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Purification and Characterization of the Tween-Hydrolyzing Esterase of *Mycobacterium smegmatis*

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An esterase hydrolyzing Tween 80 (polyoxyethylene sorbitan monooleate) was purified from sonicated cell lysates of *Mycobacterium smegmatis* ATCC 14468 by DEAE-cellulose, Sephadex G-150, phenyl Sepharose, and diethyl-(2-hydroxypropyl) aminoethyl column chromatography and by subsequent preparative polyacrylamide gel electrophoresis. The molecular weight was estimated to be 36,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 41,000 by gel filtration on a Sephadex G-150 column. The esterase contained a single polypeptide. The esterase was stable to heat treatment at 100°C and to a wide range of pH. The temperature and pH optima for the hydrolysis of Tween 80 were 50°C and 8.3, respectively. The esterase had a narrow substrate specificity; it exhibited a high activity only on compounds having both polyoxyethylene and fatty acyl moieties, such as Tweens. Monoacylglyceride was hydrolyzed more slowly by this esterase and this enzyme exhibited a nonspecific esterase activity on *p*-nitrophenyl acyl esters, especially those having short chain acyl moieties. The K_m and V_{max} were 19.2 mM and 1,670 μmol/min per mg of protein for Tween 20, 6.6 mM and 278 μmol/min per mg of protein for Tween 80, and 0.25 mM and 196 μmol/min per mg of protein for *p*-nitrophenyl acetate, respectively. Observations of the effects of various chemical modifications on the activity of the esterase indicated that tyrosine, histidine, arginine, and methionine (with tryptophan) residues may be active amino acids which play important roles in the expression of Tween 80-hydrolyzing activity of the enzyme.

Bacteriocin-like substances (mycobacteriocins) produced by rapidly growing mycobacteria were found to require Tween 80 (polyoxyethylene sorbitan monooleate) for expression of their antimycobacterial activity against indicator strains of *Mycobacterium diernhoferi* and *Mycobacterium chitae*. The mycobacteriocins are Tween-hydrolyzing esterases, and their antimycobacterial actions are mediated by oleic acid generated by Tween 80 hydrolysis (25). The present paper describes purification and properties of the Tween 80-hydrolyzing esterase produced by *Mycobacterium smegmatis* ATCC 14468 (26). A 90% pure preparation of the esterase (esterase 14468) was obtained. This enzyme proved to be a simple protein consisting of a single subunit (molecular weight, 36,000) and is highly stable to heat and to a wide range of pH. Esterase 14468 had a narrow substrate specificity, showing a high activity only on polyoxyethylene fatty acyl esters and *p*-nitrophenyl acyl esters.

MATERIALS AND METHODS

The following mycobacterial strains were used: *M. smegmatis* ATCC 14468 (producer of esterase 14468) and *M. diernhoferi* ATCC 19340 (indicator for the antimicrobial substance produced from Tween 80 by esterase 14468).

Anti-*M. diernhoferi* activity of esterase. Antimicrobial activity of esterase 14468 and Tween 80 was assayed by the following method. About 5 μl of serially twofold diluted esterase solution was spotted onto a plate of heart infusion agar containing 4% glycerol and 0.1% Tween 80. After drying, the plate was exposed to chloroform vapor and was overlaid with 3 ml of 0.5% heart infusion agar containing 4% glycerol, 0.1% Tween 80, and 10^6 *M. diernhoferi* ATCC 19340 cells per ml, unless otherwise specified. After incubation at 37°C for 3 days, the maximum dilution of the esterase solution giving a clear zone of growth inhibition was recorded in arbitrary units (AU) per milliliter. Alternatively, a paper disk (8 mm in diameter) dipped into the esterase solution was placed onto a plate of heart infusion-glycerol-Tween 80 agar overlaid with 3×10^6 indicator cells. After incubation at 37°C for 3 days, the diameter of the growth inhibitory zone was measured.

Assay for esterase activity. Tween 80-hydrolyzing activity of esterase 14468 was measured as follows. A 0.2-ml reaction mixture consisting of 1 or 1.25% Tween 80 and 10 μl of enzyme solution (70 ng of protein) in 50 mM Tris-hydrochloride buffer (pH 7.5 or 8.0) was incubated at 37°C for 10 to 15 min, unless otherwise specified. Free fatty acid liberated in the reaction mixture was measured by using a nonesteri-

fied fatty acid test kit (Wako Pure Chemical, Osaka, Japan). When water-insoluble substances such as long-chain acyl glycerols were tested, these substances were sonicated in Tris-hydrochloride buffer containing 10% dimethyl sulfoxide and 1% Triton X-100 (7) or 10% gum arabic (2).

Nonspecific esterase activity of esterase 14468 was measured by using *p*-nitrophenyl acyl esters, such as *p*-nitrophenyl acetate (PNPA), as substrates by the following method. A 0.2-ml portion of reaction mixture consisting of 1 mM PNPA and 10 μl of enzyme solution (100 ng of protein) in 50 mM Tris-hydrochloride buffer (pH 8.0) was incubated at 37°C for 15 min. Then, 4 ml of ice-cold 50 mM sodium fluoride in 50 mM Tris-hydrochloride buffer (pH 8.0) was added to the reaction mixture to terminate the reaction, and the increase in optical density at 400 nm was measured. The rate of hydrolysis of *p*-nitrophenyl acyl esters was calculated by using a molar absorption coefficient of $1.70 \times 10^4\ M^{-1}\ cm^{-1}$ for 4-nitrophenoxide ion in this buffer system. In some cases, the time course of *p*-nitrophenyl acyl ester hydrolysis in the reaction mixture containing 5 to 250 μM substrate and 1.0 μg of esterase 14468 in 2.0 ml of 50 mM Tris-hydrochloride buffer (pH 8.0) was monitored spectrophotometrically at 37°C.

Molecular weight estimation. The molecular weight of esterase 14468 was estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, using an electrophoresis calibration kit for low-molecular-weight proteins (Pharmacia Fine Chemicals AB, Uppsala, Sweden) according to the method of Weber and Osborn (35), with some modifications. Alternatively, the molecular weight estimation was performed by gel filtration on Sephadex G-150, using Combithek calibration proteins (Boehringer Mannheim, Mannheim, West Germany) according to the method of Whitaker (37).

