Synthesis of Triacylglycerol by Lipase in Phosphatidylcholine Reverse Micellar System

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ABSTRACT

Triacylglycerols were synthesized from 1,2-diacylglycerol and fatty acids by lipase entrapped in phosphatidylcholine reverse micelles in n-hexane. In the reaction system without reverse micelles, however, 1,2-diacylglycerol was hydrolyzed into 2-monoacylglycerol and fatty acid, and triacylglycerol was not synthesized. The maximum activity of synthetic reaction was obtained at Wo=10 (Wo = mol water/mol surfactant), which was the water content of this reverse micellar system. Though the optimal pH of the *R. delemar* lipase reaction is about pH 5.6 in a bulk water system, the enzyme was active for triacylglycerol synthesis at pH's from 5 to 9 in the reverse micellar system. For the synthesis of triacylglycerols, lauric, myristic, palmitic, stearic, oleic and arachidic acids were effectively used as the fatty acid substrate. 2-Monoacylglycerol was also effective as a substrate of triacylglycerol synthesis. Furthermore, 1,2-diacylglycerol could be replaced by several kinds of aliphatic alcohols as fatty acid acceptors in the reverse micellar system. In this case, those alcohols with chain length more than 4 carbons were effectively used for ester formation.

INTRODUCTION

Triacylglycerol is synthesized in vivo from 1,2-diacylglycerol and fatty acyl-CoA by diacylglycerol acyltransferase (E.C.2.3.1.20) bound to microsomal membrane. This reaction, however, requires ATP. Generally, triacylglycerol is hydrolyzed to diacylglycerol and free fatty acid in the first step of the lipase (E.C.3.1.1.3) reaction. Though the reaction proceeds to hydrolysis of triacylglycerol in the presence of a large amount of water, it is possible to synthesize triacylglycerol by lipase under the conditions of low amount of water and/or high amount of the hydrolyzed products. Thus, triacylglycerol may be synthesized by lipase in vitro.

Reverse micelles are formed by appropriate surfactants and organic solvents. This reverse micellar system is suitable to control water content. Up to date, a few enzymes have been studied on their characteristics in the reverse micellar system with bis(2-ethylhexyl)sodium sulfosuccinate (AOT) (1-4). However, such a synthetic chemical is limited to use in food industries, while natural phospholipids will be useful as surfactants for food systems.

Recently, we described the successful use of phospholipid reverse micelles in order to entrap enzymes and to control the amount of water (5,6). This paper treats the synthesis of triacylglycerols by lipase entrapped in phosphatidylcholine reverse micelles in n-hexane.

EXPERIMENTAL PROCEDURES

Materials

Phosphatidylcholine and phosphatidylethanolamine were purified from a commercial soybean lecithin by silicic acid column chromatography (7). AOT was purified as described in the literature (8). Lipase from *R. delemar* (6,000 U/mg protein) was the product of Nippon Seikagakukogyo, Tokyo. 1,2-Diacylglycerol was prepared from phosphatidylethanolamine by phospholipase C (E.C.3.1.4.3) treatment (9). 1-Monoolein, 2-monoolein, 1,2-diolein, and 1,3-diolein were purchased from PL Biochemicals, Milwaukee, Wisconsin. Thin-layer silica gel plates (No. 5721) were from Merck, Darmstadt. The diasolid-ZT column was obtained from Nippon Chromatokogyo, Tokyo. Other chemicals were all reagent grade of Nakarai Chemicals, Kyoto.

