

Lipase-Catalyzed Triglyceride Hydrolysis in Organic Solvent

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An investigation of lipases from *Rhizomucor miehei*, *Candida rugosa* and porcine pancreas revealed that these enzymes hydrolyzed triglycerides in an organic solvent system. The presence of secondary amines, *i.e.*, diethylamine, *N*-methylbutylamine, or the tertiary amine, triethylamine, greatly increased the extent of hydrolysis. The lipolysis of tallow took place under mild conditions, *e.g.*, room temperature, moderate shaking and within 20 hr. At 45°C, complete hydrolysis of tallow was obtained in 6 hr. Vegetable oils and a fish oil (cod liver oil) were also hydrolyzed at 20°C by *R. miehei* lipase in the presence of *N*-methylbutylamine for 20 hr. The lipases were recovered for reuse with some loss of activity. Optimum yields of free fatty acids were obtained by using *R. miehei* lipase as catalyst.

KEY WORDS: *Candida rugosa*, diethylamine, fish oil, *N*-methylbutylamine, porcine pancreas, *Rhizomucor miehei*, tallow, triethylamine, vegetable oils.

The fatty acid industry, which produced 1,155,483,000 pounds of fatty acids in 1987 (The Soap and Detergent Association), has increased dramatically with the introduction of each new method of fat hydrolysis. At present, the Colgate-Emery Steam Hydrolysis Process (1) is widely used. This process operates with super-heated steam at 250°C and 50 atm for 2 hr to recover 97% yield of fatty acids and 12% yield of glycerol. This high-energy process induces polymerization and color development and requires subsequent purification, such as distillation of the fatty acids, for many uses. It would be advantageous to develop a low-energy process that simultaneously produces a colorless product. The use of lipolytic enzymes is one potential approach to achieve these goals.

Using enzymes to split fats is not a recent idea. A.W. Ralston (2) reported that in 1890, Green and Sigmund, working independently, established the presence of a fat-splitting enzyme in castor beans. These enzymes, now known as lipases, function at the oil-water interface to hydrolyze fats to fatty acids and glycerol. Many investigators have studied the enzymatic reaction and have had difficulty isolating the fatty acids from the heavy emulsions that were formed (3). Thus, an emulsion-free process is desired.

Many recent advances have been made using enzymatic catalysts in organic synthesis. In their investigation of enzymes in organic solvents, Zaks and Klibanov (4) reported that some enzymes retain their activity (even when the amount of water is greatly reduced) and that water can be replaced by a variety of organic solvents. They found that lipases, such as those from *Candida rugosa*, *Rhizomucor miehei* and porcine pancreas, serve as catalysts for organic reactions in which the organic solvent contains a trace of water. The use of lipases in

non-aqueous solvents has been the subject of recent reviews by Gillis (5), Klibanov (6) and Wong (7). The lipase-catalyzed formation of fatty amides was reported by Bistline, *et al.* (8). This investigation has now been extended to the hydrolysis of fats.

In order to conserve energy and obtain light-colored fatty acids, several companies have investigated industrial enzymatic fat splitting (3) by processes that involve mixing of fats with lipase, agitating for 2 to 4 days, and isolating the products. For example, Miyoshi Fat and Oil, Tokyo, Japan, uses a lipase supplied by Meito Sangyo Co., Nagoya, Japan, and splits fats at 32°C producing 1000 metric tons of fatty acids per month (9). A reduction in reaction time would result in a decrease in fat inventories now required in the production process and a decrease in energy consumption and cost. Because lipases are currently too expensive without recovery, the ability to reuse enzymes would enhance the attractiveness of an enzymatic hydrolysis.

Our objective was to determine whether lipases would catalyze the hydrolysis of fats and oils in organic solvents at mild temperatures. Such a process could reduce energy costs, facilitate recovery of the fatty acid product, and yield a product of superior quality. Three lipase sources were selected for this initial investigation: *C. rugosa* (a yeast), *R. miehei* (a fungus) and porcine pancreas. The experiments were designed to understand the effects of the reaction conditions on hydrolysis yields produced by the three lipases.