Chemical analysis of esterase. Purified esterase 14468 preparation was subjected to 12% polyacrylamide gel electrophoresis without denaturing agents (pH 8.9 gel). The migrating band of the esterase was stained by using periodic acid-Schiff reagents for carbohydrate, diphenylamine for DNA, acridine orange for RNA, and Coomassie brilliant blue R 250 for protein, as described by Maurer (20). The lipid moiety of the esterase was stained with Sudan black B and then subjected to 12% polyacrylamide gel electrophoresis according to the method of Maurer (20).

Protein determination. Protein concentration was measured by the method of Lowry et al. (17).

Special agents. Polyoxyethylene sorbitan monolaurate (Tween 20), polyoxyethylene sorbitan monopalmitate (Tween 40), polyoxyethylene sorbitan monostearate (Tween 60), polyoxyethylene sorbitan monooleate (Tween 80), sorbitan monooleate (Span 80), and Triton X-100 were purchased from Wako Pure Chemical. Polyoxyethylene monooleate, polyoxyethylene dioleate, and polyoxyethylene monostearate were donated by Nihon Yushi Co., Tokyo, Japan. Glyceryl monooleate (monoolein; 90% is α isomer), 1,2-diolein, 1,3-diolein, triolein, *S*-palmitoyl coenzyme A, oleoylcoenzyme A, palmitic acid ethyl ester, L-α-phosphatidylcholine, L-α-phosphatidylethanolamine, DL-α-phosphatidylethanolamine dipalmitoyl, *p*-nitrophenyl acyl esters, phenylmethylsulfonyl fluoride, tryosinase (grade III), glucose oxidase (type II), galactose oxidase (type VI), neuraminidase (type V), and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, Mo. Sephadex G-150, phenyl Sepharose, and diethyl-(2-hydroxypropyl)aminoethyl (QAE) Sephadex were obtained from Pharmacia Fine Chemicals. Proflavine and 5'-isatinsulfonic acid were obtained from Aldrich Chemical Co., Milwaukee, Wis.

RESULTS

Purification of esterase 14468. A 3-day-old culture of *M. smegmatis* ATCC 14468 grown in heart infusion broth containing 4% glycerol and 0.1% Tween 80 at 37°C with reciprocal shaking (100 cycles per min) was harvested by centrifugation (10,000 × *g* for 30 min) and thoroughly rinsed with 0.05 M Tris-hydrochloride buffer (pH 7.5) containing 0.1 M NaCl and 0.01% NaN_3 (buffer A). The cell pellet (1,085 g [wet weight] from 45 liters of cultured broth) was suspended in buffer A at a concentration of 0.35 g of cells per ml and then disrupted with an ultrasonic disintegrator (Insonator, model 200M; Kubota Shoji, Tokyo, Japan) at 200 W for 20 min. After centrifugation at 15,000 × *g* for 1 h, the supernatant was dialyzed against 0.05 M potassium phosphate buffer (pH 7.5) and applied to a DEAE-cellulose column (5 by 21 cm) equilibrated with the same buffer. The bulk of esterase 14468 was eluted with 0.1 M NaCl in 0.05 M potassium phosphate buffer (pH 7.5). Flow-through fractions containing considerable amounts of esterase were again subjected to the same column chromatography. Peak fractions containing more than 50 AU of esterase activity per ml were pooled, dialyzed against distilled water, and lyophilized. The dried powder (20 g) was divided in two, and each portion (10 g) was dissolved in distilled water (ca. 30 ml), dialyzed against 0.01 M Tris-hydrochloride buffer (pH 7.5), and applied to a Sephadex G-150 column (5 by 90 cm). The esterase was eluted with 0.05 M Tris-hydrochloride buffer (pH 7.5). Peak fractions of esterase 14468 were pooled, dialyzed against 3 volumes of 0.05 M Tris-hydrochloride buffer (pH 7.5) containing 40% saturation of ammonium sulfate, and then applied to a phenyl Sepharose column (5 by 10 cm) previously equilibrated with 0.05 M Tris-hydrochloride buffer (pH 7.5) containing 30% saturation of ammonium sulfate. Esterase 14468 was eluted stepwise with 0.05 M Tris-hydrochloride buffers (pH 7.5) containing 15% saturation of ammonium sulfate and 20% ethylene glycol, 5% saturation of ammonium sulfate and 50% ethylene glycol, or 70% ethylene glycol. The peak fractions of esterase 14468 which eluted with 0.05 M Tris-hydrochloride buffer (pH 7.5) containing 5% saturation of ammonium sulfate and 50% ethylene glycol were pooled, dialyzed against 0.05 M Tris-hydrochloride buffer (pH 7.5), and

then placed on a QAE Sephadex column (2.5 by 53 cm) equilibrated with the same buffer. Esterase 14468 was eluted with 0.2 M ammonium sulfate in 0.05 M Tris-hydrochloride buffer (pH 7.5) with a considerable time lag. The peak fractions of esterase 14468 were pooled, dialyzed against 0.05 M Tris-hydrochloride buffer (pH 7.5) containing 30% saturation of ammonium sulfate, and applied on a phenyl Sepharose column (5 by 7.5 cm) equilibrated with the same buffer. Esterase 14468 was eluted with 50% ethylene glycol in 0.05 M Tris-hydrochloride buffer (pH 7.5) containing 6% saturation of ammonium sulfate (Fig. 1A). The peak fractions were combined, dialyzed against 0.02 M Tris-hydrochloride buffer (pH 8.0), and lyophilized. The dried powder (270 mg including salt) was divided in two, and each half was dissolved in a small amount (ca. 15 ml) of distilled water containing 13% glycerol and subjected to preparative polyacrylamide gel electrophoresis by using equipment (model CD-50; Tohyo Kagaku Sangyo, Tokyo, Japan) under the following conditions: 7.5% polyacrylamide gel (80 ml of pH 8.9 gel) for separating gel, 2.5% polyacrylamide gel (15 ml of pH 7.2 gel) for stacking gel, and the running current was set to 60 mA. The migrating pattern is shown in Fig. 1B. Esterase 14468 migrated slightly slower than the bromphenol blue marker. Each of the esterase fractions was mixed with 0.1 volume of glycerol and stored at −80°C until use. Before use, the thawed fraction was thoroughly dialyzed against appropriate buffer.

