APPENDIX

SUPPORTING PAPERS

1. **Sinchaikul, S.**, Sookkheo, B., Phutrakul, S., Pan, F.M., and Chen, S.T. (2001) Optimization of a Thermostable Lipase from *Bacillus stearothermophilus* P1: Overexpression, purification, and characterization. *Prot. Express. Pure.* 22(3), 388-398.

2. **Sinchaikul, S.**, Sookkheo, B., Phutrakul, S., Wu, Y.T., Pan, F.M., and Chen, S.T. (2001) Structural Modeling and Characterization of a Thermostable Lipase from *Bacillus stearothermophilus* P1. *Biochem. Biophys. Res. Com.* 283(4), 868-875.

3. **Sinchaikul, S.**, Tyndall, J.D.A., Fothergill-Gilmore, L.A., Phutrakul, S., Chen, S.T. and Walkinshaw, M.D. (2001) Expression, purification, crystallization and preliminary crystallographic analysis of a thermostable lipase from *Bacillus stearothermophilus* P1. *Acta Cryst. D*, submitted.

4. **Sinchaikul, S.**, Sookkheo, B., Phutrakul, S. and Chen, S.T (2001) Characterization and Application of Purified Lipase from *Bacillus stearothermophilus* P1. *Enzyme Microbial. Technol.*, submitted.

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 μmol 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

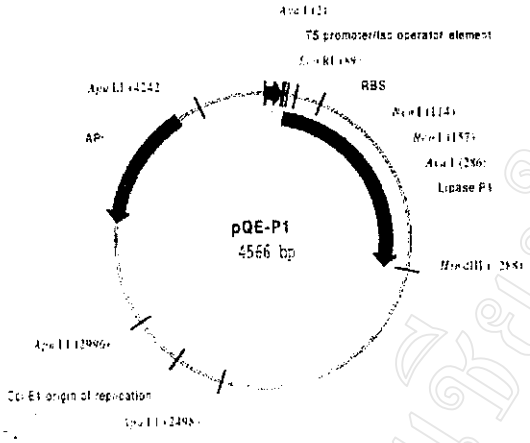


FIG. 1. Physical map of inserted lipase P1 after cloning of partial *NcoI* and *HindIII* fragments into pQE-60. The arrow indicates the region encoding the lipase gene and the direction of transcription.

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 *NcoI/HindIII* 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).

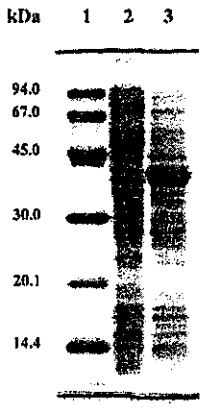


FIG. 2. SDS–PAGE shows IPTG induction of the cloned lipase from *B. stearothermophilus* P1. Lane 1, standard protein markers; lane 2, no added IPTG; lane 3, 1 mM IPTG.

```
    -110        -100            -90         -80         -70             -60         -50         -40             -30         -20         -10         -1
TTT CTC TCA CAG AAA AAC CCG ACA ATT GCC GGG ATT GAA TCA GTT GGT TGA TAT ATA TAG AAT ATT CAG GTA ATT ATG AAC AAA AAG ATT CCG TTT ATG TGA GGG GAG GAG AAG GAT AGG
                                                                                                                                            RBS

            10          20          30              40          50          60              70          80          90              100         110         120
ATG ATG AAA GGC TGC CGG GTG ATG GTT GTG TTG CTC GGA TTA TGC TTT GTG TTC GGC CTA TCG GTC CCG GGA GGG CGG ACG GAA GCG GCA TCC CTA CGC GCC AAT GAT GCG CCG ATT GTG
 M   M   K   G   C   R   V   M   V   V   L   L   G   L   C   F   V   F   G   L   S   V   P   G   G   R   T   E   A   A   S   L   R   A   N   D   A   P   I   V

            130         140         150             160         170         180             190         200         210             220         230         240
CTT CTC CAT GGG TTT ACC GGA TGG GGA CGA GAG GAA ATG TTT GGA TTC AAG TAT TGG GGC GGC GTG CGC GGC GAT ATC GAA CAA TGG CTG AAC GAC AAC GGT TAT CGA ACG TAT ACG CTG
 L   L   H   G   F   T   G   W   G   R   E   E   M   F   G   F   K   Y   W   G   G   V   R   G   D   I   E   Q   W   L   N   D   N   G   Y   R   T   Y   T   L

            250         260         270             280         290         300             310         320         330             340         350         360
GCG GTC GGA CCG CTC TCG AGC AAC TGG GAC CGG GCG TGT GAA GCG TAT GCT CAG CTT GTC GGC GGG ACG GTC GAT TAT GGG GCA GCC CAT GCG GCA AAG CAC GGC CAT GCG CGG TTT GGC
 A   V   G   P   L   S   S   N   W   D   R   A   C   E   A   Y   A   Q   L   V   G   G   T   V   D   Y   G   A   A   H   A   A   K   H   G   H   A   R   F   G

            370         380         390             400         410         420             430         440         450             460         470         480
CGC ACT TAT CCC GGC CTG TTG CCG GAA TTG AAA AGG GGT GGC CGC ATC CAT ATC ATC GCC CAC AGC CAA GGG GGG CAG ACG GCC CGC ATG CTT GTC TCG CTC CTA GAG AAC GGA AGC CAA
 R   T   Y   P   G   L   L   P   E   L   K   R   G   G   R   I   H   I   I   A   H   S   Q   G   G   Q   T   A   R   M   L   V   S   L   L   E   N   G   S   Q

            490         500         510             520         530         540             550         560         570             580         590         600
GAA GAG CGG GAG TAC GCC AAG GCG CAC AAC GTG TCG TTG TCA CCG TTG TTT GAA GGT GGA CAT CAT TTT GTG TTG AGT GTG ACG ACC ATC GCC ACT CCT CAT GAC GGG ACG ACG CTT GTC
 E   E   R   E   Y   A   K   A   H   N   V   S   L   S   P   L   F   E   G   G   H   H   F   V   L   S   V   T   T   I   A   T   P   H   D   G   T   T   L   V

            610         620         630             640         650         660             670         680         690             700         710         720
AAC ATG GTT GAT TTC ACC GAT CGC TTT TTT GAC TTG CAA AAA GCG GTG TTG GAA GCG GCG GCT GTC GCC AGC AAC GTG CCG TAC ACG AGT CAA GTA TAC GAT TTT AAG CTC GAC CAA TGG
 N   M   V   D   F   T   D   R   F   F   D   L   Q   K   A   V   L   E   A   A   A   V   A   S   N   V   P   Y   T   S   Q   V   Y   D   F   K   L   D   Q   W

            730         740         750             760         770         780             790         800         810             820         830         840
GGA CTG CGC CGT CAG CCG GGC GAA TCG TTC GAC CAT TAT TTT GAA CGG CTC AAG CGC TCC CCT GTC TGG ACA TCG ACC GAT ACC GCC CGC TAC GAT TTA TCC GTT TCC GGA GCT GAG AAG
 G   L   R   R   Q   P   G   E   S   F   D   H   Y   F   E   R   L   K   R   S   P   V   W   T   S   T   D   T   A   R   Y   D   L   S   V   S   G   A   E   K

            850         860         870             880         890         900             910         920         930             940         950         960
TTG AAT CAA TGG GTG CAA GCA AGC CCG AAT ACG TAT TAT TTG AGT TTC TCT ACA GAA CGG ACG TAT CGC GGA GCG CTC ACA GGC AAC CAT TAT CCC GAA CTC GGA ATG AAC GCA TTC AGC
 L   N   Q   W   V   Q   A   S   P   N   T   Y   Y   L   S   F   S   T   E   R   T   Y   R   G   A   L   T   G   N   H   Y   P   E   L   G   M   N   A   F   S

            970         980         990             1000        1010        1020            1030        1040        1050            1060        1070        1080
GCG GTC GTA TGC GCT CCG TTT CTC GGT TCG TAC CGC AAT CCG ACG CTT GGC ATT GAC AGT CAT TGG CTT GAG AAC GAC GGC ATT GTC AAT ACC ATT TCG ATG AAC GGT CCG AAG CGT GGA
 A   V   V   C   A   P   F   L   G   S   Y   R   N   P   T   L   G   I   D   S   H   W   L   E   N   D   G   I   V   N   T   I   S   M   N   G   P   K   R   G

            1090        1100        1110            1120        1130        1140            1150        1160        1170            1180        1190        1200
TCA AAC GAT CGG ATC GTG CCG TAT GAC GGG ACG TTG AAA AAA GGG GTT TGG AAT GAT ATG GGA ACG TAC AAT GTC GAC CAT TTG GAA ATC ATC GGC GTT GAC CCG AAT CCG TCA TTT GAT
 S   N   D   R   I   V   P   Y   D   G   T   L   K   K   G   V   W   N   D   M   G   T   Y   N   V   D   H   L   E   I   I   G   V   D   P   N   P   S   F   D

            1210        1220        1230            1240        1250
ATT CGC GCC TTT TAT TTG CGG CTT GCC GAG CAG TTG GCG AGC TTG CAG CCT TAA
 I   R   A   F   Y   L   R   L   A   E   Q   L   A   S   L   Q   P   *
```

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.

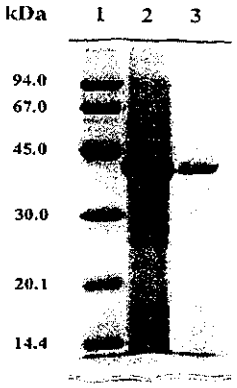


FIG. 4. SDS–PAGE of purified lipase from *B. stearothermophilus* P1. Lane 1, molecular weight markers; lane 2, crude extract; lane 3, Q HyperD10-purified lipase.

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

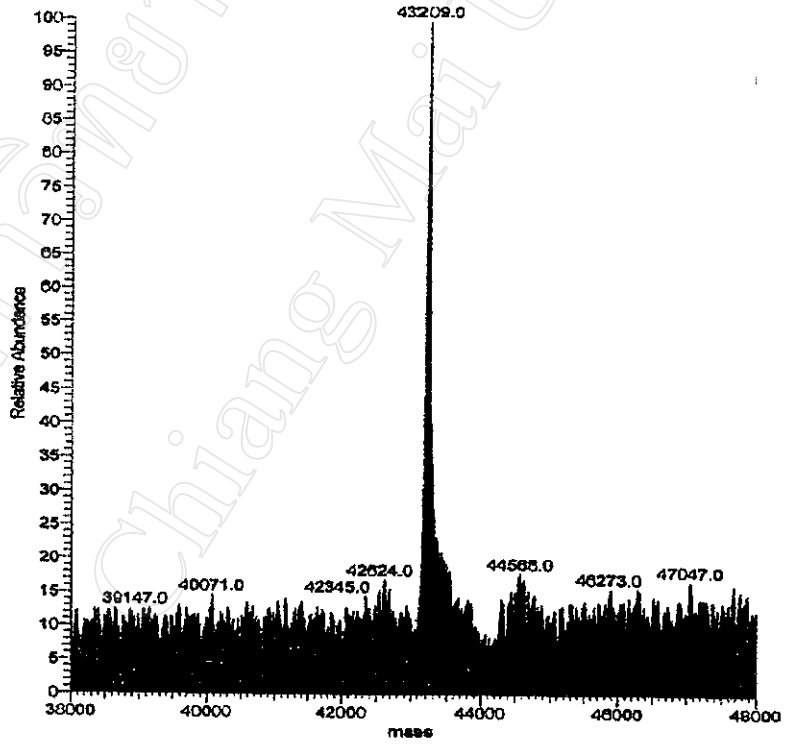


FIG. 5. Mass spectrometry of the purified *B. stearothermophilus* P1 lipase.

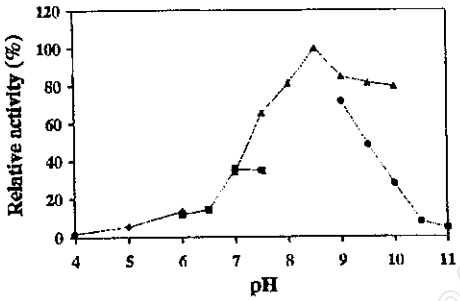


FIG. 6. Effect of pH on lipase activity. The purified lipase was assayed in various pH buffers, as described in the text. ♦, sodium acetate buffer; ■, phosphate buffer; ▲, Tris–HCl buffer; ●, Tris–glycine buffer.

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.

