1,3-Specific Lipolysis of *Lesquerella fendleri* Oil by Immobilized and Reverse-Micellar Encapsulated Enzymes[1]

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Three types of reaction systems, all batch-mode, were employed for production of hydroxy (lesquerolic and auricolic) fatty acids *via* 1,3-specific lipolysis of *Lesquerella fendleri* oil: "Free" *Rhizopus arrhizus* or immobilized *Rhizomucor miehei* lipase (Lipozyme™) in reverse micelles (System 1), Lipozyme suspended in lesquerella oil/isooctane mixture (System 2) and a suspension of water and free *R. miehei* lipase in lesquerella oil/isooctane (System 3). The objective was to find the system that best maximized yield (*i.e.*, percent hydrolysis), the proportion of hydroxy acids among the free acids liberated (hydroxy acid "purity"), and recovery/reuse of lipase activity, and that could be easily adapted into a large-scale process. System 1 provided the largest percent hydrolysis (55%) and hydroxy acid purity (85%), but of the three systems would be the most difficult to scale up. Thus, System 1 would be the most desirable reaction system only when small batch sizes are to be processed. System 3 yielded 47.2% hydrolysis, but the hydroxy acid purity was at most 73%, making it the least desirable of the three systems to employ. System 2 yielded moderate extents of hydrolyses (30–40%) and large hydroxy acid purity initially (80–83%), but the purity decreased slightly in the latter stages of the reaction due to acyl migration. System 2 was the system most easily adaptable to a large-scale process, making it the method of choice. For System 2 reactions, only when the medium was initially saturated with water and water consumed by the reaction was continuously replaced could 30–40% hydrolysis be achieved. External mass transfer limitations for Lipozyme-catalyzed reactions were not present when the solution's water content was not above saturation, and its kinematic viscosity, controlled by the temperature and the proportion of isooctane, was below 41 centistokes.

KEY WORDS: Acyl migration, hydroxy acids, *Lesquerella fendleri*, lesquerolic acid, lipolysis, Lipozyme, positional specificity, reverse micelles, *Rhizomucor miehei* lipase, *Rhizopus arrhizus* lipase.

Seed oil from *Lesquerella fendleri*, a potential new crop currently under development by the U.S. Department of Agriculture and private-sector partners, contains two hydroxy fatty acids, lesquerolic acid (53%) and auricolic acid (3%). These acids have potential industrial utility (1,2). Recently, we reported the recovery of over 80% of the hydroxy acids at 85% purity from lipolysis of lesquerella oil with the 1,3-specific lipase from *Rhizopus arrhizus* (3). In the cited work, we used reverse-micellar medium (4). Literature indicates that lipolyses have been performed with a variety of reaction systems, such as oil/water emulsion or other biphasic batch reactors (5–8) and membrane (9–12) or immobilized lipase bioreactors (13–15) operated in continuous mode. We conducted lesquerella oil lipolysis in three reactor systems (each in batch mode): System 1: "free" *R. arrhizus* lipase or immobilized *Rhizomucor miehei* lipase (Lipozyme-IM20) with water-in-oil microemulsions (reverse micelles) that contain the surfactant Aerosol-OT (AOT); System 2: lipozyme with lesquerella oil/isooctane (surfactantless) medium; System 3: "free" *R. miehei* lipase with water/oil biphasic medium. We determined which of the three gave the best combination of high recovery and purity of hydroxy acids, high recovery and reuse of lipase and the best adaptability to large-scale or continuous process.

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MATERIALS AND METHODS

Refined *L. fendleri* oil was available from previous work (16). The surfactant AOT [sodium *bis*(2-ethylhexyl)sulfosuccinate] and lipase from *R. arrhizus* were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Lipozyme IM20 and its two components, *R. miehei* lipase and Duolite A561 anion exchange resin, were generous gifts from Novo-Nordisk (Danbury, CT). Moisture removal from liquids was achieved by treatment with 4A molecular sieves from Linde (East Chicago, IN). All other materials employed were of high purity (>99%). Deionized water was used throughout.

