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Purification and Characterization of the Lipase from *Acinetobacter* sp. B2

Sung-Hwa Sohn[1,2] **& Kyeong-Ryang Park**[1]

[1]Department of Microbiology, Hannam University, 133 Ojung-dong, Taeduk-Ku, Taejon 306-791, Korea
[2]Department of Biochemistry & Molecular biology, Korea University Medical School, Seoul 136-701, Korea
Correspondence and requests for materials should be addressed to K.-R. park (Krpark@hannam.ac.kr).

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Abstract

Industrial development has increase consumption of crude oil and environmental pollution. A large number of microbial lipolytic enzymes have been identified and characterized to date.
To development for a new lipase with catalytic activity in degradation of crude oil as a microbial enzyme, *Acinetobactor* sp. B2 was isolated from soil samples that were contaminated with oil in Daejon area. *Acinetobactor* sp. B2 showed high resistance up to 10 mg/mL unit to heavy metals such as Ba, Li, Al, Cr, Pb and Mn. Optimal growth condition of *Acinetobactor* sp. B2 was confirmed 30°C.
Lipase was purified from the supernatant by *Acinetobactor* sp. B2. Its molecular mass was determined to the 60 kDa and the optimal activity was shown at 40°C and pH 10. The activation energies for the hydrolysis of *p*-nitrophenyl palmitate were determined to be 2.7 kcal/mol in the temperature range 4 to 37°C. The enzyme was unstable at temperatures higher than 60°C. The Michaelis constant (K_m) and V_{max} for *p*-nitrophenyl palmitate were 21.8 μM and 270.3 μM min^{-1} mg of $protein^{-1}$, respectively. The enzyme was strongly inhibited by Cd^{2+}, Co^{2+}, Fe^{2+}, Hg^{2+}, EDTA, 2-Mercaptoethalol.
From these results, we suggested that lipase purified from *Acinetobactor* sp. B2 should be able to be used as a new enzyme for degradation of crude oil, one of the environmental contaminants.

Keywords: lipase, *Acinetobactor* sp. B2, purification

Industrial development has increase consumption of crude oil and environmental pollution. Generally, crude oil includes aromatic hydrocarbon, aliphatic hydrocarbon, BTEX (benzene, toluene, ethylbenzene, xylene) and heavy metals. It has long been recognized that metals in the ground water, marine and soil environment have a particular significance in ecotoxicology since they are highly persistent. Lipophilicity of a chemical is a strong determinant of the risk for biomagnification. Biomagnification (and the related process bioaccumulation) refers to the transfer of a chemical from food to an organism, resulting in a higher concentration in the organism than in its diet. The result may be a concentration of the chemical as it moves up the food-chain. This will moderate the harmful effects of crude oil at higher trophic levels[1-4].

Concern should be given to microbial degradation of crude oil. A large number of microbial lipolytic enzymes have been identified and characterized to date. These lipolytic enzymes, mainly lipases and esterases, belong to the general class of carboxylic ester hydrolases (EC 3.1.1), but differ in substrate specificity and type of enzyme kinetics. All of these enzymes contain a catalytic triad that usually consists of a Ser-His-Asp. The serine is embeded in the consensus sequence G-X_1-S-X_2-G at the active site, and ester hydrolysis is mediated by a nucleophilic attack of the active serine on the carbonyl of the substrate in a charge-relay system with the two other amino acid residues[5-8]. While lipases (triacylglycerol hydrolase; E.C. 3.1.1.3) act preferentially on emulsified substrates with long-chain fatty acids, the esterases (E.C. 3.1.1.1.) hydrolyze water soluble or emulsified esters with relatively short fatty acid chains[9-15]. Lipases are enzymes widely distributed in nature, which catalyse the hydrolysis of fatty ester bonds at oil water interfaces, and are also capable to catalyse the reverse reaction in organic media, with control of the water activity. With the rapid development of enzyme technology, many new potential biotechnological applications for lipase have been identified in the areas of detergent industry, oleochemical industry, food industry, organic synthetic industry, paper manufacturing industry, biosurfactant synthesis, cosmetics and pharmaceuticals[9,16,17].

