# Lipase Catalyzed Transesterification of Unsaturated Lipids in a Microemulsion

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**A series of triglycerides having varying degrees of unsaturation of the acyl groups has been transesterified with stearic acid. It is shown that the position of the double bonds, not the degree of unsaturation, is the decisive factor in the rate of reaction. A Δ-6 double bond, which is present in petroselinic acid and γ-linolenic acid, prevents reaction. Unsymmetrical triglycerides containing only one acyl group with Δ-6 unsaturation reacts regiospecifically with the Δ-6 acyl group facing away from the enzyme. γ-Linolenic acid can be incorporated into an unhindered triglyceride through lipase catalyzed transesterification, indicating that the active site can in fact accommodate a Δ-6 fatty acid, although not the corresponding triglyceride.**

Lipase catalyzed transesterification of triglycerides in microemulsions has recently been investigated (1-3). For instance, synthetic cocoa butter, an important product for the chocolate industry, can be produced from palm oil by a partial replacement of palmitoyl groups by stearoyl groups. The reaction runs smoothly in oil-rich microemulsions (reverse micellar systems) based on either anionic or nonionic surfactants (1). A 1,3-specific lipase of either microbial or animal origin is used as catalyst.

When catalyzing hydrolysis reactions of triglycerides, lipases are known to be selective with regard to chain lengths and double bond positions of the acyl groups, in addition to showing regiospecificity with regard to the glycerol moiety. No corresponding study has been made for the transesterification of triglycerides.

The current biological interest for polyunsaturated fatty acids in fats prompted us to investigate possible limitations in the structure of the acyl group in the transesterification of polyunsaturated triglycerides. It is shown in this paper that the positions of the olefinic bonds, but not the degree of unsaturation, is the decisive factor in the rate of reaction.

## EXPERIMENTAL PROCEDURES

All reactions were carried out at 35°C under nitrogen using a magnetic stirrer. The enzyme was always used in an amount of 1.5 U/mg substrate.

Microemulsions were made by mixing isooctane (91.65 weight %), AOT (2.35%), aqueous 0.066 M phosphate buffer, pH 6 (1.0%), and substrate (5.0%). After the reaction is completed the temperature was raised to 100°C and kept at there for 10 min to denature the enzyme. The solvent was evaporated under vacuum.

The reaction product was dissolved in diethyl ether and charged on a preparative TLC plate. Elution was made with a combination of hexane, diethyl ether and formic acid (70:30:0.2). The triglyceride fraction was scraped off, dissolved in diethyl ether and collected. The triglyceride obtained was hydrolyzed and methylated with $LiOCH_3$. The methyl esters obtained were analysed by GLC using a Carbowax 1.2 μm FSOT column. Analytical grade fatty acid methyl esters were used as references. The amounts of tri-, di-, and monoglyceride present in the reaction mixture were monitored by GLC as previously described (4).

The lipases used were from *Rhizopus sp.* (68 U/mg, from Serva, W. Germany) and *Rhizopus delemar* (99 U/mg, from Fluka, Switerland).

The fatty acids and triglycerides used were all from Sigma, USA, except the tri- γ-linolenin which was synthesized from methyl-γ-linolenate and glycerol (molar ratio 3:1, 6 hr reaction at 85°C using p-toluene sulphonic acid as the catalyst and evaporating the methanol formed by a continuous nitrogen purge). Sodium bis(2-ethylhexyl)sulphosuccinate (AOT) was from Cyanamid, USA and was used without purification. The isooctane was reagent grade.

## RESULTS

*Transesterification of Unsaturated Triglycerides with Stearic Acid*: Figure 1 shows the results of reactions of five different triglycerides with stearic acid. The reactions were carried out in the oil-rich isotropic region of the system AOT (sodium bis[2-ethylhexyl]sulphosuccinate), isooctane, aqueous buffer (pH 6), and reagents (triglyceride: fatty acid, molar ratio 1:2). The reaction mixtures remained isotropic throughout the reaction period. A 1,3-specific enzyme, from *Rhizopus delemar*, was used as catalyst.

Using a molar ratio of triglyceride to stearic acid of 1:2 and assuming no specificity of the enzyme with regard to fatty acid, the stearoyl content in the triglyceride at equilibrium should be 33%. As can be seen from Figure 1, triolein, trilinolein, and tri-α-linolenin all exhibit considerable reactivity giving values of stearoyl incorporation which are above 90% of the theoretical value.