Purity of esterase 14468. A summary of the purification of esterase 14468 is shown in Table 1. I measured esterase activity by two different methods, that is, anti-*M. diernhoferi* activity in the presence of Tween 80 and Tween 80-hydrolyzing activity. These two activities increased in an almost parallel manner during the course of the purification, indicating that the anti-*M. diernhoferi* action of esterase 14468 in the presence of Tween 80 is based on its inherent activity of Tween 80 hydrolysis, as discussed in a previous report (25). On the basis of Tween 80-hydrolyzing activity, esterase 14468 was purified 2,300-fold. The last fraction in Table 1 was subjected to 12% polyacrylamide gel electrophoresis (Fig. 2). Gel electrophoresis yielded a main band with esterase activity and two minor bands of contaminating protein. From this densitometry pattern, the purity was calculated to be 90%.

Molecular weight and chemical properties. The esterase 14468 preparation was denatured by heating in the presence of 2.5% SDS and 5% 2-mercaptoethanol and subjected to SDS-polyacrylamide gel electrophoresis (10 or 12.6% polyacrylamide gel). Only one major band was detected at the R_f value corresponding to a molecular weight of 36,000. In separate experiments, this esterase activity was found to be sensitive to digestion with proteases such as pronase, chymotrypsin, and trypsin but was

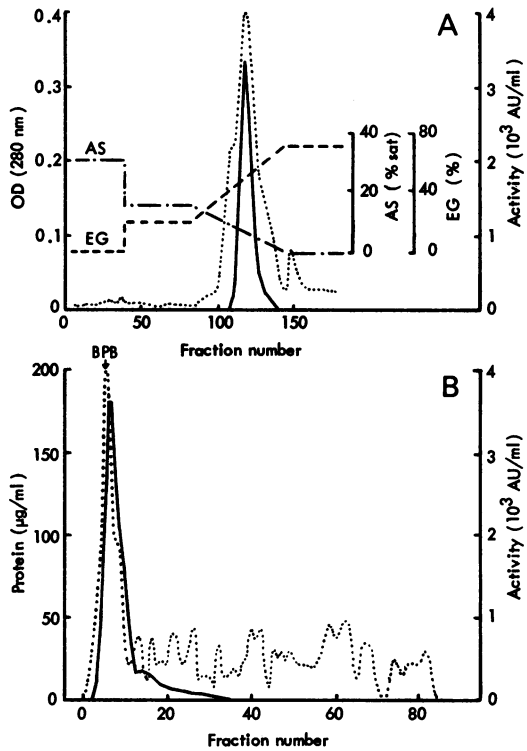

FIG. 1. Second phenyl Sepharose column chromatography (A) and preparative polyacrylamide gel electrophoresis (B) of esterase 14468. (A) Esterase 14468 fraction after QAE Sephadex column chromatography was applied on a phenyl Sepharose column under the buffer condition of 0.05 M Tris-hydrochloride (pH 7.5) containing 30% saturation of ammonium sulfate (AS), washed with 0.05 M Tris-hydrochloride buffer (pH 7.5) containing 15% saturation of ammonium sulfate and 20% ethylene glycol (EG), and then eluted with a linear gradient of ammonium sulfate and ethylene glycol. (B) The lyophilized powder of esterase 14468 after the second phenyl Sepharose column chromatography was dissolved in distilled water containing 13% glycerol and subjected to preparative polyacrylamide gel electrophoresis as described in the text. In the migrating pattern, the apparent protein peak did not coincide with the esterase activity peak. This seemed to be due to a Folin phenol reagent-reactive substance such as *N,N,N',N'*-tetramethylethylenediamine (accelerating agent for polymerization of polyacrylamide gel), which migrated at bromphenol blue (BPB) marker, since the protein concentration of each fraction was assayed by the Lowry Folin phenol method (17) and in fact *N,N,N',N'*-tetramethylethylenediamine is highly Folin phenol reagent reactive (0.01% solution gives an optical density at 750 nm of 1.4). Solid line, activity; dotted line, protein concentration.

TABLE 1. Purification of esterase 14468

Step	Total protein (mg)	Tween 80-hydrolyzing activity[a]			Anti-*M. diernhoferi* activity[b]		
		Total activity (U)	Sp act (U/mg)	Purification	Total activity (10^3 AU)	Sp act (AU/mg)	Purification
Sonication	74,000	5,880	0.080	1	2,370	32	1
DEAE-cellulose	7,670	5,220	0.68	8.6	941	123	3.8
Sephadex G-150	2,020	2,230	1.10	13.8	928	459	14.3
Phenyl Sepharose	517	1,710	3.32	41.8	445	861	26.9
QAE Sephadex	42.5	850	19.5	245	441	10,100	318
Phenyl Sepharose	23.2	648	27.9	351	251	10,800	338
Preparative polyacrylamide gel electrophoresis	1.47	268	183	2,300	37.8	25,700	794

[a] For Tween 80-hydrolyzing activity, 1 U is the amount which liberates 1 μmol of oleic acid per min from Tween 80 in the standard reaction system consisting of 1.25% Tween 80 and 0.05 M Tris-hydrochloride buffer (pH 7.5).

[b] For anti-*M. diernhoferi* activity, 1 AU/ml equals the amount of activity which gives a clear growth inhibitory zone of indicator bacteria at a 1× dilution of esterase solution.

resistant to DNase and RNase (data not shown). Thus, esterase 14468 seems to be a single polypeptide. The molecular weight of esterase 14468 was again estimated by Sephadex G-150 gel filtration. From the elution volume compared with the standard proteins, the molecular weight was calculated to be 41,000. This value is consistent with that estimated by SDS-polyacrylamide gel electrophoresis. Esterase 14468 was subjected to 12% polyacrylamide gel electrophoresis (pH 8.9 gel), and the band of esterase was stained for carbohydrate, DNA, and RNA moieties. The band was stained only with Coomassie brilliant blue or amide black (for protein) but not with periodic acid-Schiff reagent, diphenylamine, or acridine orange. Sudan black B also failed to stain this esterase.

