersome sample preparation and subsequent methylation can be omitted by the use of a novel sequential extraction and lipase-catalyzed esterification in supercritical carbon dioxide as shown by Berg *et al.* (1) and by Jackson and King (2). Furthermore, the use of supercritical CO_2, contrary to the traditional solvent extraction/methylation technique, complies with the current trend toward more restrictive environmental regulations, particularly in a research laboratory environment where the use of organic solvents must be minimized (3). Snyder *et al.* (4,5) have shown that fatty acid methyl esters (FAME) may be synthesized by the abovementioned sequential extraction and lipase-catalyzed method in supercritical CO_2 for a wide variety of lipids, such as triglycerides, steryl esters, and phospholipids.

It has been shown that the supercritical fluid extraction/supercritical fluid reaction (SFE/SFR) method was in good agreement with solvent extraction-based methods, used to measure the nutritional fat content in meat samples (4,6). The Nutritional Labeling and Education Act (NLEA) has defined nutritional fat as the sum of fatty acids from major lipid constituents, such as mono-, di-, and triglycerides, as well as minor lipid species, phospholipids, and sterols, expressed on a stoichiometric basis as triglycerides (7). Jackson *et al.* (2,4,5) have shown the lipase derived from *Candida antarctica* B, known as Novozyme SP 435 (now Chirozyme L-2), to be highly effective in the conversion of the above three major lipid categories. However, in this study, our interest was to find a more effective lipase that could accomplish the task, and thus we initially screened 12 different commercial enzymes for their hydrolysis efficacy on model lipid substrates. Then, 10 different immobilized lipases (of which 5 were the same as in the previous study) were screened for methyl ester synthesis activity under supercritical conditions in a commercially available supercritical fluid extractor, thus providing a fast and effective method of assessing lipase activity under supercritical fluid conditions.

EXPERIMENTAL PROCEDURES

Materials. Accurel EP100 (macroporous polypropylene, particle size 200–1000 μm) was obtained from Akzo Nobel (Orenberg, Germany), and mono- and dibasic phosphate were obtained from EM Science (Gibbstown, NJ). Lipase G, Lipase PS30 (*Pseudomonas cepacia*), Lipase MAP 10, Lipase AY 30, and Lipase L were all obtained gratis from Amano Corp. (Troy, VA). Chirozyme L-1 (*Burkholderia* sp.), Chirozyme L-2 (*Candida antarctica* B), and Chirozyme E-1 (pig liver esterase) were all generous gifts from Boehringer Ingelheim Corp. (Indianapolis, IN). Lipozyme (*Mucor miehei*), a lipase derived from *C. antarctica* A, and Novozyme SP 435 (*C. antarctica*) were gifts from Novo Nordisk (Franklington, NC). Lipases ESL-001-01 through ESL-001-07 were all obtained from Recombinant BioCatalysis (Philadelphia, PA). The lipase derived from *P. cepacia* and immobilized by Sol-Gel (TMOS/PrTMS) was a kind gift from Dr. Albin Zonta (Max Planck Institut für Kohlenforschung, Mülheim/Ruhr, Germany). Cholesteryl stearate was

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purchased from Nu-Chek-Prep (Elysian, MN), and phosphatidylcholine (PC) from Avanti Polar Lipids (Alabaster, AL). Vegetable shortening was obtained from a local supplier.

Immobilization of lipases. The procedure of Bosley and Peilow (8) was utilized for immobilizing the lipases. Approximately 2.0 g of Accurel EP100 was wetted with a minimal volume of absolute ethanol (typically 12.0 mL). After a few minutes, 65 mL of 0.01 M Na_2HPO_4 buffer (pH 7) was added, followed by the required amount of lipase dissolved in 175 mL of the same buffer. The mixture was then mixed on an orbital shaker (Model G10 Gyratory Shaker @ 150 rpm; New Brunswick Scientific Co., New Brunswick, NJ) held at 25°C. After 24 h, the immobilized enzymes were filtered and dried *in vacuo*.

Hydrolysis procedure. Hydrolysis reactions on cholesteryl stearate were investigated by the addition of 9 mg cholesteryl ester to approximately 1 mg lipase and 0.5 mL buffer solution. Four hundred microliters of PC solution (25 mg/1 mL $CHCl_3$) were added to 1 mg lipase and 0.5 mL buffer solution. The phospholipid samples were allowed to react at room temperature, while the cholesteryl esters, which were not soluble under such conditions, were heated to 70°C. The reaction products were analyzed by SFE chromatography and high-performance liquid chromatography (HPLC).