Reactions in water/AOT/isooctane reverse-micellar medium (System 1) were operated in batch-mode (1–100 mL) with either "free" *R. arrhizus* lipase or Lipozyme as biocatalyst. Formation of medium and operation of reactions were described previously (3). The medium used for System 2 was lesquerella oil/isooctane mixtures presaturated with water or 50 mM phosphate buffer (pH = 6.9). Lipozyme was the biocatalyst for all System 2 runs. The System 3 reaction was conducted as follows: 15 mL of 50% lesquerella oil in isooctane and 15 mL of 50 mM phosphate buffer (pH = 6.9) containing "free" *R. miehei* lipase at a concentration of 16.4 U/mL were placed in a 100 mL beaker and mixed at 400 rev min^{-1} (rpm) and ambient temperature. Agitation, required for Lipozyme-catalyzed and System 3 reactions, was provided by a 04644 Digital Hot Plate/Magnetic Stirrer from Cole-Parmer (Chicago, IL) or a Vortex/Evaporator shaking unit from Haake-Buchler (Saddlebrook, NJ). Three methods were employed for analysis: The Lowry-Tinsley free fatty acid (FFA) assay (17), gas chromatography (GC) and supercritical fluid chromatography (SFC). The first method measured the percent hydrolysis, while the second and third were used for analysis of FFA, MG (monoglyceride), DG (diglyceride) and TG (triglyceride) species. SFC also provided the relative amounts of FFA, MG, DG and TG. GC was performed on trimethyl silane-derivatized samples with a 25 m × 0.25 mm methyl 65% phenyl silicone capillary column from Quadrex (New Haven, CT). SFC was conducted in a Model 600 chromatograph from Dionex (Salt Lake City, UT) with a 10 m × 50 μm SB-phenyl-5 column from Dionex and SFC/supercritical fluid extraction-grade

carbon dioxide from Air Products (Tamaqua, PA) as carrier fluid. Chromatographic procedures for GC and SFC have been described previously (3). Enzyme concentrations are expressed in terms of g/L medium for Lipozyme and U/mL for *R. arrhizus* and *R. miehei* lipases, with U having been defined previously (3). The specific activity of Lipozyme for the range of agitation rates employed was *ca.* 16 U/mg.

Kinematic viscosity was measured with a Cannon-Fenske viscometer (Cannon Instrument Co., State College, PA) placed in an oil bath that had precise temperature control (±0.1°C). Measurements were performed in triplicate. The water content of lesquerella oil/isooctane mixtures was determined by Karl-Fischer titration. The percent moisture of Lipozyme, controlled by storage over saturated salt solutions in a dessicator for at least 48 h, was determined gravimetrically. Lipozyme removed from the reaction medium was rinsed with hexane several times, then air-dried before being weighed for water content. Hexane was chosen as rinsate to prevent stripping of water from Lipozyme. The medium water content was increased by connecting two sealed flasks in series with tygon tubing: One flask contained the reaction medium while the second contained a saturated Na_2HPO_4 solution. The apparatus was allowed to equilibrate for 24 h before the reaction was initiated.

RESULTS AND DISCUSSION

System 1. Hydrolysis of *L. fendleri* oil was conducted in water/AOT/isooctane reverse-micellar medium with *R. arrhizus* lipase or Lipozyme. The use of immobilized enzymes in reverse micelles has been explored only sparingly (18–20). The advantage of employing immobilized lipases with reverse micelles is simplified enzyme recovery (*via* filtration). Figure 1 depicts the effect of the volume percent lesquerella oil in the medium (V_{LO}) on the extent of hydrolysis catalyzed by Lipozyme. The figure demonstrates that high levels of hydrolysis can be achieved, but only in the presence of large proportions of isooctane, *i.e.*, at small values of V_{LO} (66.7% hydrolysis would represent ideal 1,3-specific lipolysis). This trend agrees with the results of Tsai and Chiang (21) and reflects the small extent of hydrolysis obtained for solventless microemulsions (22). Isooctane is needed to increase medium hydrophobicity, which in turn leads to improved surface activity by AOT, hence greater water solubilization. We found the final percent hydrolysis is negligibly dependent upon enzyme type for 1,3-specific lipases (Fig. 1), temperature and water content, when stoichiometrically sufficient amounts of water are present. However, the major problem with System 1 was with isolation of the hydroxy acids from the reaction medium on a large scale. Moreover, we could only separate the medium components by chromatographic techniques. In addition, the difficulty in designing continuous-mode bioreactors for reverse-micellar reactions is well known. In conclusion, we recommend System 1 be employed only when small quantities of hydroxy acid are needed.