Microbial lipases are diversified in their enzymatic properties and substrate specificity, which improve their biotechnological importance and justify the search for new sources of this type of enzymes. The development of new uses for lipases as applied catalysts can be fostered by their purification and characterization. To develop for a new lipase with catalytic activity in

Table 1. Susceptibility of *Acinetobacter* sp. B2 to various heavy metals

Heavy metal	Conc. (μg/mL)	Strain
		Acinetobacter sp. B2
Al	7,200	+
	8,000	−
Ba	30,000	+
	65,000	+
	70,000	−
Cd	10	+
	50	−
Co	75	+
	100	−
Cr	1,600	+
	3,000	−
Cu	100	+
	200	−
Hg	10	+
	50	−
Li	12,800	+
	25,600	−
Mn	1,600	+
	6,400	+
	8000	+
	12,800	−
Ni	250	+
	300	−
Pb	400	+
	1,200	+
	1,500	+
	1,600	−
Zn	300	+
	400	−

+, Growth; −, No growth; W, Weak growth.

degradation of crude oil as a microbial enzyme, this paper reports the purification and biochemical properties of an extracellular lipase, produced by *Acinetobactor* sp. B2.

Heavy Metal Susceptibility

We investigated the heavy metals-susceptibility of *Acinetobactor* sp. B2 was listed in Table 1. In routine examination was colony appearance.

Growth and Lipase Activity

Growth and product formation were studied in LB medium (Fig. 1). Lipase activity was observed in the culture supernatant. Lipase activity appeared after 6h cultivation and passed through a maximum of about 1.169U/mL (18h).

Substrate Apecificity of Lipase

We investigated the hydrolytic activity of *Acinetobactor* sp. B2 by using various substrates. In the plate assay, we tested the following compounds: the tributyrin ($C_{4:0}$), tricaproin ($C_{6:0}$), tricaprylin ($C_{8:0}$), tricaprin ($C_{10:0}$), trilaurin ($C_{12:0}$), trimistyrin ($C_{14:0}$), tripalmitin ($C_{16:0}$), Triolein ($C_{18:1\ (cis)-9}$), and triarachidin ($C_{20:0}$) (Fig. 2). *Acinetobactor* sp. B2 showed good activity with substrates; the highest activities were obtained with C_4, C_6, C_8, C_{10}, C_{12}, and C_{18} as substrates. The lowest activity was observed with C_{14} and C_{20} as substrates (data not shown).

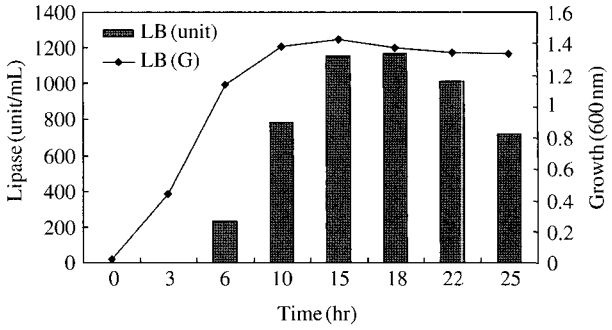

Fig. 1. Production of extracellular lipase of *Acinetobacter* sp. B2 growth in LB-broth. Extracellular lipase activity was measured with pNPP (p-nitrophenyl palmitate) as the substrate.

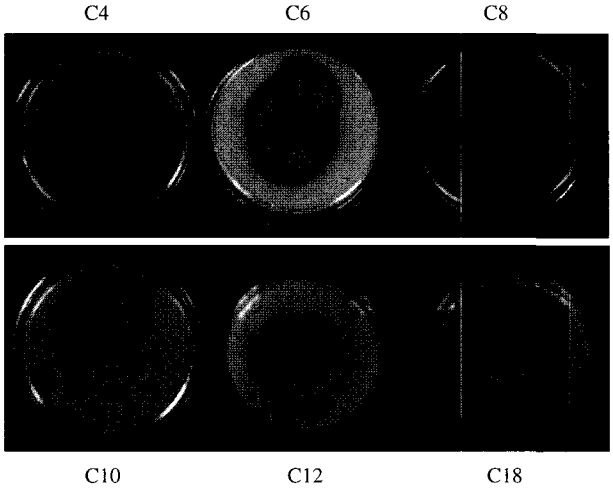

Fig. 2. Lipolytic activity with various substrates of *Acinetobacter* sp. B2. C4 to C18 indicated the triglycerides with the corresponding acyl chain length. The agar plates (2xYT) contained 1% substrate and were incubated for 2 days at 30°C in *Acinetobacter* sp. B2.

