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Purification and Characterization of a Monoacylglycerol Lipase from the Moderately Thermophilic *Bacillus* sp. H-257

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A thermostable monoacylglycerol lipase [MGLP, EC 3.1.1.23] was purified for the first time from a cell-free extract of the moderately thermophilic *Bacillus* sp. H-257. The enzyme was purified 3,028-fold to homogeneity by chromatography using Octyl-Sepharose CL-4B, Q-Sepharose FF, and Superose 12 columns. The molecular mass of the MGLP was estimated to be 25 kDa by gel filtration and 24 kDa by SDS-PAGE, suggesting a monomeric protein. The isoelectric point was determined to be 4.66 by isoelectric focusing. The MGLP retained its full activity upon incubation at 60°C for 10 min (pH 7.3), and was stable at pH 7–10. The optimal temperature for activity at pH 7.5 was 75°C, and the maximum activity was observed from pH 6–8. This enzyme hydrolyzes monoacylglycerols, with the highest activity occurring with 1-monolauroylglycerol. Di- and triacylglycerols, on the other hand, are essentially inert as substrates for the enzyme. The K_m values for the hydrolysis of 1-monolauroylglycerol, 1-monooleoylglycerol, and 2-monooleoylglycerol were determined to be 140, 83 and 59 μM, respectively. The enzyme was not inhibited by cholate, but was slightly inhibited by Triton X-100 and deoxycholate. The amino acid sequence of the N-terminal region of the enzyme (16 residues) was also determined.

Key words: moderately thermophilic bacteria, monoacylglycerol lipase, N-terminal amino acid sequence, purification, thermostable enzyme.

Lipase [triacylglycerol lipase, EC 3.1.1.3] hydrolyzes triacylglycerol to diacylglycerol, monoacylglycerol, fatty acid, and glycerol. This enzyme, which is widely distributed in animals, plants, and microorganisms, is important and useful in various industrial fields as a biocatalyst. A lipase that hydrolyzes partial acylglycerol, mono-, and diacylglycerols has been purified and characterized (*1–10*). Yamaguchi *et al.* (*11*) reported an enzyme from *Penicillium camenbertii* that hydrolyzes mono- and diacylglycerols but not triacylglycerols. Ikeda *et al.* (*12*) reported a rat liver microsomal lipase that maximally hydrolyzes long chain monoacylglycerols, and 1(3)- and 2-monoacylglycerols, but shows a little hydrolytic activity for short chain triacylglycerols, and no hydrolytic activity for long chain triacylglycerols. Until now only limited information on a specific monoacylglycerol-hydrolyzing enzyme, monoacylglycerol lipase [MGLP, EC 3.1.1.23], has been available. MGLPs from mouse (*13*) and rat (*14*) adipocytes, and from rabbit aorta (*15*) and human erythrocytes (*16*) have been reported. However, no MGLP from bacteria has yet been described.

In recent years, there has been a great demand for thermostable enzymes in industrial fields. Thus, thermostable lipases from various sources have been purified and characterized (*17–19*). Also, proteins from thermophilic organisms have proved to be more useful for biotechnological applications than similar proteins from methophiles due to their stability.

We tried to screen a thermostable MGLP from bacterial strains, and found it in the cell extract of the moderately thermophilic *Bacillus* sp. H-257 isolated from soil. In this study, we report the purification and enzymatic properties of this MGLP.

MATERIALS AND METHODS

Materials—Pharmamedia (defatted cotton seed meal) was a product of Traders Oil Mill (Fort Worth, TX, USA). Peptone was from Difco Laboratories (Detroit, MI, USA). Silicone KM-72 (anti-foaming agent) was a product of Shin-Etsu Chemical Industry (Tokyo). Adekatol SO-120 was a product of Asahi-denka Kogyo (Tokyo). 1-Monoacetylglycerol and 1-monobutyrylglycerol were products of Kanto Chemical (Tokyo). 1-Monocaprylglycerol and 1-monostearoylglycerol were products of Larodan Fine Chemicals AB (Malmo, Sweden). 1-Monolauroylglycerol, 1-monomirystoylglycerol, 1-palmitoylglycerol, 1-monolinoleoylglycerol, and 2-monooleoylglycerol were from Serdary Research Laboratories (Toronto, Ontario, Canada). 1-Monooleylglycerol, 1-monolinolenoylglycerol, and 1,2-dilinoleoylglycerol were prducts of Nu-Chek-Prep (Elysian, MN, USA). *Para*-nitro-

