Lipase-Catalyzed Alcoholysis of Triglycerides for Short-Chain Monoglyceride Production

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ABSTRACT: Lipase from *Pseudomonas fluorescens* efficiently catalyzed the alcoholysis of various TG in dry alcohols. For TG with short-chain FA, more MG were accumulated. The yields of MG were affected by the alcohols used. The maximum yields of MG were as follows: 85% for monoacetin in *n*-butanol, 96% for monobutyrin in ethanol or *n*-butanol, 50% for monocaprylin in *n*-butanol, 48% for monolaurin in isopropanol, and 45% for monopalmitin in isopropanol. The MG produced were judged to be 2-MG by TLC analysis. The presence of organic cosolvent affected the reaction rate of the lipase-catalyzed alcoholysis of TG. For the alcoholysis of various TG in ethanol and cosolvent (1:1, vol/vol), the rates had the following orders: (i) for tributyrin, hexane > toluene > acetone > ethyl acetate > chloroform > acetonitrile > pyridine; (ii) for tricaprylin, hexane > acetone > toluene > acetonitrile > ethyl acetate > pyridine > chloroform; and (iii) for trilaurin, hexane > acetonitrile = acetone > ethyl acetate > pyridine = chloroform > toluene.

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MG are useful as food flavors, pharmaceuticals, fragrances, and emulsifiers (1,2). MG of different chain lengths have different properties, e.g., HLB values, and therefore possess different biological functions and have various industrial applications (3). Conventional processes employing the direct esterification of FA with glycerol catalyzed by inorganic catalyst require high temperatures (200–250°C), which not only waste energy but also have undesirable side reactions. Lipases can be used as biocatalysts for the esterification process, and they have many advantages over the chemical process, namely, mild reaction conditions, high catalytic efficiency, and stereo- and positional specificities (2,4–8). The lipase-catalyzed production of MG has been shown to be quite successful with long-chain FA (9,10). However, it may not be applicable to the synthesis of short-chain MG owing to the relatively high acidity of short-chain FA. In a previous work (11), we discovered that *Aspergillus niger* lipase efficiently catalyzed the alcoholysis of glucose pentaacetate to prepare glucose tetraacetate in lower alcohols. Later, we discovered that *Pseudomonas fluorescens* lipase could also be used for the alcoholysis of olive oils and other long-chain TG for fatty ester production (12). Following these publications, several additional works on the alcoholysis of oils and long-chain TG were reported (8,13,14). Lipase produced by the bacterium *P. fluorescens* is used extensively for a large number of different synthetic reactions in organic chemistry, with special emphasis on the chiral production of racemic compounds (15). The *P. fluorescens* lipases have been shown to have broad substrate specificity towards TG and preferential regioselective hydrolysis of the *sn*-1 position of a TG (16,17). In the present work, we further report that the *Pseudomonas* lipase can catalyze the alcoholysis of short-chain TG in lower alcohols such as ethanol and isopropanol, which could be useful for the production of short-chain MG. The yields of MG are dependent on reaction conditions such as the chain length of TG and alcohols, the organic solvents, and the reaction times.

MATERIALS AND METHODS

Reagents. Lipase from *P. fluorescens* was a generous gift from Amano Pharmaceutical Co. Ltd. (Nagoya, Japan) and had a specific activity of 3.2 Amano units (AU) per milligram of solid. TG (triacetin, tributyrin, tricaproin, tricaprylin, trilaurin, and tripalmitin) were obtained from Sigma Chemical Co. (St. Louis, MO), and their purities were all 99% by GC. Celite 545 was purchased from Hayashi Pure Chemical Industries (Osaka, Japan). All other biochemicals and chemicals were of reagent grade.

