Optimization of a Thermostable Lipase from Bacillus stearothermophilus P1: Overexpression, Purification, and Characterization

ARTICLE *in* PROTEIN EXPRESSION AND PURIFICATION · SEPTEMBER 2001

Impact Factor: 1.7 · DOI: 10.1006/prep.2001.1456 · Source: PubMed

5 AUTHORS, INCLUDING:

Boonyaras Sookkheo
Rangsit University
12 PUBLICATIONS **197** CITATIONS
SEE PROFILE

Shyi-Jou Chen
National Defense Medical Center
175 PUBLICATIONS **2,870** CITATIONS
SEE PROFILE

Protein Expression and Purification 22, 388–398 (2001)
doi:10.1006/prep.2001.1456, available online at http://www.idealibrary.com on IDEAL®

Optimization of a Thermostable Lipase from *Bacillus stearothermophilus* P1: Overexpression, Purification, and Characterization

Supachok Sinchaikul,*,† Boonyaras Sookkheo,†,‡ Suree Phutrakul,* Fu-Ming Pan,† and Shui-Tein Chen†,[1]

**Department of Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai, 50200, Thailand; †Institute of Biological Chemistry, Academia Sinica, Taipei, 11529, Taiwan, Republic of China; and ‡Chemistry Unit, School of Science, Rangsit University, Patumthani, 12000, Thailand*

Received December 27, 2000, and in revised form March 26, 2001; published online July 17, 2001

An expression library was generated from a partial *Nco*I and *Hin*dIII digest of genomic DNA from the thermophilic bacterium, *Bacillus stearothermophilus* P1. The DNA fragments were cloned into the expression vector pQE-60 and transformed into *Escherichia coli* M15[pREP4]. Sequence analysis of a lipase gene showed an open reading frame of 1254 nucleotides coding a 29-amino-acid signal sequence and a mature sequence of 388 amino acids. The expressed lipase was isolated and purified to homogeneity in a single chromatographic step. The molecular mass of the lipase was determined to be approximately 43 kDa by SDS–PAGE and mass spectrometry. The purified lipase had an optimum pH of 8.5 and showed maximal activity at 55°C. It was highly stable in the temperature range of 30–65°C. The highest activity was found with *p*-nitrophenyl ester–caprate as the synthetic substrate and tricaprylin as the triacylglycerol. Its activity was strongly inhibited by 10 mM phenylmethanesulfonyl fluoride and 1-hexadecanesulfonyl chloride, indicating that it contains a serine residue which plays a key role in the catalytic mechanism. In addition, it was stable for 1 h at 37°C in 0.1% Chaps and Triton X-100.

Key Words: thermostable lipase; *Bacillus stearothermophilus*; cloning; sequence.

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3), which are widely distributed in animals, plants, and microorganisms, catalyze the hydrolysis of the ester bonds of triglycerides and long-chain fatty acids, generating free fatty acid, diglyceride, monoglyceride, and glycerol. They can also catalyze ester synthesis, transesterification, and interesterification in media containing a low concentration of water or in anhydrous organic solvents. Lipases are thus of particular importance in biotechnology because of diverse applications in the food industry, in biological detergents, in medical applications, in the enzymatic production of lipophilic fine chemicals, and, potentially, in waste treatment (1–5).

Recently, there has been considerable interest in the basic properties and industrial applications of thermostable lipases from mesophiles and thermophiles. Most thermostable lipases exhibit higher thermodynamic stability, both at elevated temperatures and in organic solvents, as a consequence of adaptation of the corresponding microorganisms to higher growth temperatures (5–12). Although thermostable lipases have many advantages, they are normally produced at low levels. Since the advent of protein engineering techniques, an increasing number of lipases have been commercially manufactured using recombinant bacteria and yeasts. Several thermostable lipases, such as those from *Pseudomonas fluorescens* SIK W1 (13), *Bacillus thermocatenulatus* (9), and *B. stearothermophilus* (14), have been produced by recombinant techniques for cloning and overexpression. To obtain additional potentially useful thermostable lipase, we were successful in isolating a number of thermophilic bacteria from a hot spring in

[1] To whom correspondence should be addressed. Fax: 886-2-27883473. E-mail: bcchen@gate.sinica.edu.tw.

1046-5928/01 $35.00

Chiang Mai, Thailand, that produce extracellular lipases. Among these is the bacterium *B. stearothermophilus* strain P1 that produces highly thermostable lipase and it also showed the highly stability on some organic solvents and detergents, which somewhat higher than the other lipases (15).

The present report concerns an extracellular thermostable lipase produced by a thermophilic bacterium, *B. stearothermophilus* strain P1, in which the ability of the native isolate to produce lipase is limited (16). So, P1 lipase production has been markedly increased by cloning and overexpression in *Escherichia coli* M15[pREP4] using pQE-60 as vector, and the enzyme has been purified and characterized for industrial application.

MATERIALS AND METHODS

Materials

Restriction enzymes were from New England BioLabs, Inc. (Beverly, MA) and T4-DNA ligase was from Serva Feinbiochemika (Heidelberg, Germany). Thermo Sequenase dye terminator cycle sequencing premix kit with Thermo Sequenase polymerase was from Amersham Life Science (U.S.A.). Lipase substrates were from Sigma (St. Louis, MO) and inhibitors were obtained from Roche Molecular Biochemicals (Germany). All other chemicals used were of analytical grade.

Bacterial Strains and Plasmid

B. stearothermophilus P1 was isolated from a hot spring at Chiang Mai, Thailand. *E. coli* M15[pREP4] was grown in LB2[2] medium (Scharlau) containing 25 μg/ml of kanamycin. The vector used for cloning and expression was pQE-60.

DNA Manipulation

Genomic DNA from *B. stearothermophilus* P1 was prepared using the method described by Marmur (17). Plasmid DNA was isolated using Wizard*Plus* SV Miniprep and Midiprep DNA purification systems kit (Promega). Extraction of chromosomal and plasmid DNA was performed using a gel extraction miniprep kit (Viogene). Competent *E. coli* M15[pREP4] cells were prepared using the Qiagen procedure (18).

Cloning of the Lipase Gene

Chromosomal DNA from *B. stearothermophilus* P1 was partially digested with *Nco*I and *Hin*dIII and inserted into the same restriction sites of pQE-60. After ligation, the recombinant DNA was transformed into *E. coli* M15[pREP4] and transformants selected on LB agar plates containing 100 μg/ml ampicillin, 25 μg/ml kanamycin, and 1%(w/v) tricaprylin. The colonies surrounded by a clear zone were selected. PCR amplification was used to check the inserted DNA.