Although the water content of the reaction medium is low, enzymatic hydrolysis to di- and monoglyceride will also take place. As can be seen from Figure 2, the degree of hydrolysis is highly dependent on the choice of enzyme. *Rhizopus delemar* lipase which is very active as a transesterification catalyst (equilibrium being obtained after 5-6 hr) is also effective in catalyzing hydrolysis of triolein (Fig. 2a). Lipase from *Rhizopus sp.*, on the other hand, which is also 1,3-specific, gives much less hydrolysis, as is evident from Figure 2b. Although the latter lipase is less active a catalyst for the transesterification reaction (equilibrium is obtained after about 20 hr) it is the enzyme of choice if hydrolysis is to be suppressed.

*Transesterification of Triolein with α- and γ-Linolenic Acid:* As can be seen from Figure 3, incorporation of α-linolenoyl into a model triglyceride, triolein, proceeded smoothly. Using a 2:1 molar ratio of fatty acid to triglyceride the reaction was complete, i.e., approximately one acyl group per triglyceride molecule had been replaced, after 6 hr and 24 hr with lipases from

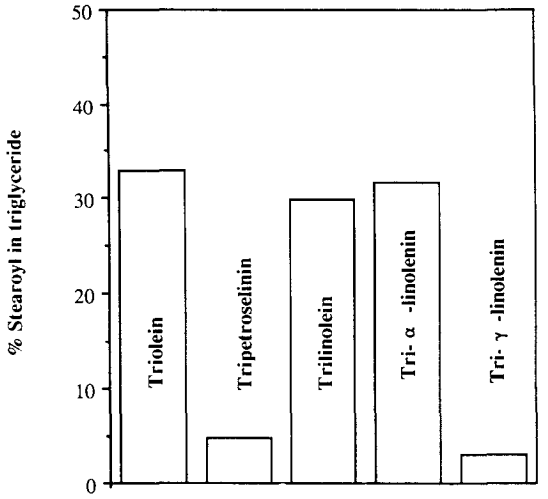


**FIG. 1. Incorporation of stearoyl into five different triglycerides through transesterification with stearic acid in the presence of *Rhizopus delemar* lipase. Reaction time was 6 hr and molar ratio of fatty acid to triglyceride was 2:1.**

*Rhizopus delemar* and *Rhizopus sp.*, respectively.

Transesterification of triolein with γ-linolenic acid proceeded less readily. As is seen in Figure 3, a large excess of fatty acid is needed in order to attain an appreciable degree of conversion. Only with the more active lipase from *Rhizopus delemar* and with a molar ratio of fatty acid to triglyceride of 7:1, can a yield corresponding to the replacement of one acyl group per triglyceride be obtained.

*Transesterification of Triglyceride Containing γ-Linolenic Acid with Oleic Acid:* 1-γ-Linoleoyl-2,3-dioleoylglycerol, prepared from triolein and γ-linolenic acid, was reacted with oleic acid in the presence of *Rhizopus delemar* lipase. Although a very large excess of oleic acid was used, the acyl group composition of the triglyceride remained essentially intact, as is shown in Table 1. It is noteworthy that the same enzyme and the same reaction conditions were used as in the reaction in the opposite direction, i.e., between triolein and γ-linolenic acid.

When stearic acid was substituted for oleic acid, transesterification took place, although also in this case it did not affect the γ-linolenoyl group.

## DISCUSSION

The outstanding characteristic of lipases is their affinity for water-oil interfaces. The surface activity of the enzyme is a prerequisite of its ability to catalyze reactions with hydrophobic substrates because it has to compete for interfaces with a multitude of other proteins. Microemulsions seem to constitute an almost ideal medium for *in vitro* reactions since they possess an enormous interface between oil and water domains.