EXPERIMENTAL PROCEDURES

Materials. The amines (diethyl, *N*-methylbutyl, and triethyl) were purchased from Aldrich Chemical Co., Milwaukee, WI. The suppliers of the triglycerides were: bleached tallow, Chemol Inc., Greensboro, NC; cottonseed oil, Sigma Chemical Co., St. Louis, MO; soybean oil, NPB Marketing, Inc., Memphis, TN; corn oil, Best Foods, CPC International, Inc., Englewood Cliffs, NJ; olive oil, Pompeian, Inc., Baltimore, MD; and cod liver oil, E. R. Squibb & Sons, Inc., Princeton, NJ. The triglycerides were used as received. Thin-layer chromatography (TLC) Silica Gel G plates were furnished by Analtech, Inc., Newark, DE. Sigma Chemical Co. supplied the following standards: triolein, 1,2-diolein, 1,3-diolein, monoolein, and oleic acid. *N*-butylpalmitamide was prepared according to the procedure of Krafft and Stauffer (10).

Enzymes used. *C. rugosa* lipase, 792 μ eq. free fatty acid released/hr/mg protein, and porcine pancreatic lipase, 25.9 μ eq/hr/mg protein, Sigma Chemical Co.; *R. miehei* lipase, immobilized on anion exchange resin, Lipozyme IM-20, 225 μ eq/hr/mg solid, Novo Nordisk Bioindustrials, Inc. (Danbury, CT). Lipase activity was measured at room temperature according to the method of Baillargeon and Sonnet (11) at pH 7.7 instead of pH 7.3. The rate of free fatty acid release was followed by addition of 0.1 N NaOH under nitrogen by means of the Radiometer titration system in pH stat mode. Enzyme-free blanks showed no uptake of base.

Instrumentation. Shaker-water bath incubator Model

66722, Precision-Dubnoff, Thomas Scientific (Swedesboro, NJ); shaker-incubator Model G76, New Brunswick Scientific Co., Inc. (Edison, NJ); Radiometer titration system, Radiometer, Inc. (Copenhagen, Denmark).

Lipolysis of tallow. Solutions of *N*-methylbutylamine (0.09 g in 10 mL hexane) and tallow (0.89 g in 10 mL hexane) were prepared. Lipase (100 mg) was added to a mixture containing 1.0 mL of amine solution and 1.0 mL of tallow solution in 15 × 100 mm test tubes. The amounts correspond to 0.1 mmole each of tallow and amines. The tubes were sealed and shaken at 125 rpm in an incubator at 20°C or 45°C for 24 hr.

Analysis of tallow lipolysis products. The release of fatty acids (FA) was monitored by thin-layer chromatography (TLC) by comparison with pure standards. The TLC plate (20 × 20 cm, 250 μ) was subjected to a two-stage development procedure; (a) toluene, ether, ethyl acetate, acetic acid (80:10:10:1 by vol); and (b) hexane, ether, formic acid (80:20:2 v/v/v). Spots were detected by spraying the plate with 60% aqueous sulfuric acid, then heating on a hot plate at 150°C for 15 min. Quantitative analysis was performed on 500 μ-thick silica gel plate with the two-stage development procedure. The individual TLC bands were located visually by spraying the plate with 0.1% alcoholic solution of 2',7'-dichlorfluorescein. The acid bands were scraped, extracted with ethyl acetate/methanol (80:20 v/v) and dried under nitrogen. The amount of product was determined gravimetrically. A large-scale hydrolysis experiment was conducted in which Lipozyme 25 g; tallow, 25 g (0.028 moles); *N*-methylbutylamine, 2.4 g (0.028 moles) and hexane, 100 mL were placed in a 250-mL flask and stirred for 24 hr at room temperature. The following recoveries were obtained: Lipozyme, 23 g (92%); tallow fatty acids, 20 g (89%); glycerol, 3 mL; hexane/amine solvent, 85 mL (85%). The latter solution contained 87.8% of the original amine.

RESULTS AND DISCUSSION

The discovery that lipases can serve as catalysts in organic solvents has led to an enthusiastic response by scientists to determine their applicability in a wide variety of chemical reactions. In a previous study, we reported that the reaction of fatty acid methyl esters (FAMES) with primary amines in "wet" hexane to form fatty amides was catalyzed by lipases (8). However, when