Expression in E. coli

Expression in *E. coli* M15[pREP4] containing the recombinant plasmid was tested by preparation of cell lysates as follows. Cells were grown at 37°C to late log phase in 3 ml of LB broth containing 100 μg/ml ampicillin and 25 μg/ml kanamycin and then IPTG (MDBio Inc.) was added to a final concentration of 1 mM. After 3 h of growth, the cells were centrifuged at 6500*g* for 20 min, the pellet was resuspended in 50 ml of 20 mM Tris–HCl buffer, pH 8.5, containing 10 mM EDTA, and the cells were lysed by sonication. After centrifugation at 12,000*g* for 20 min, the supernatant was checked for lipase by SDS–PAGE and lipase assay.

Nucleotide Sequencing

The DNA sequence was determined by cycle sequencing using the Thermo Sequenase dye terminator cycle sequencing pre-mix kit with Thermo Sequenase polymerase (Amersham Life Science). The synthetic oligonucleotides, pQE-F (5′-GGCGTATCACGAGGCCCTTTCG-3′) and pQE-R (5′-CATTACTGGATCTATCAACAGG-3′), synthesized using the expression vector pQE-60 as template, were used as primers to sequence both strands. The nucleotide and amino acid sequence data were analyzed using the MacVector 6.5 program. Homology searches were performed against the sequences in the GenBank/EMBL/DDBJ databases using the BLAST program (19).

Lipase Purification

The cell pellet from a 4-liter culture was suspended in 50 ml of 20 mM Tris–HCl buffer, pH 8.5, containing 10 mM EDTA. After sonication, the cell lysate was centrifuged at 12,000*g* for 30 min, and then streptomycin sulfate (Sigma) was added to the supernatant to a final concentration of 1% (w/v) and the precipitate formed was removed by centrifugation at 12,000*g* for 15 min. The crude enzyme preparation was simultaneously partially purified and concentrated by ultrafiltration using a membrane with a molecular weight cutoff of 10,000 Da. The concentrated protein sample (5 ml) was purified by FPLC using strong anion-exchange chromatography on a Q HyperD10 prepacked column (3 × 15 cm, Biosepra). The column was equilibrated with 20 mM Tris–HCl buffer, pH 8.5, and eluted with a linear gradient of 0–1.0 M NaCl in the same buffer at a flow rate

[2] Abbreviations used: LB, Luria broth; IPTG, isopropyl-β-D-thiogalactopyranoside; *p*-NP, *p*-nitrophenyl ester; PDVF, polyvinylidene difluoride; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; Chaps, 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate.

of 1 ml/min. Each fraction was assayed for lipase activity. The lipase-containing fractions were pooled and dialyzed overnight against 20 mM Tris–HCl buffer pH 8.5. The purified lipase was checked for purity and molecular mass with SDS–PAGE and mass spectrometry (LCQ, Finnigan).

Lipase Assay

Unless otherwise stated, lipase activity was measured at 55°C.

Lipase activity was measured by titration of the free fatty acids released by hydrolysis of olive oil using the pH stat method (20). An olive oil emulsion was prepared by emulsifying 5 ml of olive oil in 495 ml of a solution of 13 mM NaCl, 0.7 mM $CaCl_2$, and 0.5%(w/v) gum arabic for 2 min at maximum speed in a Waring blender. After adjusting the pH of the substrate emulsion (50 ml) to 8.0 by addition of 0.01 N NaOH, 0.1 ml of enzyme solution was added and the rate of fatty acid release was measured at 55°C for 5 min using a pH titrator (718 Stat Titrino, Metrohm). One lipase unit is defined as the amount of enzyme releasing 1 _mol of fatty acid per minute.

Lipase activity was also assayed using the synthetic substrate, *p*-NP caprate (Sigma) (21, 22). Twenty microliters of lipase solution was added to 880 μl of reaction buffer (20 mM Tris–HCl buffer, pH 8.5, 0.1% gum arabic, and 0.2% sodium deoxycholate) and the reaction mixture was prewarmed to 55°C and then mixed with 100 μl of freshly prepared 8 mM *p*-NP caprate in isopropanol. The reaction mixture was incubated at 55°C for 2 min, and then the reaction was stopped by addition of 0.5 ml of 3 M HCl. After centrifugation, 333 μl of supernatant was mixed with 1 ml of 2 M NaOH and the absorbance at 405 nm was measured against an enzyme-free blank. One enzyme unit is defined as the release of 1 nmol of *p*-nitrophenol per milliliter per minute. Under the conditions described, the extinction coefficient of *p*-nitrophenol is $\varepsilon = 1.85$ liters $mmol^{-1}$ mm^{-1}.

Protein Determination

The protein concentration was measured spectrophotometrically at 280 nm or by using a dye-binding assay based on the method of Bradford (23).

Polyacrylamide Gel Electrophoresis

SDS–PAGE on a 12.5% polyacrylamide slab gel (25 mA per gel) was used to determine the purity and apparent molecular weight of the lipase by the method of Laemmli (24). The molecular mass of the lipase was calibrated by using a low-molecular-mass calibration kit (Pharmacia AB, Sweden) containing phosphorylase b (94 kDa), serum bovine albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

NH_2-Terminal Amino Acid Sequence Analysis

The purified lipase was separated by SDS–PAGE and electroblotted onto a PVDF membrane, as described by Matsudaira (25). The lipase band was cut out and analyzed by Edman degradation using an Applied Biosystems Model 492 procise sequencer (Applied Biosystems, Weiterstadt, Germany).

Effect of pH on Lipase Activity

To determine the optimal pH, enzymatic activity was assayed at 55°C at various pH values (4.0–11.0). The buffers used for the pH ranges of 4.0–6.0, 6.0–7.5, 7.0–10.0, and 9.0,11.0 were, respectively, 50 mM sodium acetate, 50 mM phosphate, 50 mM Tris–HCl, 50 mM Tris–glycine.

Effect of Temperature on Lipase Activity and Stability

To determine the effect of temperature, enzymatic activity was measured at 30, 40, 50, 55, 60, 65, 70, 75, 80, and 90°C in the usual assay at pH 8.5. Thermostability of the lipase was investigated by measuring the remaining activity after incubating the enzyme in 20 mM Tris–HCl buffer, pH 8.5, at various temperatures for times up to 15 h and then assaying a 0.1-ml sample at 55°C.