Lipase-catalyzed alcoholysis. Lipase-catalyzed alcoholysis was carried out as described previously (9–11). Typically, the reactions were initiated by the addition of 0.3 g Celite-immobilized lipase to 2 mL of a 25-mM TG solution in alcohols predried with 3A molecular sieves (Merck Chemical Co., Darmstadt, Germany). The mixture was placed in a stoppered glass vial and shaken in an orbital shaker incubator at 250 rpm and 28°C. The lipase was immobilized as follows: 40 g of Celite 545 washed with l0 mM potassium phosphate buffer (pH 7.0) was mixed with 20 g of *Pseudomonas* lipase (Amano P), which was previously dissolved in 120 mL of 10-mM phosphate buffer (pH 7.0) and stirred at 4°C. A fivefold volume excess of cold (–20°C) acetone was added to precipitate the enzyme on the Celite surface. The immobilized lipase was filtered and dried under vacuum at room temperature.

TLC. Positional specificity of the enzyme was examined by TLC of the products of the enzymatic reaction with pure TG (Sigma) as substrates (18). Aliquots of the reaction mixtures

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were applied on a Silica gel 60 plate (Merck) and developed with a solvent mixture of chloroform, acetone, and acetic acid (96:4:1, by vol). Pure 1(3)-monocaprylin, 1(3)-monolaurin, 1,2-dicaprylin, 1,3-dilaurin, tricaprylin, and trilaurin (Sigma) were used as reference glycerides. Spots were visualized by heating at 180°C for 25 min after spraying with 20% sulfuric acid.

GC. The reaction products were analyzed by GC as described previously (11). One milliliter of sample was dried by a rotary evaporator and derivatized with 1,1,1,3,3,3-hexamethyldisilazane according to the procedures of Brobst and Lott (19). A 2-mL glass column packed with 3% OV-17 (Supelco, Bellefonte, PA) on 80/l00 Chromosorb WHP (Supelco) was used to analyze the reaction products of triacetin, tributyrin, tricaprylin, and trilaurin. The packing material for the analysis of tripalmitin reaction products was 1% Dexsil 300 (Dexsil, Hamden, CT) on 100/120 Supelcoport (both from Supelco).

RESULTS AND DISCUSSION

Effect of solvent polarity. It has been proposed that biocatalytic activity in organic media correlates with the hydrophobicity index, log*P* (20). However, the rate of TG alcoholysis catalyzed by the *Pseudomonas* lipase did not correlate with log*P*. Among the organic cosolvents tested, the velocity of the alcoholysis reaction was increased only when hexane was added to the reaction mixture (data not shown). The effect of the organic cosolvent appeared to be different with different TG as substrates. For the alcoholysis of various TG in ethanol and cosolvent (1:1, vol/vol), the rates of lipase-catalyzed alcoholysis decreased in the following order: (i) for tributyrin, hexane > toluene > acetone > ethyl acetate > chloroform > acetonitrile > pyridine (Table 1); (ii) for tricaprylin, hexane > acetone > toluene > acetonitrile > ethyl acetate > pyridine > chloroform (Table 2); (iii) for trilaurin, hexane > acetonitrile = acetone > ethyl acetate > pyridine = chloroform > toluene (Table 3).

Effect of various substrates. In agreement with Sugihara *et al.* (18), the results of the present TLC analysis (Fig. 1) showed

TABLE 1
Effect of Solvent Polarity on the Lipase-Catalyzed Alcoholysis of Tributyrin[a]

		Product composition[c] (%)		
Solvent	Log*P*[b]	Tributyrin	Dibutyrin	Monobutyrin
Acetonitrile	–0.33	55	38	7
Acetone	–0.23	46	42	11
Ethyl acetate	0.68	49	43	8
Pyridine	0.71	69	28	2
Chloroform	2.00	52	41	7
Toluene	2.50	30	55	15
Hexane	3.50	5	48	47

[a]Celite-immobilized *Pseudomonas* lipase (0.3 g Amano P; Amano Pharmaceutical Co. Ltd., Nagoya, Japan) was incubated with 0.16 mM tributyrin in 2 mL of organic solvent/ethanol mixture (l:l) in a stoppered glass vial and shaken on an orbital shaker at 250 rpm and 28°C for 1 h.
[b]Log*P* is defined as the logarithm of the partition coefficient in a standard octanol/water two-phase system.
[c]The product composition was analyzed by a Hitachi 263-30 gas chromatograph (Tokyo, Japan) equipped with a 2-m glass column packed with 3% OV-17 (Supelco, Bellefonte, PA) on 80/l00 Chromosorb WHP (Supelco).

