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Characteristics of a Monoacylglycerol Lipase Isolated from Pseudomonas sp. LP7315 -Hydrolysis and Synthesis of Monoglycerides

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A monoacylglycerol lipase (MGL) was purified from *Pseudomonas* sp. LP7315 by ammonium sulfate precipitation, anion-exchange chromatography, and preparative electrophoresis. The purified enzyme was homogeneous on an SDS-polyacrylamide gel with a molecular mass of 59 kDa. Its hydrolytic activity was confirmed to be specific for monoglycerides: the enzyme did not hydrolyze di- and triglycerides. MGL was found to be stable even after 1-h incubation at 65°C. The hydrolytic activity depended not only on temperature and pH but also on the type of monoglyceride used. MGL also catalyzed monoglyceride synthesis at 65°C in a solvent-free two-phase system, in which fatty acid droplets were dispersed in the glycerol phase with a low water content. The synthetic reaction proceeded at a constant rate for approximately 24 h and reached an equilibrium after 48 h of reaction. The initial rate of the synthetic reaction depended on several factors: the type of fatty acid used as the substrate, the amounts of fatty acid and glycerol, and the concentration of MGL in the glycerol phase. To analyze the effects of these factors, a kinetic model was developed based on the assumption that the adsorption equilibrium of MGL molecules at the interface between the two phases is the rate-determining factor for the synthetic reaction. The model was found to yield a good approximation of the initial synthetic rate under various reaction conditions. The analysis suggests that the adsorption behavior of MGL onto the interface had a large effect on the initial rate of the monoglyceride synthesis.

1. INTRODUCTION

Monoglycerides are widely used as emulsifiers in food, pharmaceutical, and cosmetic industries. They are manufactured by chemical glycerolysis of triglycerides catalyzed by inorganic catalysts. The glycerolysis reaction has a high demand for thermal energy because it is conducted at elevated temperatures (200-250°C) to overcome the low solubility of glycerol in oil phases[1]. It is often followed by molecular distillation to enhance the purity of monoglycerides because the reaction mixture usually contains considerable amounts of diglycerides.

Monoglycerides may be produced under much milder conditions by enzymatic processes. Lipases have been tested for this purpose[2]. Depending on the reaction condition, lipases can catalyze glycerolysis of glycerides and esterification of fatty

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acids with glycerol in addition to hydrolysis of glycerides. Monoglycerides can be obtained by any of the three types of lipase-catalyzed reaction. To enhance the yield and/or purity of monoglycerides, the use of a lipase with narrow substrate specificity may be efficient. For example, the use of a 1,3-specific lipase is expected to provide a high yield of monoglycerides following hydrolysis of triglycerides[3]. Mono- and diacylglycerol lipases (MDGLs), which can catalyze the hydrolysis of mono- and diglycerides but not that of triglycerides, may be efficient in reducing by-products in monoglyceride synthesis. MDGLs have been isolated from several fungi[4-6]. Yamaguchi and Mase[7] studied the glyceride synthesis catalyzed by a MDGL isolated from *Penicillium camembertii* U-150[5]. Monoacylglycerol lipases (MGLs) have a more strict substrate specificity than MDGLs. Although several MGLs were isolated from mammalian tissues[8-11] and a bacterial strain, *Bacillus* sp. H-257[12], glyceride synthesis catalyzed by MGL has not been studied to date.

Here, we report a thermostable MGL from a bacterial strain, *Pseudomonas* sp. LP7315, which we isolated by screening. We purified it and studied some characteristics of the purified enzyme. Furthermore, we studied its synthetic activity in a solvent-free two-phase reaction system to examine its applicability to monoglyceride synthesis.

2. EXPERIMENTAL PROCEDURE

2.1 Purification of MGL from *Pseudomonas* sp. LP7315

Strain LP7315 was isolated from soil obtained from Yokaichi, Shiga. The strain was selected from among many candidates isolated from soil because of its hydrolytic activity against monoglycerides. The strain is a rod-shaped bacterium with polar flagella. Moreover, the strain was found to be gram negative and strictly aerobic. Based on these characteristics, the strain was identified as a bacterium belonging to the genus *Pseudomonas*.

