Enzymatic Fat Hydrolysis and Synthesis

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ABSTRACT

The hydrolysis of tallow, coconut oil and olive oil, by lipase from *Candida rugosa*, was studied. The reaction approximates a first-order kinetics model. Its rate is unaffected by temperature in the range of 26-46 C. Olive oil is more rapidly hydrolyzed compared to tallow and coconut oil. Hydrolysis is adversely affected by hydrocarbon solvents and a nonionic surfactant. Since amounts of fatty acids produced are almost directly proportional to the logarithms of reaction time and enzyme concentration, this relationship provides a simple means of determining these parameters for a desired extent of hydrolysis. All three substrates can be hydrolyzed, almost quantitatively, within 72 hr. Lipase from *Aspergillus niger* performs similarly. The lipase from *Rhizopus arrhizus* gives a slow hydrolysis rate because of its specificity for the acyl groups attached to the α-hydroxyl groups of glycerol. Esterification of glycerol with fatty acid was studied with the lipase from *C. rugosa* and *A. niger*. All expected five glycerides are formed at an early stage of the reaction. Removal of water and use of excess fatty acid reverse the reaction towards esterification. However, esterification beyond a 70% triglyceride content is slow.

INTRODUCTION

Although pancreatic lipase has been available commercially for some time, it is only recently that purified preparations of microbial lipases have been produced industrially. It is therefore likely that in the foreseeable future, industrial processes will be developed for enzymatic hydrolysis of triglycerides and possibly for the enzymatic esterification of fatty acids with glycerol or other alcohols. Lipases from *Candida rugosa, Aspergillus niger* and *Rhizopus arrhizus* are commercially available in most industrialized countries. While these are still high in price, increased usage will undoubtedly result in a lower price and possibly also higher enzymatic activity. The literature on lipolysis, particularly of a biochemical orientation, is voluminous, but there is a paucity of information on procedures for large-scale lipolysis or esterification. The literature up to about 1973 (1) is well covered, and a recent review (2) covers the even broader area of fat biotechnology. A study of the major reaction parameters of lipolysis with the lipase from *C. rugosa* was undertaken in this laboratory (3) and some of the highlights of this research are repeated here.

The published information on enzymatic esterification with various lipases is quite sketchy and somewhat contradictory. From a thermodynamic point of view, lipolysis is a reversible reaction and studies of esterification with the lipase from castor beans go back to the early part of this century (4). The use of lipases from *Staphylococcus aureus* and *Candida lipolytica* (5) did not produce triglyceride when they attempted to esterify oleic acid with glycerol, but esterification of mono- and diolein resulted in some triolein formation. Syntheses with four microbial lipases (6), *A. niger, R. delamar, Geotrichum candidum* and *Penicilium cyclopium* have been studied. Extent of esterification by disappearance of fatty acid was determined by titration with standard KOH solution. Esterification was done on a small scale (0.1 g fatty acid), and the large amount of water present would not favor esterification. Thin layer chromatography (TLC) analyses showed the presence of mono- and diglycerides, but no triglycerides when lipases from *A. niger* or *R. delamar* were used. The same authors also reported the synthesis of esters of fatty and other alcohols (7). Here the methodology used was similar to that described in reference 6. Finally the authors reported that they esterified oleic acid with ethanol and then deactivated the enzyme with ethanol.

The present study covers lipolysis with lipases from *C. rugosa, A. niger* and *R. arrhizus*, as well as esterification of oleic acid with glycerol and a few other alcohols in the presence of *C. rugosa* and *A. niger* lipases.