Lipase Purification

The *p*-nitrophenyl palmitate as the substrate, a Lipase specific activity of 1352.1 mmol/min/mg of

Table 2. Purification step of lipase from *Acinetobacter* sp. B2

Fraction	Total protein (mg)	Total activit[a] (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
Crude extract[b]	179.7	242953	1352.1	100	1
$(NH_4)_2SO_4$ precipitate	7.5	11657.7	1556.5	4.8	1.2
DEAE-toyopearl 650 M	0.107	550.3	5143.3	4.7	3.3
Sephadex-G200	0.003	67.8	22595.1	0.6	14.5

[a]Lipase activity was measured by using pNPP as the substrate, [b]Supernatant from a 1-liter culture grown for 18h in LB medium.

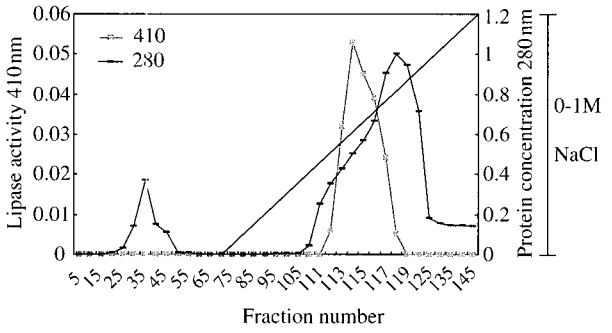

Fig. 3. Column chromatographic purification of *Acinetobacter* sp. B2 lipase from culture supernatant. Activity and absorbance were measured for each fraction. Ion-exchange chromatography was performed with a DEAE-Toyopearl 650 M column, and proteins were eluted in a 600 mL 50 mM Tris-HCl (pH 8.0).

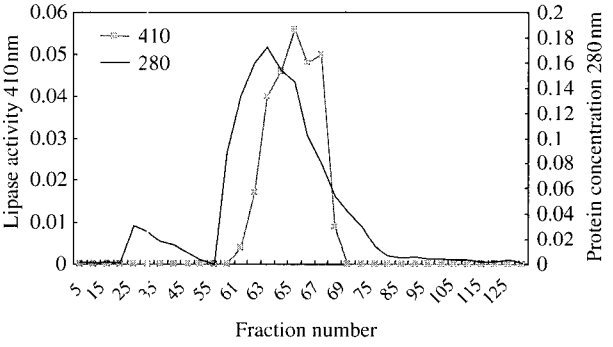

Fig. 4. Chromatography of pooled lipase fractions from DEAE-Toyopearl 650 M on DEAE-Sepharose column. Proteins were eluted in a 200 mL 50 mM Tris-HCl (pH 8.0).

protein was obtained when the organism was grown in LB medium (Table 2). In our attempt to purify lipase, the cell free supernatant, was subjected to ammonium sulfate precipitation, ion exchange chromatography on DEAE-Toyopearl 650 M column followed by gel filtration on Sephadex G-200 column. The lipase was finally purified 14.5-fold (Table 2; Figs. 3 and 4). Lipase was purified from culture

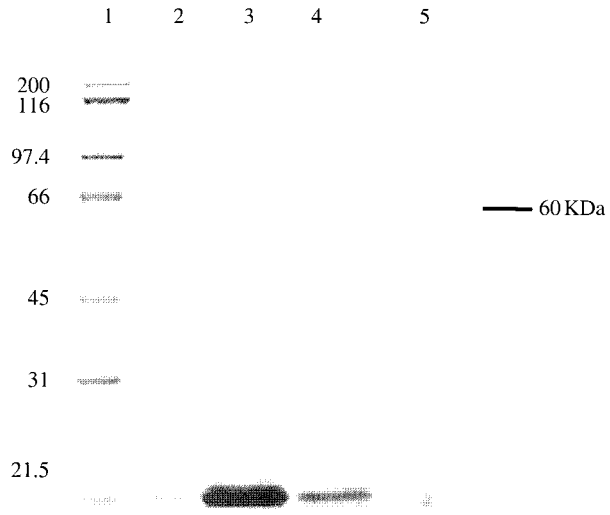

Fig. 5. Coomassie blue-stained SDS-polyacrylamide gel after electrophoresis of lipase samples. Lanes: 1, marker proteins; 2, Crude extract; 3, $(NH_4)_2SO_4$-precipitated protein; 4, the preparation after DEAE-toyopearyl 650 M chromatography; 5, the preparation after Sephadex G-200 chromatography.

supernatant of *Acinetobactor* sp. B2, as indicated in Materials, and was revealed on SDS-PAGE as a molecular mass of 60 KDa (Fig. 5).

