Invert Emulsion as a Medium for Fungal Lipase Activity

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ABSTRACT: An extracellular lipase from the fungus *Pythium ultimum* was active in an invert [water-in-oil] emulsion consisting of 4% water emulsified into edible oils with taurocholic acid as the surfactant. The pH range for optimum lipolytic activity was 7.5–8.5, and the optimum temperature for activity was 45°C. Specific activity of the purified lipase was 919.5 μmol/min/mg protein in the invert emulsion. Water content of the invert emulsion influenced activity of the lipase differently, depending on the substrate. The rate of olive oil hydrolysis with the *Pythium* lipase decreased with time, possibly due to inactivation of the enzyme and inhibition by free fatty acid products of the reaction. Total hydrolysis of olive oil by the *Pythium* lipase was compared with that by lipases from *Candida rugosa* and *Rhizopus arrhizus* in the invert emulsion. Hydrolysis essentially ceased within 24 h or less for the lipases from each source. However, the addition of aqueous solution at 8 h from the beginning of incubation stimulated hydrolysis by *C. rugosa* and *R. arrhizus* lipases by 1.8-fold and 2.5-fold, respectively, but not by the *P. ultimum* lipase, over corresponding controls after 48 h.
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KEY WORDS: *Candida rugosa* lipase, invert emulsion, lipase, *Pythium ultimum* lipase, *Rhizopus arrhizus* lipase, taurocholic acid, water-in-oil emulsion.

Lipases (EC 3.1.1.3; triacylglycerol hydrolases) are exceedingly versatile enzymes in that they can catalyze not only the hydrolysis of triacylglycerols, they also can carry out interesterification and esterification reactions (1–4). These enzymes by definition are active at oil/water interfaces, and their versatility is compounded by the fact that they are active not only in normal phase [oil-in-water (o/w)] emulsions but also in reverse-micelle systems including those containing organic solvents such as isooctane (5,6) and hexane (7). This versatility greatly expands opportunities for industrial applications of microbial lipases.

Although there has been considerable recent interest in reverse-micelle systems (5,8,9), a system that has not received much attention as a medium for lipase activity is the water-in-oil (w/o), or invert, emulsion. While in both cases the substrate oil, or organic solution of the oil, is the continuous phase of the system, the primary distinction between the invert emulsion and reverse-micelle is the size of the particles containing the emulsified aqueous solution representing the discontinuous phase, i.e., invert emulsions have particles greater than 0.1 μm in diameter (10). The volume fraction of discontinuous phase in the reverse-micelle system is low compared to an invert emulsion at generally less than 1% for optimal lipase activity (8), but the interfacial area is relatively high. Another major difference between w/o emulsions and reverse-micelles may be dynamicity. Reverse-micelles are thermodynamically stable and electrically neutral in their exterior shell, so they frequently collide, fuse, and create new reverse-micelles (11).

In this report, we describe some properties of the extracellular lipase from the fungus *Pythium ultimum* (12) in an organic solvent-free invert emulsion, and compare the activity of this lipase against olive oil with commercially available lipases from *Candida rugosa* and *Rhizopus arrhizus.* The purpose of this study was to establish an organic solvent-free improved reaction medium for hydrolyzing edible oils.

MATERIALS AND METHODS

Materials. Oils from olives, soybeans, peanuts, sunflower seeds, corn, cottonseeds, coconuts, and Menhaden fish were purchased from the Sigma Chemical Co. (St. Louis, MO). Lard oil, taurocholic acid sodium salt, and lipase from *C. rugosa* were obtained from the same source. Lipase from *R. arrhizus* was obtained from Boehringer Mannheim (Mannheim, Germany). Butter oil was prepared by heating locally purchased butter to 50°C and filtering it through cheese cloth. All reagents were of the highest purity available.