System 2: effect of stir rate. Batch reactions with Lipozyme required agitation to disperse the biocatalyst. Stirring rates must be low enough to prevent abrasion of the biocatalyst. For example, Kim and Rhee (23) encountered mechanical breakage of Lipozyme at 400 rpm. In addition, literature suggests operation at speeds above 100 rpm to avoid pore diffusion limitations (24) and above 200 rpm to prevent external mass transfer limitations (25). The effect of stir rate on the initial rate of lesquerella oil lipolysis (solvent-free basis) is depicted in Figure 2. For a medium consisting of 50% lesquerella oil/50% isooctane (*i.e.*, $V_{LO} = 50$), external mass transfer limitations (indicated by dependence of reaction rate on stir rate) existed only below 150 rpm (Fig. 2). Visually, Lipozyme appeared poorly dispersed below 150 rpm. In contrast, external mass transfer limitations were apparent for the $V_{LO} = 75$ reaction at room temperature (Fig. 2). Moreover, the

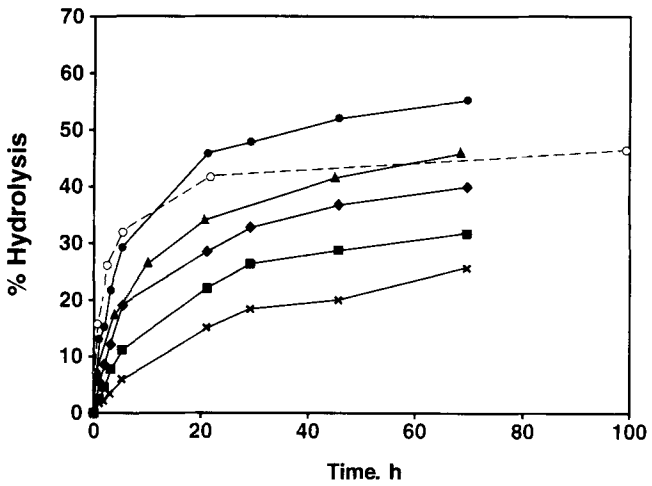

FIG. 1. Effect of % lesquerella oil (V_{LO}) on the course of hydrolysis in water/AOT/isooctane reverse micellar medium at 23°C. Reactions with Lipozyme ([Aerosol-OT(AOT)] = 100 mM, w_o = 10.1, 4.38 g Lipozyme/L), V_{LO} = closed circle, 10 (%); closed triangle, 20; closed diamond, 35; closed box, 50; ×, 65; open circle, with *Rhizopus arrhizus* ([AOT] = 125 mM, w_o = 11.2, 3.75 U/mL), V_{LO} = 20.

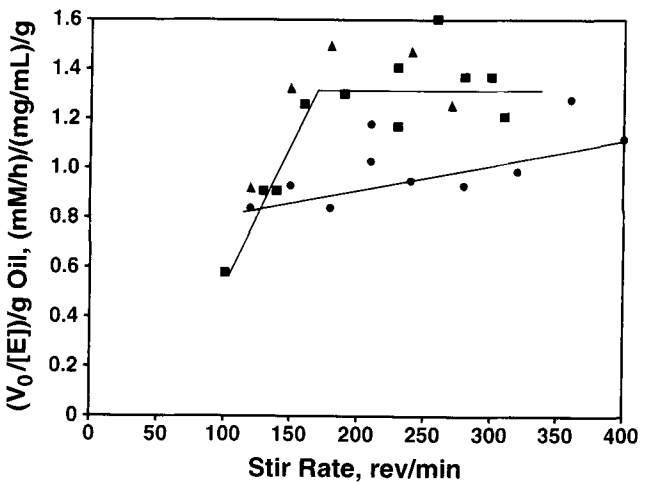

FIG. 2. Effect of stirring rate on the initial rate of hydrolysis by Lipozyme (3.3–8 mg/mL) at 23°C for various combinations of V_{LO} values and % moisture of Lipozyme. Closed box, V_{LO} = 50 (%); % moisture, = 11.5 (%); closed triangle, V_{LO} = 50; % moisture = 20.0; closed circle, V_{LO} = 75; % moisture = 11.5. See Figure 1 for abbreviation.

initial rate in this case increases linearly with stir rate (Fig. 2), which is indicative of external mass transfer limitation. In addition, the moisture content of Lipozyme had no effect on external mass transfer limitation (Fig. 2). For most cases, we chose to operate at stir rates where no external mass transfer limitations occurred (*ca.* 150–250 rpm). We recovered most of the enzyme activity from the batch reactor when operating at this range of speeds.

For this range of stir rates, poor dispersion of Lipozyme was encountered when the water content of the solution was above saturation, *i.e.*, when a second aqueous phase was present. These cases were avoided. Thus, water-saturated medium was optimal for System 2 reactions.