TABLE 2
Effect of Solvent Polarity on the Lipase-Catalyzed Alcoholysis of Tricaprylin[a]

	Product composition (%)			
Solvent	Tricaprylin	Dicaprylin	Monocaprylin	Glycerol
Acetonitrile	49	35	6	10
Acetone	41	37	9	13
Ethyl acetate	47	36	5	12
Pyridine	63	26	3	8
Chloroform	88	12	0	0
Toluene	43	33	7	17
Hexane	4	30	29	37

[a]The initial tricaprylin concentration was 94 mM. The amount of Celite-immobilized lipase (Amano P; Amano Pharmaceutical Co. Ltd.) was 0.1 g and the reaction time was 30 min. Other conditions are the same as described in Table 1.

TABLE 3
Effect of Solvent Polarity on the Lipase-Catalyzed Alcoholysis of Trilaurin[a]

	Product composition (%)			
Solvent	Trilaurin	Dilaurin	Monolaurin	Glycerol
Acetonitrile	10	36	20	34
Acetone	18	33	20	29
Ethyl acetate	38	30	8	24
Pyridine	60	19	7	14
Chloroform	71	12	7	10
Toluene	65	17	6	12
Hexane	12	31	26	31

[a]The initial trilaurin concentration was 25 mM. The amount of Celite-immobilized lipase (Amano P; Amano Pharmaceutical Co. Ltd.) used was 0.1 g and the reaction time was 30 min. Other conditions are the same as described in Table 1.

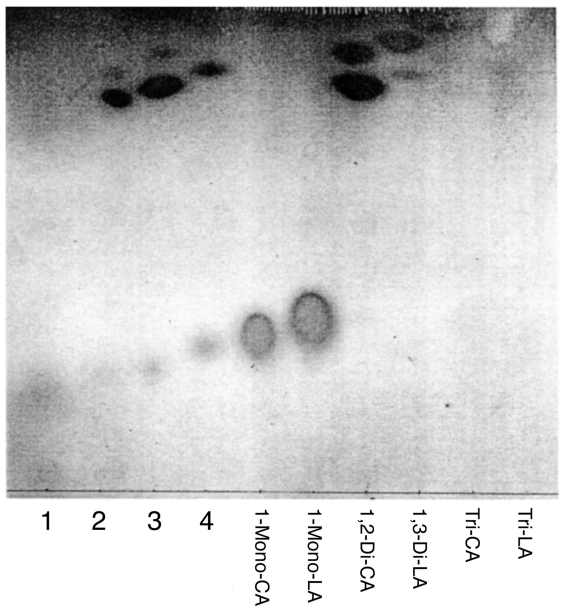

FIG. 1. TLC plate showing the products of *Pseudomonas fluorescens* lipase-catalyzed alcoholysis in ethanol. The TG substrates were tributyrin (lane 1), tricaproin (lane 2), tricaprylin (lane 3), and trilaurin (lane 4), respectively. Abbreviations: 1-Mono-CA, 1(3)-monocaprylin; 1-Mono-LA, 1(3)-monolaurin; 1,2-Di-CA, 1,2-dicaprylin; 1,3-Di-LA, 1,3-dilaurin; Tri-CA, tricaprylin; Tri-LA, trilaurin.

that 1,3-DG and 1(3)-MG migrated faster than 1,2(2,3)-DG and 2-MG, respectively. The 1,2- and 2,3-DG were not distinguishable by TLC. The MG produced by the *P. fluorescens* lipase were likely 2-MG, and the DG were probably 1,2(2,3)-DG. As shown in Table 4, the maximal yield of 2-MG depended on the TG and alcohols used in the lipase-catalyzed alcoholysis of TG. For TG with short-chain FA, more MG were accumulated. The maximal yields of 2-MG were as follows: 85% for monoacetin in *n*-butanol, 96% for monobutyrin in ethanol, 50% for monocaprylin in *n*-butanol, 48% for monolaurin in isopropanol, and 45% for monopalmitin in isopropanol.