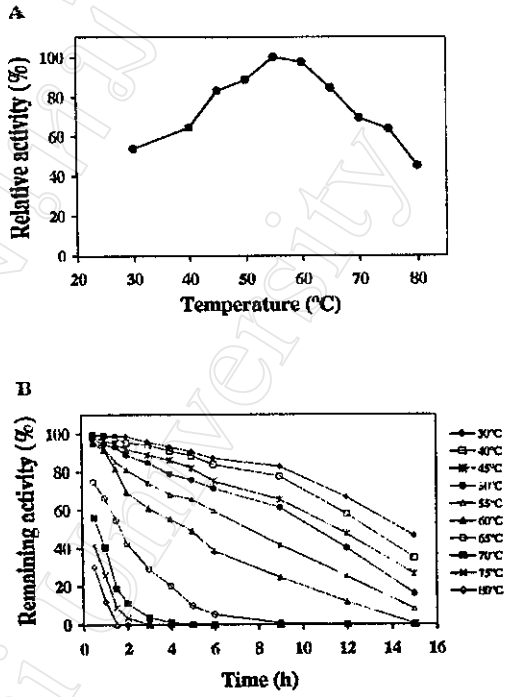


FIG. 7. Effect of temperature on lipase activity and stability. (A) The effect of temperature on lipase activity was determined at various temperatures, as described in the text. (B) The effect of temperature on lipase stability was determined by incubating the pure lipase at various temperatures for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

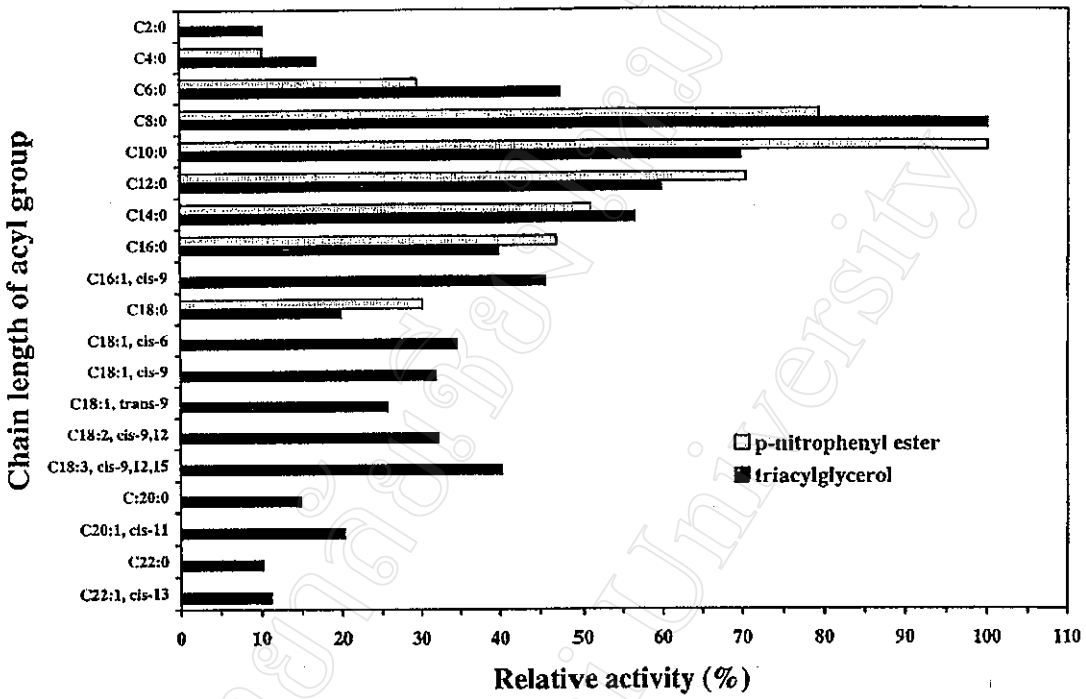


FIG. 8. Substrate specificity of the lipase using several *p*-nitrophenyl esters and triacylglycerols.

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

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	59
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 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.

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 Stucknann, 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 quantitration 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 α/β hydrolase fold. *Protein Eng.* 5, 197–211.

Biochemical and Biophysical Research Communications 283, 868–875 (2001)
doi:10.1006/bbrc.2001.4854, available online at http://www.idealibrary.com on IDEAL®

Structural Modeling and Characterization of a Thermostable Lipase from *Bacillus stearothermophilus* P1

Supachok Sinchaikul,*,† Boonyaras Sookkheo,†,‡ Suree Phutrakul,*
Ying-Ta Wu,† 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, 115-29, Taiwan, Republic of China; and
‡Chemistry Unit, School of Science, Rangsit University, Patumthani, 12000, Thailand

Received April 16, 2001

The moderate thermophilic bacterium *Bacillus stearothermophilus* P1 expresses a thermostable lipase that was active and stable at the high temperature. Based on secondary structure predictions and secondary structure-driven multiple sequence alignment with the homologous lipases of known three-dimensional (3-D) structure, we constructed the 3-D structure model of this enzyme and the model reveals the topological organization of the fold, corroborating our predictions. We hypothesized for this enzyme the α/β-hydrolase fold typical of several lipases and identified Ser-113, Asp-317, and His-358 as the putative members of the catalytic triad that are located close to each other at hydrogen bond distances. In addition, the strongly inhibited enzyme by 10 mM PMSF and 1-hexadecanesulfonyl chloride was indicated that it contains a serine residue which plays a key role in the catalytic mechanism. It was also confirmed by site-directed mutagenesis that mutated Ser-113, Asp-317, and His-358 to Ala and the activity of the mutant enzyme was drastically reduced. © 2001 Academic Press

Key Words: **thermostable lipase; *Bacillus stearothermophilus;* structural modeling.**

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3), which are lipolytic enzymes, catalyze the hydrolysis of the ester bonds of triglycerides and long chain fatty acids. 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, 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 (6–12).

Over the years crystal structure determinations of various lipases have shown that all lipases contain the α/β-hydrolase fold, a structural motif common to a wide variety of hydrolases (13). Their active sites contain a catalytic triad, Ser-His-Asp/Glu similar to those of serine proteases. Despite the fact that the amino acid sequences of the lipases, for which the structures were determined, reveal no homology (except around the active site serine), there exist clear similarities in their 3D structures. According to the lipase structures, 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 β-sheet. Serine, one part of the catalytic site, is embedded in a tight bend between an α-helix and a β-stand (14–16). The His residue is often part of a special sequence pattern, and further the active site Asp residue can be found in the triacylglycerol lipases approximately in the mid between the Ser and the His residues.

The present report concerns an extracellular thermostable lipase produced by a thermophilic bacterium, *Bacillus stearothermophilus* strain P1, isolated from a hot spring in Chiang Mai, Thailand. It has been cloned and overexpressed in *E. coli* M15[pREP4] using pQE-60 as an expression vector. In current absence of 3-D structure for this thermostable lipase from *B. stearothermophilus* P1, we decided to exploit a combined approach of secondary structure predictions and molecular modeling to investigate the structural features of this enzyme as the first time. In addition, we searched for an inhibitor with a higher affinity for the serine residue in the active site of this lipase and then

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

0006-291X/01 $35.00

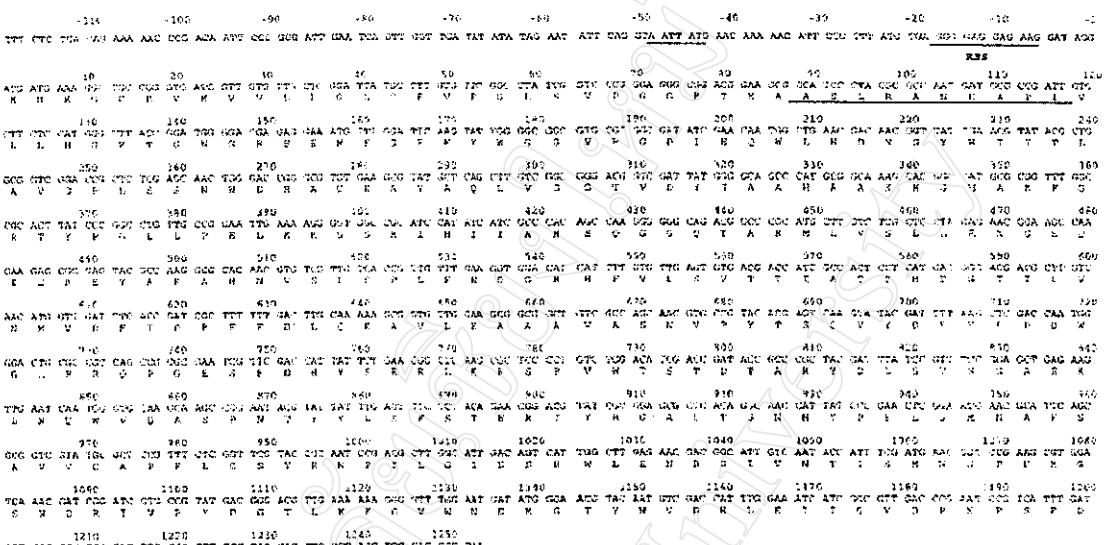


FIG. 1. Nucleotide sequence of the lipase gene from *B. stearothermophilus* P1 and its deduced amino acid sequence. The numbering of nucleotides starts at the 5′ end of the lipase gene and that of amino acids at the NH_2-terminus of mature lipase. The putative −35, −10, ribosomal binding sites (RBS), and stop codon (*) are shown. The NH_2-terminal amino acid sequence from the purified lipase P1 is underlined.

studied the serine-reactive inhibition. The site-directed mutagenesis was also used for determining the catalytic triad of this enzyme.

MATERIALS AND METHODS

Materials. Thermo Sequenase dye terminator cycle sequencing pre-mix kit with Thermo Sequenase polymerase was from Amersham Life Science (Ohio). LB broth was obtained from Scharlau (Taiwan). Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from MDBio Inc. and streptomycin sulfate was obtained from Sigma (U.S.A.). Lipase inhibitors were obtained from Roche Molecular Biochemicals (Germany).

Bacterial strains and cultivation. The cloned thermostable lipase from *B. stearothermophilus* P1 which has been cloned into the expression vector pQE-60 and transformed into *Escherichia coli* M15[pREP4] was grown at 37°C to late log phase in LB broth containing 100 μg/ml of ampicillin and 25 μg/ml of kanamycin, then isopropyl-β-D-thiogalactopyranoside (IPTG) 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 resuspended in 50 ml of 20 mM Tris–HCl buffer, pH 8.5, containing 10 mM EDTA, and the cells lysed by sonication. After centrifugation at 12,000*g* for 20 min, streptomycin sulfate was added to the supernatant to a final concentration of 1% (w/v) and the precipitate formed removed by centrifugation at 12,000*g* for 15 min.

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. The synthetic oligonucleotides, pQE-F (5′-GCGCGTATCACGAGGCCCTTTCG-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.

Lipase assay. Lipase activity was assayed using the *p*-nitrophenyl caprate as the synthetic substrate (17, 18). 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 prewarmed to 55°C, then mixed with 100 μl of freshly prepared 8 mM *p*-nitrophenyl 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 measured against an enzyme-free blank. One enzyme unit is defined as the release of 1 μmol of *p*-nitrophenol per ml per min. Under the conditions described, the extinction coefficient of *p*-nitrophenol is $\varepsilon = 1.85\ \mathrm{Lmmol^{-1}.mm^{-1}}$.

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 (19). The molecular mass of the lipase was calibrated by using a low molecular mass calibration kit (Pharmacia AB, Sweden).

NH_2-terminal amino acid sequence analysis. The purified lipase was separated by SDS–PAGE and electroblotted onto a polyvinylidene difluoride (PVDF) membrane, as described by Matsudaira (20). The lipase band was cut out and analyzed by Edman degradation using an Applied Biosystems Model 492 procise sequencer (Applied Biosystems, Weiterstadt, Germany).

Secondary structure prediction and hydrophobicity profile. Secondary structure prediction using Mac Vector sequence analysis algorithm software (International Biotechnologies, Inc., New Haven, CT) was carried out on the Macintosh computer. The conformational analysis of the lipase gene structure was based on the methods of Chou and Fasman (21) and Garnier *et al.* (22). The surface hydro-

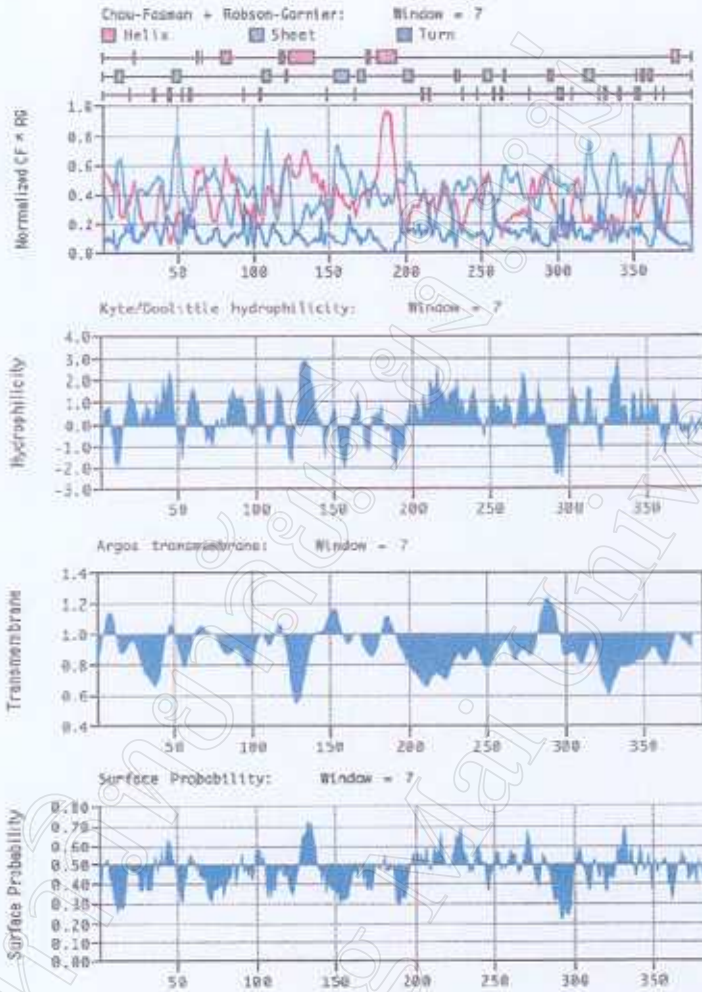


FIG. 2. Secondary structure prediction, hydropathy profile, transmembrane, and surface probability of a thermostable lipase gene from *B. stearothermophilus* P1. The analysis of the local hydrophilicity and conformational analysis of the lipase structure were based on the methods of Chou and Fishman (21), Garnier *et al.* (22), and Kyte and Doolittle (23).

phobicity profile based on the empirical Kyte–Doolittle hydropathy scale (23) of peptide segments in a protein was plotted along the amino acid sequence of the lipase from *B. stearothermophilus* P1. For graphing purpose, the signs of the hydropathy values have been reversed in order to plot the hydrophilicity instead of hydrophobicity scale. A window of size $N = 7$ is run along the length of the protein; for each window, the hydropathy values of 7 amino acids are summed and divided by 7 to obtain the average hydrophilicity per residue for the window. Values above the axis denote hydrophilic regions, which may be exposed on the outside of the analyzed molecule whereas those values below the axis indicate the hydrophobic regions, which tend to be buried inside the lipase structure molecule.