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Abbreviations: *B.* sp., *Bacillus* sp.; GK, glycerol kinase; GPO, glycerol-3-phosphate oxidase; MGLP, monoacylglycerol lipase; TOOS, *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine.

phenyl acetate, *p*-nitrophenyl butylate, *p*-nitrophenyl caplylate, *p*-nitrophenyl laurate, and *p*-nitrophenyl palmitate were products of Sigma Chemical (St. Louis, MO, USA). Glycerol kinase (GK) and glycerol-3-phosphate oxidase (GPO) were products of Asahi Chemical Industry (Tokyo). Peroxidase was from Sigma Chemical (St. Louis, MO, USA). TOOS [*N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine] was obtained from Dojindo Laboratories (Kumamoto). Octyl-Sepharose CL-4B, Q-Sepharose FF, Superose 12 columns, and Ampholine were from Pharmacia (Uppsala, Sweden). The TSK gel G3000SWXL column was from Tohso (Tokyo). Molecular weight marker proteins for gel filtration and yeast extract were from Oriental Yeast (Tokyo). All other chemicals were of analytical grade and commercially available.

Microorganism and Growth Condition—B. sp. H-257 originally isolated from soil was used as a source of MGLP. A medium of the following composition (pH 7.0) was used for the growth of the cells: glucose; 0.2% (w/v); Pharmamedia; 0.5%; peptone, 1.0%; yeast extract, 0.5%; NaCl, 0.3%; KH_2PO_4, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.05%; and Silicone KM-72, 0.05%. The bacterium was cultivated at 50°C for 12 h in a 30-liter jar fermentor under aeration (20 liters per min).

Enzyme Assay and Protein Determination—Lipase activity was determined spectrophotometrically as follows. The reaction mixture, containing 100 μl of 0.2 M PIPES-NaOH (pH 7.3), 50 μl of 0.3% 4-aminoantipyrine, 50 μl of 0.2% TOOS, 50 μl of 45 U/ml peroxidase, 25 μl of 20 mM $MgCl_2$, 25 μl of 20 mM ATP, 10 μl of 25 U/ml GK, 15 μl of 1,000 U/ml GPO and 75 μl of H_2O, and 50 μl of substrate solution (10 mM monolauroylglycerol containing 0.5% Triton X-100), was kept at 37°C, and the reaction was started by adding enzyme solution (50 μl). After incubation at 37°C for 10 min, the reaction was stopped by adding 2.5 ml of 0.5% SDS solution. The glycerol generated was measured by the absorbance at 550 nm. One unit (U) of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of glycerol per min at 37°C under the conditions specified above. Specific activity was expressed as U/mg protein. Protein concentration was determined with a Bio-Rad protein assay kit (Richmond, CA, USA) using BSA as a standard protein.

Purification of MGLP—All operations were performed at room temperature and potassium phosphate buffer was used as a standard buffer throughout the purification.

Step 1. Preparation of crude extract: B. sp. H-257 cells obtained from a 20-liter medium culture were suspended in 2 liters of 10 mM potassium phosphate buffer (pH 7.0) containing 0.2% (w/v) lysozyme and 0.2% Triton X-100, then incubated at 37°C for 2 h. After centrifugation (15,000 ×*g* for 20 min), the supernatant (1,960 ml) was concentrated by ultrafiltration to 550 ml. This solution was used as a crude extract for the further purification procedures.

Step 2. Acetone fractionation and precipitation: Cold acetone (275 ml) was added to the crude extract, and the precipitate formed was removed by centrifugation. Then cold acetone (671 ml) was added again to the supernatant solution (746 ml), and the precipitate formed was collected by centrifugation (15,000 ×*g* for 15 min). The precipitate was dissolved in 250 ml of 10 mM potassium phosphate buffer (pH 7.0). The supernatant (236 ml) was obtained by centrifugation (15,000 ×*g* for 10 min), and cold acetone (472 ml) was added. The precipitate formed was collected by centrifugation (15,000 ×*g* for 10 min) and dissolved in 130 ml of 10 mM potassium phosphate buffer (pH 7.0) containing 10% ammonium sulfate. The undissolved matter was removed by centrifugation (15,000 ×*g* for 10 min), and the supernatant obtained was used for the next purification procedure.