EXPERIMENTAL

Materials

Enzeco lipase concentrate (30,000 U/g) from *C. rugosa*, EC 3.1.1.3, was supplied by Enzyme Development Corporation, New York, NY. Palatase lipase (815 U/g) from *A. niger*, EC 3.1.1.3, and Novozyme 206 (8,150 U/g) were furnished by courtesy of Novo Laboratories, Inc., Wilton, CN. Lipase concentrate from *R. arrhizus* (46,000 U/g), EC 3.1.1.3, was kindly supplied by GB Fermentation Industries, Inc., Charlotte, NC. Refined and bleached edible beef tallow (free fatty acid [FFA] 2.5%) was obtained from the Inolex Corporation, Philadelphia, PA; edible coconut oil (FFA 1.2%) from the Capital City Division, Stokely-Van Camp Corporation, Harrison, NJ; olive oil (FFA 1.7%), imported from Filippo, Berio, Lucca, Italy, was purchased locally. Oleic acid, USP/NF, Emersol 221 was supplied by Emery Industries, Cincinnati, OH. "Analyzed" grade solvents were purchased from J. T. Baker Chemical Co., Phillipsburg, NJ. All other chemicals were purchased from Aldrich Chemical Company, Milwaukee, WI. Thin layer chromatography plates were purchased from Analtech Laboratories Incorporated, Newark, DE.

Hydrolysis with Lipase from *A. niger*

The substrate (3.01 g olive oil or tallow, or 2.52 g coconut oil) was placed in a 25-mL glass-stoppered Erlenmeyer flask provided with a 1-in. Teflon coated magnetic stir bar. Sodium phosphate buffer solution (0.1 M, pH 5.4) containing 0.01% sodium azide was added (3.7 mL) to the flask, followed by the enzyme solution. Sufficient distilled water was added to bring the total aqueous phase to 6.5 mL. The flask was placed on a stirring plate which was set in a constant temperature oven for a prescribed reaction period. A 10-mL portion of a neutralized (phenolphthalein) 50:50 v/v mixture of acetone and ethanol was added to the flask and shaken thoroughly to inactivate the enzyme. The mixture was then emptied into a titration flask, the reaction vessel was rinsed with a total of 30 mL of the solvent mixture, and the rinsings added to the titration flask. The solution was then titrated with standard 0.10 N NaOH solution. All samples were run in duplicate. Blank determinations were made on the mixture of the reactants which was inactivated immediately with the acetone/ethanol mixture, rinsed, and titrated as described above.

Hydrolysis with Lipases from *C. rugosa* and *R. arrhizus*

The experimental methodology was the same as above, except that powdered lipase was dissolved in 3.5 mL of buffer solution and no additional water was used. All tests were run in duplicate.

Enzymatic Esterification of Oleic Acid and Glycerol with Lipase from *C. rugosa*

A 1.0-g sample of the lipase from *Candida rugosa* was dissolved in 10 mL distilled water and stirred for 15 min. Glycerol (30.7 g, 0.333 mol) was then dissolved in the enzyme solution and the resulting stock solution was used in subsequent esterification experiments. The stock solution was stored in a deep freeze chest at −10 to −15 C.

To 42.3 g (0.15 mol) of oleic acid was added 4.07 g of the above stock solution containing 0.033 mol of glycerol. After stirring the mixture for 24 hr, water was distilled out with the aid of a vacuum pump (0.1 mm Hg) without application of external heat. After 1.7-1.8 g water had been removed, the reaction mixture was passed three times through a hand homogenizer. Vacuum was applied again after 4, 8, 24 and 48 hr from the start of the reaction, and every 2-3 days thereafter. The course of reaction was followed by FFA titration, by TLC and infrared (IR) spectrophotometry. As the fatty acid content decreased, the 1740 cm^{-1} ester peak increased, and the acid peak at 1710 cm^{-1} decreased.

Thin Layer Chromotography of Esterification Reaction Mixture

This was a modification of the procedure by Stevenson et al. (8). An accurately weighed sample of ca. 50 mg of sample dissolved in 400 μL of dichloromethane was streaked out on an Analtech 250 micron Silica Gel G with 5% boric acid Uniplate with the aid of a mechanical TLC streaker. The developing solution consisted of freshly prepared mixture of 210 mL petroleum ether, 90 mL anhydrous ethyl ether and 0.4 mL formic acid. The separated bands were made visible by immersing the plate in iodine vapor. Proceeding from the origin, the sequence of bands was as follows: (i) 2-monoglyceride, (ii) 1-monoglyceride, (iii) 1,2-diglyceride, (iv) 1,3-diglyceride, (v) fatty acid, and (vi) triglyceride. The two monoglycerides may not be clearly separated by this technique. For each band a column was prepared consisting of a 15 cm × 8 mm diameter Pasteur pipet, the bottom of which was fitted with a cotton plug. A $^1/_8$ in. layer of Celite was placed on top of the plug and one or two 10-mL portions of ethyl ether run through the column to prevent loose Celite particles from running through the plug. Each band was scraped off the plate into one of the columns. Ethyl ether, 20 mL, was then run through each column, and the filtrates from each column evaporated to dryness in a stream of dry nitrogen at room temperature and collected in weighed vials. The vials were ultimately dried at room temperature in a vacuum oven and the weight of each fraction was determined. All analyses were run in duplicate.