Assay of Enzyme Reaction

The enzyme solution (60,000 units of lipase from *R. delemar* dissolved in 0.1 M potassium phosphate buffer, pH 7.0) was added to 1 ml of n-hexane containing 50 μmol of phosphatidylcholine. The desired water content, Wo (mol water/mol surfactant), was obtained by addition of the required volume of an aqueous solution of the enzyme, 60,000 units of lipase, to n-hexane. The mixture was sonicated for one minute at 37 C to make reverse micelles. 15 μmol of 1,2-diacylglycerol or 30 μmol of aliphatic alcohol and 30 μmol of oleic acid were added to 1 ml of n-hexane in a vial. The enzyme activity in a non-polar solvent was assayed at 37 C by mixing equal volumes of enzyme and substrate solutions. At various intervals, an aliquot (0.5 ml) was taken out and vigorously mixed with 4 ml of chloroform-methanol (2:1, v/v) and 1 ml of 2.5% trichloroacetic acid solution for one minute. The product, triacylglycerol, in the chloroform layer was analyzed by thin-layer chromatography (TLC) with developing solvent of n-hexane/diethyl ether/acetic acid (150:150:3, v/v/v) and gas-liquid chromatography using a Diasolid-ZT column with N_2 flow rate 60 ml/min and temperature programmed from 180 to 330 C and by using an Iatroscan Analyzer with developing solvent of benzene/chloroform/formic acid (70:30:2, v/v/v). A sample was applied by using a capillary pipet on Chromarod-SII, which had been preconditioned in Iatroscan just before use. Calibration curves were obtained with different amounts of lipids above 0.5 μg. Accuracy was within 5%.

RESULTS AND DISCUSSION

Reaction

The lipase reaction in reverse micellar system in n-hexane produced triacylglycerol from 1,2-diacylglycerol and oleic acid (Fig. 1). The product was confirmed to be triacylglycerol by TLC. The formation of triacylglycerol increased gradually during the reaction, and 60% of 1,2-diacylglycerol was changed into triacylglycerol after 4 hr reaction time. Partial hydrolysis of 1,2-diacylglycerol occurred and 2-monoacylglycerol was found to be formed during the reaction. This may be caused by the enzyme leaking from reverse micelles into n-hexane.

When n-hexane was replaced by water and ether (1:1, v/v), which are usually used for phospholipase reaction, 2-monoacylglycerol and fatty acid were produced from 1,2-diacylglycerol, but no triacylglycerol was found (Table I). This shows that only hydrolysis occurred in this system. A decrease in phosphatidylcholine during the reaction suggests that phospholipases were contaminated by the *R. delemar* lipase preparation, or that the *R. delemar* lipase itself had phospholipase activity as noted by Slotboom et al. (10,11). Likewise, triacylglycerol was not formed when the reaction proceeded in n-hexane without phosphatidylcholine reverse micelles. Under these conditions, when triolein was used as a substrate instead of 1,2-diacylglycerol and oleic acid, 1,2-diolein and 2-monoolein were produced. These results indicate that phosphatidylcholine reverse micelles are necessary to carry out the synthetic reaction of triacylglycerol by lipase.

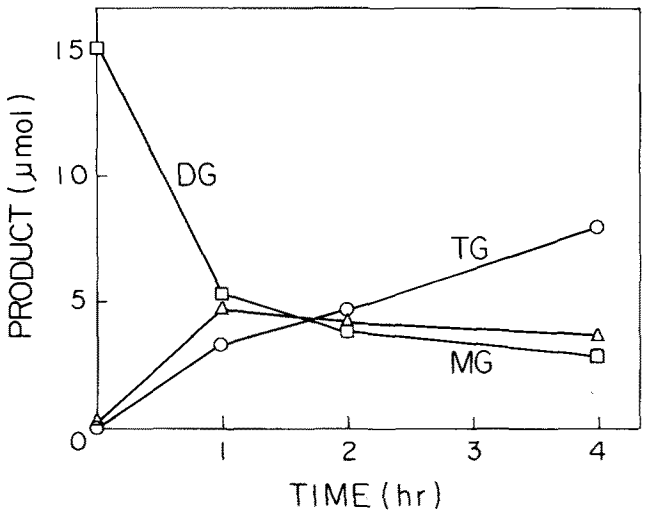

FIG. 1. Time course of lipase reaction in reverse micellar system. 15 μMol of 1,2-diacylglycerol and 30 μmol of oleic acid dissolved in 1 ml of n-hexane were incubated at 37 C by mixing with 1 ml of the enzyme solution, which was prepared by sonication of the mixture of 1 ml of 50 mM phosphatidylcholine in n-hexane and 60,000 units of lipase from *R. delemar* dissolved in 0.1 M potassium phosphate buffer, pH 7.0. The products were determined by using Iatroscan. TG, triacylglycerol; DG, diacylglycerol; MG, monoacylglycerol.