Step 3. Octyl-Sepharose CL-4B column chromatography: The enzyme solution (125 ml) was applied to an Octyl-Sepharose CL-4B column (2.5 cm × 21 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 10% ammonium sulfate. Washing of the column was carried out stepwise as follows: 100 ml of 10 mM potassium phosphate buffer (pH 7.0) containing 10% ammonium sulfate, 100 ml of the same buffer containing 5% ammonium sulfate, 100 ml of the same buffer containing 2.5% ammonium sulfate, 100 ml of the same buffer containing 1.0% ammonium sulfate, 200 ml of 10 mM potassium phosphate buffer (pH 7.0). The enzyme was eluted with 10 mM potassium phosphate buffer (pH 7.0) containing 0.5% Adekatol SO-120 at a flow rate of 100 ml per h. The active fractions were collected.

Step 4. Q-Sepharose FF column chromatography: The active fractions (160 ml) were applied to a Q-Sepharose FF column (2.5 cm × 10 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.0). After the column was washed with the same buffer (200 ml), the enzyme was eluted with a linear gradient of KCl from 0 to 0.3 M in a total volume of 600 ml. Fractions of 20 ml were collected at a flow rate of 100 ml per h.

Step 5. Superose 12 column chromatography: After dialysis of the enzyme solution against 20 volumes of buffer, the enzyme solution (1 ml) concentrated by Centricon (Amicon) was applied to a Superose 12 column (2.0 cm × 52 cm), equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 0.5 M KCl at a flow rate of 1 ml per h. Fractions of 1 ml were collected and the active fractions were dialyzed.

Determination of Molecular Mass—The molecular mass of the native enzyme was estimated by gel filtration on a TSK gel G3000SWXL column (30 cm) using 50 mM sodium phosphate buffer (pH 7.5) containing 0.2 M Na_2SO_4. The following standard proteins were used to create a calibration curve: glutamate dehydrogenase (M_r 290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), adenylate kinase (32 kDa), and cytochrome *c* (12.4 kDa). Molecular mass of the subunit was determined by SDS-PAGE using a 12.5% polyacrylamide gel. The marker proteins used were as follows: bovine serum albumin (67 kDa), ovalbumin (45 kDa), bovine pancreas α cymotrypsinogen A (25.7 kDa), cytochrome *c* (12.4 kDa). Proteins were visualized by Coomassie Brilliant Blue staining.

Substrate Specificity—Substrate specificity toward different partial acylglycerols (C_2–C_{18} acyl groups) at 1 mM was determined under the standard assay conditions described above. Substrate specificity toward different *p*-nitrophenyl esters (C_2–C_{16} acyl groups) was determined as follows. The reaction mixture, containing 100 μl of 0.2 M PIPES-NaOH (pH 7.3), 50 μl of substrate solution (1 mM *p*-nitrophenyl ester containing 1% Triton X-100), and 350 μl of H_2O, was kept at 37°C, and the reaction was started by adding enzyme solution (25 μl). After incubation at 37°C for 10 min, the reaction was stopped by adding 1 ml of 2% SDS solution. The absorbance at 420 nm was determined. The activity of MGLP toward different triacylglycerols was deter-

mined by alkaline titration according to a method described previously (*20*).

Measurement of Isoelectric Point (pI)—Isoelectric focusing was carried out at 25°C for 40 h with Ampholine carrier ampholytes giving a pH gradient of 3.5 to 10 in a 110-ml electrofocusing column.

Effects of pH and Temperature on Enzyme Activity—The pH effect on activity was identified by measuring the enzyme activity at various pH in the range of 4–9.5, using 1-monolauroylglycerol (1 mM) as a substrate. The temperature effect on activity was identified by measuring the relative activity at specified temperatures in the range of 30°C –85°C.