Substrate Specificity

Substrate specificities for different *p*-NP esters and triacylglycerols were determined by using the spectrophotometric assay (21, 22, 26). The *p*-NP esters between C2 and C18 were determined using *p*-NP–acetate, *p*-NP–butyrate, *p*-NP–caproate, *p*-NP–caprylate, *p*-NP–caprate, *p*-NP–laurate, *p*-NP–myristate, *p*-NP–palmitate, and *p*-NP–stearate as the synthetic substrate, and triacylglycerols between C2 and C22 were also determined using triacetin, tributyrin, tricaproin, tricaprylin, tricaprin, trilaurin, trimyristin, tripalmitin, tripalmitin, tripalmitolein, tristearin, tripetroselinin, triolein, trielaidin, trilinolein, trilinolenin, triarachidin, tri-11-eicosenoin, tribehenin, and trierucin. The highest activities of enzyme assay using the substrates were defined as the 100% level.

Effect of Metal Ions on Lipase Activity

Various metal ions ($CaCl_2$, $CuCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$, CsCl, LiCl, KCl, NaCl, and $FeSO_4$) at final concentrations of 1 and 10 mM were added to the enzyme in 20 mM Tris–HCl buffer, pH 8.5, and the solution was preincubated at room temperature for 5 min and then assayed for lipase activity. The lipase activity of

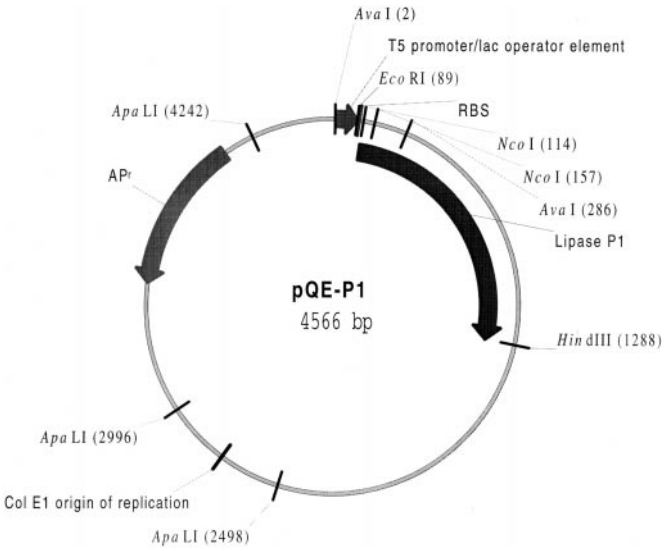

FIG. 1. Physical map of inserted lipase P1 after cloning of partial *Nco*I and *Hin*dIII fragments into pQE-60. The arrow indicates the region encoding the lipase gene and the direction of transcription.

the enzyme without added metal ion was defined as the 100% level.

Effect of Inhibitors on Lipase Activity

The effect of inhibitors on lipase activity was determined using DTT, 1-dodecanesulfonyl chloride, EDTA, 1-hexadecanesulfonyl chloride, _-mercaptoethanol, and PMSF at final concentrations of 10 mM. It was examined as a function of incubation time of enzyme with each inhibitor in 20 mM Tris–HCl buffer, pH 8.5, at 37°C for 5, 10, and 30 min. The enzyme/inhibitor mixture was then taken to assay the lipase activity. Enzyme solution without inhibitor was used as reference.

Effect of Detergents on Lipase Activity and Stability

This was determined by incubating the enzyme for 1 h at 37°C in 20 mM Tris–HCl buffer, pH 8.5, containing 0.1% (w/v) or 1% (w/v) of the detergents Chaps, SDS, sodium deoxycholate, Triton X-100, and Tween 20. Lipase activity was measured at the beginning and end of the incubation period. The activity of the enzyme preparation in the absence of detergent before incubation was defined as the 100% level.

RESULTS

Cloning and Overexpression in E. coli of the Lipase Gene from B. stearothermophilus P1

E. coli M15[pREP4] transformed with the vector containing the *B. stearothermophilus* P1 lipase coding sequence were selected by plating on LB agar containing 100 μg/ml ampicillin, 25 μg/ml kanamycin, and 1% (w/v) tricaprylin; colonies surrounded by a clear zone were selected and grown in the LB medium containing 100 μg/ml ampicillin, 25 μg/ml kanamycin. The recombinant plasmid DNA was isolated and amplified with the primers and shown to contain a 4.5-kb insert on agarose gel electrophoresis. This plasmid, designated as pQE-P1, was sequenced and shown to contain a 1.2-kb sequence coding for lipase. Its physical map is shown in Fig. 1. Overexpression of the cloned lipase P1 induced by IPTG addition resulted in a high expression of soluble lipase activity of 212×10^3 U/liter compared with 8.1 U/liter using *B. stearothermophilus* P1, i.e., a 26×10^3-fold increase. This high level of expression was confirmed by SDS–PAGE analysis (Fig. 2).

Nucleotide Sequence and NH_2-Terminal Amino Acid Sequence

The lipase gene from *B. stearothermophilus* P1 was cloned on a 1.2-kb *Nco*I/*Hin*dIII fragment into plasmid pQE-60. The nucleotide sequence of the gene, submitted to GenBank under Accession No. AF237623, revealed an open reading frame of 1254 bp encoding a 417-amino-acid polypeptide (Fig. 3), consisting of a 29-amino-acid signal sequence and a mature lipase of 388 amino acid residues, with a cleavage site between the two alanine residues at positions 29 and 30. This was confirmed by NH_2-terminal amino acid sequence analysis of purified lipase which showed that the first 15-amino-acid residues had the sequence A-S-L-R-A-N-D-A-P-I-V-L-L-H-G. These results show that the mature lipase lacks a signal peptide as a consequence of secretion across the outer membrane (27).

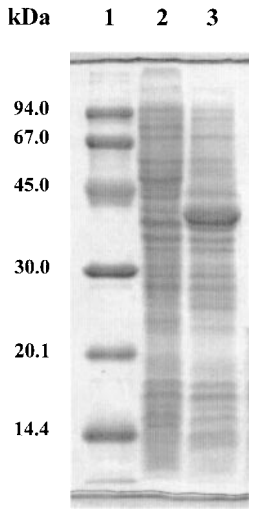

FIG. 2. SDS–PAGE shows IPTG induction of the cloned lipase from *B. stearothermophilus* P1. Lane 1, standard protein markers; lane 2, no added IPTG; lane 3, 1 mM IPTG.