Methanolysis. SFE, followed sequentially by the supercritical lipase-catalyzed reaction (SFE/SFR), was accomplished with a Hewlett-Packard Model 7680T SFE unit (Wilmington, DE), interfaced with a Hewlett-Packard 5890 II gas chromatograph (4,5). The candidate lipase (500 mg) was added to a 7-mL extraction cell, followed by insertion of a glass wool plug and 10 mg of sample. Shortening samples were added directly to the cell while 400 µL of a solution of cholesteryl stearate or PC in chloroform (25 mg/mL) was added to the cell. The residual solvent was evaporated with the aid of a nitrogen stream. SFE/SFR conditions were 17.2 MPa and 50°C for 80 min, at a CO_2 flow rate of 0.5 mL/min, with 0.5% by volume of methanol addition.

Analyses. The extent of hydrolysis and methanolysis for the shortening and cholesteryl stearate samples was assessed in a Model 600 supercritical fluid chromatograph (Dionex, Inc., Salt Lake City, UT). A Dionex SB-Octyl-50 capillary column was utilized (10 m × 100 µm × 0.5 µm film thickness). The pressure gradient program was: 120 atm for 5 min, then ramped to 300 atm at 8 atm/min. The column temperature was initially held at 100°C for 5 min, then programmed to 190°C at 8°C/min. Injection of the samples was accomplished by a time/split automatic injector with a Valco valve (Valco, Inc., Houston, TX) for 1.8 s with a 200 nL loop. The flame-ionization detector was held at 350°C.

PC conversion was measured by HPLC, in a Spectra Physics Model SP8800 liquid chromatograph (San Jose, CA), interfaced with a Varex evaporative light scattering detector Model Mark III (Alltech, Inc., Deerfield, IL). A LiChrospher Si-60 column (5 µm, 250 mm × 4.6 mm) (Alltech, Inc.) was held isothermally at 30°C. A linear-gradient solvent program was used to affect the separation of the lipid species: 40% of solvent A consisting of $CHCl_3/CH_3OH/H_2O/NH_4OH$, 60:34:5.5:0.5 (by vol), plus 60% of solvent B consisting of $CHCl_3/CH_3OH/NH_4OH$, 80:19.5:0.5 (vol/vol/vol), to 100% solvent B over a 12-min run (5). The solvent flow rate was 1 mL/min. Fatty acid and methyl ester formation from PC were also monitored by the previously described SFC program.

RESULTS AND DISCUSSION

Previously, we have shown Novozyme SP 435 (Chirozyme L-2) to be a highly effective and nonselective catalyst in the methanolysis of triglycerides, phospholipids, and sterol esters under supercritical conditions (2,4,5). However, we were interested in testing the increasing number of "commercial" lipases available today for their effectiveness as transesterification agents under supercritical conditions. Because lipases by definition exhibit hydrolytic activity on triglycerides (9,10), we were interested in their effective activity on cholesterol esters and phospholipids.

Hence, 12 lipases were monitored for their hydrolytic activity toward cholesteryl stearate (C_{18}CE) and PC (Table 1). Table 1 overall reveals limited enzymatic activity of the lipases toward the steryl ester moiety; in fact, 10 of the lipases showed no reaction under the listed hydrolytic conditions. By contrast, Lipase PS30 and Lipase MAP totally hydrolyzed PC to its fatty acids, while variable activity was exhibited by five of the other lipases, and five exhibited no reactivity toward the phospholipid.

Methanolysis under supercritical conditions showed a much different picture of reactivity (Table 2). Five lipases from the previous hydrolysis, now on a polypropylene support, showed drastically altered substrate specificity. The most effective methanolysis of triglycerides was obtained by using Chirazyme L-1, Lipozyme IM, and Novozyme SP435 (Chirazyme L-2), as shown previously (4,5). Respectable triglyceride conversions, 90 and 81%, respectively, were also achieved with Lipase G and a lipase derived from *P. cepacia* (sol-gel), as shown in Table 2. These trends were also found for the same enzymes against the steryl ester. Both Novozyme SP435 and Chirazyme L-1 converted PC at 90% or higher lev-

TABLE 1
Hydrolysis of Cholesteryl Stearate (C_{18}CE) and Phosphatidylcholine (PC)

Lipase	Conversion to fatty acid from C_{18}CE (%)	Conversion to fatty acid from PC (%)
ESL-001-01	N.R.[a]	10
ESL-001-02	N.R.	11
ESL-001-03	N.R.	N.R.
ESL-001-04	N.R.	N.R.
ESL-001-05	N.R.	N.R.
ESL-001-06	N.R.	N.R.
ESL-001-07	N.R.	15
Lipase PS30	11	100
Lipase AY	7	30
Lipase MAP10	N.R.	100
Lipase G	N.R.	50
Lipase L	N.R.	N.R.