Effects of pH and Temperature on Enzyme Stability—The pH effect on stability was evaluated by measuring residual activity after incubation of the enzyme at 60°C for 10 min at various pH, using the following buffers: DMG-NaOH (pH 4.5–7), PIPES-NaOH (pH 6.5–7), Tris-HCl (pH 7–9), Glycine-NaOH (pH 9–10). For the determination of the effect of temperature on stability, residual activities were measured after incubation of the enzyme at various temperatures for 10 min.

Analysis of N-Terminal Amino Acid Sequence—The amino acid sequence of the N-terminal region of the purified MGLP was identified by automated Edman degradation in a gas-phase protein sequencer (Applied Biosystems, USA).

RESULTS AND DISCUSSION

We isolated several MGLP-producing bacterial strains from soil samples in Kagoshima prefecture. Among them, strain H-257 was selected for its MGLP activity, which hydrolyzes monoacylglycerols but not di- and triacylglycerols. On the basis of strain characteristics, strain H-257 was identified as a moderately thermophilic bacterium of the genus *Bacillus*.

Culture Conditions for the High Production of MGLP—*B.* sp. H-257 was cultured in medium containing different carbon and nitrogen sources to find the conditions leading to the highest production of the enzyme. The highest production was observed in the cell extract when the strain was cultured for 12 h under the conditions and in the medium described in "MATERIALS AND METHODS".

Most microbial lipases are extracellular, and secreted through the external membrane into the culture medium (*21*). Lipases produced by bacteria of the genus *Bacillus* (*22–24*), *Pseudomonas* (*25*), and *Candida* (*26*) are well

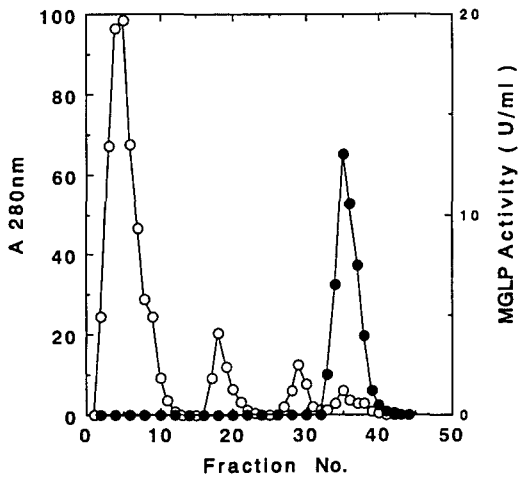

Fig. 1. **Column chromatography on Octyl-Sepharose CL-4B.** The enzyme solution (125 ml) after acetone fractionation (33.3–77.7%) was applied to an Octyl-Sepharose CL-4B column (2.5 cm × 21 cm) previously equilibrated with 10 mM phosphate buffer (pH 7.0) containing 10% ammonium sulfate. The column was washed with 100 ml of the same buffer, 100 ml of 5% ammonium sulfate, 100 ml of 2.5% ammonium sulfate, 1% ammonium sulfate, and 200 ml of 10 mM phosphate buffer (pH 7.0) and then eluted with 10 mM phosphate buffer containing 0.5% Adekatol SO-120 at a flow rate of about 100 ml per h. Fractions of 20 ml were collected. All procedures were carried out at 25°C. Other experimental conditions are described in the text. ○, absorbance at 280 nm; ●, MGLP activity.