Pseudomonas sp. LP7315 was incubated at 30°C with reciprocal shaking for 48 h in a medium containing 1% Polypepton, 0.5% beef extract, 0.1% K_2HPO_4, and 0.02% $MgSO_4 \cdot 7H_2O$. Cells (9.1 g on the wet basis) were harvested from the culture broth (270 ml) by centrifugation and washed with 25 mM Tris-HCl buffer, pH 8.0. The cells were suspended in 270 ml of 25 mM Tris-HCl buffer, pH 8.0, and incubated with 27 mg of lysozyme and 9 mg of ribonuclease A at 38°C for 16 h with agitation. The supernatant (cell-free extract) was obtained by centrifugation at 14,650×*g* for 20 min. Into 260 ml of the cell-free extract, ammonium sulfate was added up to 45% saturation with stirring at 4°C. After allowing the mixture to stand overnight at 4°C with gentle stirring, it was centrifuged at 26,000×*g* for 15 min. The precipitate thus recovered was dissolved in 20 ml of 20 mM Tris-HCl buffer, pH 8.0, and dialyzed against the same buffer. After the addition of Triton X-100 to a final concentration of 0.1%, half of the dialyzate was loaded onto a glass column packed with DEAE-Toyopearl 650M (46 mm inner diameter and 120 mm depth), which had been equilibrated with 20 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100. The column was first washed with 20 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100, at a flow rate of 0.70 ml/min. Then, the elution was performed with a linear increase in NaCl concentration from 0 M to 1 M in 20 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100, at a flow rate of 0.70 ml/min. The fractions exhibiting lipase activity were pooled, concentrated, and dialyzed against 20 mM Tris-HCl buffer, pH 8.0. Then, the dialyzate was subjected to preparative electrophoresis in the presence of 1% sodium dodecylsulfate (SDS) using model 491 Prep Cell (Bio-Rad Laboratories, Hercules, CA, USA). In an annular-shaped space (37 mm outer diameter) of the cylindrical apparatus, a 10% polyacrylamide separation gel containing 0.1% SDS (9 mm radial thickness, 100 mm height) was prepared and a 4.5% polyacrylamide stacking gel containing 0.1% SDS (12 mm height) was overlaid on it. The system was based on the method of Laemmli[13] with a few modifications. We used 25 mM Tris/192 mM glycine buffer, pH 8.3, containing 1% SDS as the reservoir buffer. A 2.5-ml portion of the sample was mixed with 2.0 ml of 125 mM Tris-HCl buffer, pH 6.8, containing 2.25% SDS, 30% glycerol, and 0.0025% bromophenol blue as a tracking dye. The final solution (4.5 ml) thus prepared was applied onto the stacking gel without heat treatment. Electrophoresis was conducted at a constant electric current of 60 mA at 4°C. An SDS-free buffer

(25 mM Tris/192 mM glycine) flowing at 0.7 ml/min was used to transport proteins from the bottom of the separation gel to the outlet of the apparatus. Ten-minute fractions were collected at the outlet starting immediately before the tracking dye emerged (approximately 10 h after the sample loading). Fractions with high lipase activities thus obtained were pooled, concentrated, and dialyzed against 20 mM Tris-HCl buffer, pH 8.0.

The purity of MGL was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 7.5% gel according to the method of Laemmli[13]. The protein bands were stained with Coomassie brilliant blue R-250.

2.2 Hydrolytic Activity of MGL

During purification, the hydrolytic activity of MGL was monitored using *p*-nitrophenyllaurate (PNPL) as a substrate (referred to as the PNPL assay). Twenty milligrams of PNPL was dissolved in 25 ml of 50 mM acetate buffer, pH 6.0, containing 2% Triton X-100. Into 0.95-ml portion of this substrate solution, 0.05 ml of the enzyme solution was added. After 15-min incubation at 40°C, 2 ml of acetone was added to the mixture to terminate the reaction. *p*-Nitrophenol produced in the reaction mixture was quantified spectrophotometrically at 410 nm. One unit was defined as the amount of enzyme that produced 1 μmol of *p*-nitrophenol per 1 min under the conditions mentioned above. In some experiments, the incubation temperature was varied to study the effect of temperature on the hydrolytic activity.