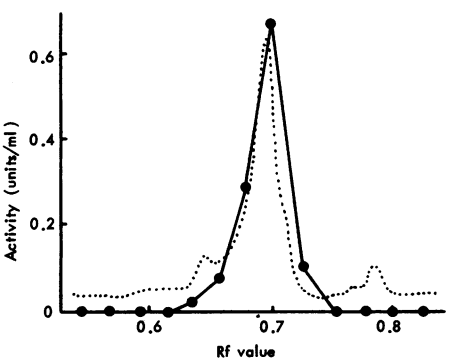

FIG. 2. Pattern of esterase 14468 on polyacrylamide gel electrophoresis without a denaturing agent. The peak fraction obtained by preparative polyacrylamide gel electrophoresis (Table 1 and Fig. 1B) was subjected to 12% polyacrylamide gel electrophoresis (pH 8.9 gel) without a denaturing agent. After electrophoresis at 25 mA for 3 h (model SJ-1060 SDH; Atoh Co., Tokyo, Japan), sections of the slab gel were stained with Coomassie brilliant blue or sliced transversely into 2-mm slices and extracted with 0.5 ml of 0.05 M Tris-hydrochloride buffer (pH 7.5) for estimation of esterase activity (●). The stained slab gel was scanned with a Soft Laser scanning densitometer (Biomed Instruments Inc., Chicago, Ill.) and the scanning pattern is indicated by the dotted line. The esterase preparation gave no protein bands in the R_f ranges of 0 to 0.6 and 0.8 to 1.0. In the migrating pattern, the protein and activity peaks did not coincide in such a strict manner due to an artifact of slicing the gels.

Stability. Both the Tween 80-hydrolyzing activity and anti-*M. diernhoferi* activity gradually decreased in a parallel manner by heating at 100°C (Fig. 3A). About 40% of the esterase activity was retained even after 30 min of heating at 100°C. Fig. 3B shows the pH stability of esterase 14468. This enzyme was stable over a wide range of pH yet the bulk of the esterase activity was lost after a 3-h incubation at pH 4 to 5. In a separate experiment, the isoelectric precipitation point of this esterase was found to be pH 4.5 (data not shown). The effects of various agents on the thermostability of esterase 14468 were also studied (Table 2). When esterase 14468 was heated at 100°C for 20 min in 0.1 M Tris-hydrochloride buffer (pH 8.0) without any additions, the residual activity was 25%. Bovine serum albumin exhibited some protective effect, whereas EDTA and high concentrations of NaCl considerably accelerated the inactivation of esterase activity. Glycerol, sucrose, and thiol compounds had no effect on the stability of this polypeptide. Mn^{2+} augmented the heat stability of the esterase, whereas Fe^{2+} markedly enhanced the heat inactivation. Neither Mg^{2+} nor Cu^{2+} affected the heat stability of esterase 14468.

Effects of temperature and pH on Tween 80-hydrolyzing activity. The hydrolysis rate of Tween 80 by esterase 14468, under standard

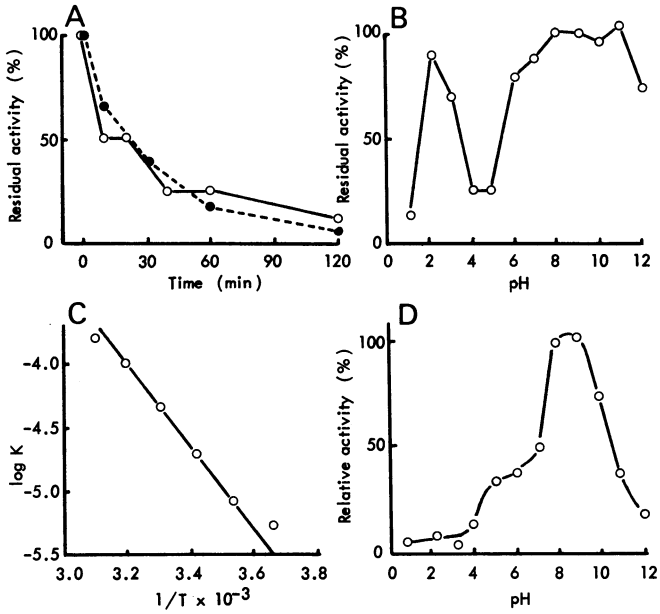

FIG. 3. Effects of temperature and pH on the activity of esterase 14468. (A) Heat stability of esterase 14468. The esterase in 0.1 M Tris-hydrochloride buffer (pH 8.0) was heated at 100°C. The residual antimycobacterial (○) and Tween 80-hydrolyzing (●) activities of esterase 14468 are shown. (B) pH stability of esterase 14468. The esterase was dissolved in 0.2 M glycine hydrochloride (pH 1 to 3), sodium acetate (pH 4 to 5), potassium phosphate (pH 6 to 7), Tris-hydrochloride (pH 8 to 9), or sodium borate (pH 10 to 12) buffers and incubated at 37°C for 3 h. After dialysis against 0.1 M Tris-hydrochloride buffer (pH 7.5) at 4°C, the residual antimycobacterial activity was measured. (C) Arrhenius plot of esterase 14468 activity for Tween 80. K is the velocity constant and T is absolute temperature. (D) Dependence of esterase 14468 activity on pH. The Tween 80-hydrolyzing activity (substrate 1.25%) of the esterase at various pH values was measured with the same buffer (0.17 M each) system as (B). In (A) and (C), Tween 80 hydrolysis by esterase was performed in 0.05 M Tris-hydrochloride buffer (pH 8.0) at a substrate concentration of 1.25%.

conditions, was fairly linear for at least 10 min. The esterase had an optimum temperature of about 50°C, and the activation energy for Tween 80 hydrolysis was calculated to be 3.74 Kcal (from the Arrhenius plot in Fig. 3C). Fig. 3D shows the pH dependency of Tween 80-hydrolyzing activity of esterase 14468. The esterase activity was highest around pH 8 to 8.5.

Substrate specificity. Table 3 shows the substrate specificity of esterase 14468. This enzyme exhibited a high carboxylic ester hydrolase activity against compounds having both the polyoxyethylene and fatty acyl moieties but the existence of sorbitan residue in these polyoxyethylene acyl esters was not essential. Glyceryl oleoyl esters, particularly monoolein, also served as substrates, but their hydrolysis rate was markedly lower than that of polyoxyethylene fatty acyl esters. Sorbitan monooleate, fatty acyl-CoAs, and phospholipids (phosphatidylcholine, phosphatidylethanolamine, and phosphatidylethanolamine dipalmitoyl; data not shown) could not serve as substrates for esterase 14468. On the contrary, esterase 14468 exhibited a nonspecific esterase activity on *p*-nitrophenyl acyl esters at a reaction rate comparable to that seen with Tween 80 hydrolysis (Fig. 4).