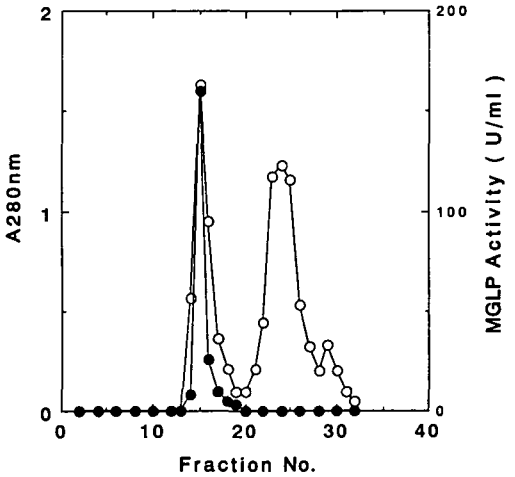

Fig. 2. **Column chromatography on Q-Sepharose FF.** The enzyme solution (160 ml) obtained from the Octyl-Sepharose CL-4B column was applied to a Q-Sepharose FF column (2.5 cm × 10 cm) previously equilibrated with 10 mM phosphate buffer (pH 7.0). The column was washed with 200 ml of the same buffer and eluted with 600 ml of a linear gradient of 0 to 0.3 M KCl containing 10 mM phosphate buffer (pH 7.0) at a flow rate of 100 ml per h, and fractions of 20 ml were collected. All procedures were carried out at 25°C. Other experimental conditions are described in the text. ●, MGLP activity; ○, absorbance at 280 nm.

TABLE I. **Summary of the purification of MGLP from *Bacillus* sp. H-257.**

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Cell free extract	82,288	3,197	0.04	1	100
Acetone fractionation	41,412	2,888	0.07	1.7	90
Acetone precipitation 1st	20,691	1,736	0.08	2.0	54
2nd	13,728	1,244	0.09	2.3	39
Octyl-Sepharose CL-4B	271	947	3.5	88	30
Q-Sepharose	8.8	819	93.0	2,325	26
Superose 12	5.3	642	121.1	3,028	20

known to be secreted extracellularly. However, the novel MGLP produced by *B.* sp. H-257 is not secreted extracellularly and accumulates in cells in an active form, making MGLP a unique enzyme.

Purification of MGLP—MGLP from *B.* sp. H-257 was purified 3,028-fold in 5 steps to a measured final specific activity of 121.1 U/mg-protein with a yield of 20% (Table I). The specific activity of the enzyme was increased 2.3-fold by acetone precipitation. The next step, hydrophobic chromatography on Octyl-Sepharose CL-4B, was effective for the purification of the enzyme with an increase in the specific activity of about 88-fold. Anion exchange chromatography on Q-Sepharose was successfully employed to increase the specific activity 2,325-fold with a recovery greater than 25%. Elution profiles from the Octyl-Sepharose CL-4B, Q-Sepharose FF and Superose 12 columns are shown in Figs. 1–3.

Molecular Mass and pI—The molecular mass of purified MGLP was determined to be about 25 kDa by gel filtration.

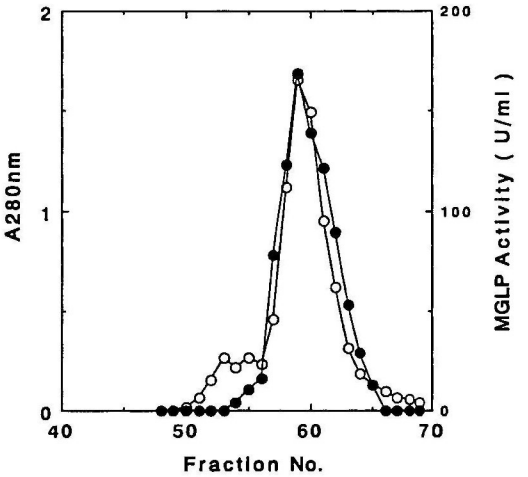

Fig. 3. **Column chromatography on Superose 12.** The enzyme solution (1 ml) obtained from the Q-Sepharose FF column was applied to a Superose 12 column (2.0 cm × 52 cm) at 25°C previously equilibrated with 10 mM phosphate buffer (pH 7.0) containing 0.5M KCl. Fractions of 1ml were collected at a flow rate of 1 ml per min. Other experimental conditions are described in the text. ●, MGLP activity; ○, absorbance at 280 nm.

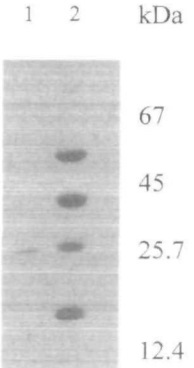

Fig. 4. **SDS-PAGE of MGLP.** Electrophoresis was carried out as described under "MATERIALS AND METHODS". After electrophoresis, the gel was stained with Coomasie Brilliant Blue. Lane 1, *Bacillus* sp. H-257 MGLP; lane 2, molecular marker proteins.