Lipase production. Pythium ultimum strain 144 was obtained from D.J.S. Barr of the Biosystematic Research Center, Central Experimental Farm (Ottawa, Canada). It was cultivated for 6 d at 18°C in 250-mL Erlenmeyer flasks containing 100 mL Vogel's medium (13) and 1 mL soybean oil using a Lab-line rotary incubator–shaker at 120 rpm as described previously (12). Mycelia were separated from the medium by suction filtration in a Buchner funnel, and proteins were precipitated from the culture medium with ammonium sulfate at 85% saturation. The precipitate was dissolved in and then dialyzed against 50 mM sodium phosphate buffer (pH 7.5) containing 10% glycerol (assay buffer). Unless noted otherwise, the enzyme prepared in this manner was used for experiments in the present study. In addition, a highly purified enzyme preparation obtained from media by $(NH_4)_2SO_4$ precipitation, and purified by diethylaminoethyl Sepharose CL-6B (Sigma Chemical Co.) chromatography and dialysis as described previously (12) was used.

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Lipase assay. The standard reaction mixture used in this study contained 5 mL oil, 0.1 mL of a 520 mM taurocholic acid solution of the assay buffer, and 0.1 mL enzyme preparation containing 0.072 mg protein in the assay buffer. When *C. rugosa* and *R. arrhizus* lipases were used, 0.1 mL of the enzyme preparation contained 0.027 and 0.007 mg protein, respectively. The final concentration of taurocholic acid and water in the 5.2 mL of reaction mixture as 10 mM and 4%, respectively. The oil was first mixed with the taurocholic acid solution, and then enzyme solution was added. The assay components were mixed (emulsified) by vortexing (*ca.* 30 s) and incubated in a constant temperature (45°C) waterbath without shaking. Variations of this standard assay procedure are noted in the table and figure legends as appropriate. After 30 min incubation, 20 mL of acetone/ethanol (1:1, vol/vol) were added to stop the reaction, and the free fatty acid content was determined by titration with 0.05 N aqueous NaOH. The reaction mixture without enzyme (blank) also was incubated as described above, and the enzyme solution was added prior to titration. The amount of product formed during incubation was calculated by subtracting the amount of free fatty acid in the blank from that of the reaction mixture after incubation with the enzyme. One unit of lipase activity was defined as the amount of enzyme required to liberate 1 μmol of fatty acid per min under the conditions specified. The assay was standardized using olive oil as the substrate. With *Pythium* lipase, the reaction was linear over time for 15–30 min where between 60 to 125 μmol of fatty acid were released. Thirty minutes was selected as the incubation time for experiments in this study. The degree of oil hydrolysis was calculated using Equation 1 (5):

$$\text{degree of hydrolysis (\%)} = \frac{\mu\text{moles fatty acids liberated}}{(\text{saponification value})(1000/56.1)(\text{oil in g})} \times 100 \quad [1]$$

The temperature for optimum activity of the *Pythium* lipase was determined by measuring activity toward olive oil as described above except at temperatures between 20 to 55°C. The reaction mixtures without lipase were equilibrated 5 min at the respective temperatures prior to starting the reaction by adding the enzyme.

The following buffers were used to determine pH dependence of the enzyme at 45°C: 50 mM citrate sodium–phosphate for pH 5.0 and 6.0, 50 mM sodium phosphate for pH 7.0 to 8.5, and 50 mM sodium phosphate NaOH for pH 10.5. The optimum concentration of taurocholic acid for lipase activity was determined using 0.25 to 20 mM of the surfactant in the aqueous phase using the standard assay described above.

Reaction mixtures having different water contents (4 to 12%) were prepared by injecting buffer (50 mM sodium phosphate pH 7.5) into the oil substrates (5.0 mL) and mixing; otherwise the standard assay was used as described above.

Reverse-micelle system. Pythium lipase activity in the sodium *bis*-(2-ethylhexyl)sulfosuccinate (AOT)–isooctane system (Fluka, Ronkokoma, NY) was determined according to Han and Rhee (5). Olive oil content of the reaction mixture was 10% (vol/vol), and the R value (defined as the molar ratio of water to AOT) was 10. The reaction was conducted at 25°C with *Pythium* lipase (11 units) dissolved in assay buffer.