Homology modeling and computer graphic study of structural lipase. The structure of the *B. stearothermophilus* P1 lipase was modeled by comparison with members of the triacylglycerol lipase subfamily (EC 3.1.1.3). The sequence of the mature lipase was analyzed initially with the PredictProtein Server (CUBIC, Columbia

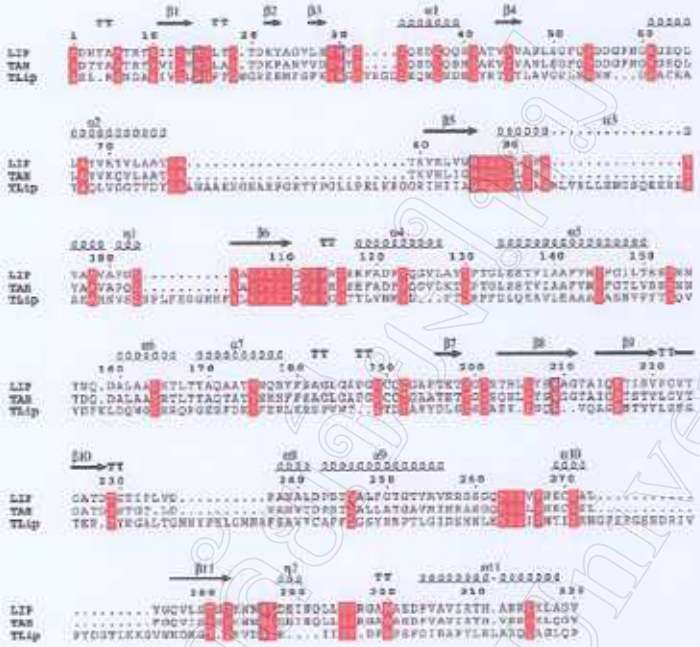


FIG. 2. The pairwise alignment of model sequence of *B. stearothermophilus* P1 lipase (TLip with reference models of lipases from *P. glumae* (1TAH), and *P. cepacia* (3LIP). The red boxes show conserved residues. α, TT and β are helix, turn, and extended sheet, respectively.

University, New York) (24–27). MAXHOM alignment of the lipase sequences was based on published multiple sequence alignment which in turn on secondary structure correspondences and confirmed completely with the WU-blast2 program (Washington University, St. Louis, MO) using EMBL database for studying the reference structure models (28–30). Computer modeling was performed on a Silicon Graphics O2 workstation using the commercial software packages Insight II (Insight II User Guide, October 1995, Biosym/MSI, San Diego, CA) and manually adjusted with information based on the result from 3D SPPM (Imperial Cancer Research Fund, London, UK) (31). Using the homologous module of the Insight II package, the atomic coordinates of the structurally conserved regions (SCRs) backbone of the reference models were taken directly. The loops connecting the SCRs or variable regions (VRs) were modeled with the function "search loop," which search a set of selected PDB structures for loops that best fit the given structural environment. The final molecular structure was refined using energy minimization. The Modeler program built in Homology/Insight II was also used to build automatically all-atom models. In general, the average RMS deviations for most of the backbone atoms of the lipase molecules are estimated in order to assess the validity and relative accuracy of homology modeling and computer simulating graphics.

Effect of inhibitors on lipase activity. The effect of inhibitors on lipase activity was determined using dithiothreitol (DTT), 1-dodecanesulfonyl chloride, ethylenediaminetetraacetic acid (EDTA), 1-hexadecanesulfonyl chloride, β-mercaptoethanol and phenylmethanesulfonyl fluoride (PMSF) at final concentrations of 10 mM. The enzyme solutions were pre-incubated at 37°C for 5 min with each inhibitor in 20 mM Tris-HCl buffer, pH 8.5, and then the lipase activity was measured. Enzyme solution without inhibitor was used as reference.

Site-directed mutagenesis. The mutant lipase genes were constructed by site-directed mutagenesis with the PCR overlap extension method (32) using a 5′ and 3′ mutagenic primers containing the desired mutations at Ser-113, Asp-317 and His-358 to Ala as nonpolar amino acid residue. After the digestion by *Nco*I and *Hin*dIII, the PCR fragments were ligated to the pQE-60 as an expression vector. The overproducing strains were constructed by transforming in *E. coli* M15[pREP4]. The mutants were selected and confirmed by DNA sequencing.

RESULTS AND DISCUSSION

Nucleotide Sequence and NH_2-Terminal Amino Acid Sequence

The nucleotide sequence of the lipase gene from *B. stearothermophilus* P1, submitted to GenBank under Accession No. AF237623, revealed an open reading frame of 1254 bp encoding a 417 amino acid polypeptide (Fig. 1), 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 (33).

Secondary Structure Prediction and Solvent Accessibility

The secondary structure prediction and solvent accessibility of the lipase gene are shown in Fig. 2. It consisted of α-helix, β-sheet and turns in a different ratio. The hydropathy profile was shown the ratio of hydrophilicity and hydrophobicity along the sequence of the lipase. The hydrophilicity of the lipase was slightly rather than its hydrophobicity and the nonpolar amino acids were slightly rather than the polar amino acids in the amino acid composition of the lipase gene structure. In addition, the surface probability and transmembrane of the lipase gene structure were showed the low level. So, it indicated that the lipase molecular structure was turned the nonpolar group of amino acid into its core structure and turned the polar group of the amino acid outside the molecule.

Homology Modeling and Computer Graphics of Lipase

Results retrieved from the PredictProtein server indicated that 3LIP (*Pseudomonas cepacia*) (34), 1CVL (*Chromobacterium viscosum*) (35), 1OIL (*P. cepacia*) (36) and 1TAH (*P. glumae*) (37), lipases having known 3-D structures appear to have the sequence identity with our model lipase about 40, 55, 40 and 55% identities, respectively. WU-blastp result also showed the sequences of 3LIP, 1CVL, 1OIL and 1TAH producing high-scoring segment pairs with score 68, 54, 68 and 54, respectively. It was indicated that the model lipase contained low full-length sequence identity to these reference sequences having known 3-D structures. Structure alignment (Homology/Insight II) of these references revealed with several structure-conserved regions (SCRs) and the variable regions (VRs) that were mostly on the surface of the protein. Using these alignments, the RMS deviations of the backbone atoms of the structurally conserved regions between the model lipase and the crystal structures of 3LIP, 1CVL, 1OIL and 1TAH were 0.98, 1.08, 1.03 and 1.89 Å, respectively. Otherwise, the major differences between the model lipase and other lipases were on VRs, which are the surface loops of varying length. Although the model lipase preserved less sequence similarity in whole to triacylglycerol lipases that have known 3-D structures, the alignment of the model lipase with SCRs of references was indicated that it was generated much more structure and sequence similarity. Most of SCRs buried in the protein core and the residues with greater RMS located in the outer surface with higher flexibility. Therefore, the sequence alignment of the model lipase to SCRs of references was adopted to be the first step in building model structure.

In study of lipase structure, we used 3LIP as a key reference protein as suggested by MAXHOM showing its 3D homologous structure appeared to have significant sequence identity to the lipase P1 and WU-Blastp result showing higher-score of the sequence producing high-scoring segment pairs. The pairwise alignment of model sequence with reference models, 1TAH and 3LIP, was drawn in the Fig. 3. The similar pairwise alignment was input into Modeler (Homology/Insight II) to build initial 3-D models of modeled structure. The predicted model was built by following Homology/Insight II procedure manually and then compared with the reference lipases. The model structure was solvated in water shell and geometry optimized by 2000 steps conjugate gradient energy minimization and showed the force field energies with the total energy of −5267 kcal/mol in 2d4h28m36s, bond of 1046 kcal/mol, angle of 2911 kcal/mol, torsion, 2025 kcal/mol, improper of 1007 kcal/mol and electrostatics of −9816 kcal/mol. The constructed model was overlaid with 3LIP and 1OIL and shown the RMS deviations to be 1.30 and 1.06 Å with 1132 and 1148 backbone atoms involved, respectively. One of the finally predicted structures was drawn as shown in Fig. 4A. Similar to most basic features of lipase, the model structure included both α-helix and extended β-sheet secondary structures in the folded protein and the β-sheet was in the core region surrounding with α-helix. The unique structure feature common to the most lipase was a lid or flap composed by an amphiphilic helix peptide sequence that was posited on the top of the cleft on which bottom sited of the catalytic triad. The helix span between Phe-180 to Val-197 forms the "lid" of the model lipase as shown in Fig. 4B. Ser-113, Asp-317 and His-358 were formed the catalytic triad within a range of information H-bond as shown in Fig. 4C. The active Ser-113 residue sites on the hairpin turn, where a central β-sheet was converted to α-helix.

Effect of Inhibitors on Lipase Activity

The effect of various inhibitors on lipase activity is shown in Table 1. All inhibitors were effective at 10 mM. The lipase was strongly inhibited by the addition of 10 mM PMSF (77% inhibition) or 1-hexadecanesulfonyl chloride (93% inhibition) that have an unbranched alkyl sulfonyl chloride structure similar to monoglycerides and a high affinity for the serine-active site. Therefore, this result shows that a serine residue plays a key role in the catalytic mechanism.

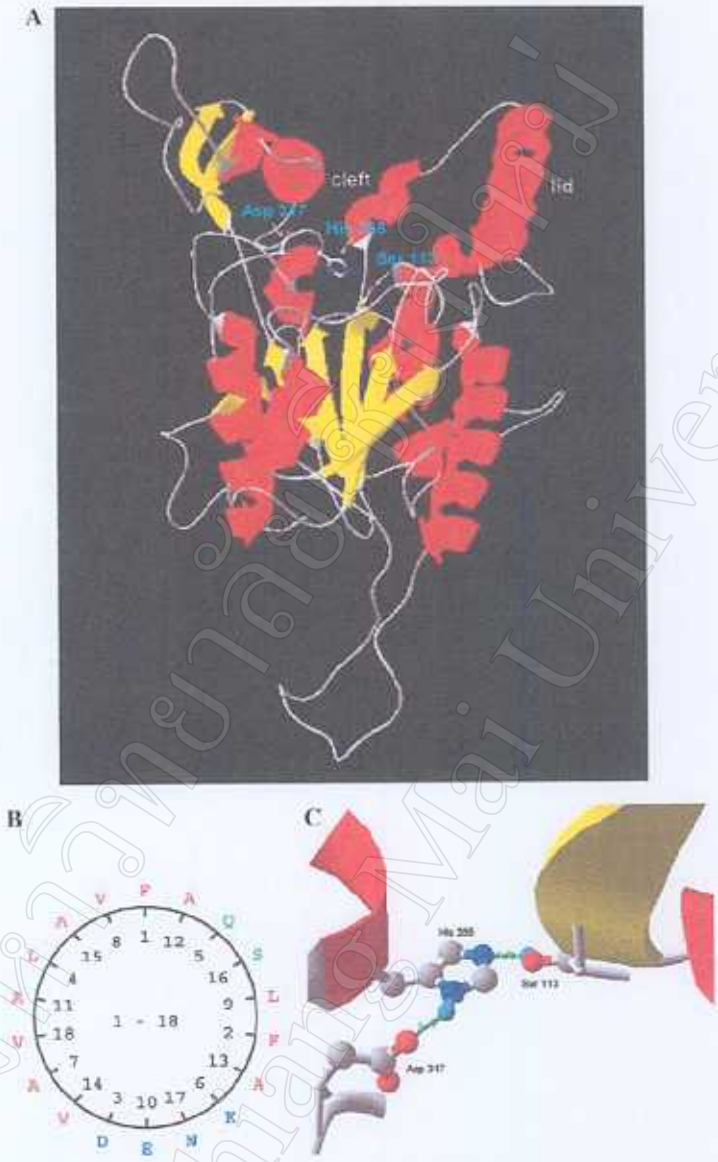


FIG. 4. The 3D structures of modeled lipase from *B. stearothermophilus* P1. (A) The modeled structure possess common features of lipase, including both α-helix and extended β-sheet secondary structures in folded protein; the β sheet was in the core and surrounded by α-helix. (B) The "lid" structure composed by an amphiphilic helix peptide sequence, Phe-180 to Val-197 of the modeled lipase. (C) Ser-113, Asp-317 and His-358 formed the catalytic triad within a range of information H-bond. Color labels: (A) red, helix; yellow, sheet; white, loop; (B) red, nonpolar amino acid; green, polar amino acid; blue, acid and basic amino acids; (C) red, oxygen atom; dark blue, nitrogen atom; blue, hydrogen atom; blond, carbon atom; green, strong hydrogen bond.