Esterification of Oleic Acid and Glycerol with *A. niger* Lipase

A 1-g sample of Novozyme 206 was dissolved in 3.13 g (0.033 mol) of glycerol and 42.3 g (0.15 mol) of oleic acid was added. After a 24-hr reaction time, vacuum was applied at room temperature as described above, and 0.5 g of water was distilled out. The sample was then homogenized and vacuum applied at fixed intervals as was described above for the esterification with the aid of lipase from *C. rugosa*.

Esterification of Oleic Acid and Various Alcohols in the Presence of Lipase from *C. rugosa*

A 0.1-g sample of the lipase was dissolved in 2 mL distilled water. The alcohol (0.1 mol if a monohydroxy compound, or 0.05 mol if a dihydroxy compound) was then added, followed by 42.3 g (0.15 mol) of oleic acid. The reaction mixture was stirred and subjected to vacuum distillation, homogenized, etc., as described above for the esterification of oleic acid with glycerol.

RESULTS AND DISCUSSION

The three lipases were not assayed for activity; it was assumed that the suppliers' assays were reasonably correct.

Enzymatic Lipolysis with *C. rugosa* Lipase

Lipolysis of tallow, coconut oil and olive oil with lipases from *C. rugosa, A. niger* and *R. arrhizus* was followed by analysis of fatty acid formed. As explained in a previous publication (3), plotting the percentage of fatty acid in the sample against the logarithm of reaction time resulted in essentially straight lines. Likewise, a plot of percentage fatty acid content vs the logarithm of enzyme concentration in the reaction mass gave straight lines. The term milliequivalent (meq) means one-third millimole of lipid. Figure 1 shows fatty acid content vs log [time] plots for coconut oil, and Figure 2 shows the fatty acid content vs log [enzyme concentration] for coconut oil. Analogous graphs for tallow can be found in reference 3. These purely empirical plots serve a practical and useful purpose. By extrapolation of the lines of Figure 1, one can determine the reaction time for a 97-98% hydrolysis at a given enzyme level. Analogously, the Figure 2 plots can be interpolated or extrapolated to determine, e.g., how much lipase is required to achieve 98% hydrolysis within a given reaction period. Graphs similar to Figures 1 and 2 were also obtained for tallow and olive oil but are not shown here. There were slight deviations from linearity. One-hour reaction time data tended to be somewhat erratic because

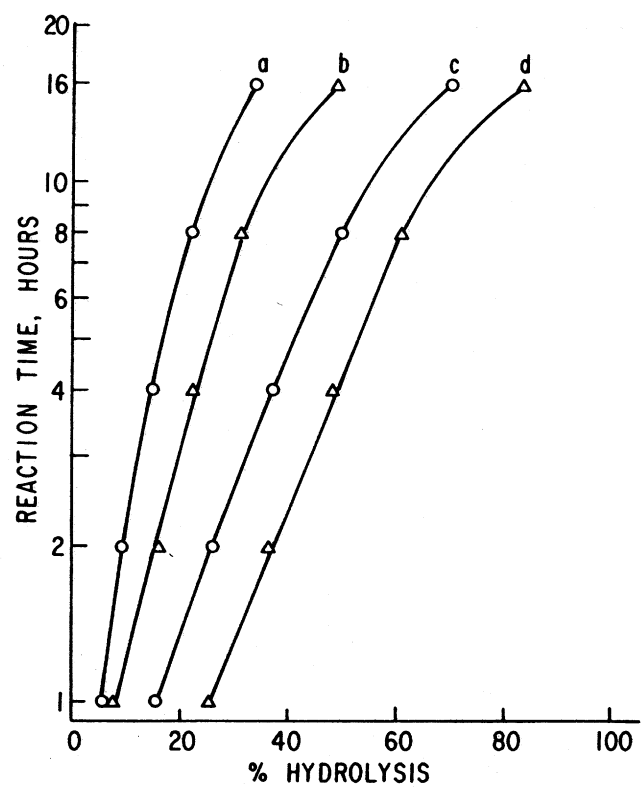

FIG. 1. Hydrolysis of coconut oil at room temperature with *C. rugosa* lipase at: (a) 3 U lipase/meq oil, (b) 6 U lipase/meq oil, (c) 15 U lipase/meq oil, (d) 30 U lipase/meq oil.