The PNPL assay mentioned above is convenient but unsuitable for studying the effect of pH on the hydrolytic activity because the absorbance of *p*-nitrophenol considerably varies with the pH of the solution. Furthermore, the substrate specificity of MGL cannot be studied by the PNPL assay. For these reasons, another method (referred to as the MPOL assay) was employed to measure the hydrolytic activity of MGL. A mixture of 6 g of monopalmitin, 3 g of olive oil, and 60 ml of 5% Triton X-100 was heated at 55°C and homogenized (DX-4, Nihonseiki Kaisha Ltd., Tokyo) at 10,000 rpm for 3 min. Olive oil was added only to prepare and stabilize the substrate emulsion. Olive oil did not undergo hydrolysis with MGL as discussed later. After the separation of foam was attained at room temperature, 2.5 ml of the mixture thus obtained was poured into a test tube and mixed with 2 ml of 100 mM Tris-HCl buffer, pH 8.0, containing 5% Triton X-100. MGL solution (0.5 ml) was added to the test tube after appropriate dilution and the reaction mixture was incubated at 55°C for 30 min. After the reaction was terminated by adding 10 ml of acetone/ethanol (1:1), the mixture was titrated with 0.05 N NaOH in the presence of phenolphthalein to quantify palmitic acid liberated during the 30-min incubation. To study the effect of pH on the hydrolytic activity, buffers at various pHs were used instead of 100 mM Tris-HCl buffer, pH 8.0. The buffers used were 100 mM sodium acetate-HCl buffer (pH 2 to 3), 100 mM acetate buffer (pH 4 to 6), 100 mM Tris-HCl buffer (pH 7 to 9), and 100 mM glycine-NaOH buffer (pH 10 to 12). Before the measurement, MGL solution was diluted 10 times with the same buffer to avoid pH deviation in the reaction mixture. To study the substrate specificity of MGL, various mono-, di-, and triglycerides were used as substrates instead of monopalmitin at pH 8.0.

2.3 Stability of MGL

To study the thermal stability of purified MGL, the hydrolytic activity was measured by the PNPL assay at 40°C after the MGL solution was pre-incubated at a prescribed temperature for 1 h. To study the pH stability, MGL solution was diluted 10 times with buffers at various pHs. The buffers used were 100 mM sodium acetate-HCl buffer (pH 2 to 3), 100 mM acetate buffer (pH 4 to 6), 100 mM Tris-HCl buffer (pH 7 to 9), and 100 mM glycine-NaOH buffer (pH 10 to 12). After the solution was incubated at 65°C for 1 h, the remaining activity was measured by the MPOL assay at pH 8.0 and 55°C.

2.4 Monoglyceride synthetic reactions

A 10-ml screw-capped glass vial containing 1.8 g of glycerol and 0.1 g of a fatty acid was pre-incubated at 65°C for 30 min. The fatty acids tested were capric acid ($C_{10:0}$), lauric acid ($C_{12:0}$), myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), and linolenic acid ($C_{18:3}$). The reaction was initiated by adding 0.1 ml of

MGL solution to the vial. During the reaction, the mixture was maintained at 65°C and stirred constantly with a magnetic stirrer. Because of the limited solubility of fatty acids in the glycerol phase containing a small amount of water, the reaction mixture consisted of two phases. After a prescribed time of reaction, the glycerides produced and the remaining fatty acids were extracted from the reaction mixture with chloroform. After chloroform was evaporated under a reduced pressure, the residue was subjected to high-performance liquid chromatography (HPLC) similar to the method described by Chi *et al.*[14]. The HPLC system was composed of a PU-980 pump (JASCO Corp., Tokyo), a Shodex RI-71 refractive index detector (Showa Denko Co. Ltd., Tokyo), a D-2500 integrator (Hitachi Ltd., Tokyo) and an ODS column (YMC-Pack ODS A-312, 4.6 mm ID × 250 mm, YMC, Kyoto). The mobile phase used was a mixture of acetonitrile, tetrahydrofuran, dichloromethane, and acetic acid (70:20:20:0.8 [v/v]). The residues obtained after evaporation of chloroform were dissolved in the same solvent mixture. Aliquots (20 μl) of these solutions were injected and eluted isocratically using the solvent mixture at a flow rate of 0.9 ml/min.

In some experiments, the amounts of glycerol and fatty acid initially supplied were varied. Even in these cases, the volume of MGL solution added was set to be 0.1 ml per 1.8 g of glycerol to maintain the initial water content of the glycerol phase constant.

3. RESULTS AND DISCUSSION

3.1 Purification of MGL

Table 1 summarizes the purification steps starting from 260 ml of the cell-free extract. As the first step of purification, the active fraction was recovered from the precipitate obtained at 45% saturation of ammonium sulfate. Through this step, the specific activity became 2.5-fold. As the second step, anion-exchange chromatography using the DEAE-Toyopearl 650M column was performed in the presence of 0.1% Triton X-100. The use of a surfactant was essential in this step to avoid the aggregation of MGL molecules. It was preliminarily confirmed that the presence of 0.1% Triton X-100 did not affect the activity and stability of MGL. Proteins were adsorbed on the column in the absence of NaCl, and eluted as NaCl concentration increased linearly. The active fractions were eluted in a range of NaCl concentration from 100 mM to 200 mM. After this step, the specific activity approximately doubled. Although several types of chromatography were tried, they were not effective for use in further purification. Thus, preparative electrophoresis in the presence of 1% SDS was attempted. It was preliminarily confirmed that MGL was stable in the presence of 1% SDS. Under the conditions employed in this study, active fractions were obtained approximately 6 h after the tracking dye emerged at the outlet of the apparatus. As shown in Fig. 1, the furified MGL was homogeneous on the SDS-PAGE gel and its molecular mass was estimated to be 59 kDa based on the position of the marker proteins. The specific activity of the purified MGL was 109 U/mg, the purification being 63-fold.