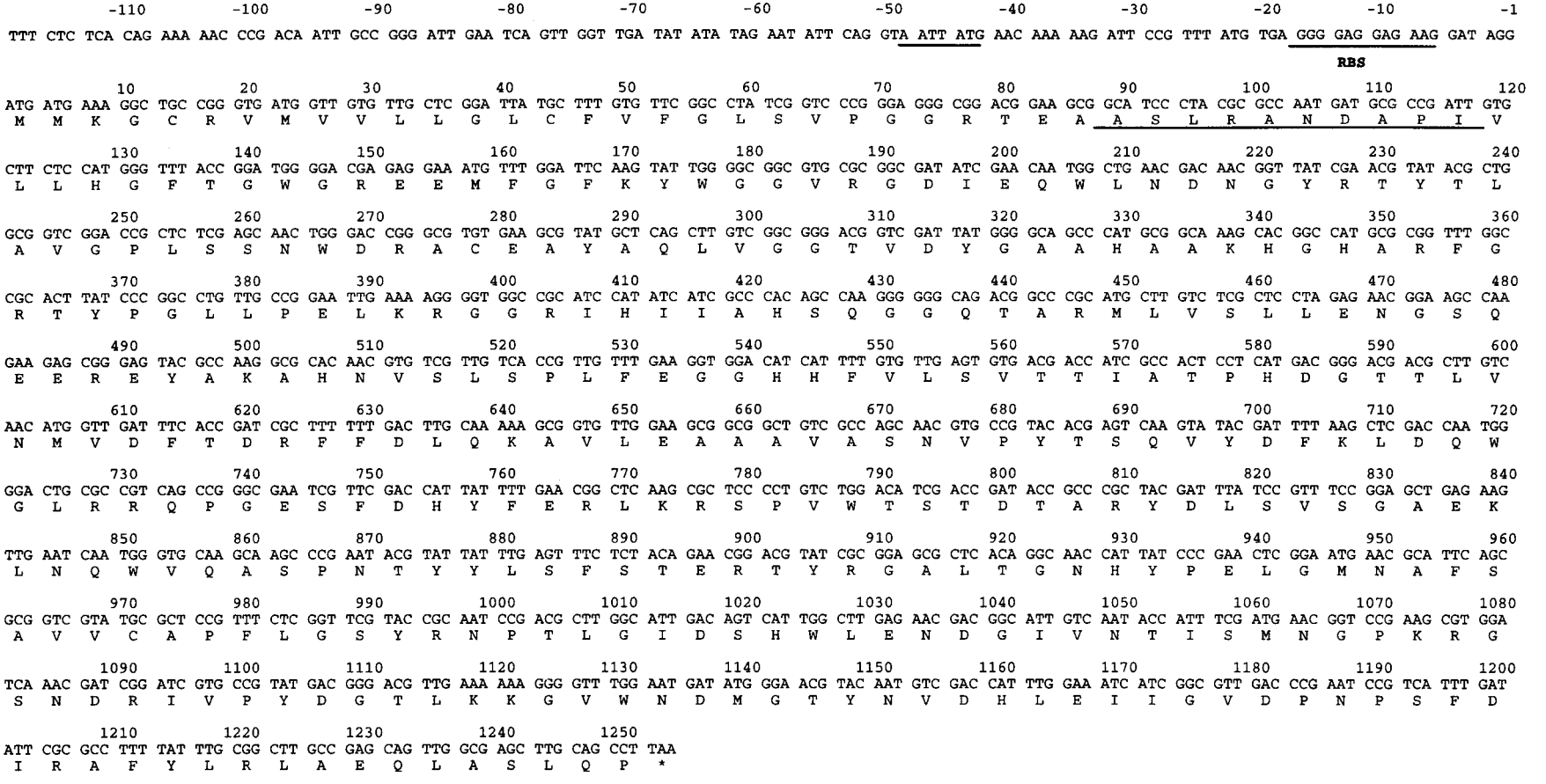

FIG. 3. Nucleotide sequence of the lipase gene from *B. stearothermophilus* P1 and its deduced amino acid sequence. The numbering of nucleotides starts at the 5′ end of the lipase gene and that of amino acids at the NH_2-terminus of the mature lipase. The putative −35, −10, and ribosomal binding sites (RBS) and stop codon (*) are shown. The NH_2-terminal amino acid sequence determined for the purified lipase P1 is underlined.

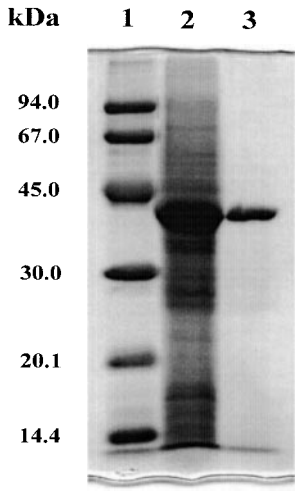

FIG. 4. SDS–PAGE of purified lipase from *B. stearothermophilus* P1. Lane 1, molecular weight markers; lane 2, crude extract; lane 3, Q HyperD10-purified lipase.

Purification of Lipase

The crude extract obtained by centrifugation of culture broth, sonication, and precipitation with 1% (w/v) streptomycin sulfate was concentrated and partially purified by ultrafiltration using a membrane with a molecular weight cutoff of 10,000 Da. The concentrated enzyme was then purified by a single ion-exchange chromatographic step by gradient elution from a strong anion exchanger (Q-HyperD10) using FPLC. Each fraction was assayed for lipase activity and the lipase pool prepared by selection of fractions with lipase activity. The pooled fractions gave a single band on SDS–PAGE with an apparent molecular mass of approximately 43 kDa (Fig. 4). The purity of the purified lipase and its molecular weight of approximately 43,209 Da were confirmed by mass spectrometer (Fig. 5). Both values for the molecular weight agree well with that of 43,203 Da calculated from the deduced amino acid sequence using MacVector sequence analysis software. The purification procedure is summarized in Table 1. The enzyme was