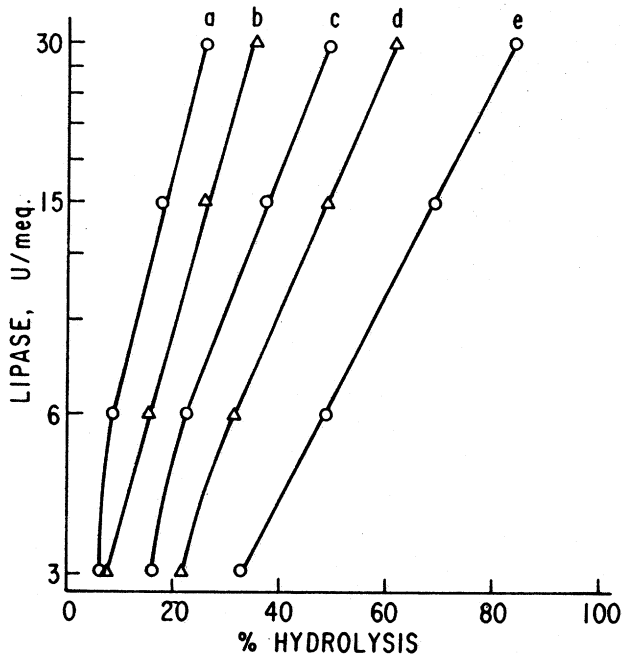

FIG. 2. Hydrolysis of coconut oil at room temperature with *C. rugosa* lipase: (a) after 1 hr, (b) after 2 hr, (c) after 4 hr, (d) after 8 hr, (e) after 16 hr.

timing was quite critical and enzyme activity would continue for a while before the enzyme was denatured either chemically or by heat. Some 16-hr data could also deviate somewhat from linearity, perhaps because mono- and diglycerides, which are good emulsifying agents for triglycerides and fatty acids, had been depleted to such an extent that the system was no longer an emulsion, and hence the interfacial area was greatly reduced.

It has been previously reported (3) that lipolysis with the *C. rugosa* lipase is not temperature-dependent in the range from 26 to 46 C. The lipolysis of a triglyceride is an inherently complex reaction involving the enzyme-catalyzed formation of di- and monoglycerides and fatty acid, as well as uncatalyzed isomerization of 1,2-diglyceride to 1,3-diglyceride, and the conversion of 2- to 1-monoglyceride, the vice versa reactions also occur. The kinetic data were found to approximate first-order kinetics (3). Tallow and coconut oil lipolysis rates were essentially the same, whereas that for olive oil was somewhat higher.

If a low boiling hydrocarbon solvent could be used in tallow hydrolysis, the reaction might be carried out at room temperature. To liquefy tallow completely at room temperature, an equal volume of hexane was required. However, addition of hexane resulted in a substantial reduction in lipolysis and did not represent a feasible approach.

The effects of other additives on enzymatic hydrolysis were also examined. Whereas sodium ion had no effect, calcium ion appeared to act as an inhibitor. The addition of 0.003% albumin gave essentially no improvement of lipolysis, nor did the addition of 0.01% of a nonionic ether alcohol type of surfactant. However, as the concentration of the latter was increased to 0.1%, strong inhibition was evident during the first 8 hr of reaction time (3).

Lipolysis with *A. niger* Lipase

Lipolysis with *A. niger* lipase is similar to that with *C. rugosa* lipase. Both lipases are nonspecific, i.e., attack both the 1- and 2-positions of triglycerides. Lipolysis with the *A. niger* lipase might give somewhat more erratic data, particularly for the 1- and 2-hr reaction periods, unless special precautions are taken. This appeared to be due to an induction period of varying duration before lipolysis began. Another source of variability was microbial contamination in the enzyme solution. Unless sodium azide, an antibacterial agent, was added to the hydrolysis vessel, the data obtained appear completely randomized.