Table 1. Summary of MGL purification

Purification step	Activity (U)	Protein† (mg)	Specific activity (U/mg)	Yield (%)
Cell-free extract	1922	1101	1.7	100
$(NH_4)_2SO_4$ precipitation	1146	247	4.6	60
DEAE-Toyopearl chromatography	873	85.7	10.2	45
Preparative electrophoresis	239	2.2	109	12

†The amount of protein was measured using a BCA assay kit (Pierce, Rockford, IL, USA) with the bovine serum albumin as a standard.

3.2 Hydrolytic activity of MGL

Figure 2 shows comparison of hydrolytic activities of MGL against different glycerides measured by the MPOL assay. When di- and trigricerides were used as the substrates, fatty acids were not detected by titration. This indicated that MGL was not able to hydrolyze di- and trigricerides including olive oil contained in

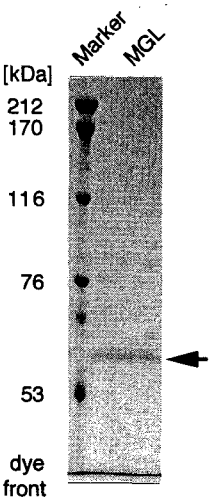

Figure 1. SDS-PAGE of the purified MGL. Marker proteins used were myosin (212 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), and glutamic dehydrogenase (53 kDa).

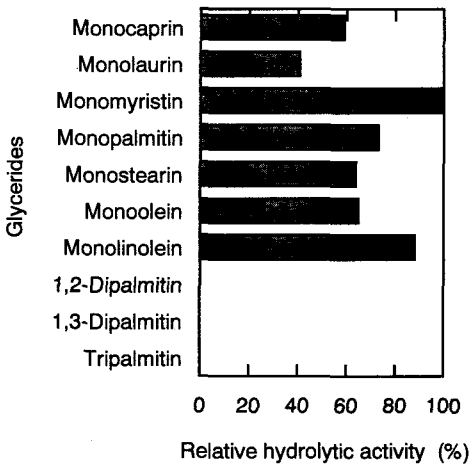

Figure 2. Relative activities of the purified MGL for the hydrolysis of various glycerides. The activity was measured by the MPOL assay at 55°C. The activity for monomyristin was taken to be 100% to express relative activity values.

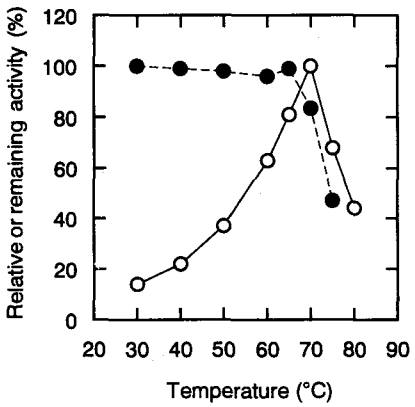

Figure 3. Effects of temperature on the activity (○) and stability (●) of the purified MGL. The activity was measured by the PNPL assay at various temperatures. The activity measured at 70°C was taken to be 100% to express relative activity values. For stability comparison, the remaining activity after 1-h incubation at 30°C was taken to be 100%. The remaining activity was measured by the PNPL assay at 40°C.

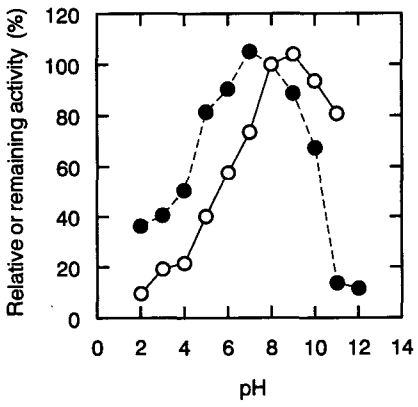

Figure 4. Effects of pH on the activity (○) and stability (●) of the purified MGL. The activity was measured by the MPOL assay at 55°C. The activity at pH 8 was taken to be 100% to express relative activity values. For stability comparison, the remaining activity measured by the MPOL assay at 55°C after 1-h incubation at pH 8 was taken to be 100%.

the substrate mixture. On the other hand, MGL showed hydrolytic activity against all monoglycerides tested here. Thus, MGL purified from *Pseudomonas* sp. LP7315 had a strict specificity for monoglycerides. The hydrolytic activity depended on the type of monoglyceride. The highest hydrolytic activity was observed against monomyristin. Compared to other MGLs, the substrate specificity seemed similar to that of rat liver microsomal MGL[9] and slightly different from that of MGL from

Bacillus sp. H-257[12], which had the highest hydrolytic activity against monolaurin.