TABLE I

Lipase Reaction in Ether-Water System

Incubation mixture	Incubation (hr)	
	0	4
	μMol	
Phosphatidylcholine	20.0	14.3
1,2-Diacylglycerol	15.0	1.1
Free fatty acid	30.0	51.6
2-Monoacylglycerol	–	14.0
Triacylglycerol	–	0

15 μMol of 1,2-diacylglycerol and 30 μmol of oleic acid dissolved in 1 ml of diethylether were incubated at 37 C by mixing with 1 ml of the enzyme solution, which contains 20 μmol of phosphatidylcholine and 60,000 units of lipase from *R. delemar* dissolved in 0.1 M potassium phosphate buffer, pH 7.0.

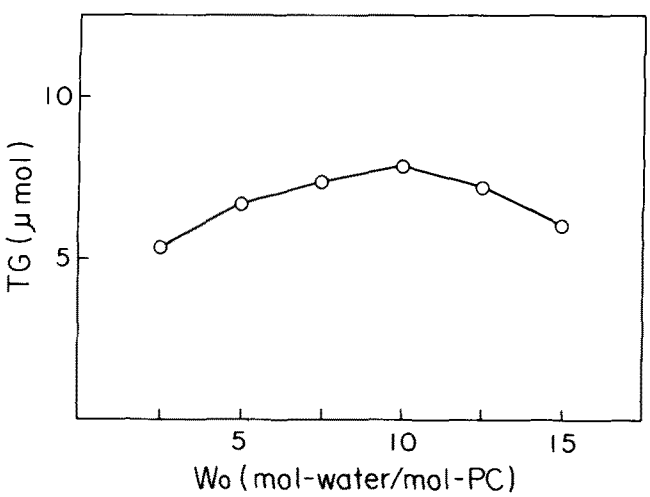

FIG. 2. Effect of water content of reverse micellar system on the synthesis of triacylglycerol. The reaction was carried out under the same conditions described in the legend to Figure 1 except that the amount of the enzyme solution was changed. TG, triacylglycerol; PC, phosphatidylcholine.

Effect of Water

Water content in the reverse micelles may be the most important factor for the promotion of the synthetic reaction of triacylglycerol. Figure 2 shows the effect of water content on the triacylglycerol synthesizing system. Wo, which means the ratio of mol water to mol phosphatidylcholine, was used as a standard to express water content in a reverse micelle. The maximum synthetic activity was obtained at Wo=10. A water content below Wo=10 is necessary for this reverse micellar system. However, excess water may be used as the substrate for the hydrolytic reaction. During the synthetic reaction, water was produced. However, the amount of water produced corresponded to about 2% of pooled water originally present in a micelle.

Effect of pH

The optimum pH for the hydrolyzing reaction by lipase from *R delemar* is about 5.6 (12) in a bulk water system. When the enzyme was entrapped in phosphatidylcholine reverse micelles at Wo=10, similar synthetic activity was observed in a pH range from 5 to 9 (Fig. 3). Two assumption may explain the result. One is that the abnormal state of enzyme may be caused by an extremely limited water environment. The other is that the nature of water entrapped in the reverse micelle may be different from that of bulk water. It has been reported that the pH of water entrapped in the reverse micelle of AOT is different from that of bulk water (13).

Substrate Specificity

For the synthesis of triacylglycerols, lauric, myristic, palmitic, stearic, oleic and arachidic acids were effectively used as the fatty acid substrate. However, 1,3-diolein could not be substituted for 1,2-diolein due to the specificity of lipase from *R. delemar* (Fig. 4). Though 1-monoolein was hydrolyzed, 2-monoolein could be used as the substrate of triolein synthesis. When 2-monoolein, palmitic and stearic acids were incubated in the reverse micellar system,stearoyl-oleoyl-palmitoyl triacylglycerol, a major component of cocoa butter, was synthesized by introducing stearic and palmitic acids into 1 and 3 positions of 2-monoolein (Fig. 5). Small amounts of dipalmitoyl-oleoyl triacylglycerol