The enzyme appeared as a single band on SDS-PAGE at a migration distance corresponding to a molecular mass of approximately 24 kDa (Fig. 4). This indicates that MGLP is catalytically active in a monomeric form. The molecular masses of MGLP from mouse/rat adipocytes and human erythrocytes were approximately 33 kDa and 60 ± 2 kDa, respectively (*13*, *14*, *16*). These were also monomeric. The apparent pI of MGLP, as determined by an electrofocusing column, was around 4.66.

Effects of pH and Temperature on Enzyme Activity—The effects of pH and temperature on MGLP activity were examined. When assayed at various pH at 37°C, MGLP

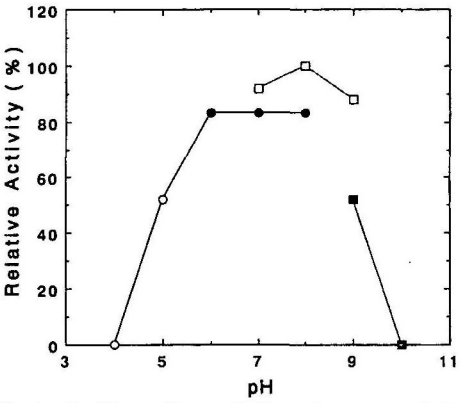

Fig. 5. **Effect of pH on the activity of monoacylglycerol lipase.** The enzyme activity at various pH values was assayed in the reaction mixture described in the text. Acetate buffer was used for pH 4 to 6 (○); phosphate buffer for pH 6 to 8 (●); Tris-HCl buffer for pH 7 to 9 (□); glycine-NaOH buffer for pH 9 to 10 (■). The enzyme source was the "Superose 12 fraction" of Table I.

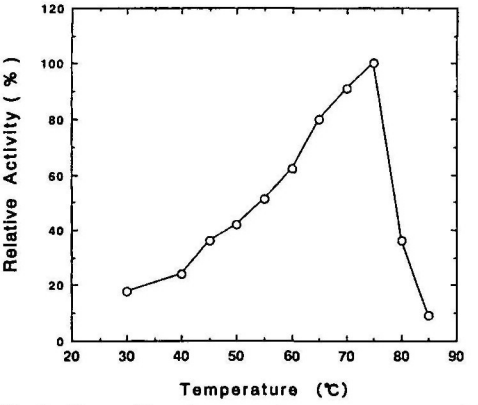

Fig. 6. **Effect of reaction temperature on monoacylglycerol lipase activity.** The reaction mixture, containing 100 µl of 0.2M PIPES-NaOH (pH 7.3), 50 µl of 10 mM monolauroylglycerol containing 0.5% Triton X-100, and 300 µl of H_2O was kept at various temperature described in Fig. 6. The reaction was started by adding 50 µl of enzyme solution, and stopped by adding 100 µl of 2 M HCl. After incubation at 37°C for 10 min, 100 µl of 2 M NaOH was added to the reaction mixture. The glycerol generated was determined colorimetrically by adding 500 µl of enzyme reagent containing 100 µl of 0.3% 4-aminoantipyrine, 100 µl of 0.2% TOOS, 100 µl of 45U/ml peroxidase, 50 µl of 20 mM $MgCl_2$, 50 µl of 20 mM ATP, 20 µl of 25U/ml glycerol kinase, 30 µl of 1,000 U/ml glycerol-3-phosphate oxidase, and 50 µl of H_2O. After incubation at 37°C for 10 min, 2 ml of 0.5% SDS solution was added, and the absorbance at 550 nm was measured.

showed high activity around pH 6–8 (Fig. 5). MGLP was most active at 75°C, when assayed for 10 min at pH 7.5 (Fig. 6).

Effects of pH and Temperature on Enzyme Stability—From pH stability tests using various buffers, it was found that the MGLP is stable from pH 7 to 10 after incubation at 70°C for 10 min, as shown in Fig. 7. Up to 60°C, the MGLP is stable for 10 min, but above 60°C it is inactivated (Fig. 8).