Particle-size determination. The diameters of dispersed aqueous (w/o) and oil (o/w) globules (particles) in the emulsions were determined by light microscopy using a Nikon Biophot light microscope (Japan) equipped with a micrometer. The o/w emulsions were prepared as described previously (12).

The total interfacial area (F) in cm^2 was calculated from the diameter and volume of the dispersed phases of the w/o (0.2 mL assay buffer plus 5.0 mL oil) and o/w (0.5 mL oil plus 9.5 mL aqueous phase) emulsions according to Equation 2:

$$F = 6\,VD^{-1} \quad [2]$$

where D is the average diameter of oil droplets in the o/w emulsion or water droplets in the w/o emulsion, in cm, and V (cm^3) is the volume of the dispersed phases (14). Treatments within each experiment were done in triplicate, and each experiment was conducted at least twice.

RESULTS AND DISCUSSION

Lipolytic activity of the *Pythium* lipase was observed over the pH range of 5.0 to 10.5 in the invert emulsion, and the optimum pH for activity was between 7.5 and 8.5. This value was 8.0 for the same lipase in an o/w emulsion (12), although other studies reported that incubation conditions and types of emulsion influence the optimum pH for the activity of different microbial lipases. For example, the pH optimum for a *C. rugosa* lipase was 7.0 to 7.5 in the presence of Triton X-100 (Sigma Chemical Co.) with various immobilized enzyme supports (15,16), and 7.1 in the AOT–isooctane reverse-micelle system (8), but shifted to the acid range (pH 4 to 6) with lecithin as the emulsifier and butter oil as the substrate (17).

The optimum temperature for activity of the *Pythium* lipase in the invert emulsion was 45°C. Enzymatic activity gradually decreased above and below that temperature. The activation energy calculated from the slope of an Arrhenius plot was 11.4 kcal/mole. These values are higher than those for the o/w emulsion system, where the optimum temperature for activity was 30°C (12) and the activation energy was 7.3 kcal/mole, respectively (Mozaffar, Z., and J.D. Weete, unpublished data). In the case of a lipase from *C. rugosa*, enhanced optimum temperature (55°C) and activation energy (15.4 kcal/mole) were reported in a reverse-micelle system consisting of lecithin–water–butter oil compared with the enzyme incubated in AOT–isooctane reverse-micelle or normal phase emulsion system with olive oil as the substrate (17). The same lipase from *C. rugosa* in an AOT–isooctane or normal phase system showed an optimum temperature of 35°C and activation energy 8 kcal/mole (8,16). It was observed that inactivation of the *C. rugosa* lipase in the AOT–isooctane or o/w emulsion began above 35°C (8,15), whereas it began at 55°C in the butter oil–lecithin reverse-micelle system (17).

The amount of water in the invert system is exceedingly low compared to that in the o/w emulsion. In this study, ac-

tivity of the *Pythium* lipase as a function of water content at 4 to 12% of the total incubation mixture volume was determined. The effect of water content on lipase activity varied, depending on the substrate. For example, activity against olive and peanut oils increased by 40% at 8% water over that at 4% (Table 1). With lard oil as the substrate, activity increased 3.7 times at 8% over that at 4% water content. However, there was little difference in the lipase activity at 4 and 8% water with Menhaden fish oil as the substrate, and activity was 33% higher at 10% water compared to that at 12%. At up to 6% water content, the emulsions were stable during 30 min incubation. Above 6%, a thick, creamy gel-like layer formed at the bottom of the reaction mixture. Although lipase activity was higher with water contents at 8% water and above, in most experiments with olive oil the water content was kept at the 4% level to avoid creaming. When the influence of water on the activity of *C. rugosa* lipase was examined in an invert emulsion system of olive oil and water with no emulsifier, activity was found to increase sharply with increasing water reaching a maximum at a water-to-oil volume ratio of 1 and gradually decreasing thereafter (14).

The influence of water content on lipase activity in the invert emulsion system is unlike that with the lipase from *C. rugosa* in the AOT–isooctane reverse-micelle system where activity decreased with increasing water content over 1% (8). In the invert emulsion system, activity of the *Pythium* lipase decreased at water content below 4% (Mozaffar, Z., and J.D. Weete, unpublished data).