Lipase Characterization

The optimal pH and temperature were determined in the range from pH 2 to 12 and 10 to 70°C, respectively. The optimal pH and temperature were pH 10 and 40°C, respectively (Figs. 6 and 7). The activation energy required for the hydrolysis of pNPP catalyzed by lipase. It was about 2.7 kcal/mol at 4 to 37°C. The Michaelis constant (K_m) was 21.8mM pNPP, and the maximal velocity (V_{max}) was 270.3 $\mu Mmin^{-1}mg^{-1}$. For the determination of thermal stability, temperatures of 30, 40, 50, 60 and 70°C were used as samples were incubated for 60 min, with measurement of residual activity at approximate intervals (Fig. 8). The enzyme was stable at 50°C for at least 20 min. The effects of metal ions on the lipases were evaluated as shown in

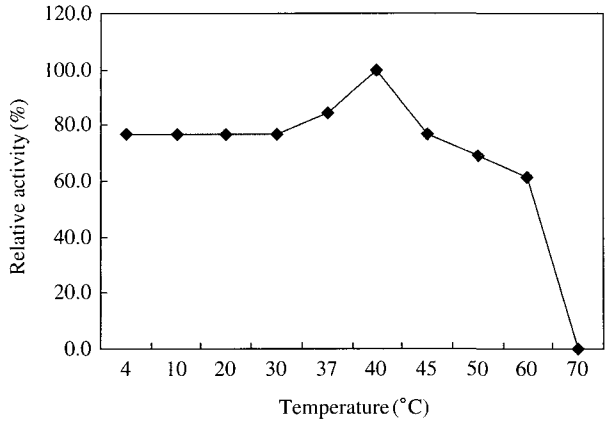

Fig. 6. Effect of temperature on the activity of lipase. The enzyme was incubated with a mixture containing 20 mM phosphate buffer (pH 7.25), and 8 μM p-nitrophenyl palmitate at various temperatures for 10 min, and p-nitrophenol formed was measured. The value obtained at 40°C was taken as 100%.

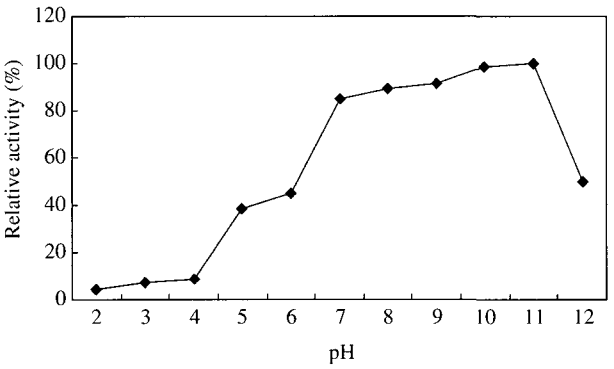

Fig. 7. Effect of pH on the activity of lipase. The activity was measured at various pH values, and presented as a percentage of the maximum activity, taken as 100%. Used buffer (final concentration, 20 mM) were glycine-HCl (pH 2.2 to 3.5); sodium acetate (pH 3.5 to 5.5); Tris-malate (pH 5.5 to 7.0); Tris-HCl (pH 7.5 to 9.0); and glycine-NaOH (pH 9.5 to 12).

Table 3. In the presence of Li^{2+} (5 and 10 mM), Ni^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+} and Ba^{2+} (5 mM), the activities of lipases were enhanced to approximately 109-165%. The lipase activities were decreased by 10 mM $CdCl_2$, $CoCl_2$, $HgCl_2$, NaN_3.

Discussion

Growth and Lipase Activity

Lipase activity appeared after 6 h cultivation and passed through a maximum at 18 h. *Acinetobactor* sp. B2 growth increased while the lipase activity decreased. This lower activity was probably due to the presence of proteases in the culture. Similar results were reported by Berto *et al.* and Bompensieri *et al.*[18,19].