Figure 3 shows temperature dependence of the hydrolytic activity of the purified MGL together with its thermal stability. Both were evaluated by the PNPL assay at pH 8. The optimum temperature for the hydrolytic activity was found to be 70°C. The purified MGL was stable during 1-h incubation at temperatures up to 65°C. Even after the incubation at 70°C for 1 h, it exhibited no less than 80% of its original activity. None of MGLs or MDGLs isolated thus far was reported to have such a high thermal stability. Most of MGLs[8, 10, 11] and MDGLs[4, 6] were reported to lose their activity rapidly at temperatures above 45°C. Even for MGL from *Bacillus* sp. H-257 having a relatively high thermal stability, loss of the activity at temperatures above 60°C was reported[12]. Figure 4 shows pH dependence of MGL activity measured by the MPOL assay at 55°C using monopalmitin as the substrate. The optimum pH for hydrolytic activity was in between 8 and 9. In Fig. 4, the effect of pH on the stability of MGL at 65°C was also shown. Its stability was highest in the range of pH from 7 to 8. It was reported that the optimum pH for MGL from rat adipocytes was in the range of pH from 7 to 9[10]. MGL from *Bacillus* sp. H-257 was also reported to show high activity and stability in the same range of pH[12].

The effect of some reagents on the hydrolytic activity of MGL was also studied using the PNPL assay. In general, the hydrolytic activity was scarcely affected by the presence of metal ions, EDTA, and sulfhydryl reagents (data not shown). Thus, it was suggested that metal ions and sulfhydryl groups were not essential for the activity. Several metal ions (Mg^{2+}, Ca^{2+}, and Mn^{2+}) were also reported to have no significant effect on the activity of MGL from *Bacillus* sp. H-257[12]. In contrast, MGL from rat adipocytes was reported to be sensitive to inhibition by *p*-chloromercuribenzoic acid and to activation by dithiothreitol[8, 10]. Thus, MGLs may have different modes of catalytic action.

3.3 Synthetic activity of MGL

Using the purified enzyme as a catalyst, synthesis of monoglycerides from glycerol and various fatty acids was attempted at 65°C. The reaction temperature exceeded melting points of all fatty acids tested here, including palmitic acid (mp. 63.1°C). Thus, the reaction system was composed of liquid droplets of a fatty acid dispersed in the glycerol phase containing small amounts of water and MGL. Figure 5 shows the time courses of monoglyceride synthesis from palmitic acid and palmitoleic acid, as examples. The ordinate shows relative molar amounts of monoglyceride produced and residual fatty acid based on the amount of fatty acid initially added. It was clear that MGL catalyzed the synthetic reactions in the two-phase system. In each case, the synthetic reaction proceeded at a constant rate for approximately 24 h, and then the reaction rate gradually decreased. The reaction mixture approximately reached an equilibrium after 48 h of reaction. The existence of equilibrium is probably ascribed to the promotion of reverse reaction, that is hydrolysis of the monoglyceride, because the water content increases as the synthetic reaction proceeds. The sum of mole fractions of monoglyceride and fatty acid was approximately 100% at any time of the reaction. During each course of the reaction, di- and triglycerides were not detected. This is a noteworthy feature of monoglyceride synthesis catalyzed by MGL. In general, when a lipase with broader substrate specificity is used, considerable amounts of di- and triglycerides are produced in addition to monoglyceride. For example, diolein amounted to more than 10% of total glycerides when MDGL from *Penicillium camembertii* U-150 was used for monoolein synthesis under conditions similar to this study[7]. When lipases (from *Geotricum candidum* and *Penicillium cyclopium*) without positional specificity were used as catalysts, the combined amount of di- and triolein was shown to be comparable to the amount of monoolein after nearly 50 h of synthetic reaction[15]. It was also reported that the amount of monoolein produced was much less than the combined amount of di- and triolein when an immobilized *Mucor miehei* lipase was used for synthetic reaction in a solvent-free system[16].