Medium kinematic viscosity, ν, was also a major factor for dispersion of Lipozyme. We measured ν as a function of temperature (T), between 25 and 60°C for lesquerella oil/isooctane mixtures of various values of V_{LO}. For each mixture, ν varied logithmatically with T, as predicted for Newtonian fluids whose densities do not change appreciably with T (26). Such is the case for lesquerella oil (27) and isooctane in the temperature range studied. From these measurements, the following relationship was derived, which predicts kinematic viscosity (centistokes, cSt) as a function of temperature (°C) and V_{LO} with 99% precision (Equation 1):

$$\ln \nu = (2.87 \times 10^{-3} - 6.36 \times 10^{-4} V_{LO})T + 0.778\ V_{LO} - 0.646 \quad [1]$$

Viscosity values obtained for $V_{LO} = 100$ agree favorably with recently published data (28). For agitation rates between *ca.* 150–200 rpm, we found a critical value of ν (*ca.* 41 cSt) above which Lipozyme dispersed poorly in the medium (visual observation). By substituting 41 cSt into Equation 1, the range of T and V_{LO} values for suitable operation of the Lipozyme reactions was determined. This is displayed in Figure 3. According to the figure, reactions with 100% lesquerella oil must be operated above 57°C, and for reactions performed at room temperature, V_{LO} must be less than 67%. This explains the external mass transfer limitations observed for $V_{LO} = 75$ medium at room temperature (Fig. 2).

System 2: effect of water content. As suggested above, it is desirable to operate with water-saturated medium. Because water is consumed during the reaction, the solution must be continuously replenished with moisture. To determine the amount of additional water needed, water-saturated lesquerella oil/isooctane was hydrolyzed with Lipozyme. After 10 h for the $V_{LO} = 50$ case, hydrolysis slowed (Fig. 4). At 23 h, the solution was removed from Lipozyme, resaturated with water and then returned to Lipozyme to allow further hydrolysis. Two additional water resaturations took place at 80 and 142 h, as indicated by the vertical lines in the figure. Table 1 lists the water *content* of the medium for the $V_{LO} = 50$ case before and after each resaturation and the immobilization matrix moisture content before and after the first resaturation. The results confirm that hydrolysis slows before 23, 80 and 142 h due to water depletion from both the medium and the matrix. But at 190 h, the water content is sufficient to allow further hydrolysis. Thus, the slowing of reaction at 190 h is probably due to the onset of thermodynamic equilibrium. Hence, the highest percent

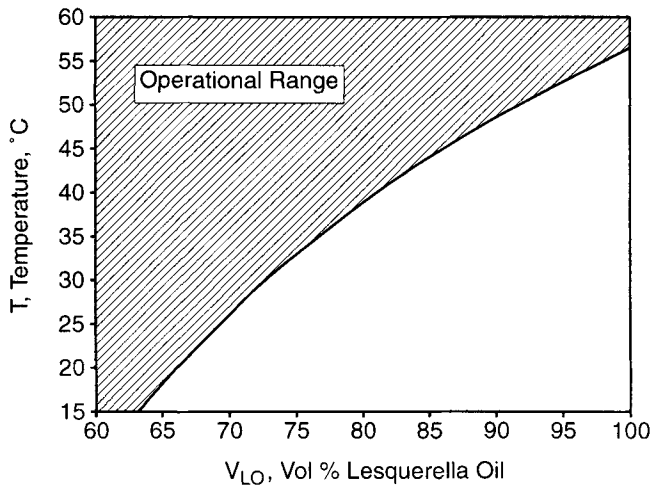

FIG. 3. T-V_{LO} operating space for lipozyme-catalyzed hydrolysis of lesquerella oil. See text for further detail, and Figure 1 for abbreviation.

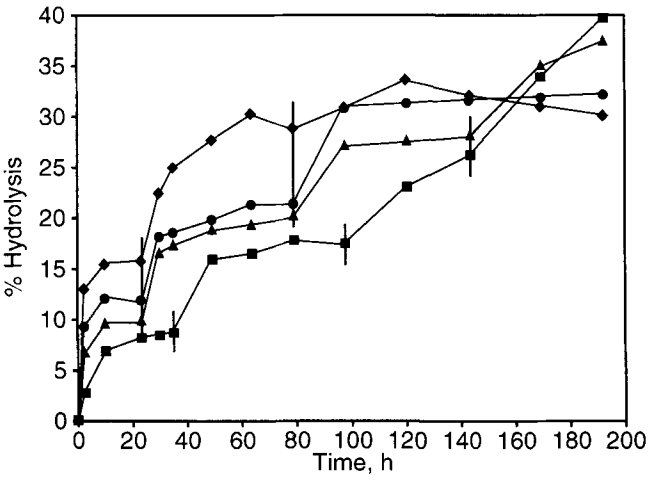

FIG. 4. Extent of hydrolysis as a function of time and V_{LO} for System 2 reactions at 23°C. Closed diamond, $V_{LO} = 10$ (%); closed circle, 25; closed triangle, 50; closed box, 75. Lipozyme concentration was 4.46 g/L. Thick vertical lines represent times where medium was resaturated with water. See Figure 1 for abbreviation.