Direct comparison between the reverse-micelle and invert emulsion systems with respect to lipase activity is difficult. In the AOT–isooctane reverse-micelle system, the continuous hydrophobic phase is an organic solution of the substrate, whereas in the invert emulsion system used in this study the continuous phase contained only the substrate oil. Also the volume fraction of the aqueous phase is less in the reverse-micelle system than the invert system, but the surface area is higher. Differences in the reaction environment are known to influence lipase activity and the optimum water requirement for activity (6,14,18,19).

Lipase activity in the invert emulsion increased with increasing taurocholic acid content from 0.5 to 10 mM concentration with no further change in activity up to 20 mM. This result was quite different from that observed for this enzyme in the o/w emulsion, where considerably smaller concentrations of the surfactant were required for optimum activity, i.e., 0.1 mM was optimum and activity was progressively reduced at up to 0.8 mM (20).

Activity of the *Pythium* lipase against different oils in the invert emulsion (Table 2) was determined according to the standard lipase assay described in the Materials and Methods section. In general, the hydrolytic activity against the oils in the invert system was at least 3.5 times higher than for the same enzyme and substrates in the o/w emulsion system (12). As in the o/w emulsion (12), the enzyme shows a slight preference for substrates having unsaturated fatty acids when incubated in the invert emulsion (Table 2). An exception to this for both the o/w and w/o emulsions is the relatively low activity toward Menhaden fish oil. Similar results were reported for a lipase from *C. rugosa* against triglycerides containing docosahexaenoic acid (DHA) (21). Menhaden oil triglycerides contain a high percentage of saturated and monounsaturated fatty acids in the 1 and 3 positions, and polyunsaturated fatty acids such as eicosapentaenoic acid and DHA in the 2-position (22). The difficulty for lipases in attacking DHA-containing substrates (e.g., fish oil) relative to oleic acid or linoleic acid containing substrates (e.g., olive oil, soybean oil) is related to the position of the double bonds in the acyl group; moreover, double bonds located near the carbonyl terminus of acyl groups impose "stiffness" which probably makes them less accessible to either the active site or other

TABLE 1
Effects of Water Content on *Pythium* Lipase Activity in an Invert Emulsion

Water (%)	Lipase activity[a] (μmol/min/mg protein) Olive oil[b]	Corn oil[b]	Peanut oil[b]	Lard	Menhaden fish oil[c]
4	57.9 (919.5)	57.9	57.2	10.4	44.0
6	57.9 (862.5)	53.2	54.4	35.9	48.6
8	81.0 (919.5)	60.2	79.9	38.2	46.3
10	88.0 (977.0)	64.8	85.6	41.7	46.3
12	88.0 (1092.0)	85.6	85.6	41.7	34.7

[a]Except as shown in parentheses where the activity is for a purified preparation of the enzyme, a crude lipase preparation was used in these experiments. Values are the means of two experiments. Variation among experiments was less than 5%. The reaction mixture contained 5 mL oil and aqueous solution (4 to 12%) composed of lipase and taurocholic acid (10 mM). The lipase activity was determined at 45°C and pH 7.5 as described in the Materials and Methods section.
[b]With 8, 10, and 12% buffer (50 mM sodium phosphate, pH 7.5), a thick creamy gel was formed at the bottom of the test tube during incubation.
[c]There was no gelation but partial separation of oil and water phases at 10 and 12% water during incubation.