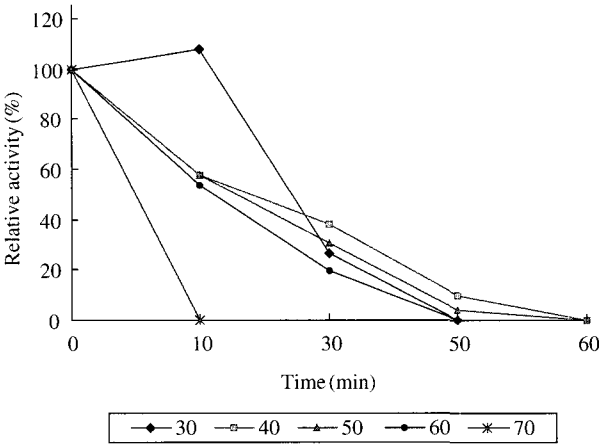

Fig. 8. Thermostabilities of lipase. The enzyme solution was incubated at 30, 40, 50, 60, 70°C for various times in the absence of substrate. The remaining activity was determined by adding 8 μM p-nitrophenyl palmitate. The activity of the enzyme at time zero was used as 100%.

Table 3. Effects of lipase by various compounds

Compound	Remaining activity (%)[a] at a concn (mM) of:	
	5.0	10
Chelating agent		
EDTA	33	30
Reducing agent		
2-Mercaptoethanol	54	8
Metal ions		
$NiCl_2 \cdot 6H_2O$	142	58
$CaCl_2$	111	61
$CdCl_2$	24	26
$LiCl_2$	134	109
$CoCl_2$	47	49
$FeCl_2$	141	36
$MnCl_2$	165	68
$HgCl_2$	96	24
$BaCl_2$	143	76
NaN_3	80	77

[a]Lipase (0.001 mg/mL) was incubated in 20 mM Tris-Cl buffer (pH 8.0) containing each compound at 37°C for 1hrs. Remaining activity was determined with 8 μM *p*-nitrophenyl palmitate at 37°C and expressed as the percent of the control value (with no addition).

Lipase Purification

The majority of the lipase activity was observed in

the culture supernatant, and only a small amount was cell associated. A typical procedure for purification of the lipase is summarized in Table 2. The yield of protein was 0.003 mg from 1L of culture; the specific activities were 22,595 U/mg. By this purification procedure, the lipase was purified 14.5-fold, with a recovery of 0.6%. A 14.5-fold purification was a little higher than the extracellular enzymes of orher organisms. For instance, results were obtained for an extracellular lipase from *Alternaria brassicicola* (3.45-fold purification)[18].

Lipase Characterization

The optimal pH for lipase activity was 10.0 (Fig. 7). The activity was reduced drastically at pH below 4.0 and lost 76% of the maximal activity at pH 10.0. The optimal pH value of lipase is similar to those reported for other microorganisms, such as oil-degrading bacterium *P. pseudoalcaligenes* F-111 (pH 6.0-10.0)[20]. The activation energy of an enzyme reaction reflects the catalytic efficiency of the enzyme. Low activation energy is due to the high catalytic efficiency of the enzyme. We determined the activation energy required for the hydrolysis of pNPP catalyzed by lipases. It was about 2.7 kcal/mol at 4°C to 37°C, and constant over the assay below 37°C. These features suggest that lipase does not undergo structural changes in this temperature range, although at higher temperatures lipase became irreversibly inactive. At temperatures above 45°C, enzyme activity fell to a inactivation energy of 12.8 kcal/mol. A low level of activation energy of lipase is comparable to the value of 8.63 kcal/mol (*Staphylococcus haemolyticus*)[11], 12 kcal/mol (Antarctic bacteria)[11], 25 kcal/mol (Mesophilic *Pseudomonas aeruginosa*)[21]. The initial velocity of lipase was determined in Tris-HCl buffer (pH 8.0) at 37°C over the substrate concentration range of 0.8-12 μM pNPP. A Lineweaver-Burk plot showed a linear response over this concentration range. The Michaelis constant (K_m) was 21.8 μM pNPP, and the maximal velocity (V_{max}) was 270.3 $\mu Mmin^{-1}mg^{-1}$. The K_m value of 21.8 μM determined for the purified lipase is lower than the value of 2.2 mg/mL (*Bacillus* sp. RSJ-1)[16]. The enzyme was stable at 50°C for at least 20 min. The enzyme was incubated with various compounds that may inhibit the enzyme, and the remaining activity was measured with pNPP as the substrate at 37°C. According to Zaliha *et al.*[22], nearly one-third of all known enzymes require the presence of metal ions for catalytic activity. This group of enzymes includes the metal enzymes, which contain tightly bound metal ion cofactors, most commonly transition metal ion such as Fe^{2+}, Fe^{3+}, Cu^{2+}, Mn^{2+}, and Zn^{2+}. The effect of metal ions and reducing, chelating agents was tested at 20 mM Tris-HCl buffer at pH 8.0 (Table 3). Lipase was completely inhibited by EDTA, 2-Mercaptoethanol, Cd^{2+}, Co^{2+} and Hg^{2+}, after 1h incubation. This suggested that lipase is a metal activated enzyme (metalloenzyme). In this group of enzymes, the ions often play a structural role rather than a catalytic one. The ions bind to the enzyme and change the conformation of the protein to counter greater stability to the enzyme[20, 22].