Plots of percentage fatty acid vs the logarithm of reaction time, or plots of logarithm enzyme concentration vs percentage of hydrolysis, were essentially linear for all three substrates, as shown for coconut oil at 26 C in Figures 3 and 4. The similarity between Figures 1 and 3, and 2 and 4, respectively, is obvious.

The *A. niger* lipase was quite heat-sensitive, as shown in Table I. Deactivation occurred at 52 C, presumably due to denaturation of the lipase.

Both lipases were capable of hydrolyzing all three substrates completely in 72 hr. The minimum amounts of lipase required to attain essentially complete hydrolysis (97-99%) at room temperature for olive and coconut oil and at 43-46 C for tallow are shown in Table II.

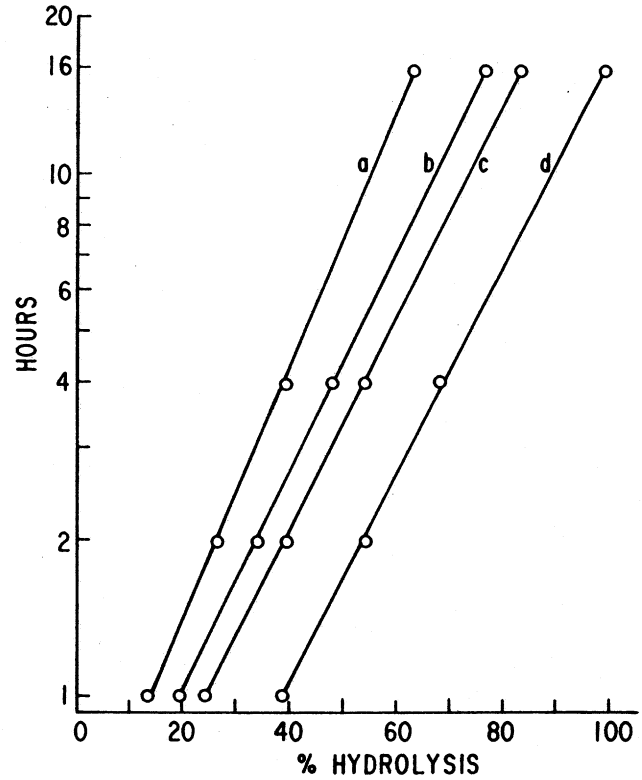

FIG. 3. Hydrolysis of coconut oil at room temperature with *A. niger* lipase at: (a) 29.3 U lipase/meq oil, (b) 58.7 U lipase/meq oil, (c) 146.7 U lipase/meq oil, (d) 29.3 U lipase/meq oil.

Lipolysis with *R. arrhizus* Lipase

The lipase from *R. arrhizus*, unlike those from *C. rugosa* and *A. niger*, is specific and will not readily hydrolyze the acyl group in the 2-position. Since the 1,2-diglyceride will isomerize to the 1,3-isomer until equilibrium is attained, and the unstable 2-monoglyceride will largely convert to the more stable 1-monoglyceride, glycerol will be formed in the course of hydrolysis with *R. arrhizus* lipase, and presumably the triglyceride can be hydrolyzed completely in due time. Because of the specificity of this lipase, it hydrolyzed triglycerides more slowly than the *C. rugosa* lipase. When percentage of hydrolysis was plotted against logarithm of reaction time, an almost linear graph was obtained