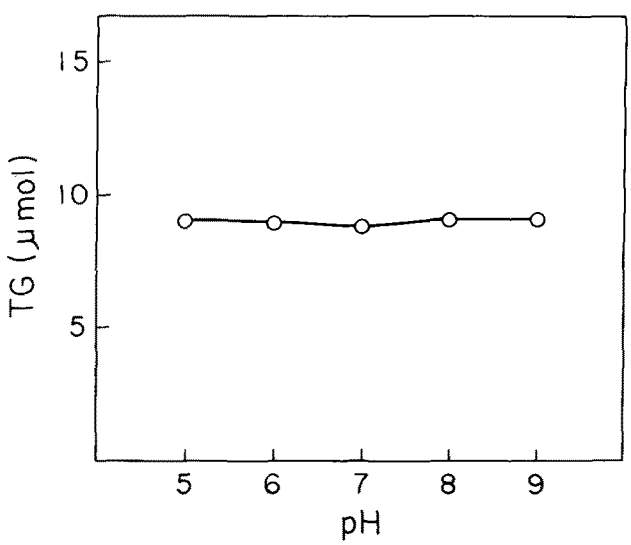

FIG. 3. Effect of pH of reverse micellar system on the synthesis of triacylglycerol. The reaction was carried out under the same conditions described in the legend to Figure 1 except that pH was changed. TG, triacylglycerol.

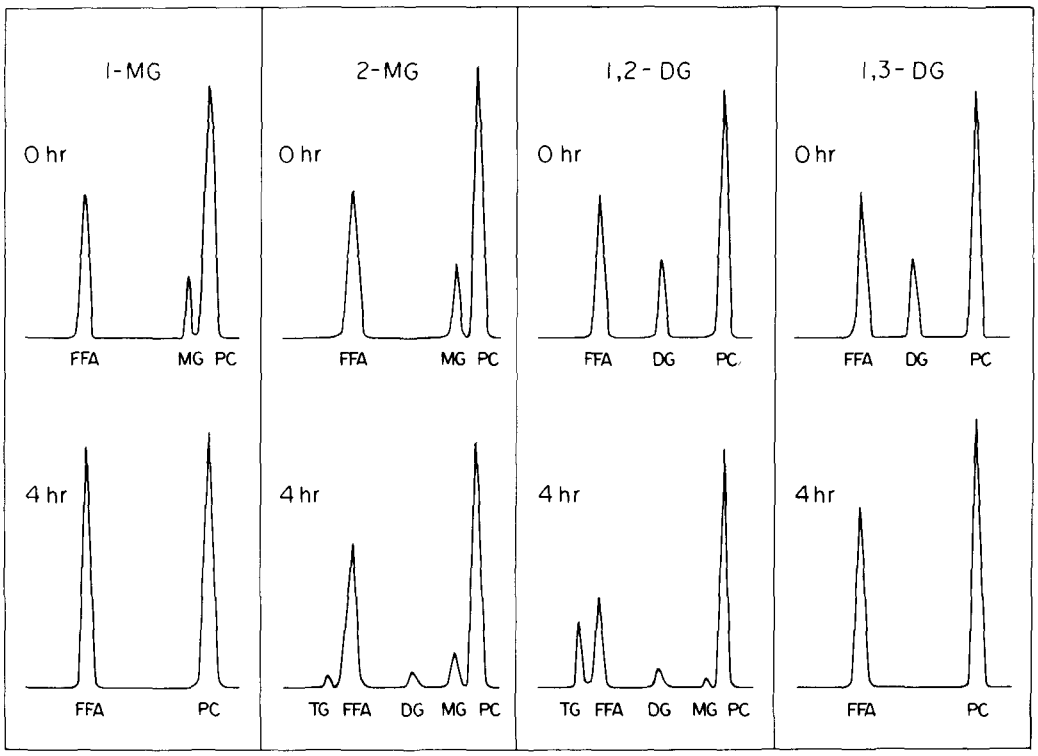

FIG. 4. Iatroscan chromatogram of the reaction products in triacylglycerol synthesis with two kinds of diacylglycerols and monoacylglycerols. The conditions of the reaction were the same as that described in the legend to Figure 1. TG, triacylglycerol; FFA, free fatty acid; DG, diacylglycerol; MG, monoacylglycerol; PC, phosphatidylcholine.