TABLE 1

Weight Percentage Water of Medium and Lipozyme Before and After Water Resaturation for the $V_{LO} = 50$ Reaction of Figure 4[a]

Time (h)	Medium, before resaturation[b]	Medium, after resaturation[b]	Lipozyme, before resaturation[c]
0	—	0.19	11.5
23	0.00	0.23	3.62
80	0.05	0.37	
142	0.15	0.53	
190	0.25	—	

[a]V_{LO} = the volume percent lesquerella oil in the medium.
[b]Determined by Karl-Fischer titration.
[c]Determined gravimetrically.

hydrolysis that can be achieved with System 2 is 35–40%, which is lower than that obtained for System 1. Moreover, at 190 h, according to SFC analysis, there is still a large proportion of DG and TG present (*ca.* 30 wt% each) and only a small percentage of MG (*ca.* 5%). Thus, it is crucial that a method be found that separates FFA from MG/DG/TG, so that the latter can be recycled as lipolysis substrate. Such a method has been developed (Hayes and Kleiman, in preparation). Also, as the reaction proceeds and more FFA is released, the level of water saturation increases (Table 1). From the experiments described in Figure 4, we were able to design a lipolysis reaction in which solution water saturation could be maintained during the course of the reaction by the addition of discrete amounts of water. Figure 5 depicts the change in percent hydrolysis and water content with time for such an experiment. (At later stages of the reaction, the medium water content was slightly greater than saturation. However, Lipozyme remained well dispersed.) Each vertical increase in percent moisture reflects the addition of water. As expected, the final extent of hydrolysis in Figure 5 matches Figure 4 results, confirming that reaction stoppage is due to thermodynamic equilibrium. Figure 5 also indicates that the rate of reaction for System 2 can approach the value obtained for System 1. In conclusion, a design for a continuous reactor scheme with immobilized lipases must include significant oil/water contacting, such as found with continuous stirred reactors (13) and multistage, countercurrent fluidized-bed reactors (14). By using the reaction scheme described for Figure 5, we have processed lesquerella oil batches as large as 200 g while achieving the same extent of hydrolysis indicated in Figure 5.

Another approach for adding water to the reaction system is to increase the water content of Lipozyme. This can be achieved by equilibrating Lipozyme with the airspace over saturated salt solutions in sealed containers (29). The percent moisture of Lipozyme is then controlled by the salt solution's water activity, which is a function of the ionic properties of the salt used (30). A similar approach was employed to control water content for lipase immobilized onto Sephadex (Pharmacia, Piscataway, NJ) in the hydrolysis of olive oil (31). Table 2 lists the salts used, their water activities and the Lipozyme moisture contents they promote. These results agree with recently published data (29,32). Fifty percent lesquerella oil (water-saturated) was hydrolyzed by Lipozyme for each of three Lipozyme water contents: 4.3, 11.5 and 20.0 mg/mL. No further water was added to the medium at any time during the reaction's course. The results, shown in Figure 6, indicate that the final percent hydrolysis increases with Lipozyme moisture content, but that the initial rate is not affected. But for all three cases, additional water would be required to obtain maximum (35–40%) conversion. An easier method for water replenishment was desired. We applied the same principle used to increase the water content of Lipozyme, namely, contact of the reactor air headspace with saturated salt solutions, to maintain medium water saturation during the course of reaction. Our apparatus, which was tightly sealed, consisted of two rubber-stoppered Erlenmeyer flasks connected together with tygon tubing. One flask contained a saturated Na_2HPO_4 aqueous solution (high water activity), the other contained

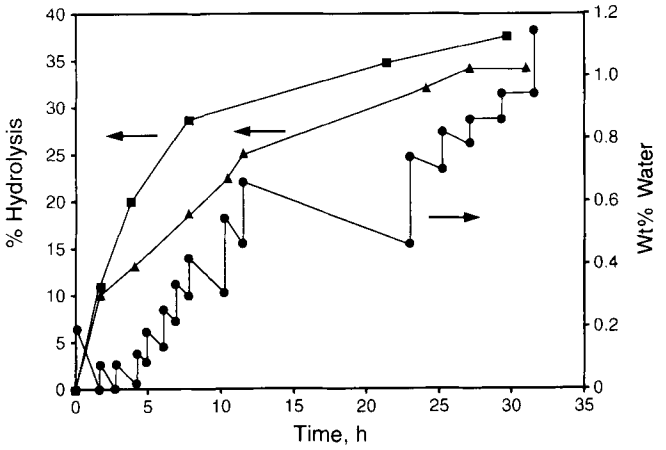

FIG. 5. A comparison between reverse micelles (closed box) and surfactantless medium (closed triangle) for the Lipozyme-catalyzed hydrolysis of lesquerella oil at V_{LO} = 50 (%) and 23°C. For the latter reaction, water was added during its course in discrete amounts. Lipozyme concentration was 16.7 g/L for each. Initial conditions for reverse micelles: w_o = 9.48, [AOT] = 100 mM. Closed circle, water concentration for surfactantless medium. See Figure 1 for abbreviation.