However, the enzyme-substrate complex is not believed to involve hydrophobic bonding of aliphatic chains (5). Hydrolysis reactions with various branched and bulky ester substrates have made it clear that lipases

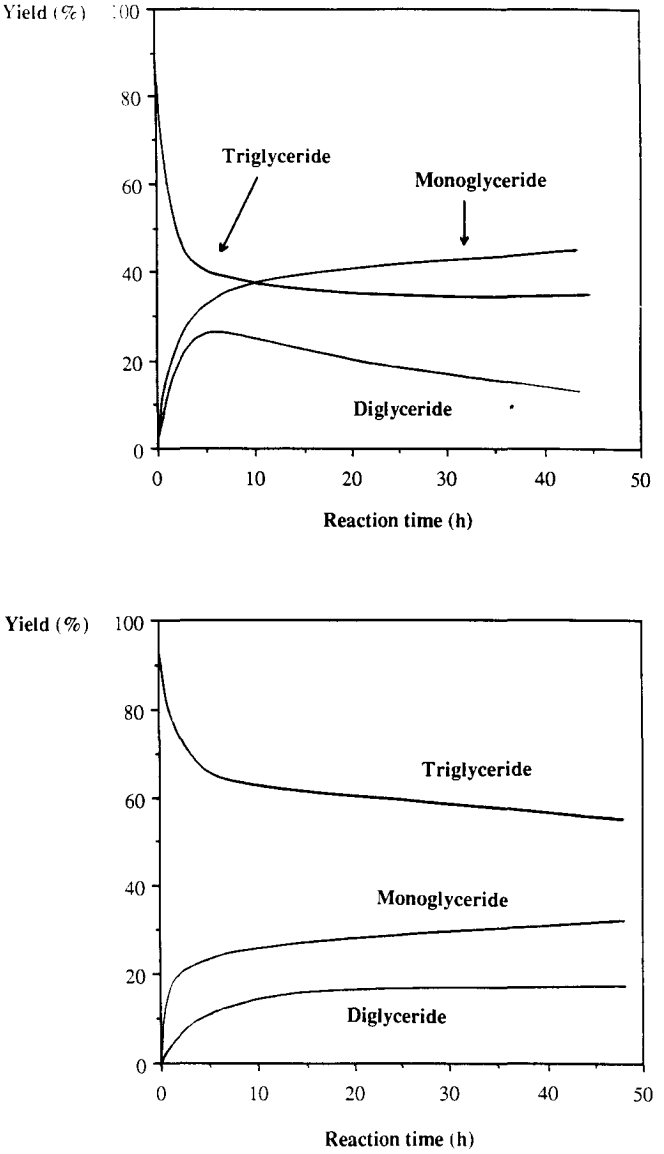


**FIG. 2. Yield of tri-, di- and monoglyceride in reaction between stearic acid and triolein (molar ratio 2:1) using lipase from *Rhizopus delemar* (upper) and *Rhizopus sp.* (lower) as catalyst.**

are sensitive to steric effects on the acid side but rather insensitive on the alcohol side. It has been postulated that fixation of the substrate occurs through hydrogen bonding to the C-O-C oxygen of the ester bond and possibly also to the carbonyl oxygen (5). The enzyme nucleophile, which may be a serine hydroxyl group (6), will attack the carbonyl carbon and the alcoholate released will be protonated, as is illustrated in Figure 4. The acylated nucleophile is subsequently hydrolyzed to fatty acid and regenerated nucleophile.

The reaction is reversible and at low water activity equilibrium is shifted towards condensation, i.e., ester synthesis. In the presence of an added fatty acid the deacylation-acylation sequence at low water content will lead to the formation of a mixture of esters. In the ideal case the ratio of esters formed can be determined from the ratio of fatty acid to starting ester compound.

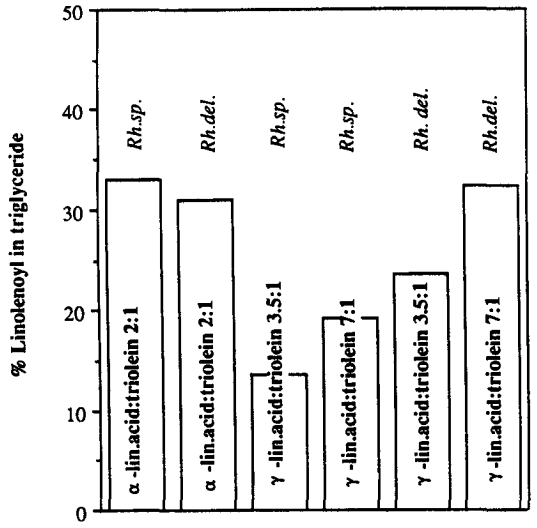


**FIG. 3. Incorporation of linolenoyl into triolein through transesterification with α- and γ-linolenic acid. The times for *Rhizophus delemar* and *Rhizopus sp.* catalyzed reactions are 6 and 24 hr, respectively.**

However, steric effects similar to those encountered in hydrolysis reactions are likely to be present in the transesterifications as well.