TABLE 2
Hydrolytic Activity of an Extracellular Lipase from *Pythium ultimum* Against Different Oils in an Invert Emulsion

Oils	Activity[a] (μmol/min/mg protein)
Olive	57.5 ± 0.7[b]
Soybean	54.4 ± 3.1[b]
Peanut	57.5 ± 0.7[b]
Sunflower	50.5 ± 0.6[b,c]
Corn	55.6 ± 2.4[b]
Cottonseed	55.9 ± 2.9[b]
Coconut	43.2 ± 0.7[d,e]
Lard oil	10.0 ± 0.6[f]
Menhaden fish	42.4 ± 8.1[d,e]
Menhaden fish (hydrogenated)	45.5 ± 8.7[c,d]
Butter oil	37.8 ± 1.4[e]

[a]Each value is the mean ± SD of three determinations. Means with the same letter are not significantly different. Significance was calculated by the Duncan's Multiple Range Test ($\alpha = 0.05$ level). The reaction mixture contained 5 mL oil and 4% aqueous soluton composed of lipase and taurocholic acid (10 mM). The lipase activity was determined at 45°C and pH 7.5 as described in the Materials and Methods section.

binding sites on the lipase (21). The extremely low activity toward lard oil, which contains substantial amounts of unsaturated substrates, may be due mainly to inherent properties of the w/o invert emulsion rather than substrate preference (Table 2). With the o/w emulsion system, *Pythium* lipase activity toward lard oil was the same as with soybean or sunflower oil (Mozaffar, Z., and J.D. Weete, unpublished data).

The experiments described above were conducted with a crude enzyme preparation obtained by ammonium sulfate precipitation of proteins from the growth medium. However, a highly purified preparation of the enzyme was used for direct comparison of activity in o/w and w/o emulsions. The specific activity of the purified *Pythium* lipase was 4.3 times higher in the invert emulsion, at 919.5 μmol/min/mg protein, than in the normal phase emulsion when both systems contained taurocholic acid as the surfactant (Table 3). In a previous study (20), we found that *Pythium* lipase had similar activities in the o/w emulsion when 0.1 mM taurocholic acid or 0.8 mM (3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate were added to the lipase assay mixture, with specific activities of 215.5 and 194.0 μmol/min/mg protein, respectively (Table 3).

Since lipase-catalyzed reactions are essentially interfacial processes (23), measurements were made to determine if differences in surface area may explain the differences in activity of the lipase in the two types of emulsions. In this study, the invert emulsion of olive oil with 4% water and 10 mM taurocholic acid contained emulsified water globules ranging from about 1 to 20 μm in diameter with an average diameter of 7.0 ± 5.3 μm. Oil globules in the o/w emulsions used for assaying the *Pythium* lipase (12) ranged from 1 to 15 μm in diameter with an average diameter of 5.9 ± 3.1 μm. Although the average globule diameters were not significantly different between the two emulsion systems, the total mean interfacial surface areas were 5100 cm^2 and 1700 cm^2 for the o/w and w/o emulsions, respectively (Table 3). The widely different surface areas in the two emulsion systems are accounted for by the difference in water content. These results suggest that the 4.3-fold higher lipase activity in the w/o invert emulsion compared to that in the o/w emulsion (Table 3) is due mainly to inherent properties of the invert system rather than to emulsified globule size or surface area. Previous results from our laboratory on the effects of surfactants on lipase activity also support this conclusion (20).

TABLE 3
Activity of Purified *Pythium* Lipase Under Different Assay Conditions

Assay system[a]	Lipase activity (μmol/min/mg protein)	Interfacial surface area (cm^2)
o/w Emulsion (0.1 mM taurocholic acid)[b]	215.5 ± 20.4	5100
o/w Emulsion (0.8 mM CHAPS)[b]	194.0 ± 10.1	—
w/o Invert emulsion (10 mM taurocholic acid)[c]	919.5 ± 27.5	1700

[a]Values in parentheses show concentrations of surfactants in the reaction mixture.
[b]Assay conditions were optimum for activity using the conventional oil-in-water (o/w) emulsion system as described by Mozaffar *et al.* (Ref. 20). w/o, Water-in-oil. CHAPS, (3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.
[c]Assay conditions were as described in the Materials and Methods section.