TABLE 2

Moisture Content of Lipozyme Stored over Saturated Salt Solutions, as Determined Gravimetrically[a]

Salt	Water activity at 20°C[a]	% Moisture
Untreated	—	11.5
$CaSO_4$	NA	4.3
KCl	0.86	20.0
Na_2HPO_4	0.98	23.3

[a]From Reference 34. NA indicates data not available.

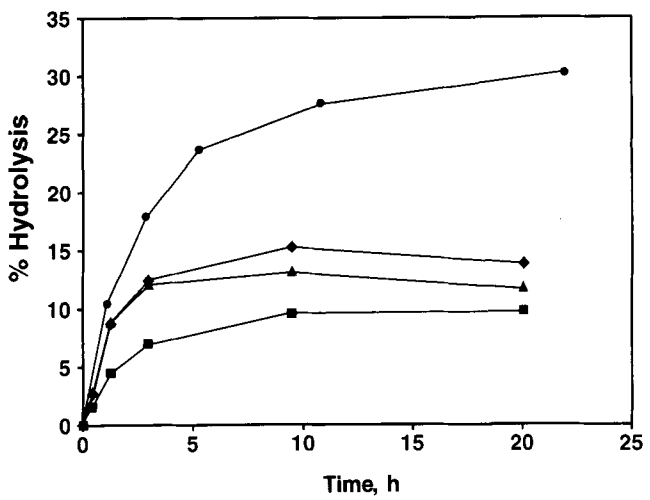

FIG. 6. Effect of Lipozyme water content and water-saturation of reactor headspace on hydrolysis at V_{LO} = 50% and 23°C. Closed box, 4.3% moisture; [Lipase] = 23.5 mg/mL; closed triangle, 11.5% and 23.5 mg/mL; closed diamond, 20.0% and 23.5 mg/mL; closed circle, 23.3% and 10.1 mg/mL with reactor headspace saturated with moisture (see text for more detail). See Figure 1 for abbreviation.

TABLE 3

The Distribution Among DG and TG During Lesquerella Oil Lipolysis at 23°C

System	V_{LO} (%)	Time (h)	% Hydrolysis	DG (%)[a]			TG (%)[b]		
				18,18	18,H	H,H	18,18,H	H,18,H	H,H,H
	10[c]	0.0					11.5	82.3	0.0
2		11.1	15.6	10.9	84.6	3.2	16.9	76.6	0.0
2		48.8	27.7	12.7	67.9	18.4	26.6	63.7	1.5
2		97.0	30.8	15.1	61.0	22.8	33.7	51.7	3.0
2		192.0	30.2	14.2	49.0	33.4	35.8	38.3	8.5
2	50[c]	11.1	9.7	9.4	86.4	1.9	12.9	81.3	0.0
2		48.8	18.8	11.5	77.3	9.1	19.0	73.2	0.0
2		97.0	27.1	14.3	74.9	8.6	24.6	67.4	0.0
2		192.0	37.4	17.2	64.8	16.3	31.6	54.5	1.9
2	75[c]	11.1	7.0	10.5	87.0	0.0	12.0	82.5	0.0
2		48.8	15.9	11.2	86.7	0.0	13.5	81.8	0.0
2		97.0	17.4	12.8	81.8	3.1	15.9	78.0	0.0
2		192.0	39.6	15.6	79.2	3.1	18.7	78.3	0.0
2	100[d,e]	54.4	51.5	16.2	51.3	31.4	29.5	39.4	9.2
1	35[f,g]	68.2	39.9	14.7	81.4	2.2	17.9	73.2	0.0
1	10[h]	200.0	61.0	30.7	69.3	0.0	36.1	45.2	0.0
3	50	29.6	47.2	26.1	72.2	0.0	32.5	49.8	0.0

[a]Diglyceride (DG) species, percent among DG; 18, H refer to C_{18}, hydroxyl acyl groups, respectively. [b]Triglyceride (TG) species, percent among TG. [c]Other conditions noted in Figure 4. [d]Temperature (T) = 60°C. [e]Other conditions noted in Figure 7. [f]Lipozyme. [g]Other conditions and abbreviations noted in Figure 1. [h]T = 23°C, w_o = 948, [Aerosol-OT] = 0.1 M, [Lipozyme] = 16.7 g/L.

the reaction mixture, Lipozyme, and a magnetic stirring bar. Karl-Fischer titration measurements demonstrated that the water content of the isooctane/lesquerella oil in the apparatus increased with time, but slowly. Lipolysis in this apparatus was nearly complete (*ca.* 30% hydrolysis) in 24 h without the addition of water (Fig. 6). A better apparatus design, with larger air–water and air–medium interfacial area, would improve this method.