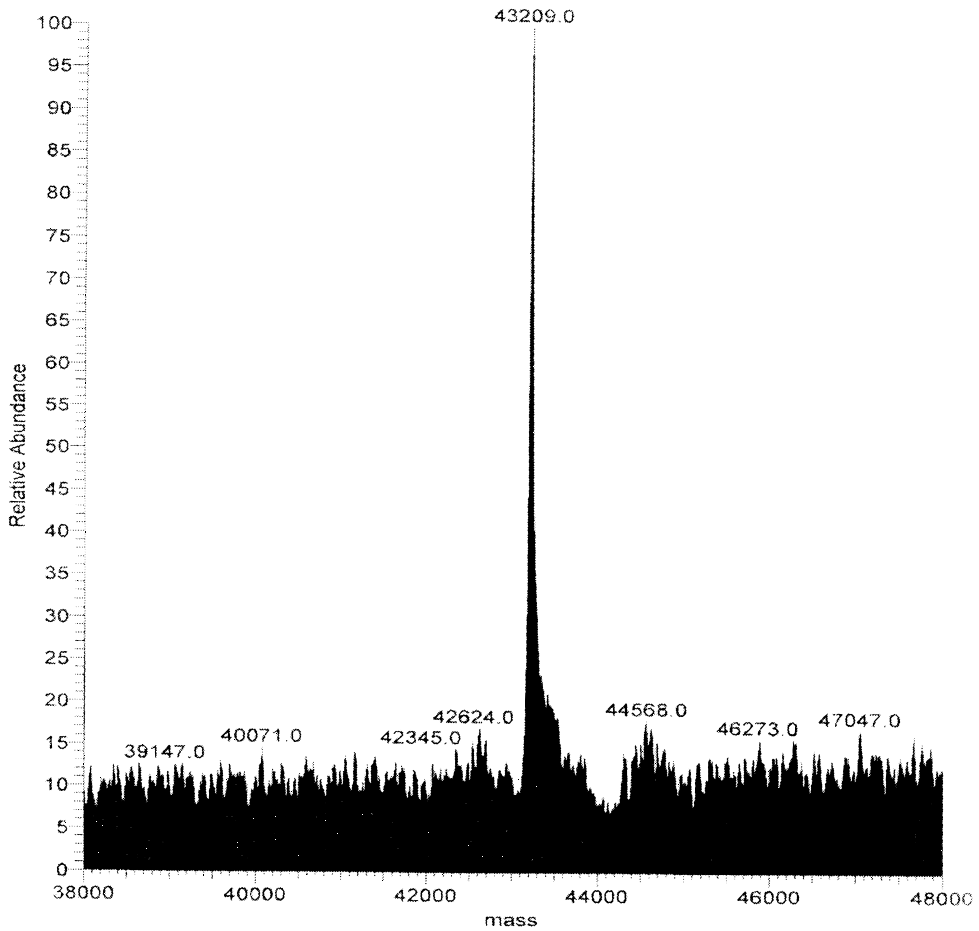

FIG. 5. Mass spectrometry of the purified *B. stearothermophilus* P1 lipase.

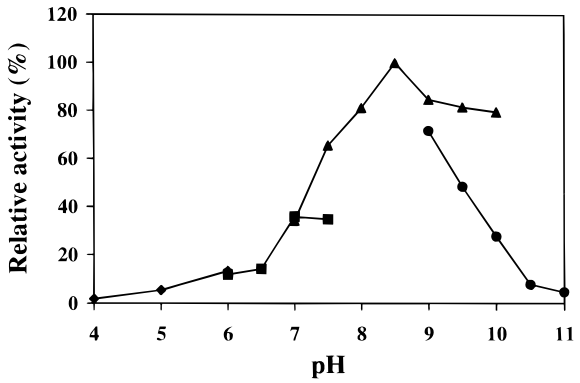

FIG. 6. Effect of pH on lipase activity. The purified lipase was assayed in various pH buffers, as described in the text. ♦, sodium acetate buffer; ■, phosphate buffer; ▲, Tris–HCl buffer; ●, Tris–glycine buffer.

purified 18-fold with a yield of 71% from the crude extract.

Effect of pH on Lipase Activity

The effect of pH on lipase activity at 55°C with *p*-NP–caprate as substrate was examined at various pH values. The enzyme was active in the pH range 7.5–10.0 and the optimal pH was shown to be 8.5 in 50 mM Tris–HCl buffer (Fig. 6). Consistently higher activity was observed with the Tris–HCl buffer than with sodium acetate, phosphate, and Tris–glycine buffer.

Effect of Temperature on Lipase Activity and Stability

To test the effect of temperature on lipase activity, assays were performed for 1 h at various temperatures. The lipase was most active in the temperature range 45–65°C, with maximal activity at 55°C (Fig. 7A). The thermostability of the enzyme was examined by measuring the residual activity at different times of incubation for up to 15 h at various temperatures at pH 8.5. After incubation for 1 h, the enzyme was stable at 30–65°C, with a residual activity greater than 50% of the initial activity (Fig. 7B). At 55°C, the optimal temperature for activity, it was stable for more than 6 h and had a half-life of about 7.6 h. Prolonged incubation at all temperatures resulted in loss of activity.

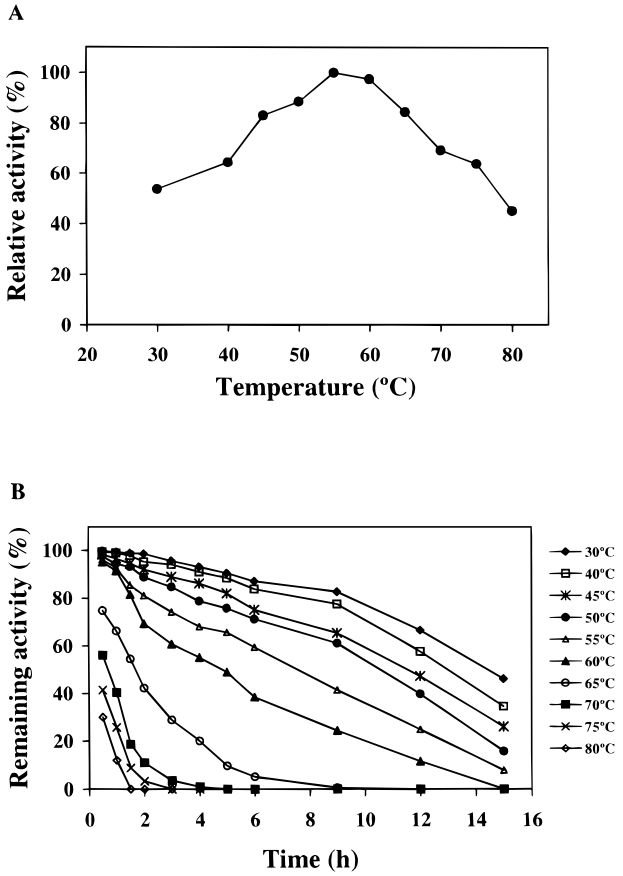

FIG. 7. Effect of temperature on lipase activity and stability. (A) The effect of temperature on lipase activity was determined at various temperatures, as described in the text. (B) The effect of temperature on lipase stability was determined by incubating the pure lipase at various temperatures for up to 15 h and measuring the remaining activity.