TABLE 1

Effect of Inhibitors on the Purified Lipase

Inhibitors	Remaining activity (%)
Control	100
DTT	83
EDTA	76
β-Mercaptoethanol	88
PMSF	23
1-Dodecanesulfonyl chloride	42
1-Hexadecanesulfonyl chloride	7

Note. The lipase was preincubated at 37°C with various inhibitors at concentrations of 10 mM and then the remaining activity assayed.

Site-Directed Mutagenesis

The mutant lipases, changing Ser-113, Asp-317 and His-358 to Ala as non-polar amino acid were expressed in *E. coli* M15[pREP4] with the same size of the lipase gene and same molecular mass of 43 kDa on SDS–PAGE of wild-type. They were then determined the nucleotide sequences and found that the position at Ser-113, Asp-317 and His-358 of each mutant were changed to Ala correctly. In addition, the lipase activities of all mutant enzymes decreased significantly 3441-, 2925- and 3858-fold of the wide-type enzyme, respectively. The present work corroborates that this mutant could possibly cause conformational changes of the surrounding residues in the active catalytic center. Therefore, Ser-113, Asp-317 and His-358 are the active site residues of a thermostable lipase from *B. stearothermophilus* P1. In addition, the active site Ser is most often found in the consensus sequence Gly-X-Ser-X-Gly, but the consensus sequence of this lipase was found to be Ala-X-Ser-X-Gly which also appeared in the homologous sequence of the other lipases such as *B. thermocatenulatus* (8). *B. subtilis* (38) and *B. thermoleovorans* ID-1 (39). A more extensive kinetic and structural analysis of the mutant is currently under way.

CONCLUSION

This study is the first report of a predicted 3-D structure model of a thermostable lipase from *B. stearothermophilus* P1. The secondary structure of this lipase was predicted and aligned with the other lipases, which have known the 3-D structure. We found that the major differences between this lipase and the other lipases are on VRs which are surface loops of varying length and may related to the characterization in the high temperature. In addition, we correctly predicted the amino acids belonging to the catalytic triad, Ser-113, Asp-317, and His-358 that was also confirmed by serine-reactive reagent and site-directed mutagenesis. Therefore, the *B. stearothermophilus* P1 lipase is definitively member of the α/β hydrolase fold family.

ACKNOWLEDGMENTS

This project is part of the Royal Golden Jubilee Ph.D. project of Mr. Surpachok Sinchaikul supported by the Thailand Research Fund (TRF), Bangkok, Thailand. We 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. Ollis, D., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., Harel, M., Remington, S. J., Silman, I., Schrag, J. D., Sussman, J. L., Verscheuren, K. H. G., and Gold man, A. (1992) The α/β hydrolase fold. *Protein Eng.* **5**, 197–211.
14. Chapus, C., Rovery, M., Sarda, L., and Verger, R. (1988) Minireview on pancreatic lipase and colipase. *Biochimie* **70**, 1223–1234.
15. Cygler, M., Schrag, J. D., and Ergan, F. (1992) Advances in structural understanding of lipases. *Biotechnol. Genet. Eng. Rev.* **10**, 143–184.
16. 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.
17. Lesuisse, E., Schanck, K., and Colson, C. (1993) Purification and preliminary characterization of the extracellular lipase of *Bacil-*

lus subtilis 168, an extremely basic pH-tolerant enzyme. *Eur. J. Biochem.* **216,** 155–160.

18. 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.
19. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.
20. Matsudaira, P. (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **262**(21), 10035–10038.
21. Chou, P. Y., and Fasman, G. D. (1974) Prediction of protein conformation. *Biochemistry* **13,** 222–245.
22. Garnier, J. (1978) Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J. Mol. Biol.* **120,** 97–120.
23. Kyte, J., and Doolittle, R. F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157,** 105–132.
24. Rost, B., and Sander, C. (1993) Prediction of protein secondary structure at better than 70% accuracy. *J. Mol. Biol.* **232,** 584–599.
25. Rost, B., and Sander, C. (1994) Conservation and prediction of solvent accessibility in protein families. *Proteins* **20,** 216–226.
26. Sander, C., and Schneider, R. (1991) Database of homology-derived structures and the structural meaning of sequence alignment. *Proteins* **9,** 56–68.
27. Rost, B. (1996) PHD: Predicting one-dimensional protein structure by profile based neural networks. *Methods Enzymol.* **266,** 525–539.
28. 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.
29. Altschul, S. F., Thomas, L. M., Alejandro, A. S., Jinghui, Z., Zheng, Z., Webb, M., and David, J. L. (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* **25,** 3389–3402.
30. Kim, C. W., Brent, A. W., and Randall, F. S. (1995) BEAUTY: An enhanced BLAST-based search tool that integrates multiple biological information resources into sequence similarity search results. *Genome Res.* **5,** 173–184.
31. Kelly, L., Maccallum, R., and Sternberg, M. Biomolecular Modeling Laboratory, Imperial Cancer Research Fund, UK. http://www.bmm.icnet.uk/servers/3dpssm.
32. Urban, A., Neukirchen, S., and Jaeger, K. E. (1997) A rapid and efficient method for site-directed mutagenesis using one-step overlap extension PCR. *Nucleic Acids Res.* **25**(11), 2227–2228.
33. Wandersman, C. (1992) Secretion across the bacterial outer membrane. *TIG* **8**(9), 317–322.
34. Schrag, J. D., Li, Y., Cygler, M., Lang, D., Burgdorf, T., Hecht, H. J., Schmid, R., Schomburg, D., Rydel, T. J., Oliver, J. D., Strickland, L. C., Dunaway, C. M., Larson, S. B., Day, J., and McPherson, A. (1997) The open conformation of a *Pseudomonas* lipase. *Structure* **5**(2), 187–202.
35. Lang, D. A., Hofmann, B., Haalck, L., Hecht, H. J., Spener, F., Schmid, R. D., and Schomburg, D. (1996) Crystal structure of a bacterial lipase from *Chromobacterium viscosum* ATCC 6918 refined at 1.6 angstroms resolution. *J. Mol. Biol.* **259**(4), 704–717.
36. Kim, K. K., Song, H. K., Shin, D. H., Hwang, K. Y., and Suh, S. W. (1997) The crystal structure of a triacylglycerol lipase from *Pseudomonas cepacia* reveals a highly open conformation in the absence of a bound inhibitor. *Structure* **5**(2), 173–185.
37. Noble, M. E., Cleasby, A., Johnson, L. N., Egmond, M. R., and Frenken, L. G. (1993) The crystal structure of triacylglycerol lipase from *Pseudomonas glumae* reveals a partially redundant catalytic aspartate. *FEBS Lett.* **331**(1–2), 123–128.
38. Dartois, V., Baulard, A., Schanck, K., and Colson, C. (1992) Cloning, nucleotide sequence and expression in *Escherichia coli* of a lipase gene from *Bacillus subtilis* 168. *Biochim. Biophys. Acta* **1131,** 253–260.
39. 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.

Expression, purification, crystallization and preliminary crystallographic analysis of a thermostable lipase from *Bacillus stearothermophilus* P1

Supachok Sinchaikul[a,b], Joel D. A. Tyndall[b], Linda A. Fothergill-Gilmore[b] Suree Phutrakul[a], Shui-Tein Chen[c] and Malcolm D. Walkinshaw[b]

[a]Department of Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai, 50200, Thailand, [b]Structural Biochemistry Group, Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh, EH9 3JR, UK, and [c]Institute of Biological Chemistry, Academia Sinica, Taipei, 11529, Taiwan, R.O.C.

correspondence email: malcolm.walkinshaw@ed.ac.uk

Running title: Thermostable lipase from *B. stearothermophilus* P1

Abstract

The gene encoding a thermostable lipase secreted by *Bacillus stearothermophilus* P1 (isolated from a hot spring in Chiang Mai, Thailand) has been cloned and over expressed in *Escherichia coli*. The recombinant lipase was purified to homogeneity using ammonium sulphate precipitation, strongly anion-exchange chromatography (Poros 20 HQ) and Sephacryl S-200HR. The molecular mass was shown to be 43,209 Da by mass spectrometry. Crystals suitable for X-ray diffraction analysis were obtained by the hanging drop method of vapour diffusion with ammonium sulphate as the precipitating agent. Determination of the structure by molecular replacement with existing mesophilic lipase structures has proved unrewarding because of structural divergence, but preliminary results with heavy atom soaking indicate that this strategy will allow the structure to be solved. The availability of this new lipase structure will be of particular significance because it will be the first thermostable lipase to be described.

1. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are found in various organisms, including animals, plants, fungi, and bacteria. They catalyze the hydrolysis and transesterification of triacylglycerols, although some will degrade a fairly broad range of compounds containing an ester linkage. Microbial lipases are of considerable interest for biotechnological applications such as detergents, oleochemistry, cheese production, pharmaceuticals and the industrial synthesis of fine chemicals (reviewed by Jaeger

& Reetz, 1998). In particular, thermostable lipases isolated from thermophilic bacteria are playing an increasingly important role in industrial processes because they exhibit relatively high thermodynamic stability both at elevated temperatures and in organic solvents (Omar *et al.*, 1987; Iizumi *et al.*, 1990; Sugihara *et al.*, 1992; Schmidt-Dannert *et al.*, 1996; 1997; Kim *et al.*, 1998).

Numerous lipase structures have been investigated by X-ray crystallography, including mammalian pancreatic lipase (Lombardo *et al.*, 1989; Winkler *et al.*, 1990; Moreau *et al.*, 1992); fungal lipases (Brady *et al.*, 1990; Brzozowski *et al.*, 1992; Schrag & Cygler, 1993; Grochulski *et al.*, 1993; Derewenda *et al.*, 1994; Uppenberg *et al.*, 1994; Lewis *et al.*, 1997) and mesophilic bacterial lipases (see review by Arpigny & Jaeger, 1999). A characteristic feature of these enzymes is the presence of a catalytic triad consisting of Ser-His-Asp/Glu. The catalytic serine is embedded in a signature pentapeptide sequence, Gly-X-Ser-X-Gly, that is located at the C-terminal portion of a parallel -sheet. The serine is positioned in a tight bend between an -helix and a -strand (Brenner, 1988; Cygler *et al.*, 1992; Derewenda & Derewenda, 1991; Ollis *et al.*, 1992).

We now report the over-expression, purification and first crystallographic analysis of a thermostable lipase, that from *Bacillus stearothermophilus* P1.

2. Methods and results

2.1. Cloning and expression

Chromosomal DNA from *Bacillus stearothermophilus* P1 (isolated from a hot spring in Chiang Mai, Thailand) was partially digested with *Nco* I and *Hind* III, and the fragments inserted into the same restriction sites of plasmid pQE-60 (Qiagen). After ligation, the recombinant plasmids were transformed into *E. coli* M15[pREP4] (Qiagen), and screening was done by plating on LB agar containing 100 μg/ml of ampicillin, 25 μg/ml of kanamycin, and 1% (w/v) tricaprylin. Colonies surrounded by a clear zone were chosen and grown in LB medium containing 100 μg/ml of amplicillin and 25 μg/ml of kanamycin.

The recombinant plasmid DNA was isolated and amplified by PCR, and shown to contain a 4.5 kb insert by agarose gel electrophoresis. Both strands of the lipase gene were sequenced automatically. The nucleotide sequence of the gene, submitted to GenBank under accession number AF237623, revealed an open reading frame of 1,254 bp encoding a 417 amino acid polypeptide (Sinchaikul *et al.*, 2001). The sequence was found to be 92% identical to lipases from the thermophilic bacteria *B. thermocatenulatus* and *B. stearothermophilus* L1 (Schmidt-Dannert *et al.*, 1996; Kim *et al.*, 1998), but only 15-17% identical to mesophilic bacterial lipases for which crystal structures are available (*Burkholderia glumae, Chymobacterium viscosum, Burkholderia cepacia* and *Pseudomonas aeruginosa*) (Noble *et al.*, 1993; Lang *et al.*, 1996; Kim *et al.*, 1997; Schrag *et al.*, 1977; Nordini *et al.*, 2000).

Expression of the lipase enzyme was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) when the culture grown at 310 K had attained late log phase (OD_{600} of 0.6). The expression was optimized by testing various concentrations of IPTG (0.2, 0.4, 0.6, 0.8, 1, 2,

3, and 5 m*M*) and various times of induction (1, 2, 3, 4 and 5 h). Expression levels were measured by SDS-PAGE (total cells lysed by SDS-PAGE sample buffer) and by assay of lipase activity in cell lysates (see below). The best expression was achieved with 0.4 m*M* IPTG for 3 h at 310 K.

2.2. Purification of recombinant lipase

Cells were collected from four litres of culture by centrifugation at 6,500*g* for 30 min, and suspended in 50 ml of 20 m*M* Tris-HCl buffer, pH 8.5 containing 10 m*M* EDTA, 5m*M* 3,4-dichloroisocoumarin, 1 m*M* E64 and 100 m*M* 1,10-phenanthroline. After sonication, the cell lysate was centrifuged at 12,000*g* for 30 min and the precipitate discarded. Streptomycin sulphate was added to the supernatant to a final concentration of 1% (w/v), and the precipitate was removed by centrifugation at 12,000*g* for 15 min. Protein precipitated between 20% saturated and 40% saturated ammonium sulphate was collected by centrifugation at 12,000*g* for 30 min, and suspended in 20 m*M* Tris-HCl buffer, pH 8.5. The protein was concentrated, and desalted and solvent exchanged into the same Tris buffer with a Vivaspin 20 ml centrifugal concentrator (Vivasciences) with a molecular weight cutoff of 10,000.