Catalytic Properties—The MGLP showed the highest activity toward 1-monolauroylglycerol (C_{12} acyl group) among the substrates examined (C_2–C_{18} acyl groups), as shown in Table II. When the acyl chain length of the substrate was increased above C_{12} or decreased below C_{12}, there was a steep decrease in enzyme activity. It has been reported that the MGLP from rat adipocytes (*14*) and rabbit aorta (*15*) hydrolyze 1(3)- and 2-monooleoylglycerols ($C_{18:1}$) similarly. Thus, we examined the positional specificity of MGLP for monooleoylglycerols. The enzyme had 1.25 times higher activity toward 2-monooleoylglycerol in comparison with 1(3)-monooleoylglycerol. On the other hand,

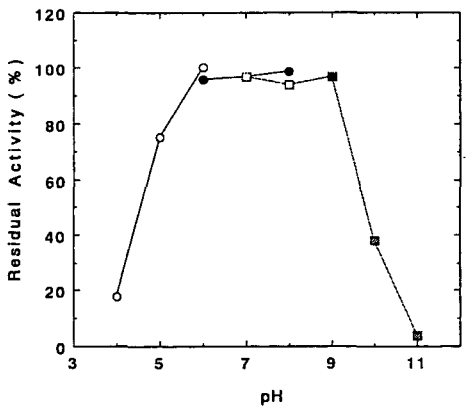

Fig. 7. **Effect of pH on the stability of monoacylglycerol lipase.** The enzyme solution (10 U/ml) in 10 mM buffer was incubated for 60 min at 45°C. Dimethylglutarate-NaOH buffer was used for pH 4 to 6 (○); PIPES-NaOH buffer for pH 6 to 8 (●); Tris-HCl buffer for pH 7 to 9 (□); glycine-NaOH buffer for pH 9 to 11 (■). The enzyme source was the "Superose 12 fraction" in Table I.

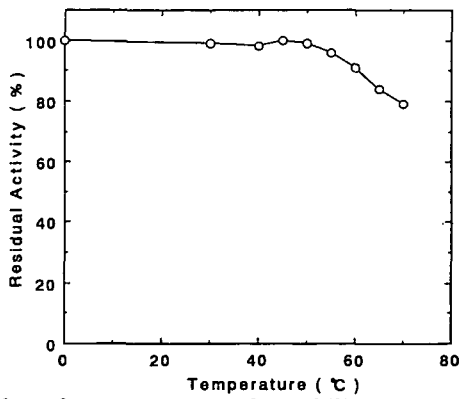

Fig. 8. **Effect of temperature on the stability of monoacylglycerol lipase.** The enzyme solution (10 U/ml) in 10 mM PIPES-NaOH (pH 7.5) buffer was incubated for 10 min at various temperatures. The residual activity was assayed in the reaction mixture described in the text.

no measurable activity was detected for 1,2-diacylglycerols, diacylglycerol from egg yolk phosphatidylcholine, on triacylglycerols.

The substrate specificity toward *p*-nitrophenyl esters (1 mM) was also determined. The MGLP showed the highest activity toward *p*-nitrophenyl butylate (C_4 acyl group) among the substrates examined, which is 23.9% of the activity toward 1-monolauroylglycerol. Not only monoacylglycerols (alcoholic hydroxyl esters) but also *p*-nitrophenyl esters (phenolic hydroxyl esters) were substrates for MGLP. MGLP had higher activity toward 1-monolauroylglycerol (C_{12} acyl group) among partial acylglycerols than the activity toward *p*-nitrophenyl butylate (C_4 acyl group) among *p*-nitrophenyl esters. The enzyme also showed activity toward *p*-nitrophenyl laurate (C_{12} acyl group) and *p*-nitrophenyl palmitate (C_{16} acyl group), suggesting that it has different substrate specificity from esterases.