System 2: effect of V_{LO} and T. Unlike the case for reverse micelles (Fig. 1), in System 2 reactions neither the equilibrium extent of conversion, nor the initial rate of reaction was strongly dependent on V_{LO} (Fig. 4). In addition, SFC analysis indicated that the reactions of Figure 4 all underwent a similar kinetic path. Table 3 describes the distribution of DG and TG species while Table 4 contains the FFA distribution at several times for the experiments depicted in Figure 4. The formation of TG(20-OH,20-OH,20-OH) (mostly trilesquerolin), which is not present initially, DG(20-OH,20-OH), and the decrease in percent lesquerolic/auricolic acid among FFA demonstrate acyl migration of C_{18} unsaturates from the 2-glycerol position to the 1-(3-) position and of 20-OH acyl groups from the 1-(3-) to the 2-position (see below).

Lipolysis of lesquerella oil was also conducted at 60°C with discrete amounts of water being added to the medium during the course of reaction. The results are shown in Figure 7. Over 45% hydrolysis was obtained in just over two days. The reaction time can be reduced by optimizing water addition and increasing the proportion of Lipozyme. The 45% hydrolysis level being slightly higher than the level achieved at room temperature (35–40%) is due to acyl migration, as shown by the dramatic decrease in the proportion of hydroxy acids among FFA (Fig. 7).

The final percent hydrolysis (given the presence of stoichiometrically sufficient water and the absence of acyl migration) was not affected significantly by temperature within the range 23–60°C (data not shown). Lipozyme has been shown to retain activity at 70°C (33), and we detected no significant activity loss during operation at 60°C.

TABLE 4

The Distribution of Free Fatty Acids During Lipolysis of Lesquerella Oil at 23°C

System	V_{LO}	Time (h)	% Hydrolysis	C_{16}	C_{18}	Hydroxy fatty acids
2	10[a]	23.9	15.8	2.8	16.7	80.5
2		79.7	28.7	1.8	20.0	78.2
2		168.6	32.1	2.2	28.0	69.9
2	50[a]	23.9	9.7	1.6	15.7	82.7
2		79.7	20.0	1.7	14.9	83.5
2		168.6	34.8	1.6	20.3	78.1
2	75[a]	23.9	8.3	1.9	15.1	83.0
2		79.7	17.8	2.1	16.0	82.0
2		168.6	33.7	1.9	20.0	78.1
1	50[b]	69.6	31.8	2.0	18.4	79.6
1	20[c,e]	7.0	25.3	2.2	13.1	84.5
1	20[c,e]	33.1	39.2	1.8	13.1	84.8
1	20[c,e]	79.1	42.8	1.8	17.0	80.9
1	20[d,e]	7.0	30.1	1.9	10.7	87.2
1	20[d,e]	33.1	33.4	1.8	11.9	86.4
1	20[d,e]	79.1	41.9	2.6	13.7	82.5
3	50[c]	6.3	33.7	2.7	44.6	50.0
3		22.2	42.4	1.9	26.0	70.8
3		29.6	47.2	1.6	24.3	73.0
—	—	—	100.0[f]	2.0	40.0	57.0

[a]Other conditions noted in Figure 4. [b]Other conditions noted in Figure 1. [c]Free *Rhizomucor miehei* lipase. [d]Free *Rhizopus arrhizus* lipase. [e]T = 23°C, w_o = 10.1, [AOT] = 0.1 M, [Lipase] = 2.7 U/mL. [f]Values for complete hydrolysis. See Tables 2 and 3 for abbreviations.