In summary, the activation energies for the hydrolysis of *p*-nitrophenyl palmitate were determined to be 2.7 kcal/mol in the temperature range 4 to 37°C. The enzyme was unstable at temperatures higher than 60°C. The Michaelis constant (K_m) and V_{max} for *p*-nitrophenyl palmitate were 21.8 μM and 270.3 $\mu M\ min^{-1}$ mg of $protein^{-1}$, respectively. The enzyme was strongly inhibited by Cd^{2+}, Co^{2+}, Fe^{2+}, Hg^{2+}, EDTA, 2-Mercaptoethalol. From these results, we suggest that lipase purified from *Acinetobactor* sp. B2 was able to be used as a new enzyme for degradation of crude oil, one of the environmental contaminants.

Methods

Strains and Growth Condition

The *Acinetobacter* sp. B2 was isolated in our laboratory from soil that was contaminated with waste lubricating oil at garages and gas stations in Taejon city. It was grown in Luria-Bertani (LB) medium [1% tryptone, 0.5% yeast extract, and 0.5% NaCl (pH 7.4)] or 2xYT [1.6% tryptone, 1% yeast extract, and 0.5% NaCl (pH 7.4)]. For plates, medium was solidified with 1.8% (wt/vol) agar. *Acinetobacter* sp. B2 was grown at 30°C.

Characterization and Biochemical Test

The *Acinetobacter* sp. B2 was tested for heavy metal susceptibility by rich media contain heavy metals. The following heavy metals were tested: $BaCl_2$, $CdCl_2$, $CoCl_2$, $CrCl_3$, $CuSO_4$, $HgCl_2$, $ZiCl_2$, $MnSO_4$, $7H_2O$, $NiCl_2$, $Pb(NO_3)_2$, and $ZnSO_4$. All tests were read after incubation at 37°C for 2-3day.

Lipase Detection and Characterization

Three types of lipase indicator plates were used. (i) For tricaprylin plates, tricaprylin (1%, vol/vol) was added to 2xYT agar. Lipase production on these plates was indicated by a zone of clearance around the colonies. (ii) For Egg-yolk plates, 1.5% egg-yolk emulsion, 0.3% beef extract, 0.5% peptone and 1% NaCl were added to 1.8% agar. Lipase production on these plates was indicated by turbidity halo around

the colonies. (iii) For olive oil plates, 2% olive oil, 0.04 mM rhodamine B, 0.3% beef extract, 0.5% peptone and 1% NaCl were added to 1.8% agar. Production of lipase on these plates was detectable only after at least 1days of incubation and is detected by the formation of a orange colored fluorescent halo (under UV light at 350 nm) around the colonies. For determination of the substrate specificity, the following compounds were used: tributyrin ($C_{4:0}$), tricaproin ($C_{6:0}$), tricaprylin ($C_{8:0}$), tricaprin ($C_{10:0}$), trilaurin ($C_{12:0}$), trimistyrin ($C_{14:0}$), tripalmitin ($C_{16:0}$), Triolein ($C_{18:1\ (cis)-9}$), and triarachidin ($C_{20:0}$). The substrates were emulsified with sonifier (output watts 14W; SONICS & MATERIAL INC., Vibra-CellTM, U.S.A.) for 10 min.

General Protein Techniques

Protein were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% polyacrylamide resolving gel. For estimation of the molecular weight, myosin (M_r, 200,000), β-galcatosidase (M_r, 116,250), phosphorylase b (M_r, 97,400), serum albumin ovalbumin (M_r, 66,200), ovalbumin (M_r, 45,000), carbonic anhydrase (M_r, 31,000), trypsin inhibitor (M_r, 21,500), lysozyme (M_r, 14,400) and aprotinin (M_r, 6,500) were used as standard proteins. Proteins were visualized in the gels by staining with Coomassie brilliant blue. Protein concentrations were measured according to Bradford [23] with bovine serum albumin as a standard.