Kinetics. The saturation kinetics of esterase 14468 with Tween 20, Tween 80, and PNPA gave hyperbolic curves (data not shown). From double reciprocal plots (Fig. 4A and B), the K_m and V_{max} values of the esterase against Tween 20, Tween 80, and PNPA were estimated as follows: Tween 20 hydrolysis, K_m = 19 mM and V_{max} = 1,670 μmol/min per mg of protein; Tween 80 hydrolysis, K_m = 6.6 mM and V_{max} = 278 μmol/min per mg of protein; PNPA hydrolysis, K_m = 0.25 mM and V_{max} = 196 μmol/min per mg of protein. Figure 4C shows that *p*-nitrophenyl short-chain acyl esters, especially *p*-nitrophenyl butyrate, served as much better substrates than *p*-nitrophenyl esters having long-chain acyl moieties (C12 to C16).

Effects of metal ions and chelating agents. Table 4 shows the effects of various metal ions and chelating agents on the hydrolysis of Tween 80 by esterase 14468. Cu^{2+}, Fe^{2+}, and Fe^{3+} were markedly inhibitory against the esterase, whereas Ca^{2+}, Mg^{2+}, Mn^{2+}, and Ag^{+} were

TABLE 2. Effects of various agents on the heat stability of esterase 14468[a]

Addition	Concn	Residual activity (%)
None		25
Bovine serum albumin	200 μg/ml	50
Glycerol	25%	25
Sucrose	25%	25
2-Mercaptoethanol	10 mM	25
Dithiothreitol	10 mM	25
EDTA	10 mM	12.5
NaCl	500 mM	12.5
$CaCl_2$	10 mM	12.5
$MgSO_4$	10 mM	25
$FeSO_4$	10 mM	0
$CuSO_4$	10 mM	25
$MnCl_2$	10 mM	100

[a] Esterase 14468 was heated at 100°C for 20 min in the presence or absence of indicated agents in 0.1 M Tris-hydrochloride buffer (pH 8.0). The residual activity was measured on the basis of anti-*M. diernhoferi* activity.

noneffective or weakly inhibitory in high doses. Metal chelating agents did not affect the esterase activity; however, EDTA did exhibit a weak inhibitory action. Partial inhibition of esterase by *o*-phenanthroline and α,α'-dipyridyl is thought to be due to their nonspecific binding to the hydrophobic moiety of esterase 14468 rather than due to chelation to metals, as reported by Yamamoto et al. (40) in the case of salicylate hydroxylase. Similar results were also obtained in the case of PNPA hydrolysis by esterase 14468 (Table 4), but it should be noted that the inhibition due to Fe^{2+} and Fe^{3+} was somewhat lower than that in the case of Tween 80 hydrolysis and that Mn^{2+} ion inhibited considerably the PNPA hydrolysis.

Table 4 also shows the effects of some chemicals which are specific inhibitors of certain carboxylic ester hydrolases, such as carboxylesterase and arylesterase (28), on the hydrolyzing activity of esterase 14468 against Tween 80 and PNPA. Phenylmethylsulfonyl fluoride (1 mM), eserine (physostigmine) (1 mM), 5,5'-dithiobis(2-nitrobenzoic acid) (1 mM), and sodium fluoride (10 mM) had no significant effect on either activities of esterase 14468.

Effects of substrate analogs. To confirm the specificity of esterase 14468 for polyoxyethylene and long-chain acyl moieties, the effects of some substrate analogs having either polyoxyethylene or acyl moieties but without the ability to serve as substrate for the Tween 80-hydrolyzing activity of esterase 14468 were examined (Table 5). Polyoxyethylene caprylphenol ether caused a marked inhibition of Tween 80 hydrolysis at concentrations over 1.25%. Polyoxyethylene alkyl ether (Brij 58) and sorbitan monooleate (Span 80) also showed a considerable inhibitory action against esterase 14468-mediated Tween 80 hydrolysis. High concentrations of ethanol were markedly inhibitory to esterase 14468, presumably because of the protein denaturing action.

Effects of various chemical modifications. Esterase 14468 was subjected to various types of chemical modifications and the residual activity was measured (Table 6). Treatments with *N*-bromosuccinimide, diethyl pyrocarbonate, 2,3-butanedione, and trinitrobenzene sulfonate; iodination, acetylation, or photooxidations with proflavine or methylene blue; and oxidation of tyrosine residues with tyrosinase caused an almost complete deactivation of esterase 14468. On the other hand, treatments of esterase 14468 with 5'-isatinsulfonic acid, monoiodoacetic acid, hydroxylamine, *p*-nitrophenol, or *p*-chloromercuribenzoate or oxidation of tryptophan residues with hydrogen peroxide and dioxane had no significant effect on the esterase activity. Treatment of esterase 14468 with glucose oxi-

TABLE 3. Substrate specificity of esterase 14468[a]

Substrate	Relative activity (%)
Tween 80	100
Tween 20	181
Tween 40	130
Tween 60	103
Polyoxyethylene monostearate	150
Polyoxyethylene monooleate	64
Polyoxyethylene dioleate	100
Sorbitan monooleate	0
Monoolein	23
1,2-Diolein	9
1,3-Diolein	2
Triolein	4
Palmitoyl-CoA	0.0 (0.3)[b]
Oleoyl-CoA	0.4 (0.3)[b]
Palmitate ethyl ester	6

[a] Esterase activity was assayed by measuring the amount of fatty acid liberated from the indicated substrates. The concentrations of substrates tested were as follows: Tweens, polyoxyethylene acyl esters, and sorbitan monooleate, 1.25%; glycerides and palmitate ethyl ester, 2.5 mM; fatty acyl-CoA, 1.0 mM. Relative activity was calculated by fitting the hydrolysis rate of Tween 80 at the same concentration with that of each substrate (135 to 171 μmol/min per mg of protein) at 100%.

[b] As shown in parentheses, no significant hydrolysis of these substrates were observed, even with the following assay system. The reaction mixture in 0.05 M Tris-hydrochloride buffer (pH 8.0) containing 0.5 mM 5,5'-dithiobis(2-nitrobenzoic acid) and 100 μM acyl-CoA was incubated at 37°C for 2 h, and then the increase in optical density at 412 nm was measured.