Figure 6 shows the initial rates of monoglyceride synthesis from various fatty acids. Among the saturated fatty acids tested, myristic acid ($C_{14:0}$) was found to be the most efficient acyl donor. Comparable initial rates were observed when palmitoleic acid ($C_{16:1}$) and oleic acid ($C_{18:1}$) were used as substrates. In glyceride synthesis catalyzed by other lipases at 30 or 40°C, saturated fatty acids with shorter chains ($C_{10:0}$ or $C_{12:0}$) were reported to be better acyl donors than $C_{14:0}$[7, 15]. The

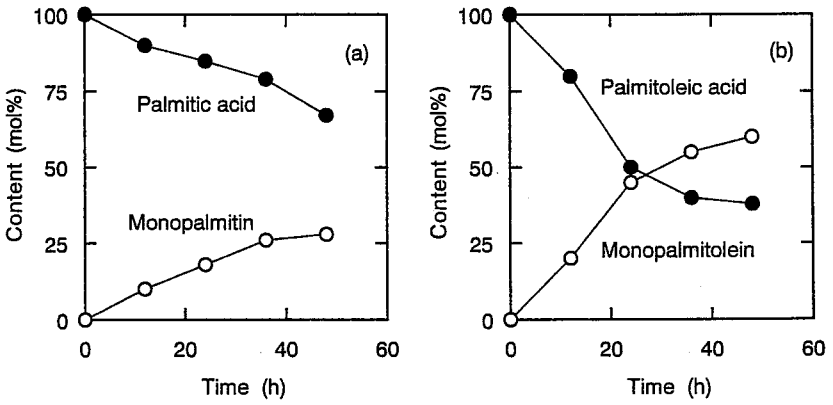

Figure 5. Time courses of (a) monopalmitin and (b) monopalmitolein syntheses catalyzed by MGL at 65°C. The initial amounts of glycerol and fatty acid were 1.8 g and 0.1 g, respectively. The initial concentration of MGL in the glycerol phase was 0.392 U/ml.

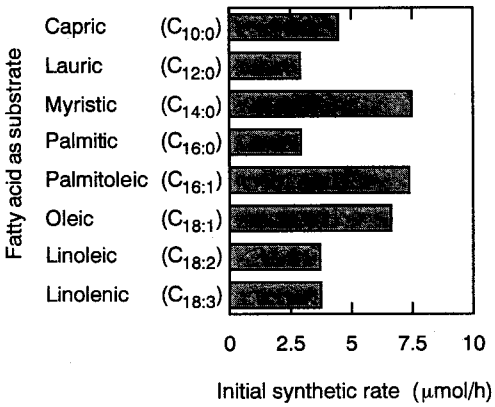

Figure 6. Initial rates of monoglyceride synthesis with various fatty acids as substrates. Reaction conditions were the same as those in Fig. 5.

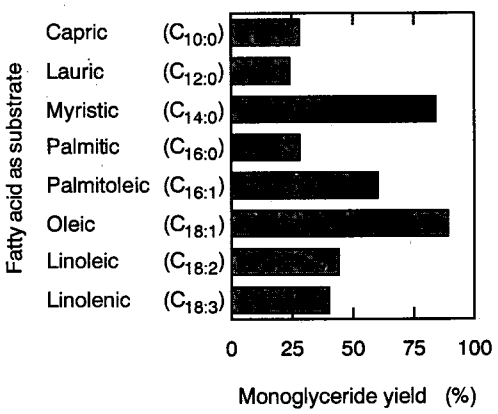

Figure 7. Final yields of monoglycerides produced from various fatty acids. Reaction conditions were the same as those in Fig. 5.

reaction temperature may be the factor responsible for the difference, because the physical state of a fatty acid varies with the temperature particularly within the range of its melting point. Figure 7 shows equilibrium yields of monoglyceride synthesis from various fatty acids. The equilibrium yield also depended on the type of fatty acid used as the substrate. It is noteworthy that the yields exceeded 80% for monomyristin and monoolein syntheses.