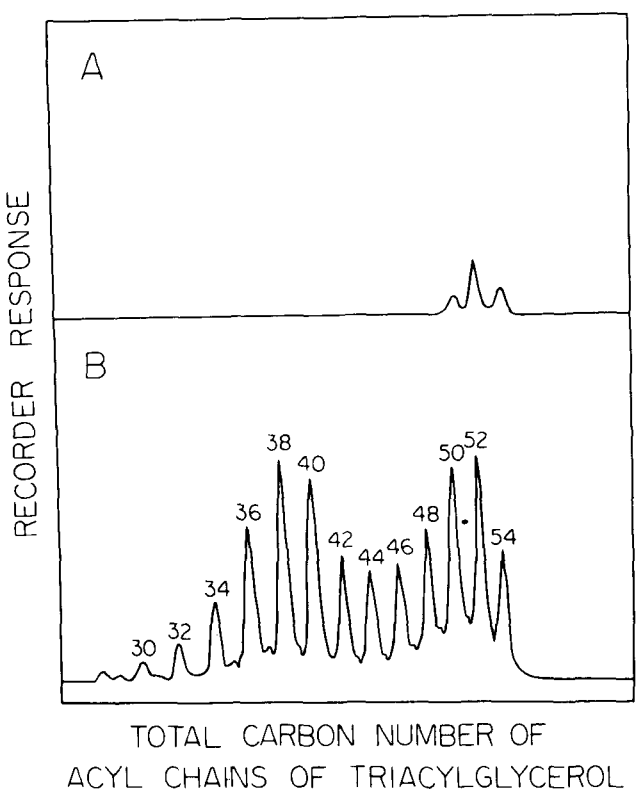

FIG. 5. Synthesis of stearoyl-oleoyl-palmitoyl triacylglycerol. Molecular species of triacylglycerols were analyzed by Diasolid-ZT column. A, triacylglycerols synthesized; B, triacylglycerol molecular species of butter.

(total carbon number of acyl chains is 50) and distearoyl-oleoyl triacylglycerol (total carbon number of acyl cains is 54) also were synthesized.

It was found that 1-monoacylglycerol and 1,3-diacylglycerol were formed from glycerol and fatty acid, but that 1,2-diacylglycerol and triacylglycerol were not formed (data not shown). This selective synthesis is due to the substrate specificity of the lipase from *R. delemar*, which cannot cleave the ester linkage at the C-2 position. Therefore, various kinds of triacylglycerols may be synthesized

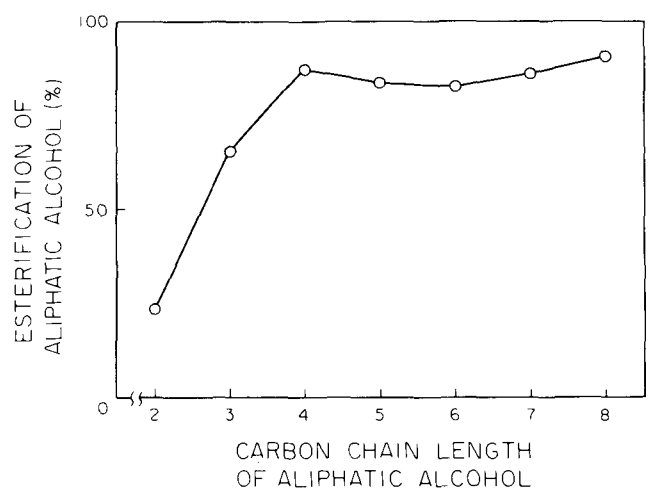

FIG. 6. Effect of chain length of aliphatic alcohols in the ester synthesis in reverse micellar system catalyzed by lipase. The reaction was performed under the same conditions as Figure 1 except that 30 μmol of aliphatic alcohol instead of 15 μmol of 1,2-diacylglycerol was used.

from glycerol and fatty acids by using a lipase preparation without the substrate specificity for 1,2 or 3 position.