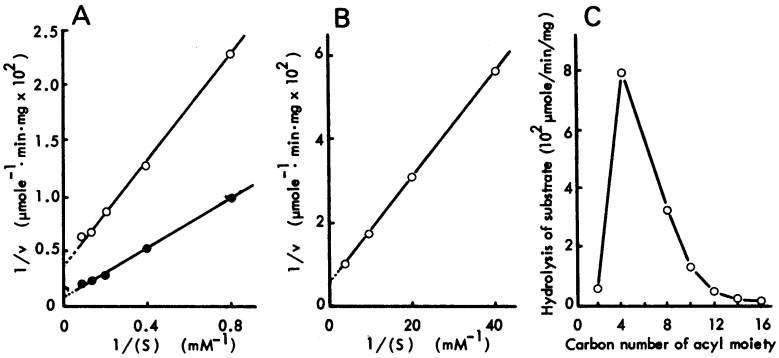

FIG. 4. Kinetics of the enzyme activity of esterase 14468 against Tweens and *p*-nitrophenyl acyl esters. (A) Double-reciprocal plots of hydrolysis of Tween 20 (●) and Tween 80 (○) by esterase 14468. (B) Double-reciprocal plot of PNPA hydrolysis by esterase 14468. (C) Dependence of hydrolysis rate of various *p*-nitrophenyl acyl esters by esterase 14468 on the chain length of acyl moieties. In this case, the reaction was performed at pH 7.0.

dase (20 U per ml, 37°C for 60 min), galactose oxidase (20 U per ml, 37°C for 60 min), neuraminidase (2.5 U per ml, 37°C for 6 h), or 25 mM sodium periodate (0°C for 2 h) did not significantly alter the activity of esterase 14468.

DISCUSSION

The bacteriocin-like substance of *M. smegmatis* (smegmatocin) was purified on the basis of its anti-*M. diernhoferi* activity in the presence of Tween 80 in the assay medium. Tween 80-hydrolyzing activity of this protein was copurified with its antimycobacterial activity, and the 90% pure smegmatocin had a high specific activity of Tween 80 hydrolysis. Thus, the Tween 80-hydrolyzing activity is thought to be an inherent activity of this bacteriocin-like protein. We have already reported that rapidly growing mycobacteria other than *M. smegmatis* also produce bacteriocin-like proteins having Tween 80-hydrolyzing activity (25). Tween 80-hydrolyzing activity of whole bacterial cells, cell lysate, or partially purified enzyme preparation of rapid (1, 11, 34, 36) and slow (29, 36) growers of mycobacteria has been discussed. Mycobacteria utilize Tween 80 as a carbon source by incorporating free oleic acid generated by Tween 80 hydrolysis (9, 29). The present Tween 80-hydrolyzing enzyme (esterase 14468) seem to play an important role in Tween 80 utilization by *M. smegmatis*. Hydrolysis and utilization of Tweens have been reported for *Pseudomonas* species (12, 21) and *Micrococcaceae* (3).

Esterase 14468 had a specific activity of Tween 80 hydrolysis to generate 183 μmol of oleic acid per min per mg protein. This Tween 80-hydrolyzing rate is much the same as that of palmitoyl CoA-hydrolyzing thioesterase from *M. smegmatis* (230 μmol/min per mg for palmitoyl CoA) (39), but higher than those of acyl-CoA-hydrolyzing esterase from *Pseudomonas aeruginosa* (32 μmol/min per mg for Tween 80) (21), triacylglycerol lipase from *Mycobacterium phlei* (12 to 14 μmol/min per mg for triacylglyceride) (23), and adipose tissue lipoprotein lipase (57 μmol/min per mg for triolein) (5). Considerably higher rates of acyl ester hydrolysis than that for Tween 80 hydrolysis by esterase 14468 have been reported for monoglyceride lipase from adipose tissue (350 μmol/min per mg for monoolein) (32) and milk lipoprotein lipase (600 μmol/min per mg for triglyceride) (14).

The molecular weight of esterase 14468, a single polypeptide, was estimated to be 36,000 by SDS gel electrophoresis and 41,000 by Sephadex G-150 gel filtration. Similar molecular weights have been reported for adipose tissue monoacylglyceride lipase (32,000) (32), undecyl acetate esterase from *Pseudomonas cepacia* (34,500) (27), acyl-CoA thioesterase from *M. smegmatis* (40,000) (39), triacylglycerol lipase from *M. phlei* (40,000 for active subunit) (23), acyl-CoA hydrolyzing thioesterase from *P. aeruginosa* (55,000) (21), and rat liver lysosomal lipase (58,000) (30).

The protein band of esterase 14468 on polyacrylamide gel electrophoresis was a simple protein not containing carbohydrate, DNA, RNA, or lipid moiety. In this respect, esterase 14468 is different from bovine milk lipoprotein lipase, which contains a carbohydrate residue (14).

Most lipases and esterases are known to be heat labile (19, 23, 27, 31, 39) and many mammalian lipases are very unstable even at room temperature (2, 5, 14, 30, 32). Esterase 14468 was stable to heat and retained about 25 to 40% residual activity, even after 30 min of heating at

TABLE 4. Effects of various metal ions, chelating agents, and some esterase inhibitors on the activity of esterase 14468[a]

Addition	Concn (mM)	Relative activity (%)	
		Tween 80 hydrolysis	PNPA hydrolysis
None		100	100
$CaCl_2$	10	67	97
$MgSO_4$	10	105	98
$CuSO_4$	1, 5[b]	48	16
	10	8	
$FeSO_4$	1, 5[b]	23	66
	10	9	
$FeCl_3$	1, 2.5[b]	8	49
$MnCl_2$	10	85	32
$AgNO_3$	10	54	
EDTA	10	73	91
KCN	10	108	
NaN_3	10	112	99
8-Hydroxyquinoline	10	88	
o-Phenanthroline	10	41	
α,α'-Dipyridyl	10	53	
Phenylmethylsulfonyl fluoride	0.01	73	99
	1	66	92
Eserine	0.01	122	97
	1	134	97
5,5'-Dithiobis(2-nitrobenzoic acid)	0.01	111	99
	1	104	94
NaF	1	59	
	10	62	96
	100	4	64

[a] Hydrolyzing activity of esterase 14468 for Tween 80 and PNPA was measured under the following conditions. For Tween 80 hydrolysis, the reaction mixture consisting of 0.05 M Tris-hydrochloride buffer (pH 7.5), 1.25% Tween 80, and purified esterase 14468 (70 ng of protein), with or without addition of indicated agents, was incubated at 37°C for 15 min (100% activity values for two separate experiments were 172 and 202 μmol/min per mg of protein). For PNPA hydrolysis, the reaction mixture consisting of 0.05 M Tris-hydrochloride buffer (pH 8.0), 1 mM PNPA, and esterase 14468 (100 ng of protein), with or without addition of indicated agents, was incubated at 37°C for 15 min (100% activity value was 236 μmol/min per mg of protein).