The time course of olive oil hydrolysis conducted in the invert emulsion at 45°C was examined using the crude *Pythium* lipase preparation to determine the stability of this enzyme over time and establish its capacity to hydrolyze the oil. In a preliminary experiment, only 2.3% of the oil was hydrolyzed after 24 h (Mozaffar, Z., and J.D. Weete, unpublished data), and separation of the emulsion began after 4 h incubation with a cloudy layer appearing at the bottom of the reaction mixture. The low lipolytic activity may have been due to thermal inactivation of the lipase or modification of emulsion properties. To test this, the incubation temperature was decreased to 35°C, and the olive oil emulsion was stirred continuously to prevent separation. Up to 10% of the olive oil was hydrolyzed at 24 h, but no further hydrolysis occurred thereafter (Fig. 1A). In previous experiments we observed that the initial rate of hydrolysis of olive oil by the *Pythium* lipase was increased with increasing aqueous phase (Table 1), and Kaimal *et al.* (24) reported enhanced hydrolysis of mustard oil with *C. rugosa* lipase as a function of water content in an emulsion-free system containing oil/*tert*-butanol/enzyme and water. During the course of the reaction in a mixture containing 8 units of enzyme and 4% assay buffer initially, additional buffer (0.2 mL) was added to the reaction mixtures at 4, 8, 12, and 24 h, but there was no difference in the amount of hydrolysis between reaction mixtures with or without additional buffer (Fig. 1A).

The time course of olive oil hydrolysis in the invert emulsion using lipases from *C. rugosa* and *R. arrhizus* was also conducted to determine their ability to hydrolyze oil in an invert system. These enzymes were selected because they exhibit some properties similar to the *Pythium* lipase in the taurocholic acid–olive oil emulsions (o/w), e.g., increased lipase activity at low taurocholic acid concentration (0.1 mM) and decreased activity with increasing taurocholic acid concentration (20). Initially, the time course of the reaction with *C. rugosa* lipase was conducted in a medium containing 4% assay buffer (aqueous solution) as before with the *Pythium* lipase (Fig. 1A). At 4 h, 8% of the oil was hydrolyzed and after 24 h 49% was degraded (Fig. 1B); however, no further hydrolytic activity was observed thereafter. Subsequently, during the course of the reaction, additional buffer (0.2 mL) was added to the reaction mixtures at 4, 8, 12, and 24 h. The degree of hydrolysis was increased by the addition of buffer when added at 8 h, and 90% conversion was achieved after 48 h incubation (Fig. 1B). The reason olive oil hydrolysis was increased by adding additional aqueous phase to the reaction mixture is not known; however, it may have been due to changes in emulsion properties where more substrate became available to the enzyme (14,29,30). Whatever may be the case, the enzyme from *C. rugosa* was very active in the invert