From the product concentration vs. reaction time profiles (Figs. 2–4), it is clear that the alcoholysis reaction followed a sequential kinetic process:

$$\text{TG} \xrightarrow{k_1} \text{DG} \xrightarrow{k_2} \text{MG} \xrightarrow{k_3} \text{glycerol} \qquad [1]$$

where k_1, k_2, and k_3 represent the rate constants of the sequential alcoholysis step. The acyl group was transferred to the respective alcohols used, and led to the production of FA esters such as ethyl butyrate (Fig. 2), isopropyl laurate (Fig. 3), and isopropyl palmitate (Fig. 4). Figure 2 shows that tributyrin was completely converted into dibutyrin and monobutyrin in 2 h in the presence of 0.3 g Celite-immobilized lipase at 28°C. After 12 h almost all of the dibutyrin was converted into monobutyrin, but no significant amount of glycerol appeared. We concluded that the alcoholysis rate of monobutyrin (k_3) was very slow compared with k_1 and k_2. Therefore, a high concentration of monobutyrin can be accumulated. However, for TG with medium- and long-chain FA such as trilaurin and tripalmitin, glycerol appeared before the TG was completely alcoholyzed (Figs. 3 and 4). The alcoholysis rate of MG (k_3) appeared to increase as the chain length of FA in MG increased. Therefore, the alcoholysis of TG catalyzed by *P. fluorescens* lipase was the most suitable for the production of MG with short-chain FA such as monoacetin and monobutyrin. Since by-products such as ethyl acetate and ethyl butyrate are low-b.p. compounds, they are easily separated from the MG.

TABLE 4
Effect of Various Alcohols on the Yield of MG in the Lipase-Catalyzed Alcoholysis of Various TG[a]

	Maximal yield of 2-MG (%)[b]			
Substrates	Ethanol	Isopropanol	*n*-Butanol	Amyl alcohol
Triacetin	30 (3.3)[c]	50 (2.6)	85 (3.3)	75 (2.0)
Tributyrin	96 (8.0)	90 (4.0)	95 (8.0)	92 (4.0)
Tricaproin	23 (8.0)	48 (7.0)	65 (3.0)	10 (8.0)
Tricaprylin	10 (0.3)	45 (0.3)	50 (0.4)	42 (0.3)
Trilaurin	38 (2.0)	48 (0.3)	35 (0.3)	35 (0.3)
Tripalmitin		45 (2.0)		

[a]Celite-immobilized lipase (0.3 g Amano P; Amano Pharmaceutical Co. Ltd.) was incubated with 25 mM of various TG in various alcohols (2 mL) in stoppered glass vials and shaken on an orbital shaker at 250 rpm and 28°C for various time intervals; the product composition was then assayed by GC.
[b]The yield of MG = (MG)/[(TG) + (DG) + (MG) + (glycerol)].
[c]The numbers in parentheses denote the reaction time (hours) to reach the maximal production of MG under the specified conditions.

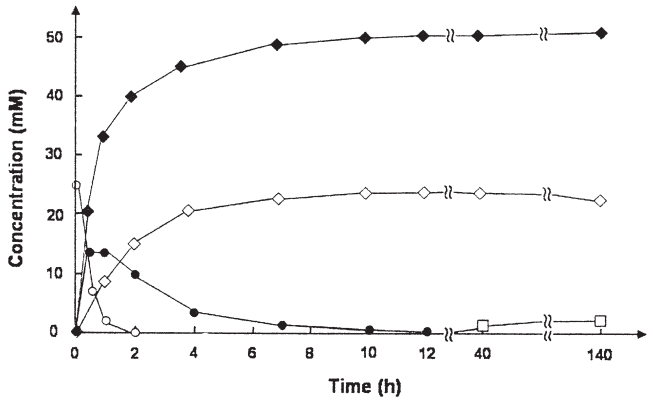

FIG. 2. The concentration vs. reaction time profile for the *P. fluorescens* lipase-catalyzed alcoholysis of tributyrin in ethanol. The reaction conditions are the same as described in Table l. (◆) Ethyl butyrate; (○) tributyrin; (●) dibutyrin; (◇) monobutyrin; (□) glycerol.