[b] Concentrations of agents added to the incubation mixture for PNPA hydrolysis are indicated.

100°C without addition of any protective agents (Fig. 3 and Table 2). Other heat-stable esterases have been reported: the lipase-like protein from *M. phlei* (11) and acyl-CoA thioesterase of *P. aeruginosa* (21). Esterase 14468 was stable especially in alkaline pH, and a similar pH stability has been observed in esterase A from *Bacillus subtilis* (24). Although many mammalian lipoprotein lipases and lysosomal lipases are stabilized by glycerol (5, 14, 30), glycerol at 25% concentration failed to stabilize the esterase activity at 100°C. Storage of this esterase without any protecting agents at −80°C for more than 6 months produced no appreciable loss of the activity (data not shown). From these findings, esterase 14468 is a most stable carboxylic acyl hydrolase. The optimum pH of esterase 14468 was approximately 8.3 (Fig. 3D). This feature is consistent with findings in common mammalian and bacterial lipolytic enzymes (5–7, 10, 15, 21, 32, 33).

Esterase 14468 had a considerably narrow substrate specificity restricted to esters having both polyoxyethylene and long-chain acyl moieties, such as Tweens (Table 3). This enzyme could also hydrolyze glyceryl acyl esters, but the hydrolysis rates were much lower than those for polyoxyethylene acyl esters. The Tween-hydrolyzing activity has also been noted for the mycobacterial lipase from *M. phlei* (11), nonspecific lipase from rat pancreas (2), acyl esterase from *P. aeruginosa* (21), and membrane-bound esterase from *M. smegmatis* (1). Esterase 14468 hydrolyzed monoolein much more rapidly than diolein and triolein. Thus, this enzyme seems to be a monoacylglyceride acyl hydrolase, such as monoacylglyceride lipase of chicken adipose tissue (6) and monoacylglyceride-hydrolyzing enzyme of rat adipose tissue (32). Long-chain acyl esterase from *P. aeruginosa* reported by Ohkawa et al. (21) shows similar substrate specificity except for its action on acyl-CoA and monoacyl sucrose (esterase 14468 could not hydrolyze acyl-CoA and sorbitan monooleate). Tween 80 hydrolysis by esterase 14468 was significantly inhibited by substrate analogs having either polyoxyethylene or long-chain acyl moieties. Therefore, it is thought that esterase

TABLE 5. Effects of various substrate analogs on Tween 80-hydrolyzing activity of esterase 14468[a]

Addition	Concn (%)	Inhibition (%)
Ethylene glycol	1.25	21
Polyethylene glycol	1.25	17
	2.5	9
Polyoxyethylene alkyl ether (Brij 58)	1.25	29
	2.5	27
Triton X-100	1.25	43
	2.5	52
Sorbitan monooleate	0.75	29
Ethanol	10	73
	20	92

[a] The Tween 80-hydrolyzing activity of esterase 14468 was assayed under the following conditions. The reaction mixtures consisting of 0.05 M Tris-hydrochloride buffer (pH 8.0), 1.25% Tween 80, and esterase 14468 (70 ng of protein), with or without addition of indicated substrate analogs or ethanol, were incubated at 37°C for 10 min. Reaction rate of control incubation (none added) was 140 μmol/min per mg of protein.

TABLE 6. Effects of various chemical modifications on the enzymatic activity of esterase 14468[a]

Modification with[b]:	Residual activity (%)[c]
None	100
5'-Isatinsulfonic acid	100
Hydroxylamine	100
p-Nitrophenol	100
H_2O_2 (50% dioxane)	50
Monoiodoacetic acid	50
p-Chloromercuribenzoate	50
Trinitrobenzene sulfonate	25
Tyrosinase	6
Acetic anhydride	3
Proflavine (+light)	3
Iodine	0
N-Bromosuccinimide	0
Methylene blue (+light)	0
Diethyl pyrocarbonate	0
2,3-Butanedione	0

[a] Purified esterase 14468 (35 μg of protein per ml) was treated with various chemicals or enzymes in the presence of bovine serum albumin at a concentration of 1.25 mg/ml as protecting agent. After treatment, the esterase solution was thoroughly dialyzed against 0.01 M Tris-hydrochloride buffer (pH 7.5), and the residual activity was measured on the basis of antimicrobial activity with Tween 80.