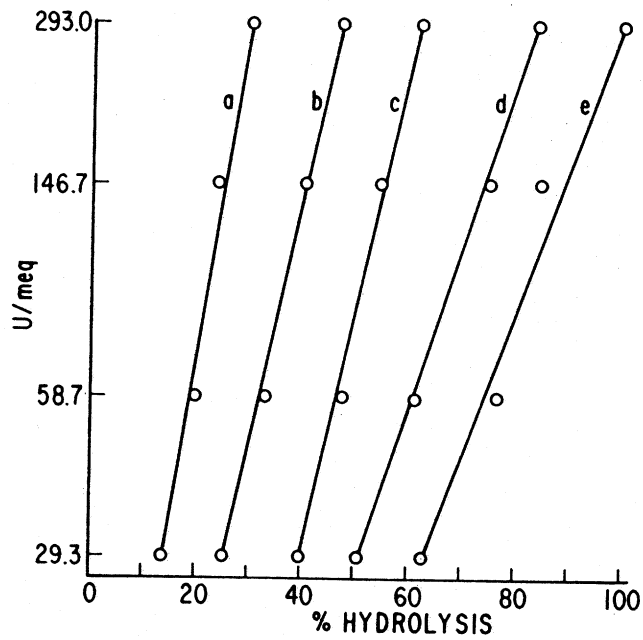

FIG. 4. Hydrolysis of coconut oil at room temperature with *A. niger* lipase: (a) after 1 hr, (b) after 2 hr, (c) after 4 hr, (d) after 8 hr, (e) after 16 hr.

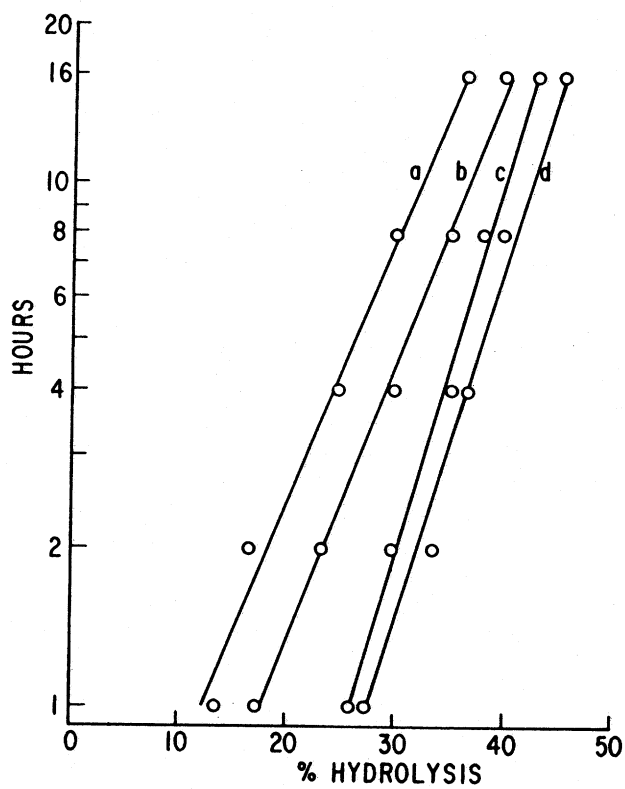

FIG. 5. Hydrolysis of olive oil at room temperature with *R. arrhizus* lipase at: (a) 2.3 U lipase/meq oil, (b) 4.6 U lipase/meq oil, (c) 11.5 U lipase/meq oil, (d) 23 U lipase/meq oil.

for olive oil at room temperature (Fig. 5). Coconut oil behaved similarly.

A class separation of the partially hydrolyzed olive oil with *R. arrhizus* lipase was carried out by TLC. The TLC procedure described above gave almost 100% recovery of starting material. The only difficulty observed was that at times some of the very fine silica gel may not be held back

TABLE I

Hydrolysis of 3.33 Millimoles of Coconut Oil at pH 5.4 with *A. niger* Lipase Solution (Palatase) at Various Temperatures

Reaction time (hr)	Percentage hydrolysis at 25 C	34 C	43 C	52 C
1	38.7	26.8	33.6	19.4
2	39.4	43.4	45.9	25.7
4	61.3	59.1	56.8	35.5
8	77.8	74.2	70.4	38.0
16	99.1	91.5	84.1	48.9

TABLE II

Minimum Lipase Requirement for Complete Hydrolysis of 100 g Substrate after 72 hr Reaction Time

Substrate	Amount of lipase from *C. rugosa* (mg)	*A. niger* (g)	Temperature (C)
Tallow	166	140	43-46
Coconut oil	198	48	23-26
Olive oil	66	40	23-26

TABLE III

TLC Class Separation of Olive Oil Partially Hydrolyzed by *R. arrhizus* Lipase at 25 C

Component	Millimoles in sample
Triglyceride	0.1516
Diglyceride	0.2710
Monoglyceride	0.1453
Fatty acid	0.7660
Glycerol (calculated)	0.0618

by the filter bed of the acid fraction, and recovery values of over 100% were obtained. In such cases the organic matter should be carefully dissolved in ether, the vials rinsed with ether, and the ether solutions evaporated in fresh vials. The silica gel particles tended to adhere to the glass and were not readily dislodged by the ether extraction.