The lipase was then purified by anion-exchange perfusion chromatography and gel filtration on Sephacryl S-200 (Fig. 1). The fractions with high lipase activity were pooled and characterised by SDS-PAGE (Fig. 2) and specific activity (Table 1). The purified lipase showed a single band by SDS-PAGE and was purified 39-fold over the crude extract with a yield of 19%. The molecular mass of the purified lipase was shown to be 43,209 Da by mass spectrometry (LCQ^{TM}, Finnigan).

Table 1. Purification of thermostable lipase from *B. stearothermophilus* P1.

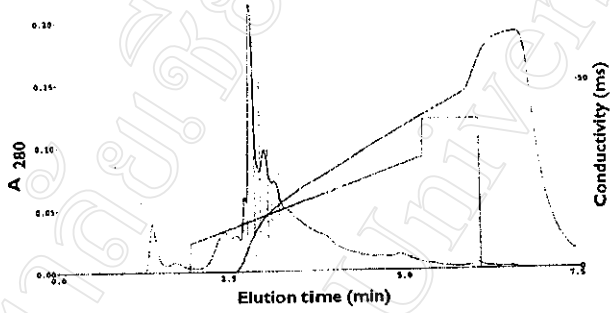
Step	Volume (ml)	Total activity ($\times 10^3$U)	Total protein (mg)	Specific activity (U.mg^{-1})	Purification (fold)	Recovery (%)
Crude extract	11.8	40.6	611	66.5	1.00	100
$(NH_4)_2SO_4$ precipitation	5.5	30.3	132	230	3.45	74.6
HQ column	14.0	12.2	14.6	836	12.6	30.0
Sephacryl S-200HR	8.7	7.8	3.0	2,600	39.1	19.2

2.3. Lipase assay

Lipase activity was measured by the release of *p*-nitrophenol from *p*-nitrophenyl caprate (Lesuisse *et* al., 1993; Winkler & Stuckmann, 1979) Lipase solution (20 µl) was added to 880 µl reaction buffer containing 20 m*M* Tris-HCl buffer pH 8.5, 0.1% (w/v) gum arabic and 0.2% (w/v) sodium deoxycholate. The reaction mixture was pre-warmed at 55 °C, and then mixed with 100 µl of 8

m*M* freshly prepared *p*-nitrophenyl caprate (Sigma) solubilized in isopropanol. The mixture was incubated at 328 K for 2 min and the reaction 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 A_{405} was measured against an enzyme-free control. One enzyme unit is defined as the amount of enzyme that releases 1 nmol of *p*-nitrophenol min^{-1}. The protein concentration was measured spectrophotometrically at 280 nm or by using a dye-binding assay (Sedmak & Frossberg, 1977) with bovine serum albumin (Sigma) as a standard.

(a)



(b)

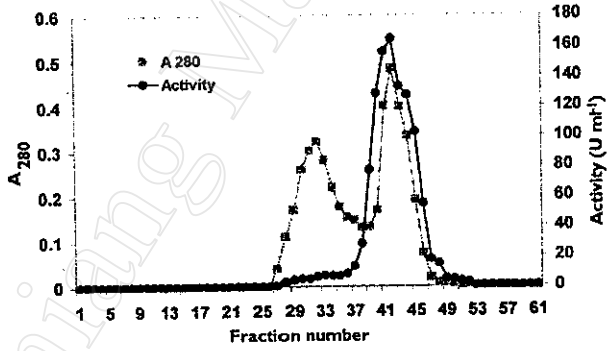


Fig. 1. Purification of lipase. (a) The ammonium sulphate fraction (see text, total volume 5.5 ml) was injected in 1 ml samples onto a Poros 20 HQ column (4.6 x 100 mm) using a BioCAD workstation (Applied Biosystems). Elution was with 20 m*M* Tris-HCl buffer, pH 8.5 containing a linear gradient of 0.15 to 0.6 *M* NaCl at a flow rate of 10 ml min^{-1}. The largest peak (eluting at 0.18 *M* NaCl) contained high lipase activity, and the corresponding fractions were pooled and concentrated with a Vivaspin tube. Separate traces indicate the NaCl gradient and conductivity. (b) The partially purified lipase was then applied to a Sephacryl S-200 HR column (1.6 x 100 cm) and eluted with 20 m*M* Tris-HCl buffer, pH 8.5 at a flow rate of 0.25 ml min^{-1}; the fraction size was 2.5 ml.

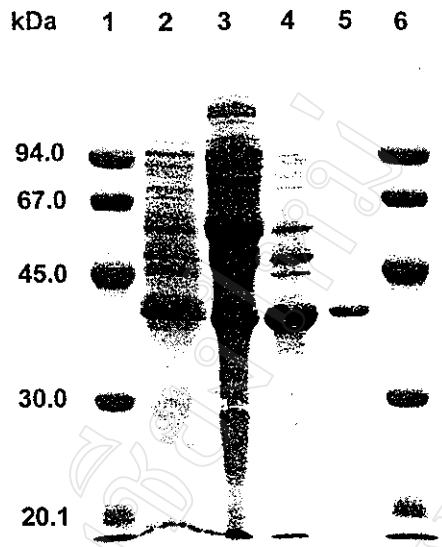


Fig. 2. SDS-PAGE analysis of lipase fractions. A 12.5 % gel was used, and marker proteins (lanes 1 and 6) were from a low Molecular Weight Calibration Kit (Amersham Pharmacia Biotech) containing phosphorylase b (94 kDa), serum bovine albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). Lane 2, crude cell extract; lane 3, 20-40% saturated ammonium sulphate precipitate; lane 4, Poros HQ pool; lane 5, Sephacryl pool.

2.4. Crystallization and data collection

The hanging-drop vapour-diffusion method was used to grow crystals in 24-well Linbro plates. Tetragonal crystals grew rapidly after 24 h at 289 K, with 20% saturated ammonium sulphate as precipitant in 0.1 *M* Hepes buffer, pH 6.8-7.0. The protein concentration was 15 mg ml^{-1} in 20 m*M* Tris-HCl buffer, pH 8.5.

Table 2. Crystallographic statistics for *B. stearothermophilus* P1 lipase

Resolution (Å)	30.0-2.5
Space group	C2
a (Å)	117.54
b (Å)	80.82
c (Å)	99.36
β (°)	96.35
R_{merge}	0.109
(top shell)	0.401
Completeness %	94.3
(top shell)	88.6
Number of observations	89044
Number of reflections	30531

Data were collected to 2.5 Å at the ESRF facility (λ = 0.931 Å), Grenoble. Data were processed using DENZO and Scalepack (Otwinowski & Minor, 1997), and the statistics are presented in Table 2. There is one molecule per asymmetric unit [Vm = 2.92 01 $Å^3$ Da^{-1} (Matthews, 1968)].

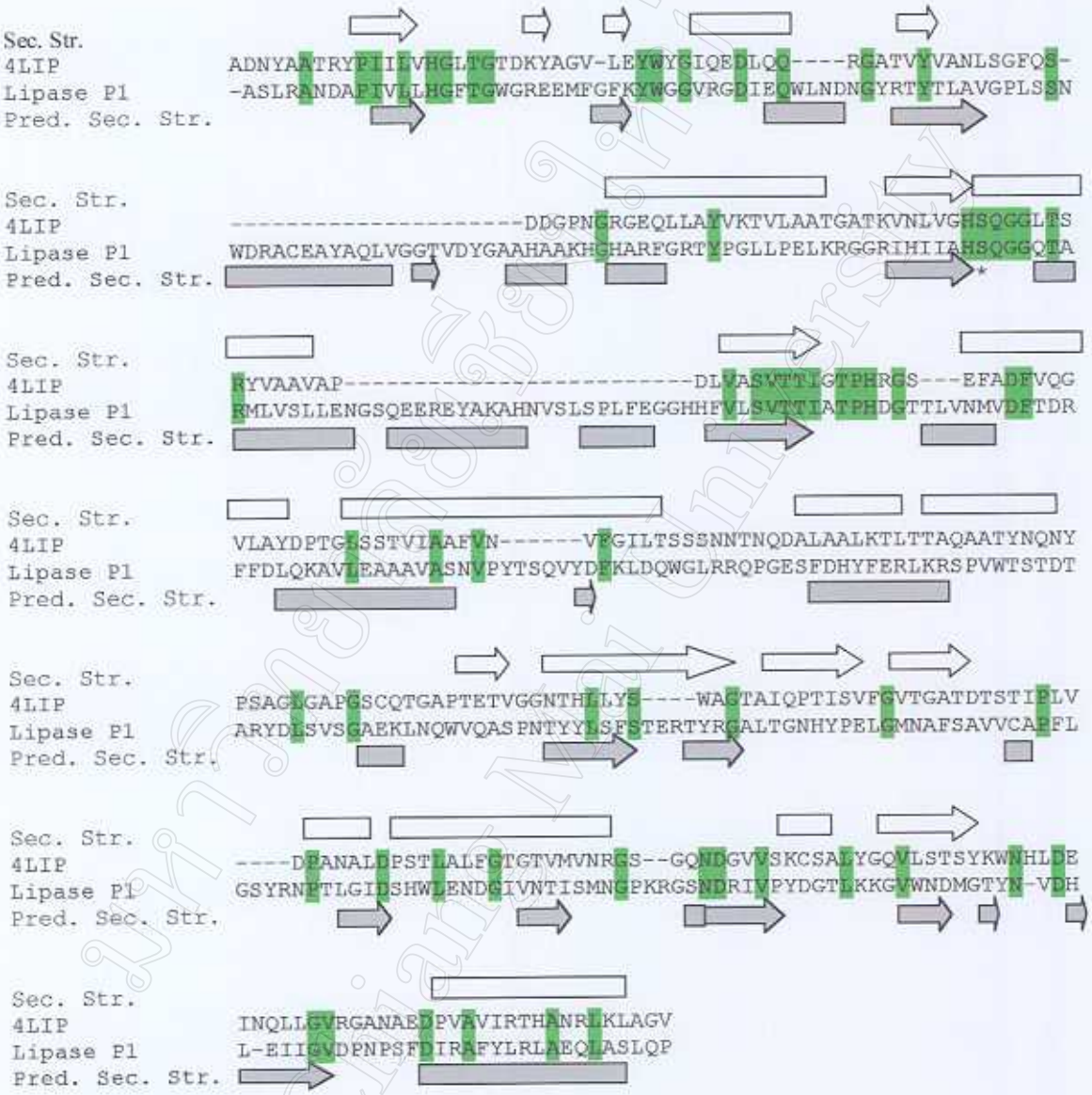


Fig. 3. Comparison of lipases from *B. stearothermophilus* P1 and *Burkhardia cepacia*. A clustalW alignment of the two sequences (designated Lipase P1 and 4LIP, respectively) is shown flanked by the known secondary structure of the *Burkhardia* enzyme (taken from the pdb entry) and the predicted structure of the *B. stearothermophilus* enzyme. Arrows indicate β-strands and bars show α-helices. The shaded residues are identical in both sequences. The catalytic serine is denoted by an asterisk.

3. Discussion

Lipases are a generally well characterised group of enzymes, with crystal structures available for enzymes from mammals, fungi and mesophilic bacteria. However, there is as yet no detailed structural information for a thermostable lipase, although this would be of considerable interest as the basis for tailoring the enzyme for a range of biotechnological applications. We have thus over-expressed, purified and crystallised a thermostable lipase from *B. stearothermophilus* P1.

The crystals were shown to diffract well, and an attempt was made to find a solution through molecular replacement. The structure of a lipase from *Burkholderia cepacia* (4lip.pdb) (Lang *et al.*, 1998) with a sequence identity of 17% was used as a search model. Fig. 3 shows a clustalW sequence alignment (Thompson *et al.*, 1994) of the two proteins with the corrresponding secondary structure of the *Burkholderia* lipase and the predicted secondary structure (PSIPRED, http://insulin.brunel.ac.uk/psipred/) of the *B. stearothermophilus* enzyme. Of note is the relatively good agreement between the predicted and known structures, despite the low sequence identity. Molrep (Vagin & Teplyakov, 1997; Vaguine *et al.*, 1999) was used to try to find the molecular replacement solution, with the search model corresponding to the apoenzyme or to a truncated model omitting the deletions shown in Fig. 3 as well as the two helices from Glu118 - Tyr129 and Leu134 - Thr150. Neither procedure was successful, and it is likely that the *Burkholderia* lipase is too divergent, especially in terms of sequence and loop structure, to permit molecular replacement to be feasible. It was therefore concluded to attempt heavy atom soaking as an alternative method.

By contrast, the sequence of the lipase studied here is very similar indeed to the lipases isolated from the thermophilic bacteria *B. thermocatenulatus* (Schmidt-Dannert *et al.*, 1996) and *B. stearothermophilus* L1 (Kim *et al.*, 1998). The availability of the detailed structure of the *B. stearothermophilus* P1 lipase will thus enable work on the other two enzymes to be placed in a structural context.

This project is a part of the Royal Golden Jubilee Ph.D. project of Mr. Supachok Sinchaikul that was supported by the Thailand Research Fund (TRF), Bangkok, Thailand. We gratefully acknowledge the University of Edinburgh for research support.

4. References

Arpigny. J.L. & Jaeger, K.-E. (1999). *Biochem. J.* 343, 177-183.

Brady, L., Brzozowski, A.M., Derewenda, Z.S., Dodson, E., Dodson, G., Tolley, S., Turkenburgh, J.P., Christiansesn, L., Jensen, B.H., Norskov, L., Thim, L. & Menge, U. (1990). *Nature* 343, 767-770.

Brenner, S. (1988). *Nature (London)* 334, 528-530.

Brzozowski, A.M., Derewenda, Z.S., Dodson, E.J., Dodson, G.G. & Turkenburg, J.P. (1992). *Acta Cryst.* B48, 307-319.