[b] Treatments were as follows (amino acid residues being modified are listed in parentheses): 5'-isatinsulfonic acid (Trp), 10 mM 5'-isatinsulfonic acid in 0.1 M acetic acid, incubated at 20°C for 14 h; hydroxylamine (Glu, Asp), 10 mM hydroxylamine in 0.2 M Tris-hydrochloride buffer (pH 8.0), incubated at 37°C for 1 h; *p*-nitrophenol (Glu), 50 mM *p*-nitrophenol in 0.2 M Tris-hydrochloride buffer (pH 8.0), incubated at 37°C for 1 h; oxidation with hydrogen peroxide (Trp), 0.1 M H_2O_2 in 50% dioxane–0.2 M Na_2CO_3 buffer (pH 9.5), incubated at 37°C for 1 h; monoiodoacetic acid (Met, R—SH, His), 20-mM monoiodoacetic acid in 0.2 M potassium phosphate buffer (pH 7.0), incubated at 37°C for 1 h; *p*-chloromercuribenzoate (R—SH), 0.25 mM *p*-chloromercuribenzoate in 0.2 M Tris-hydrochloride buffer (pH 8.0), incubated at 37°C for 1 h; trinitrobenzene sulfonate (Lys, Arg), 1 mM trinitrobenzene sulfonate in 0.2 M sodium borate buffer (pH 9.5), incubated at 37°C for 2 h; tyrosinase (Tyr), 200 U/ml of tryosinase in 0.05 M potassium phosphate buffer (pH 7.5), incubated at 37°C for 1 h; acetylation (Lys, Arg, Tyr, R—SH), acetic anhydride (final 6%) in 50% saturated sodium acetate, incubated at 0°C for 1 h; photooxidation with proflavine (Trp, Met), 1 mM proflavine in 0.2 M sodium acetate buffer (pH 5.5), incubated at 20°C for 12 h under photoirradiation (15-W lamp, 5 cm in height); iodination (Tyr, R—SH, His), 5 mM I_2 and 20 mM KI in 0.2 M Na_2CO_3 buffer (pH 7.5), incubated at 37°C for 1 h; *N*-bromosuccinimide (Trp, R—SH, Met, Tyr), 1 mM *N*-bromosuccinimide in 0.2 M sodium acetate (pH 5.0), incubated at 37°C for 30 min; photooxidation with methylene blue (His, Met, Trp, Tyr), 0.05 mg of methylene blue per ml in 0.2 M Tris-hydrochloride buffer (pH 8.5), incubated at 37°C for 1 h with photoirradiation; diethyl pyrocarbonate (His, R—SH), 8 mM diethyl pyrocarbonate in 1 mM Tris-hydrochloride buffer (pH 7.5) containing 0.2 M NaCl and 10 mM $CaCl_2$, incubated at 37°C for 1 h; 2,3-butanedione (Arg), 20 mM 2,3-butanedione in 1 M NaCl–0.05 M sodium borate buffer (pH 7.5), incubated at 37°C for 1 h.

[c] Residual activity was calculated by defining the residual activity of control incubation (buffer alone) in the same buffer as 100%.

14468 has a distinct affinity for both of the moieties. In addition, esterase 14468 also hydrolyzed *p*-nitrophenyl acyl esters comparable with its Tween-hydrolyzing activity (Fig. 4). Similar nonspecific esterase activity on *p*-nitrophenyl acyl esters including PNPA has been observed for Tween-hydrolyzing acylesterases from *P. aeruginosa* (21) and triacylglycerol lipase from *M. phlei* (23).

Esterase 14468 hydrolyzed Tween 20 and Tween 80 with apparent K_m values of 19 and 6.6 mM, respectively. These K_m values are higher than those of long-chain acyl esterase from *P. aeruginosa* (15 μM for Tween 80) (21), thioesterase from *M. smegmatis* (9 μM for palmitoyl CoA) (39), and rat liver mitochondrial phospholipase A_2 (0.6 mM) (8) but on a level similar to those of heparin-released triglyceride lipase from rat liver (1.3 mM) (4), phospholipase from *Saccharomyces cerevisiae* (1.9 mM for dipalmitoyl phosphatidylcholine) (38), Triacylglycerol lipase from *M. phlei* (8.8 mM for tripalmitin) (23), and extracellular and membrane-bound lipase of *B. subtilis* (0.5 to 22 mM for various phospholipids and glycerides) (16). The K_m for PNPA hydrolysis by esterase 14468 was 0.25 mM and much lower than the K_m values for Tween hydrolysis.

Fe and Cu ions proved to be inhibitory to the activity of esterase 14468, and it is noteworthy that Fe^{2+} markedly accelerated the heat inactivation of esterase 14468. None of the chelating agents so far tested inactivated esterase 14468. Thus, esterase 14468 is thought not to be a metalloenzyme. In this respect, esterase 14468 differs from rat liver mitochondrial phospholipase A_2, which is inactivated by EDTA (8), and from rat liver lysosomal lipase (30), rat hepatic lipase (15), rat liver cholesterol esterase (33), and membrane-bound lipase from *B. subtilis* (16), which require divalent metal ions such as Ca^{2+} and Mg^{2+} for expression of their activity or are markedly activated by these metal ions. Esterase 14468 was highly resistant to phenylmethylsulfonyl fluoride, eserine, 5,5'-dithiobis(2-nitrobenzoic acid) (sulfhydryl inhibitor as *p*-chloromercuribenzoate and mercuric chloride), and sodium fluoride, all potent inhibitors of certain types of carboxylic ester hydrolases (2, 13, 28, 32). Similar resistance to these agents has been reported for intracellular esterase from *B. subtilis* (24), but this enzyme is sensitive to

sulfhydryl inhibitor, thus differing from esterase 14468.

Chemical modifications of tryosine residues of esterase 14468 by iodination or oxygenation with tryosinase caused an almost complete loss of enzymatic activity, and modifications of amino acid residues with an NH_2 group (lysine and arginine) by trinitrobenzene sulfonate, acetic anhydride (acetylation), and 2,3-butanedione (specific to arginine residue) also depleted most of the esterase activity. Moreover, the activity of esterase 14468 was completely suppressed by photooxidation with methylene blue and by treatment with diethyl pyrocarbonate, which are largely directed to histidine or other amino acid residues having sulfhydryl groups. Inactivation of esterase 14468 by photooxidation with proflavine suggests some role of tryptophan and methionine residues in Tween 80-hydrolyzing mechanisms by the esterase, provided that two amino acid residues actually do collaborate. Thus, it may be concluded that tryrosine, histidine, arginine, and presumably tryptophan and methionine residues play important roles in the expression of enzymatic activity of esterase 14468 to hydrolyze Tween 80 and also other polyoxyethylene acyl esters. Esterase 14468 differs from rat adipose tissue monoacylglycerol-hydrolyzing enzyme (32) and rat pancreas nonspecific lipase (2), which are most sensitive to *p*-chloromercuribenzoate or *p*-hydroxymecuribenzoate.

Some microbial lipases such as that from *Aspergillus niger* can synthesize various esters from alcohols and fatty acids (22), and some mammalian esterases have an inherent aldehyde dehydrogenase activity (18) but the esterase 14468 had no such activity (data not shown). Moreover, preliminary experiments showed that esterase 14468 could hydrolyze certain lipid components of *M. smegmatis* (such as glycerophospholipid-like substances) at a rate much slower than that for Tween hydrolysis (unpublished observations). Further studies on the true function of esterase 14468 in the metabolism of *M. smegmatis* are under way.

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