The results obtained in this work show that Δ-6 double bonds in triglycerides effectively prevent transesterifications with unhindered fatty acids, such as stearic acid. Two conclusions can be drawn: First, the position of the double bonds, not the degree of unsaturation, is decisive. Tri-α-linolenin, which contains 18:3, (*cis,cis,cis*)-9,12,15 acyl groups, reacts almost as readily as triolein. Tri-γ-linolenin, which contains 18:3, (*cis,cis, cis*)-6,9,12 acyl groups, and tripetroselinin (18:1, (*cis*)-6 acyl groups), is unreactive. Similar effects of unsaturation in positions 2-6 have previously been observed for lipase catalyzed hydrolysis (5,7,8). The double bond probably imparts restrictions in chain rotation and prevents it from properly bending away from the enzyme. Second, unsymmetrical triglycerides containing only one acyl group with Δ-6 unsaturation react regiospecifically. As is shown in Table 1, with 1-γ-linolenoyl-2,3-dioleoylglycerol the 3-oleoyl group, but not the 1-γ-linolenoyl, can

**TABLE 1**

**Variations of acyl group content of the triglycerides obtained from the reaction of 1-γ-linolenoyl-2,3-dioleoylglycerol with oleic acid and stearic acid. Molar ratio of fatty acid to triglyceride was 10:1, *Rhizopus delemar* lipase was used and the reaction time was 6 hr.**

| | % Content of | | |
|---|---|---|---|
| | γ-linolenoyl | oleoyl | stearoyl |
| Starting triglyceride | 32 | 68 | — |
| After reaction with oleic acid | 30 | 70 | — |
| After reaction with stearic acid | 31 | 51 | 18 |

be replaced by stearoyl. Docking of the triglyceride with the unhindered acyl group facing the enzyme is evidently not severely hindered by the Δ-6 unsaturated acyl group.

As an extension of this work it would have been interesting to investigate transesterification of a triglyceride having an unhindered group in 1-position and Δ-6 unsaturated groups in 2- and 3-positions. However, such a product is not readily available through organic synthesis nor by enzymatic means. One may speculate that triglycerides of this type would be resistant to enzymatic catalysis because natural triglycerides of high ω-6 fatty acid content, such as fish oil, borage oil, black currant oil, etc., never contain more than 30% of these acyl groups (9,10).

Figure 3 shows that γ-linolenoyl groups can be incorporated into triglycerides through transesterification with a large excess of γ-linolenic acid. This means that fatty acids having Δ-6 double bonds can indeed approach the enzyme and acylate the nucleophile of the active site (e.g., a serine hydroxyl group). As discussed above, a triglyceride based on the same fatty acid is resistant to enzymatic transesterification or hydrolysis. Evidently, the active site can, although with some difficulty (the reaction requires a large excess of acid) accommodate a Δ-6 acid but not the corresponding triglyceride. This is not trivial, as it is generally accepted that lipases are insensitive to the structure of the alcohol moiety of the ester. In this case, however, with an extreme steric requirement of the

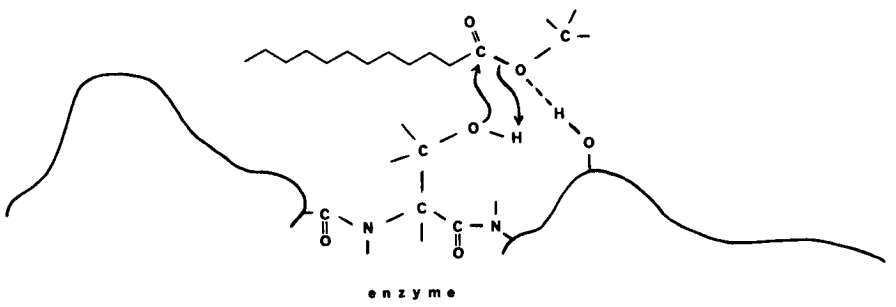


**FIG. 4. A schematic illustration of the enzyme-substrate complex.**

acyl group the alcohol part of the molecule will decide the feasibility of the reaction.

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