Cygler, M., Schrag, J.D. & Ergan, F. (1992). *Biotechnol. Genet. Eng. Rev.* 10, 143-184.

Derewenda, U., Swenson, L., Green, R., Wei, Y., Dodson, G.G., Yamaguchi, S., Haas, M.J. & Derewenda, Z.S. (1994). *Nature Struct. Biol.* 1, 36-47.

Derewenda, Z.S. & Derewenda, U. (1991). *Biochem. Cell. Biol.* 69, 842-851.

Grochulski, P., Li, Y., Schrag, J.D., Bouthillier, F., Smith, P., Harrison, D., Rubin, B. & Cygler, M. (1993). *J. Biol. Chem.* 268, 12843-12847.

Iizumi, T., Nakamura, K. & Fukase, T. (1990). *Agric. Biol. Chem.* 54, 1253-1258.

Jaeger, K.-E. & Reetz, M.T. (1998). *Trends Biotechnol.* 16, 396-403.

Kim, K.K., Song, H.K., Shin, D.H., Hwang, K.Y. & Suh, S.W. (1997). *Structure* 5, 173-185.

Kim, H.-K., Park, S.Y., Lee, J.-K. & Oh, T.-K. (1998). *Biosci. Biotechnol. Biochem.* 62, 66-71.

Lang, D., Hofmann, B., Haalck, L., Hecht, H.-J., Spener, F., Schmid, R.D. & Schomburg, D. (1996). *J. Mol. Biol.* 259, 704-717.

Lang, D.A., Mannesse, M.L., de Haas, G.H., Verheij, H.M. & Dijkstra, B.W. (1998). *Eur. J. Biochem.* 254, 333-340.

Lesuisse, E., Schanck, K. & Colson, C. (1993) *Eur. J. Biochem.* 216, 155-160.

Lewis, G., Bevan, J., Rawas, A., McMichael, P., Wisdom, R., McCague, R., Watson, H. & Littlechild, J. (1997). *Acta Cryst.* D53, 348-351.

Lombardo, D., Chapus, C., Bourne, Y. & Cambillau, C. (1989). *J. Mol. Biol.* 205, 259-261.

Moreau, H., Abergel, C., Carriere, F., Ferrato, F., Fontecilla-Camps, J.C., Cambillau, C. & Verger, R. (1992). *J. Mol. Biol.* 225, 147-153.

Noble, M.E.M., Cleasby, A., Johnson, L.N., Egmond, M.R. & Frenken, L.G.J. (1993). *FEBS Lett.* 331, 123-128.

Nordini, M., Lang, D.A., Liebeton, K., Jaeger, K.-E. & Dijkstra, B.W. (2000). *J. Biol. Chem.* 275, 31219-31225.

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. & Goldman, A. (1992). *Protein Eng.* 5, 197-211.

Omar, I.C., Hayashi, M. & Nagai, S. (1987). *Agric. Biol. Chem.* 51, 37-45.

Schmidt-Dannert, C., Rua, M., Atomi, H. & Schmid, R.D. (1996). *Biochim. Biophys. Acta* 1301, 105-114.

Schmidt-Dannert, C., Rua, M.L., Wahl, S. & Schmid, R.D. (1997). *Biochem. Soc. Trans.* 25, 178-182.

Schrag, J.D. & Cygler, M. (1993). *J. Mol. Biol.* 230, 575-591.

Schrag, J.D., Li, Y., Cygler, M., Lang, D., Burgdorf, T., Hecht, H.J., Schmid, R., Schomburg, E., Rydel, T.J., Oliver, J.D. *et al.* (1997). *Structure* 5, 187-202.

Sedmak, J.J. & Frossberg, S.E. (1977). *Anal. Biochem.* 79, 544-552.

Sinchaikul, S., Sookkheo, B., Phutrakul, S., Wu, Y.-T., Pan, F.-M. & Chen, S.-T. (2001). *Biochem. Biophys. Res. Commun.* 283, 868-875.

Sugihara, A., Ueshima, M., Shimada, Y., Tsunasawa, S. & Tominaga, Y. (1992). *J. Biochem.* 112, 598-603.

Thompson, J.E., Higgins, D.G. & Gibson, T.J. (1994). *Nucleic Acids Res.* 22, 4673-4680.

Uppenberg, J., Hansen, M.T., Patkar, S. & Jones, T.A. (1994). *Structure* 2, 293-308.

Vagin, A.A. & Teplyakov, A.J. (1977). *Appl. Cryst.* 30, 1022.

Vaguine, A.A., Richelle, J., & Wodak, S.J. (1999). *Acta Cryst.* D55, 191-205.

Winkler, F.K., D'Arcy, A. & Hunziker, W. (1990). *Nature* 343, 771-774.

Winkler, U.K. & Stuckmann, M. (1979) *J. Bacteriol.* 138, 663-670.

Characterization and Application of a Purified Thermostable Lipase from *Bacillus stearothermophilus* P1

Supachok Sinchaikul[*,†], Boonyaras Sookkheo[†,‡], Suree Phutrakul[*], 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, R.O.C.

[‡] Chemistry Unit, School of Science, Rangsit University, Patumthani, 12000, Thailand.

[1] To whom correspondence should be addressed.

Prof. Dr. Shui-Tein Chen, Institute of Biological Chemistry, Academia Sinica,

128 Yen Chiu Yuan Rd., Sec II, Nankang, Taipei, Taiwan, R.O.C.

Tel: 886-2-27855981 ext.7071 Fax: 886-2-27883473.

E-mail: bcchen@gate.sinica.edu.tw

Running title: Thermostable lipase from *B. stearothermophilus* P1

The thermostable lipase from a thermophilic bacterium *Bacillus stearothermophilus* P1 has been cloned and overexpressed in *E. coli* M15[pREP4] using pQE-60 as a vector. It was purified to homogeneity by ultrafiltration and strong anion exchange Q HyperD chromatography. The molecular mass of the purified 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 stable for 1 h at pH 8.5-9.0 and 55°C and highly stable in the temperature range of 30-65°C. The highest activity was found with *p*-NP caprate as the synthetic substrate and tricaprylin as the triacylglycerol. The K_m and V_{max} values obtained for the lipase toward *p*-NP caprate were 2.86 mM and 88 $U.ml^{-1}.min^{-1}$, respectively. It was strongly inhibited by 10 mM PMSF 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 at 37°C for 1 h in 0.1% CHAPS and Triton X-100. It was also stable in various organic solvents (30%v/v) at 37°C for 1 h except acetonitrile and butanol. The chiral separation of the lipase on 3-phenoxy-1,2-propanediol showed the lipase has a preference on S(−)-form, especially in dichloromethane.

Keywords: Thermostable lipase; *Bacillus stearothermophilus*; Characterization

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

1. Introduction

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, trans-esterification and inter-esterification in organic solvents. Lipases are thus of particular importance in biotechnology because of diverse applications in the food industry, biological detergents, medical applications, 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 because they 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 [6-12]. For these reasons, lipases have found applications in various fields of biotransformations. These can be classified according to the nature of the substrates into three main categories: i) modification of fats and oils; ii) acylation/deacylation of carbohydrates and protecting/deprotecting of peptides; and iii) synthesis of chiral compounds. In this report, the major focus will be given to the last application. To obtain additional potentially useful thermostable lipases, we were successful in isolating a number of thermophilic bacteria from a hot spring in Chiang Mai, Thailand, which produce extracellular lipases. Among there is the bacterium *Bacillus stearothermophilus* strain P1, which produces highly thermostable lipase [13].

In this report, we present the characterization and application of the purified recombinant lipase from a thermophilic *B. stearothermophilus* P1 that had been markedly increasing by cloning and overexpressed in *E. coli* M15[pREP4] using pQE-60 as an expression vector [14]. Its properties are very interesting in terms of its application, especially its highly stability. Not only it was stable in a high temperature but also stable in various pH values, detergents and organic solvents. In addition, it catalyzed the acetylation of racemic 3-phenoxy-1,2-propanediol as substrate that plays an important role as target molecules in asymmetric synthesis with enzymes as chiral catalysts.

2. Materials and methods

2.1. Bacterial strain and cultivation

A recombinant lipase from *B. stearothermophilus* P1, which had been cloned into *E. coli* M15[pREP4] using pQE-60 as an expression vector was grown at 37°C to late log phase in 4 liter of LB broth containing 100 μg/ml of ampicillin and 25 μg/ml of kanamycin, then IPTG (MDBio Inc.) was added to a final concentration of 1 mM. After 3 h of growth, the cells were centrifuged at 6,500 g for 20 min, the pellet resuspended in 50 ml of 20 mM Tris-HCl buffer pH 8.5 containing 10 mM EDTA, and the cells lysed by sonication. After centrifugation at 12,000 g for 20 min, streptomycin sulfate (Sigma) was added to the supernatant to a final concentration of 1%(w/v) and the precipitate formed

removed by centrifugation at 12,000 g for 15 min. The supernatant was checked for lipase by SDS-PAGE and lipase assay.

2.2. Lipase purification

The crude enzyme preparation from the 4-liter culture was simultaneously partially purified and concentrated by ultrafiltration using a membrane with a molecular weight cut-off of 10,000 Da. The concentrated protein sample (5 ml) was purified by FPLC using anion-exchange chromatography on a Q HyperD^{TM}10 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 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^{TM}, Finnigan).

2.3. Lipase assay

The relative hydrolytic activity of the lipase towards different triacylglycerols was determined by spectrophotometric assay using the formation of copper soaps for the detection of free fatty acids [15]. The substrate solution consisted of triglycerides (100 mM) emulsified with homogenizer in distilled water with gum arabic (0.2 mM) at maximum speed for 2 min. Copper (II)-acetate-1-hydrate aqueous solution (90 mM), adjusted to pH 6.1 with pyridine, was used as copper reagent. The chromogenic reagent contained diethyldithiocarbamic acid (sigma) (5.8 mM) dissolved in absolute ethanol. The reaction was started by addition of 0.1 ml substrate solution to 0.9 ml of enzyme solution in 20 mM Tris-HCl buffer (pH 8.5). The enzyme reaction was carried out for 2 min at 55°C. Immediately after incubation, 0.45 ml of reaction mixture was transferred to a test tube containing 0.25 ml of 3 M HCl. Fatty acids were subsequently extracted by addition of 3 ml n-hexane and vigorous vortexing for 2 min. The organic phase (2.5 ml) was transferred to a fresh test tube filled with 0.5 ml copper reagent. The mixture was vortexed for 1.5 min and phase separation was achieved by centrifugation. Then, the organic phase (2 ml) was mixed with 0.4 ml of the chromogenic reagent and the absorption was measured at 430 nm.

Lipase activity was also assayed using the synthetic substrate, *p*-NP caprate (Sigma) [16,17]. Lipase solution (20 µl) 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 pre-warmed to 55°C, 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 measured against an enzyme-free blank. One enzyme unit is defined as the release of 1 nmol of *p*-nitrophenol per ml per min. Under the conditions described, the extinction coefficient of *p*-nitrophenol is $\varepsilon = 1.85\ l.mmol^{-1}.mm^{-1}$.

2.4. Protein determination

The protein concentration was measured spectrophotometrically at 280 nm or by the method of Bradford [18] using bovine serum albumin (Sigma) as the standard.

2.5. 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 [19]. 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).

2.6. 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 [20]. The lipase band was cut out and analyzed by Edman degradation using an Applied Biosystems Model 492 procise sequencer (Applied Biosystems, Weiterstadt, Germany).

2.7. Effect of pH on lipase activity and stability

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 50 mM sodium acetate, 50 mM phosphate, 50 mM Tris-HCl and 50 mM Tris-glycine, respectively. The pH stability in the pH range 4.0-11.0 was measured by incubating the lipase at 55°C for 1 h in various buffers and assaying the remaining activity in the usual assay at pH 8.5.

2.8. 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, then assaying a 0.1 ml sample at 55°C.

2.9. Substrate specificity

Substrate specificities for different *p*-NP esters and triacylglycerols were determined by using the spectrophotometric assay. The *p*-NP esters between C2-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. The triacylglycerols between C2-C22 was 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.

2.10. Kinetic study of lipase

The reactions were carry out at 55°C by using various *p*-NP caprate concentrations of 2, 4, 6, 8, 10, 20, 30 and 50 mM and various reaction times of 30 sec, 1 min, 1.5 min, 2 min, 4 min, 6 min, 8 min and 10 min. The kinetic constants of apparent Michaelis constants (K_m) and maximum reaction rates (V_{max}) of the reactions were calculated from the Lineweaver-Burk plot.

2.11. Positional specificity

The positional specificity of the lipase was determined by use of a method modified from that described by Lesuisse et al. [21]. 40 mg of pure triolein, diolein and monolein were dissolved in 1 ml of chloroform. The enzyme sample (~20 mg) was then added and the reaction mixture was incubated at 55°C for 1 h. After incubation, the reaction products were extracted by the addition of 1.0 ml diethyl ether. The extract was concentrated by lyophilization and applied to a silica gel plate (0.2 mm, Merck). A standard mixture of tri-, di-, and monoolein (Sigma) was used as a reference. Plates were developed with a 96:4:1 mixture (by volume) of chloroform/acetone/acetic acid. The spots of glycerides and fatty acids were visualized by exposure to iodine vapor.