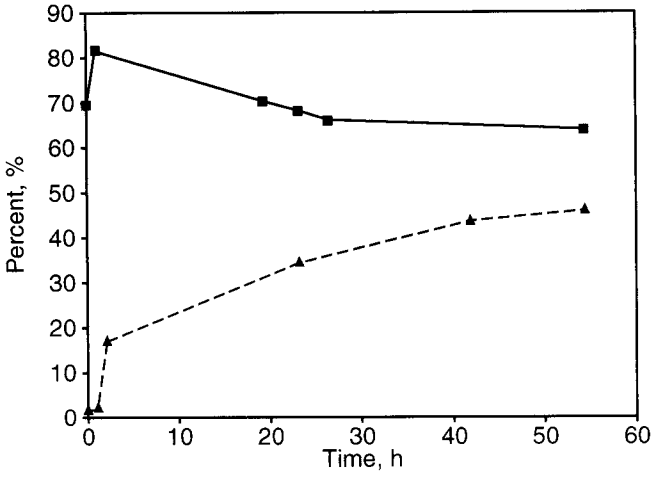

FIG. 7. The change in (closed triange) percent hydrolysis and (closed box) percent hydroxy acids among free fatty acids with time for hydrolysis of 100% lesquerella oil at 60°C by Lipozyme (52.9 g/L). Water was added in discrete amounts as performed for the experiment described by Figure 5.

Lipase specificity and acyl migration. The results mentioned above reflect the frequent occurrence of acyl migration during Lipozyme-catalyzed lipolysis of lesquerella oil. The formation of C_{18} 1-MG and accompanying loss of C_{18} 2-MG provided further evidence of acyl migration (data not shown). Other investigators have also observed acyl migration during Lipozyme-catalyzed reactions (23,32, 34). A careful examination of Table 3 shows a strong correlation between the extent and rate of hydrolysis and the formation of TG(20-OH,20-OH,20-OH) and DG(20-OH,20-OH). For example, the $V_{LO} = 10$ reaction reaches thermodynamic equilibrium the fastest and results in the largest production of these two species. In reverse micelles (System 1), catalysis by Lipozyme also led to acyl migration but to a much lesser extent. And with free *R. arrhizus* lipase in reverse micelles, acyl migration was negligible (Tables 3 and 4).

We suspected that the lower proportion of hydroxy acid for Lipozyme catalysis may partly be a function of the intrinsic activity of *R. miehei* lipase. For determination, we performed lesquerella oil lipolysis in reverse micelles with *R. arrhizus* and *R. miehei* lipases under identical conditions and with equal enzyme concentrations (U/mL). The results showed that the hydroxy acid purity among FFA was indeed lower for the *R. miehei*-catalyzed reaction throughout (Table 4). GC analysis indicated the absence of acyl migration for both reactions and the presence of larger amounts of 1-monolesquerolin and 1,3-dilesquerolin for the *R. miehei*-catalyzed hydrolysis (data not shown). Thus, *R. miehei* lipase has poorer 1,3-positional specificity than *R. arrhizus* lipase. In conclusion, both weaker positional specificity and acyl migration explain the lower percentage of hydroxy acids among FFA for Lipozyme-catalyzed hydrolysis.

Some interesting observations on the causes and factors promoting acyl migration can be derived. First, temperature catalyzes the process (32). This is evident by the results of lipolysis at 60°C (Fig. 7). Second, the water content plays a strong role because it promotes hydrolysis. To explain further, it is believed that isomerization occurs primarily among the DG (35,36) and/or MG (37,38) produced by hydrolysis. Our results do not discredit the significance of water. In addition, the presence of FFA is reported to catalyze acyl migration (35,39). It is of interest that the reactions in reverse micelles, which produce larger concentrations of FFA (and have larger water contents), promote less acyl migration (Table 3). Likewise, low levels of 2-MG were detected during the esterification of glycerol and lauric acid catalyzed by *R. delemar* lipase, a 1,3-specific biocatalyst, in reverse micelles (40). A consistent explanation for these trends has yet to be determined. Ion exchange resins have also been reported to catalyze acyl migration (32, and references therein). To examine whether the immobilization matrix played a role, we took a mixture of DG and TG, recovered from a lesquerella oil lipolysis reaction *via* extraction that contained large amounts of water, added the exchange resin component of Lipozyme and provided magnetic stirring. After one week, SFC analysis showed no evidence of acyl migration or any change in the medium composition.

System 3. Water/oil biphasic media have been commonly employed for lipolysis (5–8). Results for System 3 with "free" *Mucor miehei* lipases are contained in Tables 3 and 4. It is apparent that a larger percent hydrolysis is achieved with System 3 (47.2%) than with System 2 (30–35%). Also, recovery of enzyme activity from the medium after completion of the reaction was quite high: 82%. Furthermore, System 3 yielded low degrees of acyl migration (Table 3). However, the percent of 20-OH acyl groups among the FFA is significantly lower than that obtained from either System 1 or 2 throughout the course of reaction (Table 4), hence, System 3 is a less desirable choice. Reasons for the lower hydroxy acid purity here are probably related to the differences in the ability of hydroxy acids to adsorb at the different interfaces present in the three systems.

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