[a]N.R. = no reaction.

TABLE 2
Lipase-Catalyzed Methanolysis with SFE/SFR Conversion of Lipids (%)

Lipase	Shortening	C_{18}CE	PC
Lipase PS30[a]	2	10	1
Lipase L[a]	4	1	N.R
Lipase AY[a]	5	1	N.R.
Lipase MAP10[a]	56	31	22
Lipase G[a]	90	100	48
Pseudomonas cepacia			
Lipase (sol-gel)[b]	81	45	80
Novozyme 435[c]	100	98	99
Lipase from *Candida antarctica* A.[c]	1	N.R.	N.R.
Chirazyme L-1[c]	100	98	90
Chirazyme E-1[c]	6	2	1
Lipozyme IM[c,d]	99	96	60

[a]Immobilized on Accurel.
[b]Reaction products included 15% monoglycerides and 19% diglycerides.
[c]Carrier-fixed (not specified by manufacturer).
[d]Reaction products included 16% monoglycerides. SFE/SFR, supercritical fluid extraction/supercritical fluid reaction. See Table 1 for other abbreviations.

els. As indicated in Table 2, the other 10 enzymes exhibited varied activities against PC, ranging from no reaction to 80% conversion for a lipase derived from *P. cepacia* (sol-gel). Overall, these results indicate that the five enzymes mentioned are all suitable candidates for catalyzing methanolysis of the three listed substrates. More complete conversions could probably have been obtained by running the reaction somewhat longer or under slightly adjusted conditions of temperature and pressure. This certainly appears to be true for the methanolysis conducted with a lipase derived from *P. cepacia* (sol-gel), where incomplete conversion is indicated by the presence of residual mono- and diglyceride. It is also possible that slight adjustment of the conditions might optimize these conversions or increase the activity level of other lipases where methanolysis was not favored. Of course, lipase denaturation, with commensurate loss of catalytic activity at high pressures and temperatures, must also be considered when adjusting the SFR conditions.

Comparing the results in Tables 1 and 2 is interesting. It appears that substrate specificity changes between the two reaction conditions. For the in-common substrate, the steryl ester, it can generally be said that partial or no conversions were attained under hydrolytic conditions for all five lipases common to both sets of experiments. Lipase SP30 shows wide substrate specificity, as expected (11), by the partial hydrolysis of C_{18}CE. However, under supercritical conditions, Lipase G provided excellent methanolysis of the cholesteryl stearate (100%), while Lipase SP30 shows lower activity than under the hydrolysis conditions. Results were quite different for these five in-common lipases when used to perform hydrolysis or methanolysis, respectively, on PC. As shown in Table 1, Lipase PS30 showed promising results in the hydrolysis screening with PC. However, when immobilized and used under supercritical conditions, the lipase showed little activity against the phospholipid. In contrast, Lipase G retained its activity for PC in supercritical CO_2. This clearly indicates that some of these lipases exhibit altered substrate specificity under supercritical conditions, and that screening lipase activity under hydrolytic conditions is not an accurate measure of its effectiveness under supercritical conditions, at least for interesterification reactions. In the light of this finding, it is interesting that Lipozyme IM retained some of its 1,3-specificity (12).

The above results are testimony to the value of this supercritical fluid-based screening technique as a routine screening method for lipase activity in the presence of supercritical fluids. As indicated by the results in Table 2, assorted enzyme candidates can be evaluated in 1–2 d, automatically, against specific substrates, without resorting to laborious single determinations that are time- and labor-intensive. Such a technique has applicability not only in evaluating the formation of FAME for analytical determinations but allows the effectiveness of the enzyme moieties to be evaluated in terms of their use as industrial catalysts under supercritical fluid conditions. This is quite different from previous studies, in which the activity of one enzyme in supercritical fluid media has been compared with its performance in organic solvents (13–15). Using the approach described above also allows one to use a minimal amount of enzyme and substrate to assess the feasibility and rate of these enzymatically catalyzed reactions.

Finally, this study verifies that traditional aqueous-based hydrolysis procedures for evaluating lipase activity do not accurately reflect their catalytic activity profile (substrate specificity) in supercritical fluid media, such as compressed CO_2. Comparison of the results for in-common lipases in Tables 1 and 2 indicates that a different level of activity and substrate specificity for the same lipase exists in the buffer solution vs. the supercritical CO_2 medium. In addition, many lipases exhibit discriminatory activity against different substrates in both media, lending desired specificity or limitations, depending on the end result desired. Certainly, alternative lipase candidates which offer equivalent activity against common lipid substrates have been found and evaluated.

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