2.12. Effect of inhibitors on lipase activity

The effect of inhibitors on lipase activity was determined using DTT, 1-dodecanesulfonyl chloride, EDTA, 1-hexadecanesulfonyl chloride, β-mercaptoethanol, and PMSF at final concentrations of 10 mM. The enzyme solutions were pre-incubated at 37°C for 5 min with each inhibitor in 20 mM Tris-HCl buffer pH 8.5, and then the lipase activity was measured. Enzyme solution without inhibitor was used as reference.

2.13. 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.

2.14. Effect of organic solvents on the lipase stability

The enzyme solutions were incubated in the presence of various organic solvents such as acetone, acetonitrile, benzene, butanol, chloroform, cyclohexane, dichloromethane, diethyl ether, ethanol, n-heptane, hexane, iso-octane, iso-propanol, methanol and toluene in the final concentration of

30%(v/v) at 37°C for 1 h and the control contained no organic solvent as described by Sztajer et al. [22]. The activity was determined at the beginning and the end of incubation time.

2.15. Chiral separation of lipase

A solution of racemic 3-phenoxy-1,2-propanediol (168.2 mg, 0.1 mmol) in various organics solvents (acetone, chloroform, dichloromethane; 3 ml) was treated with vinyl acetate (0.2 ml) and recombinant lipase P1 (10 mg). The suspension was stirred at 55°C for 1 h. The monitoring of the reaction and the determination of enantiomeric excess of the remaining substrate (or the product) were conducted simultaneously by HPLC analysis using Chiralcel OD-RH (4.6×150 mm, Daicel Chemical Co.) with the conditions: mobile phase, 0.2 M H_3PO_4-KH_2PO_4 pH 2/acetonitrile (70:30 v/v); flow rate, 0.5 ml/min; detection, UV 254 nm; retention time, 7 min.

3. Results

3.1. 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 cut-off 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-HyperD^{TM}10) 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 [14]. The molecular weight of the purified lipase approximately 43,209 Da was confirmed by Mass spectrometer and calculating from the deduced amino acid sequence using MacVector sequence analysis software. The enzyme was purified 18-fold with a yield of 71% from the crude extract.

3.2. Effect of pH on lipase activity and stability

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 7.0-10.0, the optimal pH being 8.5 (Fig. 1A). At 55°C, the lipase was stable for 1 h in a wide range of buffers between pH 8.0-11.0 (Fig. 1B). It showed greatest stability at pH 8.5-9.0, the same range as the optimal pH.

3.3. 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. 2A). 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. 2B). 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.

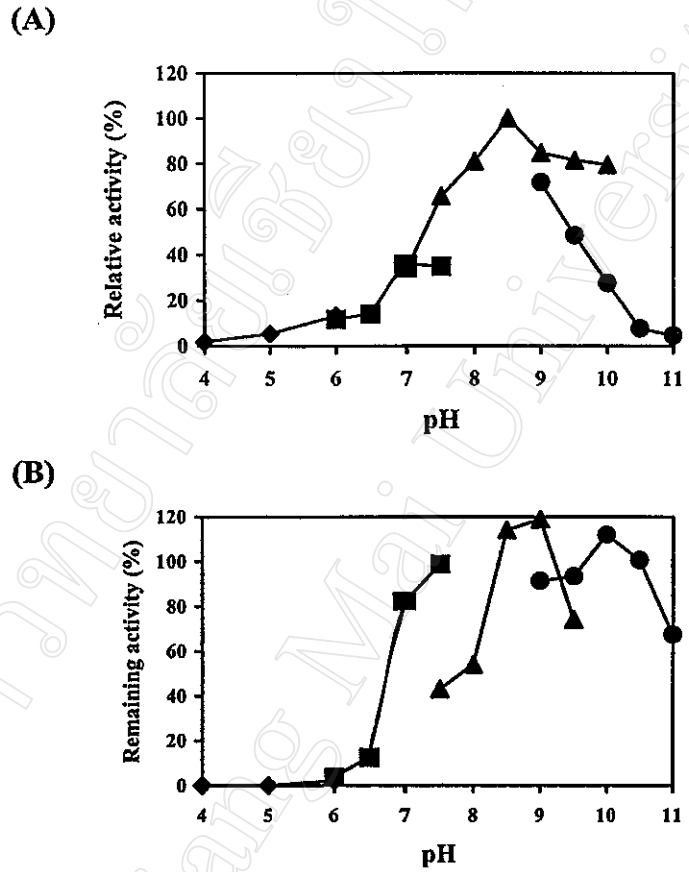
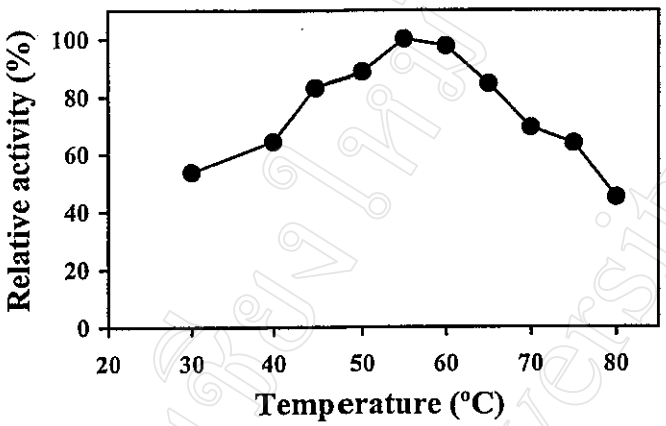


Fig. 1. Effect of pH on lipase activity (A) and stability (B). A: The purified lipase was assayed in various pH buffers, as described in the text. B: The purified lipase was incubated in various pH buffers for 1 h at 55°C, then assayed in the normal way. Symbols: ♦, Sodium acetate buffer; , Phosphate buffer; , Tris-HCl buffer; •, Tris-glycine buffer.

3.4. 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. 3). The lipase activity on long-chain of substrates was between 70-100% of optimal for C8 or C10 groups and 30-50% 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-C12, with optimal activity with C8 (tricaprylin). In addition, the lipase hydrolyzed trilinolenin more than trilinolein and triolein.

(A)



(B)

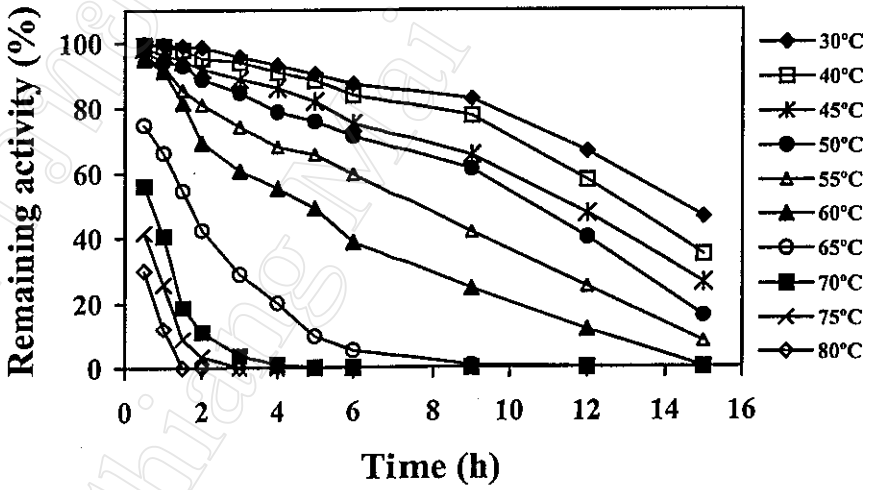


Fig 2. Effect of temperature on lipase activity and stability. A: The effect of temperature on lipase activity was determined at various temperatures, as described in the text. B: The effect of temperature on lipase stability was determined by incubating the pure lipase at various temperatures for up to 15 h and measuring the remaining activity.

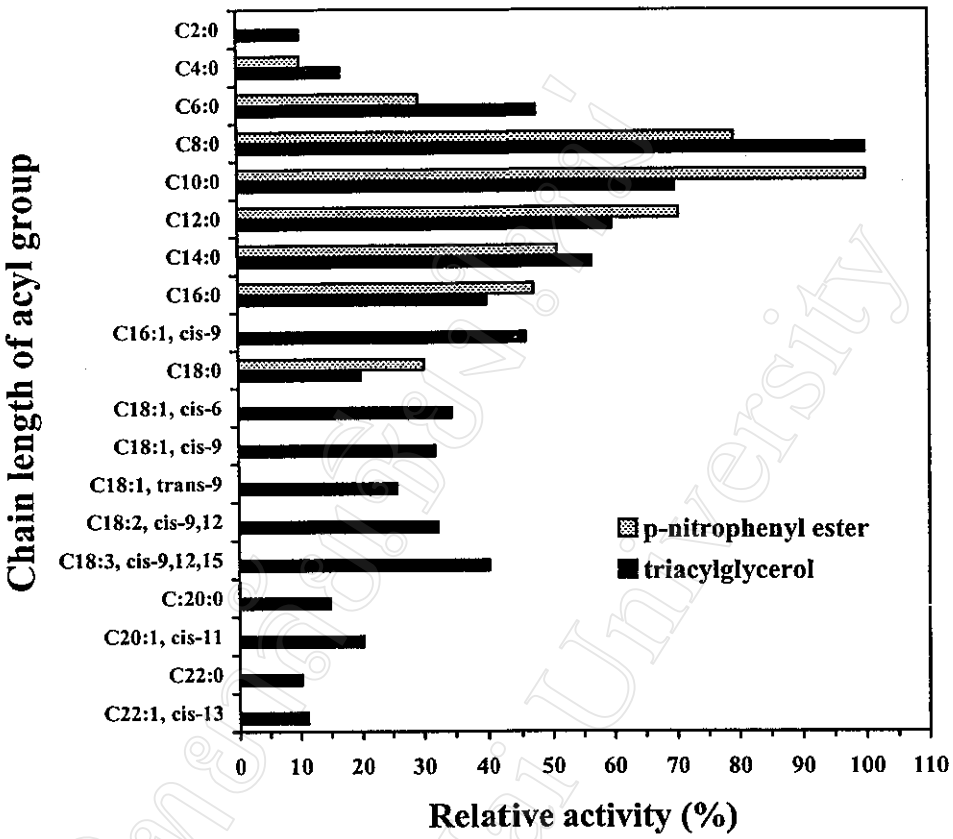


Fig. 3. Substrate specificity of the lipase using several *p*-NP esters and triacylglycerols.

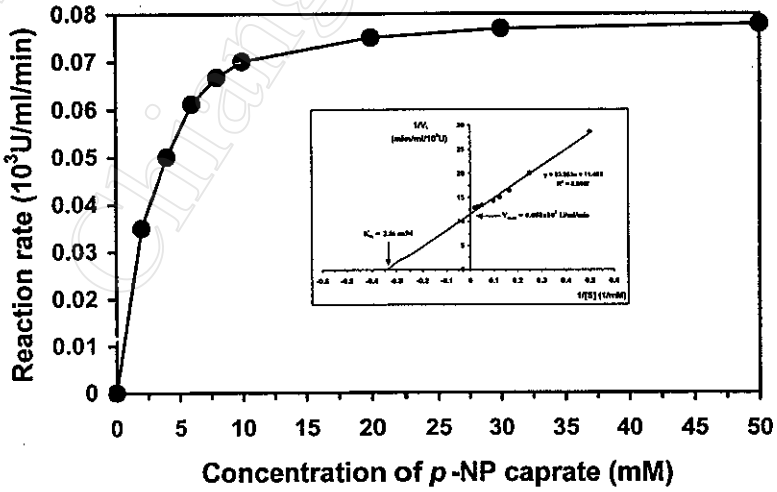


Fig. 4. Michaelis-Menten type plot of lipase hydrolysis rate at different *p*-NP caprate concentrations. Insert: Lineweaver-Burk representation of Michaelis-Menten plot.

3.5. Kinetic study of lipase

The hydrolysis of *p*-NP caprate was found to follow a Michaelis-Menten type and Lineweaver-Burk plot as shown in Fig. 4. The K_m and V_{max} values at 55°C values were found to be 2.86 mM and 88 $U.ml^{-1}min^{-1}$, respectively.

3.6. Positional specificity

All substrates; 1-mono-, 1,2-di- , 1,3-di-, and triolein, were hydrolyzed by the lipase. At 55°C, the acyl migration was occurred, which could be seen in the control (without enzyme) but containing 1,2- and 1,3-diolein substrate solutions, respectively. After 1 h of incubation, more oleic acid was released from 1,3-diolein as confirmed by running TLC (Data not shown). Otherwise, it was also checked the lipase activity and found that their activities of the hydrolysis of monoolein, diolein and triolein were 7.96, 2.39 and 6.80 U/ml, respectively. So, it indicated that the lipase P1 has a preference for digestion at 1-positional ester bonds. This is similar to the enzymes from the other strains of *Bacillus* sp. [12], *B. subtilis* 168 [16], and *B. thermocatenulatus* [10].

3.7. Effect of inhibitors on lipase activity

The effect of various inhibitors on lipase activity is shown in Table 1. All inhibitors were effective at 10 mM. The chelating agent EDTA did not high affect the activity of the lipase, and this suggested that it was not a metalloenzyme. On the other hand, 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.

Table 1. Effect of inhibitors on the purified lipase. The lipase was pre-incubated with each inhibitor in the final concentration of 10 mM at 37°C for 5 min and then the remaining activity assayed.

Inhibitors	**Remaining activity (%)**
Control	100
DTT	83
EDTA	76
β-mercaptoethanol	88
PMSF	23
1-Dodecanesulfonyl chloride	42
1-Hexadecanesulfonyl chloride	7

3.8. 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 2). 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 by more than 50%, except in the case of CHAPS and Triton X-100.