Substrate Specificity

The lipase hydrolyzed synthetic substrates with acyl group chain lengths of between C8 and C12, with optimal activity with C10 (*p*-NP–caprate) (Fig. 8). The lipase activity on long chain of substrates was between 70 and 100% of optimal for C8 or C10 groups and 30 and 50% for C12 to C18, whereas, with short-chain substrates (C2–C6), lipase activity was less than 30%. In addition, the lipase hydrolyzed triacylglycerols with acyl-group chain lengths of between C8 and C12, with

TABLE 1

Summary of the Purification Procedure for the Thermostable Lipase from *B. stearothermophilus* P1

Steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	86,300	1860	46	1	100
Ultrafiltration	79,620	1224	65	1.4	92
Q HyperD column	61,650	76	811	18	71

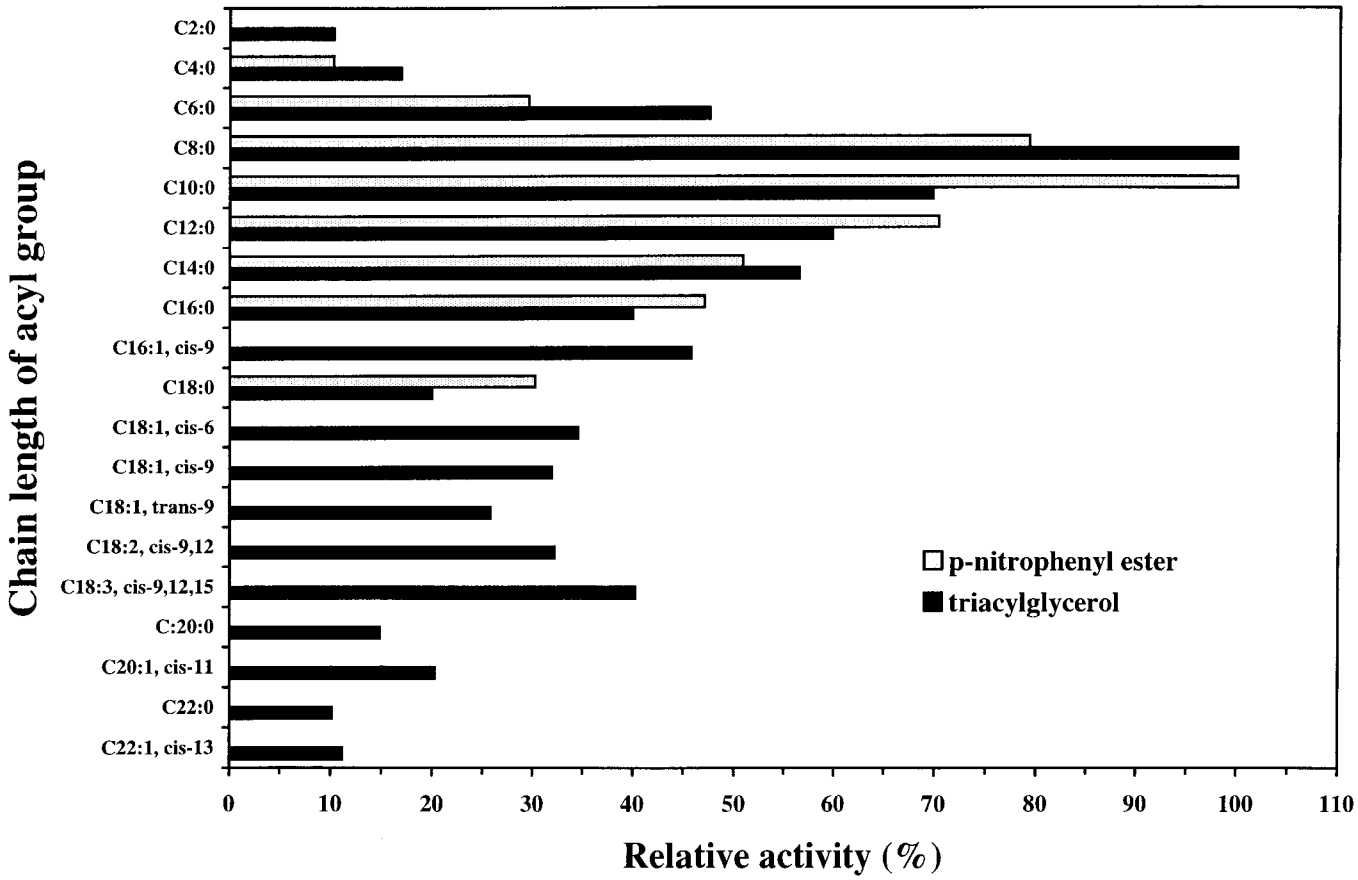

FIG. 8. Substrate specificity of the lipase using several *p*-nitrophenyl esters and triacylglycerols.

optimal activity with C8 (tricaprylin). In addition, the lipase hydrolyzed trilinolenin more than trilinolein and triolein.

Effect of Metal Ions on Lipase Activity

The effect of metal ions was tested for studying the influence of metal ions on the activity of lipase including the determination of metal ions that can activate the activity of lipase. In the presence of 1 mM most tested metal ions, activity was decreased slightly; however, $ZnCl_2$ and $FeSO_4$ reduced the lipase activity to 35 and 53%, respectively (Table 2). Using 10 mM metal ions, the inhibitory effects were greater, $ZnCl_2$ and $FeSO_4$ (10 mM) caused almost complete block of lipase activity, and the activities were about 1.6 and 0.76%, respectively.

Effect of Inhibitors on Lipase Activity

The effect of various inhibitors on lipase activity is shown in Table 3. All inhibitors were effective at 10 mM. Significant inhibition was already observed at 5 min, and the inhibition increasing depending on the time of incubation. The chelating agent EDTA did not greatly affect the activity of the lipase, and this suggested that it was not a metalloenzyme. The lipase was strongly inhibited by the addition of 10 mM PMSF (77% inhibition) or 1-hexadecanesulfonyl chloride (93% inhibition), showing that a serine residue plays a key role in the catalytic mechanism. After a 10-min incubation, the activities of lipases with PMSF, 1-dodecanesulfonyl chloride, and 1-hexadecanesulfonyl chloride were significantly decreased; in particular, 1-hexadecanesulfonyl chloride completely abolished the activity of lipase.

Effect of Detergents on Lipase Activity

On addition of 0.1% (w/v) detergents, no effect on lipase activity was seen, except in the case of SDS, sodium deoxycholate, and Tween 20 which slightly reduced activity (Table 4). At 1% detergent, a greater effect was seen, especially with SDS, which reduced the activity by about 50%, and Tween 20, which strongly inhibited lipase activity. After incubation in the presence of 0.1% detergent at 37°C for 1 h, Chaps and Triton X-100 increased enzyme stability, whereas SDS, sodium deoxycholate, and Tween 20 decreased the stability; in the presence of 1% detergents, activity was decreased

TABLE 2

Effect of Metal Ions on the Purified Lipase

Metal ions	Concentration (mM)	Relative activity (%)
Control	0	100
$CaCl_2$	1	96
	10	92
$CuCl_2$	1	84
	10	63
$MgCl_2$	1	98
	10	90
$MnCl_2$	1	84
	10	41
$ZnCl_2$	1	35
	10	1.6
CsCl	1	90
	10	84
KCl	1	87
	10	72
LiCl	1	84
	10	71
NaCl	1	97
	10	90
$FeSO_4$	1	53
	10	0.76

Note. The lipase was preincubated at room temperature with various metal ions at concentrations of 1 and 10 mM and then the activity was assayed.

by more than 50%, except in the case of Chaps and Triton X-100.