Moreover, we found that several aliphatic alcohols, instead of 1,2-diacylglycerol, could be used by the lipase as the fatty acid acceptor in a reverse micellar system. Figure 6 shows the effect of chain length of aliphatic alcohols upon the reactivity of ester formation with oleic acid, which indicates that alcohols with a chain length greater than 4 carbons were effective. However, cholesterol could not be used as the substrate in place of aliphatic alcohols (data not shown).

Triacylglycerol (or ester) synthesis in the reverse micellar system is pictured in Figure 7. The carboxyl group of the fatty acid and hydroxyl group of 1,2-diacylglycerol (or aliphatic alcohols) are in contact with water in reverse micelles in n-hexane and can be esterified by the lipase located inside the reverse micelles. Once products are synthesized, they lose amphipathic properties and are moved from the water phase into the n-hexane phase. In this way the reaction proceeds to triacylglycerol synthesis.

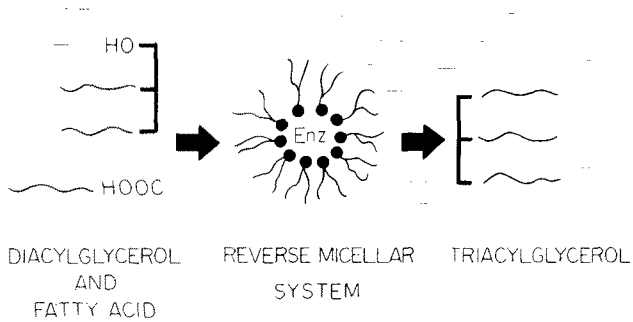

FIG. 7. The reaction scheme of triacylglycerol synthesis in reverse micellar system. See the text.

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Studies on Peroxidized Lipids. VI. Fluorescent Products Derived From the Reaction of Primary Amines, Malonaldehyde and Monofunctional Aldehydes

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ABSTRACT

Monofunctional aldehydes such as acetaldehyde, *n*-propylaldehyde, *n*-butylaldehyde, *n*-hexylaldehyde, *n*-heptylaldehyde and benzaldehyde affected the reaction between primary amines and malonaldehyde. While the reaction of primary amines and malonaldehyde at pH 7 produced fluorescent 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehydes **Ia-f**, the reaction of the primary amines, malonaldehyde and the aldehydes listed above gave fluorescent 4-substituted 1,4-dihydropyridine-3,5-dicarbaldehydes **IIa-j**. The primary amines used for this reaction included alkylamines, amino acids and alkanolamines. The optimal ratio of the amine, malonaldehyde and the aldehyde was 1:2:1-2, at which compounds **II** were produced quantitatively. Peroxidized lipids which may contain malonaldehyde and other aldehydes could react with the primary amines to produce highly fluorescent **II**. Fluorescence spectra of **II** showed excitation maxima at 386-403 nm and emission maxima at 444-465 nm in phosphate similar to those of **I**. The spectra of these 1,4-dihydropyridines **I** and **II** were roughly similar to those of lipofuscin pigment, but they exhibited different characteristics in acid and alkaline media from those of lipofuscin pigment. Compounds **II** may be useful as model compounds to elucidate the chemical structure of lipofuscin pigment.

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INTRODUCTION

The formation of fluorescent lipofuscin pigment has been suggested in the in vivo reaction of proteins and peroxidized lipids (1). The structure of fluorescent lipofuscin pigment has been believed to be the 2:1-conjugated Schiff base (N,N'-disubstituted 1-amino-3-iminopropene) of a primary amine and malonaldehyde, which might be produced by peroxidation of polyunsaturated fatty acids (2-5). In our recent studies of the reaction of primary amines with malonaldehyde, it was shown that the reaction afforded 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehydes **Ia-e** as major fluorescent products (6-9), which may be formed by the 1:3-reaction of the amines and malonaldehyde.

Peroxidation of polyunsaturated fatty acids has been shown to give various aldehydes besides malonaldehyde (10-12). We found that monofunctional aldehydes stimulated and participated in the formation of fluorescent substances in the reaction of primary amines and malonaldehyde. This paper describes the structures and the properties of the fluorescent products formed from the reaction of primary amines, malonaldehyde and monofunctional