Table 2. Effect of detergents on the purified lipase. The lipase was incubated at 37°C for 1 h in 20 mM Tris-HCl buffer pH 8.5, with detergents.

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

Table 3. Effect of organic solvents on the purified lipase. The lipase was incubated at 37°C for 30 min in several organic solvents and then the residual activities was assayed.

Organic solvents	log P*	Relative activity (%)	
		0 h	1 h
Control	-	100	97
Methanol	-0.77	83	45
Acetonitrile	-0.34	66	0.2
Ethanol	-0.32	79	66
Acetone	-0.24	72	78
Iso-propanol	0.05	52	39
Butanol	0.88	77	14
Diethyl ether	0.89	85	67
Dichloromethane	1.25	86	74
Benzene	2.00	90	92
Chloroform	2.24	92	92
Toluene	2.69	88	93
Cyclohexane	3.44	70	46
Hexane	3.48	61	54
n-Heptane	4.00	88	95
Iso-octane	4.52	69	60

*The logarithm of the solvent partition coefficient between octanol and water.

3.9. Effect of organic solvents on the lipase stability

After the enzyme was incubated in the presence of 30%v/v organic solvents at 37°C for 1 h, it was found that the enzyme could stable in the presence of ethanol, acetone, diethyl ether,

dichloromethane, benzene, chloroform, toluene, hexane, n-heptane and iso-octane (Table 3). It was highly stable in the presence of n-heptane, toluene, benzene and chloroform but it was inactive in the presence of acetonitrile and butanol. Otherwise, its thermostability did not depend on the log P of organic solvents.

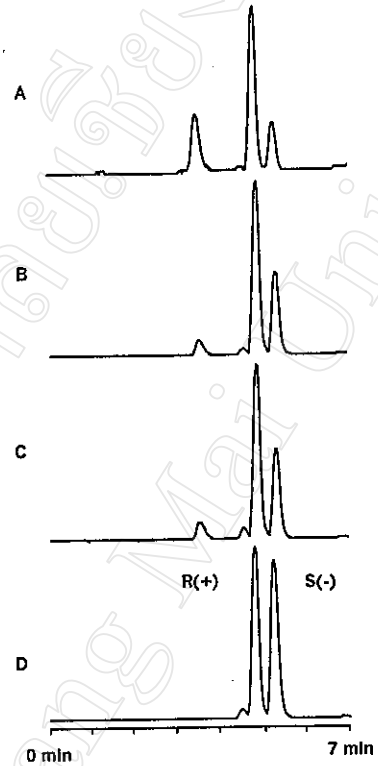


Fig. 5. Analysis of unreacted starting material of 3-phenoxy-1,2-propanediol catalyzed with lipase from *B. stearothermophilus* P1 by Chiralcel OD-RH. A, reaction in dichloromethane; B, reaction in chloroform; C, reaction in acetone; D, standard of 3-phenoxy-1,2-propanediol.

3.10. Chiral separation of lipase

The lipase P1 catalyzed the acylation of diol-substrate, 3-phenoxy-1,2-propanediol to form the first product, 1-acetyl-3-phenoxy-1,2-propanediol or 2-acetyl-3-phenoxy-1,2- propanediol and then the second product, 1,2-diacetyl-3-phenoxy-1,2-propanediol. The unreacted starting material of 3-phenoxy-1,2-propanediol was analyzed by Chiral OD-RH column. R(+) 3-Phenoxy-1,2-propanediol was eluted at the retention time of 4.776 min and the S(−) 3-phenoxy-1,2-propanediol was eluted at the retention time of 5.221 min (Fig. 5). The lipase P1-catalyzed acetylation has the enantiomeric preference of S(−)

3-phenoxy-1,2- propanediol when the reaction was performed in acetone, chloroform, and dichloromethane. The ratio of unreacted substrate R(+)/S(−) in acetone and chloroform is 2:1 whereas the ratio in dichloromethane is 3:1.

4. Discussion

The thermophilic bacterium *B. stearothermophilus* isolated from a hot spring in Chiang Mai, Thailand could produce the highly thermostable proteases [23]. In this work, the bacterium strain *B. stearothermophilus* P1 could produce a highly thermostable lipase [13]. Due to the limited production, so it had been cloned into *E. coli* M15[pREP4] using a pQE-60 as an expression vector for markedly increased production. The lipase P1 was purified by a strong ion-exchange chromatography using 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 chromatography, 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.

The characterizations of the purified lipase were very interesting, and showed that it was active over a wide range of pH from 7 to 10 and temperatures between 45 and 65°C. In addition, the enzyme was stable at pH 8-10 and temperatures between 30-65°C. Its half-life at 55°C, the optimal temperature, was about 7.6 h, showing the stability of this enzyme is high at this high temperature and somewhat higher than that of other lipases from *B. thermocatenulatus* [8] and *B. thermoleovorans* [24]. Moreover, the 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. thermoleovolans* 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. Otherwise, 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 [25,26], 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 β-sheet. Serine, one part of the catalytic site, is embedded in a tight bend between an α-helix and a β-stand [27,28]. 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.

In this report, we chose acyclic racemic 3-phenoxy-1,2-propanediol as substrate for lipase-catalyzed transesterification in order to obtain enantiomerically pure compounds. Particularly, 3-(aryloxy)-1,2-propanediols in enantiomerically pure form are of interest as pharmaceuticals, as intermediates in the synthesis of β-receptor blockers or for other synthetic purposes such as chiral ligands for transition metal complexes or building blocks for crown ethers. In the acylation of 3-phenoxy-1,2-propanediol with vinyl acetate catalyzed by lipase P1, the enantioselective adsorptions of S(−) form in acetone, chloroform, and dichloromethane were found and highly enantioselectivity adsorption of S(−) form was occurred in dichloromethane. This implied that the solvents did have much influence on the enantioselectivity of the reaction.

Acknowledgements

This project is part of the Royal Golden Jubilee Ph.D. project of Mr. Supachok Sinchaikul 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, Pandey A. *Candida rugosa* lipases: Molecular biology and versatility in biotechnology. Yeast 1998;14: 1069-87.

[2] Bjorkling F, Godtfredsen SE, Kirk O. The future impact of industrial lipases. TIBTECH 1991; 9: 360-3.

[3] Harwood J. The versatility of lipases for industrial uses. Trends Biochem Sci 1989;14: 125-6.

[4] Jaeger KE, Reetz MT. Microbial lipases form versatile tools for biotechnology. Trend Biotechnol 1998;16(9): 396-403.

[5] Schmid RD, Verger R. Lipases: Interfacial enzymes with attractive applications. Angew Chem Int Ed 1998;37: 1608-33.

[6] Iizumi T, Nakamura K, Fukase T. Purification and characterization of a thermostable lipase from newly isolated *Pseudomonas* sp. KWI-56. Agric Biol Chem 1990;54(5): 1253-8.

[7] Omar IC, Hayashi M, Nagai S. Purification and some properties of a thermostable lipase from *Humicola lanuginosa* no. 3. Agric Biol Chem 1987;51(1): 37-45.

[8] Schmidt-Dannert C, Rua ML, Atomi H, Schmid RD. Thermoalkalophilic lipase of *Bacillus thermocatenulatus*. I. Molecular cloning, nucleotide sequence, purification and some properties. Biochim Biophys Acta 1996;1301: 105-14.

[9] Schmidt-Dannert C, Rua ML, Wahl S, Schmid RD. *Bacillus thermocatenulatus* lipases: a thermoalkalophilic lipase with interesting properties. Biochem Soc Trans 1997;25(1): 178-82.

[10] Schmidt-Dannert C, Sztajer H, Stocklein W, Menge U, Schmid RD. Screening, purification and properties of a thermophilic lipase from *Bacillus thermocatenulatus*. Biochim Biophys Acta 1994;1214: 43-53.

[11] Sugihara A, Ueshima M, Shimada Y, Tsunasawa S, Tominaga Y. Purification and characterization of a novel thermostable lipase from *Pseudomonas cepacia*. J Biochem 1992;112(5): 598-603.

[12] Sugihara A, Tani T, Tominaga Y. Purification and characterization of a novel thermostable lipase from *Bacillus* sp. J Biochem 1991;109(2): 211-6.

[13] Maungprom N, Kanasawus P, Phutrakul S. Extracellular lipase activity of thermophilic bacterium from Chiang Mai hot spring. J Sci Fac CMU 1996;23: 9-13.

[14] Sinchaikul S, Sookkheo B, Phutrakul S, Pan FM, Chen ST. Optimization of a thermostable lipase from *Bacillus stearothermophilus* P1. Prot Express Pure. In press.

[15] Lee DW, Koh YS, Kim KJ, Kim BC, Choi HJ, Kim DS, Suhartono MT, Pyun TR. Isolation and characterization of a thermophilic lipase from *Bacillus thermoleovorans* ID-1. FEMS Microbiol Lett 1999;179: 393-400.

[16] Lesuisse E, Schanck K, Colson C. Purification and preliminary characterization of the extracellular lipase of *Bacillus subtilis* 168, an extremely basic pH-tolerant enzyme. Eur J Biochem 1993;216: 155-60.

[17] Winkler UK, Stuckmann M Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. J Bacteriol 1979;138: 663-70.

[18] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72: 248-54.

[19] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227: 680-685.

[20] Matsudaira P. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J Biol Chem 1987;262(21): 10035-10038.

[21] Lesuisse E, Schanck K, Colson C. Purification and preliminary characterization of the extracellular lipase of *Bacillus subtilis* 168, and extremely basic pH-tolerant enzyme. Eur J Biochem 1993;216: 155-60.

[22] Sztajer H, Lunsdorf H, Erdmann H, Menge U, Schmid R. Purification and properties of lipas from *Penicillium simplicissimum*. Biochim Biophys Acta 1992;1124: 253-261.

[23] Sookkheo B, Sinchaikul S, Phutrakul S, Chen ST. Purification and Characterization of the Highly Thermostable Proteases from *Bacillus stearothermophilus* TLS33. Protein Expression and Purification 2000;20: 142-151.

[24] Cho AR, Yoo SK, Kim EJ. Cloning, sequencing and expression in *Escherichia coli* of a thermophilic lipase from *Bacillus thermoleovorans* ID-1. FEMS Microbiol Lett 2000;186: 235-8.

[25] Brenner S. The molecular evolution of genes and proteins: a tale of two serines. Nature 1988;334: 528-30.

[26] Cygler M, Schrag JD, Ergan F. Advances in structural understanding of lipases. Biotechnol Genet Eng Rev 1992;10: 143-84.

[27] Derewenda ZS, Derewenda U. Relationships among serine hydrolases: evidence for a common structural motif in triacylglyceride lipases and esterases. Biochem Cell Biol 1991;69: 842-51.

[28] Ollis DL, Cheah E, Cygler M, Dijkstra B, Frolow F, Franken SM, Harel M, Remington SJ, Silman I, Schrag JD, Sussman JL, Verchueren KHG, Goldman A. The α-β hydrolase fold. Protein Eng 1992;5: 197-211.

VITA

Name Mr. Supachok Sinchaikul

Born 5 July 1973

Education

1991-1994 B.Sc. (Animal Science) Faculty of Agriculture, Chiang Mai University, Thailand.

1995-1998 M.Sc. (Biotechnology) Faculty of Science, Chiang Mai University, Thailand.

1998-2001 Ph.D. (Biotechnology) Faculty of Science, Chiang Mai University, Thailand.

Poster Presentation

10-11 April 1999 "Characterization of Thermostable Lipase Secreted from Thermophilic Bacterium Strain P1" The 14th Joint Annual Conference of Biomedical Sciences, Taipei, Taiwan.

21-24 November 1999 "Molecular Cloning, Expression and Purification of Thermostable Lipase from *Bacillus stearothermophilus* P1" The 8th Asian Chemical Congress and Chemical Industry & Instrument Exhibition, Taipei, Taiwan.

Oral Presentation

10-15 June 2000 "Cloning, Expression, Purification and Structural Modeling of a Thermostable Lipase from *Bacillus stearothermophilus* P1" International Conference "BIOCATALYSIS-2000", Moscow, Russia.

20-26 May 2001 "Crystallization of a Thermostable Lipase from *Bacillus stearothermphilus* P1" 1st Southeast Asia Protein Crystallography Workshop, Nakhon Ratchasima, Thailand.

Publications

1. Sinchaikul, S., Sookkheo, B., Phutrakul, S., Pan, F.M. and Chen, S.T. (2001) Optimization of a Thermostable Lipase from *Bacillus stearothermophilus* P1: overexpression, purification, and characterization. *Prot. Express. Pure.* 22(3), 388-398.
2. Sinchaikul, S., Sookkheo, B., Phutrakul, S., Wu, Y.T., Pan, F.M. and Chen, S.T. (2001) Structural Modeling and Characterization of a Thermostable Lipase from *Bacillus stearothermophilus* P1. *Biochem. Biophys. Res. Com.* 283(4), 868-875.
3. Sinchaikul, S., Tyndall, J.D.A., Fothergill-Gilmore, L.A., Phutrakul, S., Chen, S.T. and Walkinshaw, M.D. (2001) Expression, purification, crystallization and preliminary crystallographic analysis of a thermostable lipase from *Bacillus stearothermophilus* P1. *Acta Cryst. D*, submitted.
4. Sinchaikul, S., Sookkheo, B., Phutrakul, S. and Chen, S.T (2001) Characterization and Application of Purified Lipase from *Bacillus stearothermophilus* P1. *Enzyme Microbial. Technol.*, submitted.

Scholarship

1998-2001 The Royal Golden Jubilee Ph.D. Grant of the Thailand Research Fund (TRF), Thailand.