DISCUSSION

The lipase from *B. stearothermophilus* P1 is a novel enzyme found in an organism growing in a hot spring in Chiang Mai, Thailand. Due to the low lipase production of native bacteria, we markedly increased production of this enzyme by cloning it into *E. coli* M15[pREP4], using the QIAexpress system. The goal of this strategy, using pQE-60 and based on the T5 promoter transcription–translation system, was to put the gene under the control of IPTG induction. Expression of recombinant protein was rapidly induced by IPTG addition to levels that were 26,000-fold higher that those seen in cultures of either *B. stearothermophilus* P1 or of the transformed *E. coli* M15[pREP4] in the absence of IPTG.

TABLE 3

Effect of Inhibitors on the Purified Lipase

Inhibitors	Remaining activity (%)		
	5 min	10 min	30 min
Control	100	97	95
DTT	83	79	74
EDTA	76	73	69
β-Mercaptoethanol	88	87	80
PMSF	23	11	2
1-Dodecanesulfonyl chloride	42	20	5
1-Hexadecanesulfonyl chloride	7	0	0

Note. The lipase was incubated with each inhibitor in the final concentration of 10 mM at 37°C for 5, 10, and 30 min and then the remaining activity was assayed.

TABLE 4

Effects of Detergents on the Purified Lipase

Detergents	Concentration (%, w/v)	Relative activity (%)	
		0 h	1 h
Control	0	100	82
Chaps	0.1	100	91
	1.0	72	58
SDS	0.1	80	57
	1.0	57	37
Sodium deoxycholate	0.1	95	67
	1.0	71	42
Triton X-100	0.1	101	96
	1.0	74	50
Tween 20	0.1	95	68
	1.0	20	14

Note. The lipase was incubated at 37°C for 1 h in 20 mM Tris–HCl buffer, pH 8.5, with detergents.

The NH_2-terminal amino acid sequence of *B. stearothermophilus* P1 is similar to that of other lipases cloned in *E. coli* (8, 14, 28, 29), which also have a 29-amino-acid signal sequence and a cleavage site between Ala-29 and Ala-30. Its deduced mature sequence is similar to that of the thermostable lipases from *B. thermoleovorans* ID-1, *B. stearothermophilus* L1, and *B. thermocatenulatus*, with which it shows 96, 93, and 91% identity of residues, respectively. Based on the amino acid sequence similarities of several lipases in the region of the catalytic triad His, Ser, Asp (30) and on the modeled structures of several lipases, we suggest that Ser-113, Asp-317, and His-358 form the catalytic triad of the lipase from *B. stearothermophilus* P1.

The lipase P1 was purified by a single ion-exchange step on a Q HyperD column. The final product showed a single band on SDS–PAGE with an apparent molecular weight of approximately 43 kDa. The purity and molecular weight were confirmed by reverse-phase HPLC, which showed a single protein peak eluting from the HPLC column, and by mass spectrometry, which showed a single protein peak with a molecular weight of 43,209 Da. This single Q HyperD purification step makes it very easy to obtain pure lipase. The characterization of the purified lipase was very interesting, and showed that it was active over a wide range of pH values from 7 to 10 and temperatures between 45 and 65°C. In addition, the purified lipase was stable at a wide range of temperatures between 30 and 65°C. Its half-life at 55°C, the optimal temperature, was about 7.6 h,

showing that the stability of this enzyme is high at this high temperature and somewhat higher than that of other lipases from *Bacillus* sp. (8, 14, 29). Moreover, lipase P1 is different from other *Bacillus* sp. in the respect of substrate specificity. It showed high activity toward tricaprylin (C8) and *p*-NP–caprate (C10) whereas the lipases from *B. thermocatenulatus*, *B. thermocatenulatus* ID-1, and *B. stearothermophilus* L1 showed high activity toward tributyrin (C4) and *p*-NP–caprate (C10), tricaprylin (C8) and *p*-NP–caproate (C6), and trilaurin (C12) and *p*-NP–caprylin (C8), respectively. These results imply that there are some structural and functional differences between lipase P1 and other lipases from *Bacillus* sp. in spite of the significant amino acid sequence similarity. Furthermore, the effect of metal ions on the activity was that there are no metal ions that can activate the activity of lipase but $ZnCl_2$ and $FeSO_2$ strongly inactivated the activity of lipase. Comparison of the effect of metal ions on the activity of the lipase P1 with other lipases showed that lipase P1 was inactivated by 1 mM $ZnCl_2$ and $FeSO_4$ and the activity was maintained at 35 and 53%, respectively, whereas the lipase from *B. stearothermophilus* L1 was inactivated and the activity was maintained at 75 and 81%, respectively. Otherwise, the lipase from *B. thermoleovorans* ID-1 was activated in the presence of Ca^{2+} or Zn^{2+}. Moreover, the lipase P1 was stable at 37°C for 1 h in the presence of 0.1% detergents, such as Chaps and Triton X-100. This enzyme can therefore be used for environmental and industrial applications.

Most lipases have a catalytic triad consisting of Ser-His-Asp/Glu (31, 32), similar to that in serine proteases. The catalytic serine is embedded in a signature pentapeptide sequence, Gly-X-Ser-X-Gly, located at the C-terminal of a section of parallel strands of _-sheet. Serine, one part of the catalytic site, is embedded in a tight bend between an _-helix and a _-stand (32–34). We confirmed that a catalytic serine was present in the lipase molecule using 1-dodecanesulfonyl chloride or 1-hexadecanesulfonyl chloride, which have an unbranched alkyl sulfonyl chloride structure similar to monoglyceride and a high affinity for the active site. The results show that the lipase was strongly inhibited by 10 mM 1-hexadecanesulfonyl chloride and by 10 mM PMSF.

Secondary structure prediction, X-ray crystallization for three-dimensional structure determination, computer modeling, and industrial applications are in progress.

ACKNOWLEDGMENTS

We thank Dr. Linda A. Gilmore, Department of Biomedical Sciences, Edinburgh University, Edinburgh, United Kingdom, for discussion and revision of the English text. This project is part of the Royal Golden Jubilee Ph.D. project of S.S. supported by the Thailand Research Fund (TRF), Bangkok, Thailand. We also gratefully acknowledge the National Science Council, Taiwan, for funds to support part of the research in Taiwan.