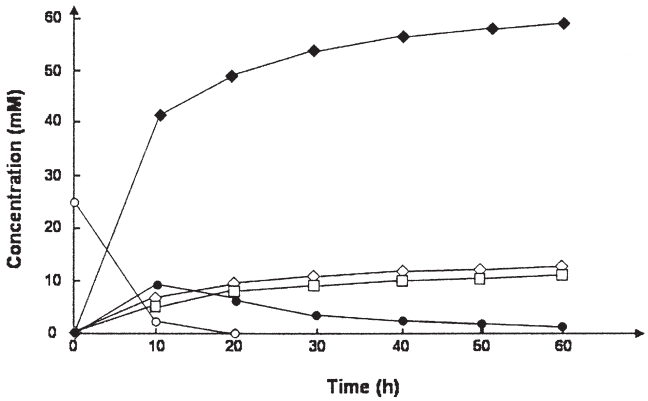

FIG. 3. The concentration vs. reaction time profile for *P. fluorescens* lipase-catalyzed alcoholysis of trilaurin in isopropanol. The reaction conditions are the same as described in Table l. (◆) Isopropyl laurate; (○) trilaurin; (●) dilaurin; (◇) monolaurin; (□) glycerol.

The catalytic efficiency and substrate specificity of reactions of this type are also presumably affected by the source of the lipase. Although lipases share a similar catalytic triad Ser-Asp/Glu-His (similar to serine proteases), their overall sequence homologies are usually quite low; therefore, their substrate binding sites are quite different. No single lipase can be the best for the synthesis of MG of all different chain lengths. For the synthesis of a particular MG, lipases from various sources should be tested to find the best one for the desired application.

In the present work, we discovered that the *P. fluorescens* lipase is excellent for the production of monoacetin and monobutyrin, with 85 and 96% yields, respectively. However, yields of other MG were not as high; therefore, other lipases should be tested for the best production yields. In a number of cases, organisms produced lipase isoforms that exhibited quite different substrate specificities (21). Crude enzyme preparations are used in most applications, and lipases obtained from different suppliers have been shown to exhibit variations in catalytic efficiency and stereospecificity (22). We have discovered multiple enzyme forms with different substrate specificities and

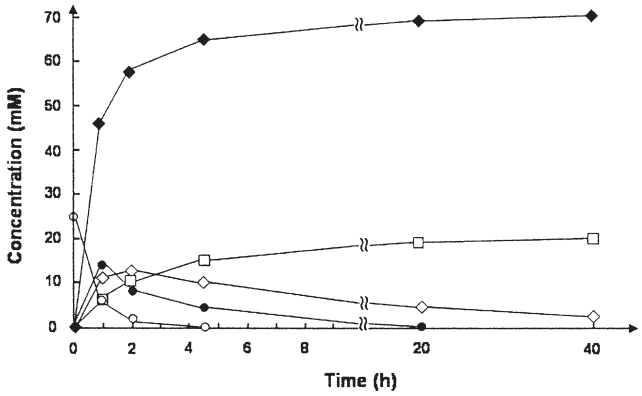

FIG. 4. The concentration vs. reaction time profile for the *P. fluorescens* lipase-catalyzed alcoholysis of tripalmitin in isopropanol. The reaction conditions are the same as described in Table l. (◆) Isopropyl palmitate; (○) tripalmitin; (●) dipalmitin; (◇) monopalmitin; (□) glycerol.

thermostabilities in a commercial *Candida rugosa* lipase preparation (23). Recently, we demonstrated that five *C. rugosa* lipase genes are differentially expressed in the presence of different inducers (24). Traditionally, the culture conditions are optimized for the maximal production of enzyme activity units. Our results indicate that different culture conditions might result in heterogeneous compositions of isozymes, which display different catalytic activities and specificities. By engineering the culture conditions, selected enzymes can be enriched for particular biotechnological applications.

Many factors affect the lipase-catalyzed synthesis of MG, including enzyme structure, immobilization methods (25), reaction media (organic solvents and supercritical fluid), temperature, pH (or pH memory in organic solvents), water activity (and log*P* in organic media), acyl donors, and receptors. To obtain the best yield of FA esters, all these factors should be optimized.

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