The initial rate of the synthetic reaction depended also on the amounts of substrates and on the concentration of MGL in the glycerol phase. To analyze the effects of these factors on the rate of monoglyceride synthesis, we developed a kinetic model. The reaction system was composed of two phases: the fatty acid phase and the glycerol phase. The glycerol phase contained MGL and water. We assume that the adsorption behavior of MGL molecules from the glycerol phase onto the interface between the two phases is the rate-determining factor for the synthetic reaction. If the Langmuir-type adsorption equilibrium is assumed, the following equation holds.

$$K_E = \theta_E / \{C_E (1 - \theta_E)\} \quad (1)$$

where K_E is the equilibrium constant, C_E the equilibrium concentration of MGL in the glycerol phase, and θ_E the proportion of

the interface area covered by MGL at equilibrium. The mass balance for MGL gives the following equation.

$$V_G C_{E0} = V_G C_E + \theta_E S / S_E \tag{2}$$

where C_{E0} is the initial concentration of MGL in the glycerol phase, the volume of which is V_G, S the total area of the interface, and S_E the area that a unit amount of MGL covers. The fatty acid phase was composed of a substrate itself. The initial concentration of glycerol, the other substrate, in the glycerol phase was constant in all experiments. Considering these conditions, the synthetic reaction rate catalyzed by a unit amount of MGL molecules adsorbed onto the interface may be assumed to be constant (k_P). Thus, the overall reaction rate, r_P, is given as follows:

$$r_P = k_P \theta_E S / S_E \tag{3}$$

Assuming $K_E C_E << 1$, the following equation is obtained from Eqs. 1, 2, and 3.

$$\frac{1}{r_P} = \frac{1}{k_P C_{E0} K_E S / S_E} + \frac{1}{k_P C_{E0}} \frac{1}{V_G} \tag{4}$$

Equation 4 predicts a linear relationship between $1/r_P$ and $1/V_G$ for each set of C_{E0} and S.

First, we determined the applicability of Eq. 4 to estimate the initial rate of monopalmitin synthesis. The initial rate was measured within 24 h of the reaction. Figure 8(a) shows the relationship between $1/r_P$ and $1/V_G$ when C_{E0}=0.392 U/ml and the initial amount of palmitic acid was 0.1 g. A constant amount of palmitic acid is a necessary condition to have a constant value of S. The initial reaction rate increased with the increase in the volume of the glycerol phase. Similar effects of the glycerol content on monoolein synthesis catalyzed by a MDGL[7] and by triacylglycerol lipases[15] were reported, although the water content in the glycerol phase seemed to vary depending on the glycerol content in those cases. As shown in Fig. 8(a), a linear relationship was found between $1/r_P$ and $1/V_G$, indicating that the model developed here can theoretically explain the effect of glycerol amount on the initial rate of monoglyceride synthesis. From the straight line shown in Fig. 8(a), we obtained the values for the following parameters: $k_P C_{E0}$=3.58 mmol/(ml·h) and $K_E S/S_E$=3.03 ml.

Based on these values, we can estimate the parameters for various C_{E0} values. The value of $k_P C_{E0}$ is directly proportional to C_{E0}, whereas $K_E S/S_E$ is constant for various C_{E0} values. Figure 8(b) shows the comparison between the experimental and estimated values for three different values of C_{E0}. Equation 4 gave a fairly good approximation for each value of C_{E0}. Thus, Eq. 4 is applicable to estimate the initial reaction rate of monopalmitin synthesis, at least for a constant amount of palmitic acid. Figure 8(c) shows the results obtained for a constant value of C_{E0} with different amounts of palmitic acid initially supplied. Assuming that the total interface area, S, is proportional to the amount of palmitic acid, the correlation between $1/r_P$ and $1/V_G$ was estimated using Eq. 4 and illustrated as a line for each case. When 0.2 g of palmitic acid was initially supplied, the experimental results were well approximated by Eq. 4. When the initial amount of palmitic acid was decreased to 0.05 g, however, the experimental values of $1/r_P$ tended to be larger than the estimated values. The reason for this deviation is not clear. It might be ascribed to the assumption $K_E C_E << 1$, under which Eq. 4 was derived. If the assumption does not hold, Eq. 4 will overestimate the amount of MGL on the interface, leading to underestimation of the value of $1/r_P$. Moreover, the diameter distribution of palmitic acid droplets might change and affect the reaction rate. To be able to discuss in further detail about the reason for the deviation between the experimental and estimated values and to develop a more reliable model, information about the total interface area is required.

The initial reaction rate of monomyristin synthesis was analyzed based on Eq. 4. Figure 9 shows the results. In all of the experiments, the amount of myristic acid initially supplied was 0.1 g and C_{E0}=0.392 U/ml. Also in this case, a linear relationship was found between $1/r_P$ and $1/V_G$, indicating that Eq. 4 was applicable. The parameter values were estimated from the straight line shown in Fig. 9: $k_P C_{E0}$=2.81 mmol/(ml·h) and $K_E S/S_E$=17.2 ml. Compared to the values obtained from Fig. 8(a), the $K_E S/S_E$ value was considerably larger (5.7 fold) in monomyristin synthesis than in monopalmitin synthesis, while