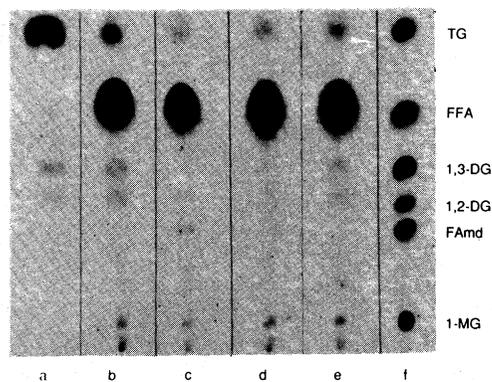


FIG. 1. Reaction of tallow with diethylamine, *N*-methylbutylamine and triethylamine catalyzed by Lipozyme in hexane, 20°C, 24-hr incubation period. Concentration: tallow (0.1 mmol) + amine (0.1 mmol) + Lipozyme, 100 mg in 2 mL hexane. a: tallow, unreacted; b: tallow with no amine; c: tallow + diethylamine; d: tallow + methylbutylamine; e: tallow + triethylamine; f: standard mixture, TG = triglycerides, FFA = free fatty acid, 1,3-DG = 1,3-diglycerides, 1,2-DG = 1,2-diglycerides, FAm = *N*-butylpalm- itamide, 1-MG = monoglyceride. The origin is at the bottom of this figure, represented by the spot below the position of 1-MG in lanes b-e.

this investigation was extended to secondary amines, complete hydrolysis of the FAMES or fatty triglycerides was observed. It was decided to study this particular reaction more fully in order to achieve a better understanding of the catalytic abilities of lipases in organic solvents.

Since it was established in our previous report that high molecular weight (mol wt) fatty substrates react preferentially with low mol wt amines, diethylamine, *N*-methylbutylamine and triethylamine were selected to react with tallow in "wet" hexane with lipase from *R. miehei* as catalyst (Fig. 1, Table 1). The lipase, in the absence of amine but otherwise under the same conditions, catalyzed 76% hydrolysis of tallow to fatty acids (Table 1). The addition of secondary and tertiary amines to the tallow solution in hexane increased the release of fatty acids under the same conditions to 95% (Table 1). No apparent reactions took place in the tallow/amine mixtures in the absence of added lipase. The secondary and tertiary amines apparently act as co-catalysts, enhancing the reactivity of the lipase catalysts. The amines may "bind" or "complex" the formed fatty acids and remove

TABLE 1

Secondary and Tertiary Amine Effect on Lipase-Mediated Tallow Hydrolysis in Hexane^a

No.	Tallow +	% Yield ^b		
		Triglycerides	Fatty acids	Others ^c
1.	Control, no additives	93.8	Trace	6.3
2.	Lipase only ^d	19.3	76.0	4.8
3.	Lipase + diethylamine ^d	0.6	95.1	4.4
4.	Lipase + <i>N</i> -methylbutylamine ^d	Trace	95.3	4.8
5.	Lipase + triethylamine ^d	1.2	95.2	3.7

^aAt 20°C, 20 hr Conc. = tallow 0.1 mmol + amine, 0.1 mmol.

^bBy prep. TLC.

^cIncludes mono- and diglycerides.

^dCatalyzed by the lipase from *Rhizomucor miehei*.

them from the equilibrium. This would drive the reaction toward completion as well as speed it kinetically. Increased water concentration decreased the rate of tallow hydrolysis by *R. miehei* lipase. The "wet" hexane used normally contained 0.02% water. In similar experiments with the lipases from porcine pancreas and *C. rugosa* and in the presence of *N*-methylbutylamine, about 60% hydrolysis of tallow to fatty acids was realized compared to the 95% hydrolysis for lipase from *R. miehei*. No adjustments were made for the different specific activities of these lipase preparations.

Trimyristin, tripalmitin, tristearin, and triolein were also hydrolyzed by *R. miehei* lipase in the presence of *N*-methylbutylamine, thus producing free fatty acids in 95% yield. Neither variation in the size or degree of unsaturation of the fatty acid moieties affected hydrolysis of the fatty substrate. The general applicability of lipase-catalyzed lipolysis in an organic solvent was further shown in a series of reactions catalyzed by *R. miehei* lipase by using

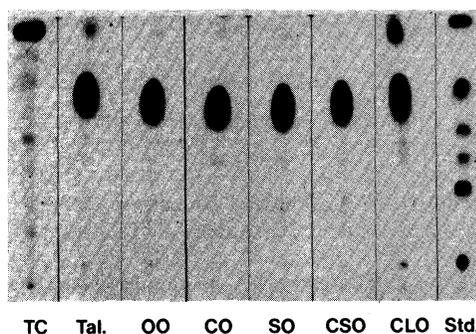


FIG. 2. Hydrolysis of tallow, vegetable oils and fish oil in the presence of *N*-methylbutylamine, catalyzed by Lipozyme in hexane, 20°C, 24-hr incubation period. Concentration: see Figure 1. TC = (tallow control) = tallow + *N*-methylbutylamine without lipase; TAL = tallow; OO = olive oil; CO = corn oil; SO = soybean oil; CSO = cottonseed oil; CLO = cod liver oil; Std. = standards (see Fig. 1).