REFERENCES

1. Benjamin, S., and Pandey, A. (1998) *Candida rugosa* lipases: Molecular biology and versatility in biotechnology. *Yeast* 14, 1069–1087.
2. Bjorkling, F., Godtfredsen, S. E., and Kirk, O. (1991) The future impact of industrial lipases. *TIBTECH* 9, 360–363.
3. Harwood, J. (1989) The versatility of lipases for industrial uses. *Trends Biochem. Sci.* 14, 125–126.
4. Jaeger, K. E., and Reetz, M. T. (1998) Microbial lipases form versatile tools for biotechnology. *Trend Biotechnol.* 16(9), 396–403.
5. Schmid, R. D., and Verger, R. (1998) Lipases: Interfacial enzymes with attractive applications. *Angew. Chem. Int. Ed.* 37, 1608–1633.
6. Iizumi, T., Nakamura, K., and Fukase, T. (1990) Purification and characterization of a thermostable lipase from newly isolated *Pseudomonas* sp. KWI-56. *Agric. Biol. Chem.* 54(5), 1253–1258.
7. Omar, I. C., Hayashi, M., and Nagai, S. (1987) Purification and some properties of a thermostable lipase from *Humicola lanuginosa* no. 3. *Agric. Biol. Chem.* 51(1), 37–45.
8. Schmidt-Dannert, C., Rua, M. L., Atomi, H., and Schmid, R. D. (1996) Thermoalkalophilic lipase of *Bacillus thermocatenulatus*. I. Molecular cloning, nucleotide sequence, purification and some properties. *Biochim. Biophys. Acta* 1301, 105–114.
9. Schmidt-Dannert, C., Rua, M. L., Wahl, S., and Schmid, R. D. (1997) *Bacillus thermocatenulatus* lipases: A thermoalkalophilic lipase with interesting properties. *Biochem. Soc. Trans.* 25(1), 178–182.
10. Schmidt-Dannert, C., Sztajer, H., Stocklein, W., Menge, U., and Schmid, R. D. (1994) Screening, purification and properties of a thermophilic lipase from *Bacillus thermocatenulatus*. *Biochim. Biophys. Acta* 1214, 43–53.
11. Sugihara, A., Ueshima, M., Shimada, Y., Tsunasawa, S., and Tominaga, Y. (1992) Purification and characterization of a novel thermostable lipase from *Pseudomonas cepacia*. *J. Biochem.* 112(5), 598–603.
12. Sugihara, A., Tani, T., and Tominaga, Y. (1991) Purification and characterization of a novel thermostable lipase from *Bacillus* sp. *J. Biochem.* 109(2), 211–216.
13. Chung, G. H., Lee, Y. P., Jeohn, G. H., Yoo, O. J., and Rhee, J. S. (1991) Cloning and nucleotide sequence of thermostable lipase gene from *Pseudomonas fluorescens* SIK W1. *Agric. Biol. Chem.* 55(9), 2359–2365.
14. Kim, H. K., Park, S. Y., Lee, J. K., and Oh, T. K. (1998) Gene cloning and characterization of thermostable lipase from *Bacillus stearothermophilus* L1. *Biosci. Biotechnol. Biochem.* 62(1), 66–71.
15. Boonsinthai, B., and Phutrakul, S. (1991–1993) Secretion of lipases from organic-solvent-tolerant bacteria and stability of enzymes in organic solvents. *J. Sci. Fac. CMU* 18–20, 2–10.
16. Maungprom, N., Kanasawud, P., and Phutrakul, S. (1995) Extracellular lipase activity of thermophilic bacterium from Chiang Mai hot spring. *J. Sci. Fac. CMU* 23, 9–13.
17. Marmur, J. (1961) A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. Mol. Biol.* 3, 208–218.
18. Qiagen Inc. (1997) "The QIA Expressionist: A Handbook for High-Level Expression and Purification of 6_His-Tagged Proteins," Qiagen Inc., Santa Clarita, CA.

19. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410.
20. Peled, N., and Krenz, M. C. (1981) A new assay of microbial lipases with trioleoyl glycerol. *Anal. Biochem.* 112, 219–222.
21. Lesuisse, E., Schanck, K., and Colson, C. (1993) Purification and preliminary characterization of the extracellular lipase of *Bacillus subtilis* 168, an extremely basic pH-tolerant enzyme. *Eur. J. Biochem.* 216, 155–160.
22. Winkler, U. K., and Stuckmann, M. (1979) Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. *J. Bacteriol.* 138, 663–670.
23. Bradford, M. M. (1976) 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.
24. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
25. Matsudaira, P. (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* 262(21), 10035–10038.
26. Lee, D. W., Koh, Y. S., Kim, K. J., Kim, B. C., Choi, H. J., Kim, D. S., Suhartono, M. T., and Pyun, T. R. (1999) Isolation and characterization of a thermophilic lipase from *Bacillus thermoleovorans* ID-1. *FEMS Microbiol. Lett.* 179, 393–400.
27. Wandersman, C. (1992) Secretion across the bacterial outer membrane. *TIG* 8(9), 317–322.
28. Dartois, V., Baulard, A., Schank, K., and Colson, C. (1992) Cloning, nucleotide sequence and expression in *Escherichia coli* of a lipase gene from *Bacillus subtilis*. *Biochim. Biophys. Acta* 1131, 253–260.
29. Cho, A. R., Yoo, S. K., and Kim, E. J. (2000) Cloning, sequencing and expression in *Escherichia coli* of a thermophilic lipase from *Bacillus thermoleovorans* ID-1. *FEMS Microbiol. Lett.* 186, 235–238.
30. Jaeger, K. E., Ransac, S., Koch, H. B., Ferrato, F., and Dijkstra, B. (1993) Topological characterization and modelling of the 3D structure of lipase from *Pseudomonas aeruginosa*. *FEBS Lett.* 332, 143–148.
31. Brenner, S. (1988) The molecular evolution of genes and proteins: A tale of two serines. *Nature* 334, 528–530.
32. Cygler, M., Schrag, J. D., and Ergan, F. (1992) Advances in structural understanding of lipases. *Biotechnol. Genet. Eng. Rev.* 10, 143–184.
33. Derewenda, Z. S., and Derewenda, U. (1991) Relationships among serine hydrolases: evidence for a common structural motif in triacylglyceride lipases and esterases. *Biochem. Cell. Biol.* 69, 842–851.
34. Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., Harel, M., Remington, S. J., Silman, I., Schrag, J. D., Sussman, J. L., Verchueren, K. H. G., and Goldman, A. (1992) The _-_hydrolase fold. *Protein Eng.* 5, 197–211.