Enzyme Purification

Bacterial cells from a 1-L culture were removed by centrifugation, and protein in the supernatant was brought to 15% saturation with ammonium sulfate. After being stirred for 30 min, the solution was centrifuged at 6,000 g for 15 min. The resultant supernatant was brought to 40% saturation with ammonium sulfate. After being stirred for 30 min, the solution was again centrifuged at 6,000 g for 30 min. The precipitate was redissolved in buffer A [50 mM Tris-HCl (pH 8.0)] and dialyzed overnight against the same buffer. The dialyzed enzyme solution was lyophilized and then dissolved in buffer A. The concentrated enzyme solution was applied to a diethylaminoethyl (DEAE)-toyoperarl 650 M column (2.5 × 20 cm) equilibrated with buffer A. Protein was eluted applying a linear gradient of 0-1 M NaCl in buffer A, by using a flow rate of 0.5 mL/min. The active fractions were collected and lyophilized. Lipase-containing fractions were pooled and lyophilized. This fraction was redissolved in buffer A and dialyzed overnight against the same buffer. The dialyzed enzyme solution was lyophilized. Protein was resuspended in 0.5 mL of buffer A and futher purified by gel filtration (Sephadex G-200; 1.5 × 100 cm), with a flow rate of 0.25 mL/min. purification was monitored by activity assays and SDS-PAGE. All protein purification steps were performed at 4°C.

Enzyme Assays and Characterization

The lipase activity was measured by Falk's method[24]. The molar absorption coefficient of p-nitrophenol (pNP) at pH 8.0 was determined to be 14,900 $M^{-1}cm^{-1}$. One unit of enzyme activity is defined as the amount of enzyme forming 1mmol pNP min^{-1}.

The optimal pH values for the lipase were determined to be in the pH range of 2.2-12 (20 mM glycine-HCl [pH 2.2-3.5], sodium acetate [pH 3.5-5.5], Tris-malate [pH 5.5-7.0], Tris-HCl [pH 7.5-9.0], glycine-NaOH [pH 9.5-12.0]), and optimal temperature values were studied in the range of 10-70°C with 8 mM p-nitrophenyl palmitate (pNPP) as the substrate. The stability against irreversible heat inactivation was analyzed by incubating the enzyme in 0.1 M phosphate buffer (pH 7.25) at 30, 50, 60, and 70°C, respectively. At appropriate intervals, aliquots were withdrawn, and the enzymatic activity was determined with *p*-nitrophenyl palmitate as a substrate. The residual activity was calculated by dividing the activity determined after incubation with that determined before incubation.

The effects of metal ions and inhibitors were tested against the *p*NPP hydrolyzing activity of purified lipase. For this assay, the enzyme was incubated with 5 mM, 10 mM inhibitor for 1 h at 37°C, respectively and the residual enzyme activity was measured.

For the kinetic analyses, the substrate concentration spanned the K_m value. The hydrolysis of the substrate with the enzyme followed Michaelis-Menten kinetics, and the kinetic parameters, K_m and V_{max}, were determined from a Lineweaver-Burk plot. K_m and V_{max} values for *p*-nitrophenyl palmitate were determined with the substrate concentration range from 0.8 to 12 μM. All experimental data are means of triplicate determinations.

Reference

1. Atlas, R.M. Effects of Temperature and crude oil composition on petroleum biodegradation. *Appl, Microbiol.* **30**(3), 396-403 (1975).
2. Jobson, A., Cook, F.D. & Westlake, D.W.S. *Microbial* Utilization of Crude Oil. *Appl, Microbiol.* **23**(6), 1082-1089 (1972).
3. Campbell *et al.* Mercury and other trace elements in a pelagic arctic marine food web (Northwater polynya,