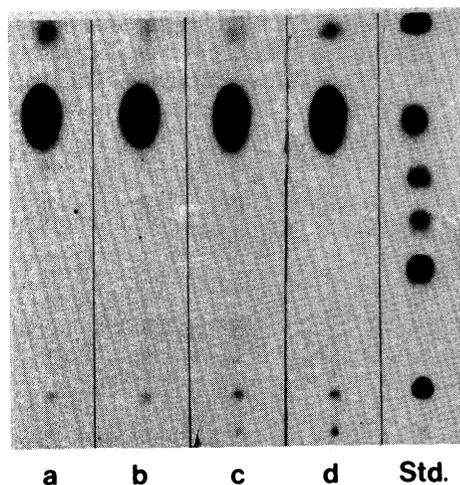


FIG. 3. Hydrolysis of tallow with recovered lipase, *R. miehei* (*R.M.*) in *N*-methylbutylamine. Concentration: see Figure 1. a: tallow + fresh *R.M.*; b: tallow + *R.M.*, first recovery; c: tallow + *R.M.*, second recovery; d: tallow + *R.M.*, third recovery; e: Std. (see Fig. 1).

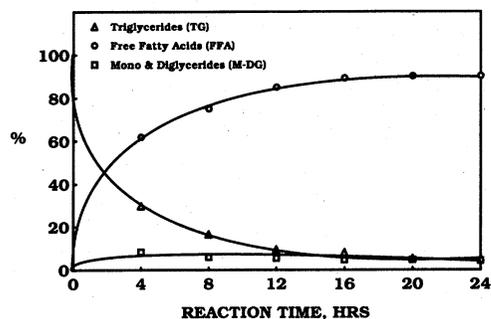


FIG. 4. Lipase catalyzed hydrolysis of tallow at 20°C. Concentration: see Figure 1.

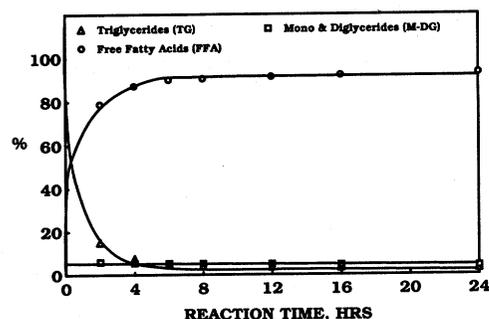


FIG. 5. Lipase catalyzed hydrolysis of tallow at 45°C. Concentration: see Figure 1.

the following oils: cottonseed, soybean, corn, olive and fish in the presence of *N*-methylbutylamine (Fig. 2).

The recovery and reuse of lipase is an important economic consideration in the application of enzymes for fat hydrolysis. A series of experiments was carried out with tallow and *N*-methylbutylamine using *R. miehei* lipase as catalyst (Fig. 3). The lipase was separated from the reaction solution by filtration, washed with hexane, and used again in subsequent reactions. The results of tallow hydrolysis with "fresh" lipase are illustrated in Figure 3, lane a. The experiment was then repeated three times sequentially with the "used" lipase, Figure 3, lanes b, c and d. These results show that the lipase may be used several times for tallow hydrolysis without significant loss of lipase activity.

The effect of temperature on lipase-catalyzed tallow hydrolysis in wet hexane was determined with the *R. miehei* lipase. The two temperatures selected were 20 and 45°C. Samples were taken every 4 hr at 20°C and every 2 hr at 45°C and analyzed by prep TLC for free fatty acids, unreacted triglycerides, and mono- and diglycerides. The results are shown in Figure 4 for 20°C and Figure 5 for 45°C. Tallow was hydrolyzed at 20°C to 90% free fatty acids in 20 hr. However, when the temperature was increased to 45°C the same result was obtained in 6 hr. No evidence of tallow hydrolysis was observed at either 20 or 45°C in the absence of lipase. Again the *R. miehei* lipase was recovered and reused. This relatively small temperature increase greatly enhances the rate of tallow hydrolysis.

A comparison with the Colgate-Emery Process shows that hydrolysis can be readily accomplished at room temperature instead of 250°C. The products should not be discolored as in the high-temperature processes. There-

fore, the distillation step to purify the fatty acids may be unnecessary. No heavy emulsions are encountered and the fatty acids are readily isolated by low-temperature techniques. The lipase, solvent and amine, with adjustment for slight losses, may be reused. A significant savings in energy should be attained. Thus, a method has been described for the high-efficiency hydrolysis of triglycerides under mild conditions by using simple reactors and reactants.

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