An assay method for lipase activity using diacylglycerol as a substrate is known to be useful (*27*). This method is based on the measurement of monoacylglycerol, which is liberated from diacylglycerol. For this purpose, a specific monoacylglycerol-hydrolyzing enzyme is essential and a small K_m for monoacylglycerol is preferable. Therefore, we examined the K_ms of the enzyme described here. Initial velocity analysis showed that the enzyme exhibits Michaelis-Menten type kinetic patterns. K_ms for the hydrolysis of 1-monolauroylglycerol, 1-monooleoylglycerol, and 2-monooleoylglycerol were determined to be 140, 83, and 59 μM,

TABLE II. **Substrate specificity of MGLP toward mono-, di-, triacylglycerols.**

Substrate	Relative activity (%)
1-Monoacetylglycerol ($C_{2:0}$)	10.5
1-Monobutyroylglycerol ($C_{4:0}$)	47
1-Monocapryloylglycerol ($C_{8:0}$)	94
1-Monolauroylglycerol ($C_{12:0}$)	100
1-Monomyristoylglycerol ($C_{14:0}$)	84.5
1-Monoparmitoylglycerol ($C_{16:0}$)	63.5
1-Monostearoylglycerol ($C_{18:0}$)	45.5
1-Monooleoylglycerol ($C_{18:1}$)	57
1-Monolinoleoylglycerol ($C_{18:2}$)	75
1-Monolinolenoylglycerol ($C_{18:3}$)	81.5
2-Monooleoylglycerol ($C_{18:1}$)	71.5
1,2-Dilinoleoylglycerol ($C_{18:2}$)	0
Triacetylglycerol ($C_{2:0}$)	0
Tributyroylglycerol ($C_{4:0}$)	0
Trioleoylglycerol ($C_{18:1}$)	0
p-Nitrophenyl acetate ($C_{2:0}$)	9.3
p-Nitrophenyl butylate ($C_{4:0}$)	23.9
p-Nitrophenyl caplylate ($C_{8:0}$)	12.7
p-Nitrophenyl laurate ($C_{12:0}$)	6.2
p-Nitrophenyl palmitate ($C_{16:0}$)	1.9

TABLE III. **Effect of detergents on MGLP activity.**

Detergent	Concentration	Relative activity (%)
None		100
Triton X-100	0.05 %	100.2
	0.25 %	90.3
	0.65 %	89.7
	1.05 %	89.5
Cholate	1 mM	97.5
	5 mM	99.5
Deoxycholate	1 mM	94.8
	5 mM	83.1

respectively, suggesting the enzyme has a high affinity for monoacylglycerols.

Pancreatic lipase is well known to be strongly activated by deoxycholate, and diacylglycerol is solubilized with non-ionic detergents or deoxycholate. Therefore, the effects of detergents on enzyme activity were also determined. As shown in Table III, the enzyme was not inhibited by cholate, but was slightly inhibited by Triton X-100 (1.05%) and deoxycholate (5 mM).

Many lipases are known to be activated by calcium ions. We examined the influence of mono- and divalent ions (10 mM) in addition to calcium ions on MGLP activity. None of the following compounds showed any appreciable influence on enzyme activity: NaCl, KCl, LiCl, $MgCl_2$, $MnCl_2$, and $CaCl_2$ (data not shown).

The results described above suggest that the enzyme is very useful for application in the assay method described above.

Analysis of the N-Terminal Amino Acid Sequence—The amino acid sequence of the N-terminal region of the enzyme was determined as follows: Ser-Glu-Gln-Tyr-Pro-Val-Leu-Ser-Gly-Ala-Glu-Pro-Phe-Tyr-Ala-Glu- (16 residues). This sequence was compared with the sequences deposited in the DDBJ database and with three other *Bacillus* lipase sequences: *B. stearothermophilus* (*19*), *B. thermocatenulatus* (*28*), and *B. subtilis* (*29*). However, no significant homology to other sequences was found.

In this study, we screened a thermostable MGLP from the moderately thermophilic *B.* sp. H-257, established a purification method for the enzyme, and showed its characteristics. To our knowledge, this is the first description of a specific monoacylglycerol-hydrolyzing enzyme from a moderately thermophilic bacterium. This MGLP accumulates intracellularly in an active form, and hydrolyzes monoacylglycerol, but not di- and triacylglycerols to an appreciable extent. It is thermostable up to 60°C, suggesting its usefulness for analytical applications such as the measurement of serum lipase levels and the analysis of monoacylglycerols in food. However, the production of MGLP by *B.* sp. H-257 is low (about 8.3 ng/ml). To increase production efficiency, molecular cloning and expression of the structural gene will be necessary.

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