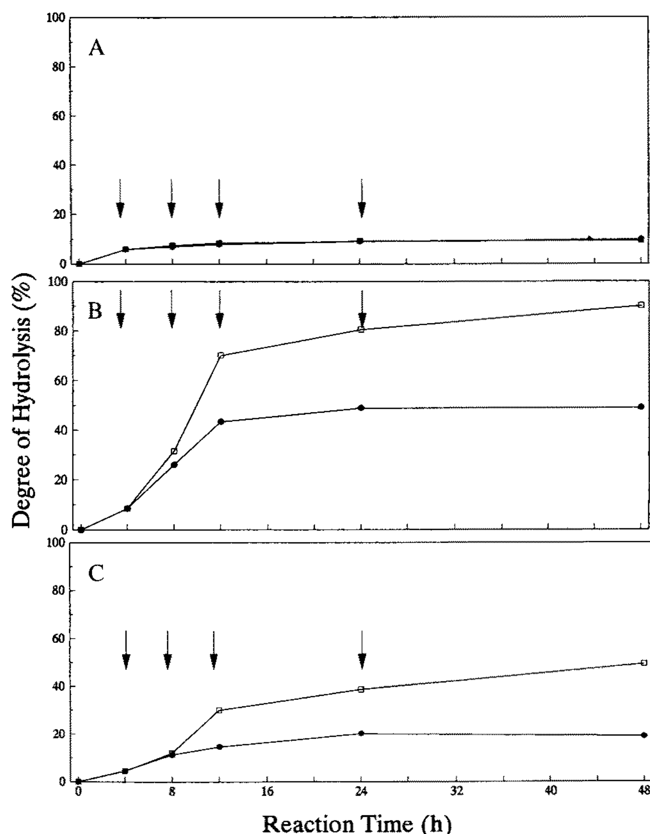

FIG. 1. (A) Time course of olive oil hydrolysis with an extracellular lipase from *Pythium ultimum* in an invert emulsion. The reaction mixtures were incubated separately for the times indicated, and the free fatty acid content was determined as described in the Materials and Methods section. The reaction mixture contained olive oil, 5 mL; taurocholic acid, 10 mM; assay buffer, 4% (vol/vol), and 0.144 mg protein (8 units). The incubation temperature was 35°C, and the pH was 7.5 (—●—). The reaction mixture was stirred magnetically throughout the incubation period. Initial aqueous phase was 4% of the total assay mixture volume, and additional assay buffer (0.2 mL) was added at 4, 8, 12, and 24 h (—□—). Arrows (↓) indicate the times when the buffer was added. (B) Time course of olive oil hydrolysis by *Candida rugosa* lipase in an invert emulsion. The conditions were the same as those described in Part A except that the temperature was 30°C and the incubation mixture contained 0.027 mg protein (8 units). (C) Time course of hydrolysis of olive oil by *Rhizopus arrhizus* lipase in an invert emulsion. The conditions were the same as those desribed in Part A except that the temperature was 30°C and the incubation mixture contained 0.007 mg protein (8 units).

emulsion when buffer was added to the reaction mixture periodically over the course of the reaction.

The time course of olive oil hydrolysis in the invert emulsion using lipase from *R. arrhizus* is shown in Figure 1C. As with the lipase from *C. rugosa,* hydrolysis was relatively slow at 4% assay buffer with only 20% of the oil being hydrolyzed after 48 h incubation (Fig. 1C). With the addition of assay buffer as described above, 49% of the oil was hydrolyzed at 48 h. In the time-course experiments shown in Figure 1, 8 units of enzyme from each source were used. However, with prolonged incubation time (96 h) and fifty times more enzyme units, 82% oil hydrolysis was achieved with *R. arrhizus* lipase (data not shown). Unlike the *C. rugosa* lipase which is nonspecific for position, the *R. arrhizus* lipase is 1,3-specific and therefore the maximum hydrolysis that might be expected is only 67% of the substrate. The fact that 82% hydrolysis was obtained after long incubation times probably indicates "acyl migration" or isomerization of 2-monoacylglycerols and 1,2-diacylglycerols to 1-monoacylglycerols and 1,3-diacylglycerols, respectively (27).

Lipases are known to be subject to inhibition by the reaction products, i.e., fatty acids (28–30). This was confirmed for the *Pythium* lipase in the w/o emulsion when enzyme activity was determined in the presence of oleic acid. Activity decreased by about 40% when 100 μL oleic acid (1.9% of the total reaction mixture) was added to the reaction mixture. This result was unlike that reported previously for a lipase from *Saccharomycopsis lipolytica* that was activated by oleic acid (31). Also, when the *Pythium* lipase was incubated at 35°C in the absence of substrate, it was found that activity gradually decreased with time and disappeared after 24 h (Mozaffar, Z., and J.D. Weete, unpublished data). Both product inhibition and heat inactivation contribute to the decreased activity of the *Pythium* lipase during the course of the reaction. Also, activity of the *Pythium* lipase in the AOT–isooctane reverse-micelle system was very low where only 5% of the olive oil was hydrolyzed at 24 h.

We have shown in this study that the lipases from *C. rugosa* and *R. arrhizus* are exceedingly active in w/o emulsions. In addition, the capacities of these enzymes, but not for the *Pythium* lipase, for total oil hydrolysis can be greatly improved by manipulating the incubation conditions over the course of the reaction by adding aqueous solution. The major advantage of using the w/o invert emulsion over the reverse-micelle system is that a high degree of oil hydrolysis can occur without the addition of an organic solvent. An additional advantage to the w/o emulsion is that the surfactants used for this system, such as taurocholic acid, are less toxic than AOT, which is commonly used for reverse-micelles (17,32).

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