Results of a TLC separation of olive oil hydrolyzed with *R. arrhizus* lipase for 4 hr are shown in Table III. A substantial amount of glycerol had been formed due to isomerization and subsequent hydrolysis. Had no isomerization occurred, the amount of fatty acid formed should have been 0.2710 + (2× 0.1453) = 0.5616 mM of fatty acid. However, the sample contained an additional 0.2044 mM of fatty acid due to complete hydrolysis to glycerol. Thus, the sample contained 0.2044/3 = 0.0681 mM of glycerol.

Enzymatic Esterification of Oleic Acid with Glycerol

Enzymatic esterification of fatty acids with glycerol as described above may lack practicality because the reaction is very slow. The major advantage of enzymatic esterification is that it can be run at room temperature, does not involve introduction of impurities as when, e.g., acyl halides are used, and does not cause degradation and formation of color bodies, which are encountered when high temperature esterification methods are used.

To bring about effective esterification of glycerol, it was necessary to develop a fixed experimental protocol. Preparation of an aqueous solution of the lipase followed by addition of glycerol gave a solution which could be stored in a freezer at −10 to −15 C without precipitation. Micro-

TABLE IV

TLC Separation of Reaction Mixture of Triolein Synthesis with *C. rugosa* Lipase after 1 hr Reaction Time at 25 C

Component	wt %
2-Monoglyceride	0.37
1-Monoglyceride	2.26
1,2-Diglyceride	0.86
1,3-Diglyceride	3.48
Triglyceride	1.76
Fatty acid	91.26

bial spoilage was essentially eliminated because of low temperature. Stock solutions thus prepared remained stable for several months without loss of enzyme activity.

To drive the reaction towards esterification, we used a 50% excess of fatty acid and reduced water activity by distilling out water periodically at room temperature with the aid of a vacuum pump. Some problems may occur with this technique. If too much water was pumped off, the enzyme precipitated, stopping catalytic activity. However, addition of a small amount of water redissolved the precipitate and restored enzyme activity. It was found that the emulsion, formed after a few hours of reaction, contains large droplets and required homogenization to form droplets of ca. 3 microns. This usually prevented precipitation.

Enzymatic esterification was readily followed by analysis of free fatty acid contents and by IR spectrophotometry, which showed a gradually growing ester peak at 1740 cm^{-1} and a correspondingly shrinking acid peak at 1710 cm^{-1}. TLC separation showed that all expected mono-, di-, and triglycerides were formed after a 1-hr reaction time as shown in Table IV, and it should be noted that triglyceride was present even at this early stage of esterification. As mentioned above, the reaction slowed down and after a 6-week reaction time esterification of all hydroxyl groups was ca. 80% complete. We were not able to make the reaction proceed further, presumably because equilibrium had been attained. A TLC analysis of a 6-week reaction product showed 1.4% monoglycerides, 19.8% diglycerides, 43.6% triglycerides and 35.3% fatty acids. The same esterification of glycerol and oleic acid could also be run with the lipase from *A. niger*, and the results obtained were essentially the same as those with the *C. rugosa* lipase. The enzymes were still highly active after 6 weeks and hydrolyzed the glyceride mixture overnight in the presence of water.

Fatty acids can be esterified with various alcohols other than glycerol (7). We also found that sorbitol would not esterify oleic acid. Ethylene glycol and diethylene glycol esterify slowly and incompletely. Benzyl alcohol esterified to 90-95% in ca. 6 weeks. We were not able to esterify methanol and oleic acid with *C. rugosa* lipase. Because of the slowness of these esterifications, as developed up to the present, this information is of scientific rather than practical industrial interest. However, conceivably more active and less costly enzymes may become available and a superior process for enzymatic esterification may be developed in the future.

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