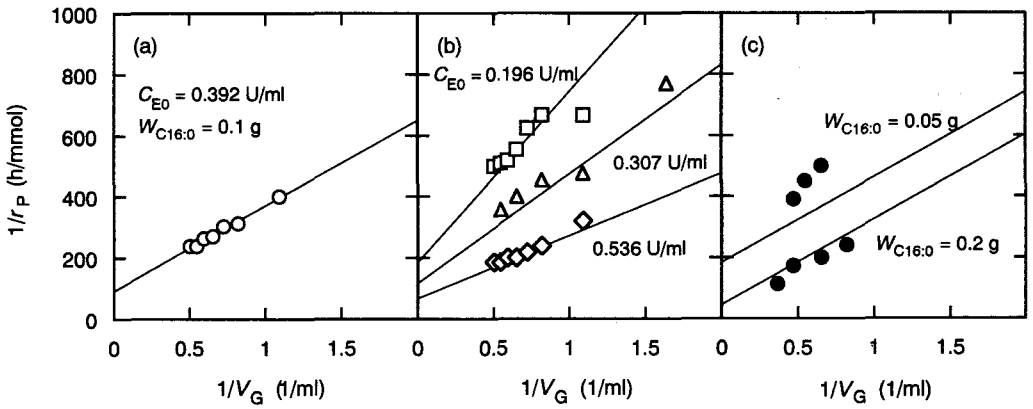

Figure 8. Effect of the glycerol phase volume (V_G) on the initial rate of monopalmitin synthesis (r_P) catalyzed by MGL at 65°C. Reaction conditions: (a) the initial concentration of MGL in the glycerol phase (C_{E0}) was 0.392 U/ml and the initial amount of palmitic acid ($W_{C16:0}$) was 0.1 g; (b) $W_{C16:0}$ =0.1 g and C_{E0} was varied as indicated; (c) C_{E0}=0.392 U/ml and $W_{C16:0}$ was varied as indicated. In (b) and (c), Eq. 4 was evaluated with the parameters obtained from the data shown in (a) and illustrated as a line for each experimental condition. Symbols represent the experimental data.

the values of $k_P C_{E0}$ in the two cases differed only slightly. This indicated that the rate of monomyristin synthesis increased due to the increase in the fraction of MGL adsorbed onto the interface where the reaction occurred. Considering the similar densities of the two melted fatty acids (d_4^{70}=0.8528 for myristic acid and d_4^{80}=0.8414 for stearic acid), the volumes of the two fatty acid phases were also similar. This suggests that the values of the total interface area (S) in the two cases did not significantly differ. Thus, the affinity of MGL to the interface of myristic acid (K_E) is probably responsible for the increase in the initial reaction rate.

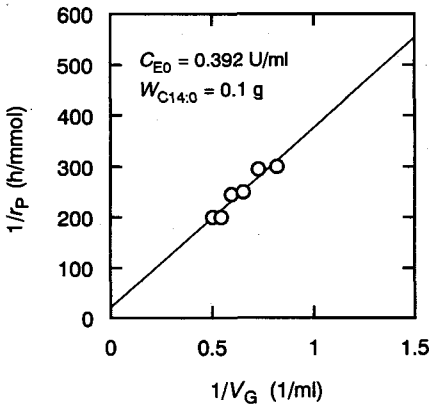

Figure 9. Effect of the glycerol phase volume (V_G) on the initial rate of monomyristin synthesis (r_P) catalyzed by MGL at 65°C. Reaction conditions: C_{E0}=0.392 U/ml and the initial amount of myristic acid ($W_{C14:0}$) was 0.1 g.

4. CONCLUSIONS

In this study, we isolated MGL from *Pseudomonas* sp. LP7315. It was confirmed that the purified MGL had a strict substrate specificity for monoglyceride and a high thermal stability. We also showed that the purified MGL catalyzed monoglyceride synthesis in a system composed of droplets of a fatty acid dispersed in a glycerol phase with a low water content. Due to its unique features mentioned above, MGL reported here is expected to be useful in monoglyceride synthesis. In particular, its high thermal stability allows synthetic reactions at temperatures higher than the melting points of many fatty acids. To analyze the effects of various factors on the initial rate of the synthetic reaction, a kinetic model was developed assuming that the adsorption of MGL molecules onto the interface between the two phases is an essential step for catalysis. The model was shown to theoretically explain the effects of several factors on the initial rate of monoglyceride synthesis. Although the effects of the reaction conditions on the total interface area remain to be studied, the model developed here may provide a basis for the analysis of many other reactions conducted in two-phase systems.

5. ACKNOWLEDGEMENT

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