Baffin bay). *Science of the Total Environment* (2005).
4. Monperrus *et al.* Determination of metal and organometal trophic bioaccumulation in the benthic macrofauna of the adour estuary coastal zone (SW France, Bay of Biscay). *J. Environ. Monit.* **7**, 693-700 (2005).
5. Kim, H.E. & Park, K.R. Purification and characterization of an Esterase from *Acinetobacter lwoffii* I6C-1. *Current Microbiology* **44**, 401-405 (2002).
6. Sullivan, E.R., Joseph, G.L. & Rita, R.C. Cloning and sequence analysis of the lipase and lipase chaperone-encoding genes from Acinetobacter calcoaceticus RAG-1, and redefinition of a proteobacterial lipase family and an analogous lipase chaperone family. *Gene* **230**, 277-285 (1999).
7. Li, X., Tetling, S., Winkler, U.K., Jaeger, K.E. & Benedik, M.J. Gene cloning, sequence analysis, purification, and secretion by *Escherichia coli* of an extracellular lipase from *Serratia marcescens. Appl. Environ. Microbiol.* **61**(7), 2674-2680 (1995).
8. Longshaw, C.M., Farrell, A.M., Wright, J.D. & Holland, K.T. Identification of a second lipase gene, *gehD*, in *Staphylococcus epidermidis*: comparison of sequence with those of other staphylococcal lipase. *Microbiology* **146**, 1419-1427 (2000).
9. Sommer, P., Bormann, C. & Gotz, F. Genetic and Biochemical characterization of a New Extracellular Lipase from *Streptomyces cinnamomeus, Appl, Environ. Microbiol.* **63**, 3553-3560 (1997).
10. Nadini, M., Lang, D.A., Liebeton, K., Jaeger, K.E. & Dijkstra, B.W. Crystal structure of *Pseudomonas aeruginosa* lipase in the open conformation. *J. Biologycal Chemistry* **275**, 31219-31225 (2000).
11. Oh, B.C., Kim, H.K., Lee, J.K., Kang, S.C. & Oh, T.K. *Staphylococcus haemolyticus* lipase: biochemical properties, substrate specificity and gene cloning. *FEMS. Microbial. Lett.* **179**, 385-392 (1999).
12. Quyen, D.T., Schmidt Dannert, C. & Schmid, R.D. High-level formation of active *Pseudomonas cepacia* lipase after heterologous expression of the encoding gene and its modified chaperone in *Escherichia coli* and rapid in vitro refolding. *Appl, Environ. Microbiol.* **65**, 787-794 (1999).
13. Yang, J., K.obayashi, K., Iwasaki, Y., Nakano, H. & Yamane, T. In vitro analysis of roles of a disulfide bridge and a calcium binding site in activation of *Pseudomonas* sp. Strain KWI-56 lipase. *J. Bacteriol.* **182**(2), 295-302 (2000).
14. Kok, R.G., Clara, B.N., Rodrigo, H.G., Inge, M.N. & Klaas, J.H. Physiological factors affecting prduction of extracellular lipase (LipA) in *Acinetobacter calcoaceticus* BD413: Fatty acid repression of lipA expression and degradation of *LipA. J. Bacteriol.* **178**, 6025-6035 (1996).
15. Muralidhar *et al.* Understanding lipase stereoselectivity. *J. Microbial Biotech. Rev.* **18**, 81-97 (2002).
16. Sharma, R., Soni, S.K., Vohra, R.M., Gupta L.K. & Gupta, J.K. Purification and characterization of a thermostable alkaline lipase from a new thermophilic *Bacillus* sp. RSJ-1, *Process Biochemistry* **37**, 1075-1084 (2002).
17. Kriger, N. *et al.* Purification of a *Penicillum citrinum* lipase by chromatographic processes. *Bioprocess Engineering* **20**, 59-65 (1999).
18. Berto, P., Belingheri, L. & Dehorter, B. Production and purification of a novel extracellular lipase from *Alternaria brassicicora. Biotechnology Letters.* **19**, 533-536 (1997).
19. Bompensieri *et al.* Purification of a lipase from Acinetobacter calcoaceticus AAC323-1 by hydrophobic-interaction. Methods **23**, 77-81 (1996).
20. Lin, S.F., Chiou, C.M., Yeh, C.M. & Tsai, Y.C. Purification and partial characterization of an alkaline lipase from *Pseudomonas pseudoalcaligenes* F-111. *Appl. Environ. Microbiol.* **62**, 1093-1095 (1996).
21. Choo, D.W., Kurihara, T., Suzuki, T., Soda, K. & Esaki, N. A cold-adapted lipase of an Alaskan Psychrotroph, *Pseudomonas* sp. strain B11-1: gene cloning and enzyme purification and characterization. *Appl. Environ. Microbiol.* **64**, 486-491 (1998).
22. Zaliha, R.N., Rahman, R.A., Baharum, S.N., Basri, M. & Salleh, A.B. High-yield purification of an organic solvent-tolerant lipase from Pseudomonas sp. Strain S5. *Anal. Biochem.* **341**, 267-274 (2005).
23. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254 (1976).
24. Falk, M.P.F., Sanders E.A. & Deckwer, W.D. Studies on the production of lipase from recombinant *staphylococcus carnosus. Appl. Environ. Microbiol.* **35**, 10-13 (1990).