CHAPTER 2

MATERIALS AND METHODS

2.1) Materials

2.1.1) Chemicals

For the chemicals used in the molecular cloning, agarose was obtained from J.T. Baker, the antibiotics such as ampicillin and kanamycin were obtained from aMRESCO and Nacalai Tesque, respectively, isopropyl-β-D-thiogalactoside (IPTG) was from Viogene, and the LB miller broth was from Scharlau. The restriction enzymes (*Nco* I and *Hind* III) and their buffers were from BioLabs, Inc. (New England). The streptomycin sulphate was obtained from Sigma. The TBE buffer (5×) and TG-SDS buffer (10×) were from aMERSCO. T4-DNA ligase was from Serva Feinbiochemika (Heidelberg, Germany). Thermo Sequenase dye terminator cycle sequencing pre-mix kit with Thermo Sequenase™ polymerase was from Amersham Life Science (Ohio, USA).

For the general chemicals used, acryl/bis 29:1 solution and 10× TG-SDS

buffer was obtained from aMERSCO, ammonium sulphate was obtained from BDH and TEMED was obtained from Sigma. The dyes such as coomassie brilliant blue R-250 and naphthol blue black were from Sigma. The cupric acetate, deoxycholate sodium salt, diethyldithiocarbamic acid and gum arabic used in the lipase assay were from Sigma. The lipase substrates such as triacylglycerols, *p*-nitrophenyl esters, monoolein, diolein and triolein were from Sigma and inhibitors such as dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), β-mercaptoethanol, phenylmethanesulfonyl fluoride (PMSF), 1-dodecanesulfonyl chloride and 1-hexadecanesulfonyl chloride were obtained from Sigma and Roche Molecular Biochemicals (Germany). The LMW electrophoresis calibration kit was obtained from Amersham Pharmacia Biotech. 3-phenoxy-1,2-propanediol was obtained from Sigma. The surfactants such as sodiumdodecyl sulphate (SDS), triton X-100 and Tween 20 were from Sigma. All other chemicals used were of analytical grade.

2.1.2) Primers

All primers were purchased from GENSET Singapore Biotech. Pte Ltd as follows in Table 2.1.

Table 2.1. All primers were used in the experiments.

Primers	Sequences	Size (mer)	Concentration (μg/μl)
Lipase-F	5′-GCCCATGGCATCCCTACGCGCC-3′	22	1.65
Lipase-R	5′-GCAAGCTTAAGGCTGCAAGCTCGC-3′	24	1.93
P1	5′-GACAAGCGTCGTCCCGTCATG-3′	21	2.34
P2	5′-GGCACAAACATGCGCTTTTC-3′	20	1.42
PQE-F	5′-GGCGTATCACGAGGCCCTTTCG-3′	22	0.98
pQE-R	5′-CATTACTGGATCTATCAACAGG-3′	22	1.05
M13-for	5′-AGGGTTTTCCCAGTCACGAC-3′	20	1.04
M13-rev	5′-ACACAGGAAACAGCTATGAC-3′	20	1.11
Lipase-1F	5′-ATCGCCCACGCACAAGGGGGG-3′	21	1.91
Lipase-1R	5′-CCCCCCTTGTGCGTGGGCGAT-3′	21	1.84
Lipase-2F	5′-CTTGAGAACGCAGGCATTGTC-3′	21	2.50
Lipase-2R	5′-GACAATGCCTGCGTTCTCAAG-3′	21	2.24
Lipase-3F	5′-AATGTCGACGCATTGGAAATC-3′	21	1.96
Lipase-3R	5′-GATTTCCAATGCGTCGACATT-3′	21	2.28

2.1.3) Equipments

For cultivation of microorganisms, autoclave (Model AS-4160) was obtained from Tomin, centrifuge Avanti™ J-25 was obtained from Beckman, incubator (Model:

1353) was obtained from Hotech and orbital shaking incubator (Model: S300R) was obtained from Firstek Scientific.

For the molecular cloning, Applied Biosystems 373 DNA Sequencer was obtained from Stretch, GeneAmp PCR System 9700 was obtained from PE Applied Biosystems, GeneQuant II was obtained from Amersham Pharmacia Biotech, Mini-M Plasmid DNA Miniprep System was obtained from Viogenic and/or Wizard™ Plus Minipreps DNA Purification System was obtained from Promega and PCR Clean UP-M System Kit was obtained from Viogenic.

For the purifications, AKTApurifier System (FPLC) was obtained from Amersham Pharmacia Biotech, electrophoresis power supply – EPS601 was obtained from Amersham Pharmacia Biotech, Flexidry (Model FD24) was obtained from Kingmech, HPLC was obtained from Hitachi, fraction collector (Model FC203B) was obtained from Gilson, Q HyperD™ 10 prepacked column (Optima™ 5/10) was obtained from Biosepra, Spectra/Por® molecularporous membrane (MWCO: 12,000-14,000) was obtained from Spectrum and viva spin concentrator was obtained from Vivascience.

For the general equipments, the analytical balance (Model AB204) was obtained from Mettler Toledo, cover glass (Borosilicate lass) 22mm was obtained

from BDH, Immobilon™-P transfer membranes (PVDF) was obtained from Millipore, microcentrifuge (KM-15200) was obtained from Kubota, micropipettes was obtained from Beckman, orbital shaker bath (Model: 902) was obtained from Hotech, pH meter (Model SP-701) was obtained from Suntex, sonicator was obtained from Sonics&Materials Inc., 24-well tissue culture plates was obtained from Falcon, U-3300 spectrophotometer was obtained from Hitachi and vortex Genie-2 was obtained from Scientific Industries.

2.1.4) Microorganisms

The microorganisms used were the cloned thermostable lipase P1 in *E. coli* DH5α, *E. coli* M15[pREP4] and *E. coli* JM109.

2.2) Cultivation

2.2.1) Cultivation of cloned lipase P1 in *E. coli* DH5α

The cloned thermostable lipase P1, which has been cloned in *E. coli* DH5α using pUC-19 as a vector was grown at 37°C to late log phase in 100 ml-LB broth containing 100 μg/ml ampicillin with shaking of 200 rpm (about 16-18 h). The cell pellet was obtained by centrifugation at 6,500 ×g for 20 min.

2.2.2) Extraction of cloned lipase

The cell pellet (~2 g) was resuspended in 10-15 ml of 20 mM Tris-HCl buffer pH 8.5 containing 10 mM EDTA and lysed by sonication (pulse: 1.0 sec-on, 1.5 sec-off; time: 30 sec; amplitude 95%). It was centrifuged at 12,000 ×g for 20 min and the supernatant was then added with 1%(w/v) streptomycin sulfate. The precipitate was removed by centrifugation at 12,000 ×g for 15 min.

2.3) Molecular cloning

2.3.1) Extraction of plasmid DNA

It was done by using Wizard®*Plus* SV minipreps DNA purification system kit (Promega).

2.3.1.1) Production of a Cleared Lysate

The 1-5 ml of bacterial culture containing high copy number plasmid or 10 ml of bacterial culture containing low copy number plasmid was centrifuged for 5 minutes at 10,000 ×g in a tabletop centrifuge. The supernatant was poured off and the tube was blotted upside down on a paper towel to remove excess liquid. The 250 µl of Wizard®*Plus* SV Cell Resuspension Solution was added and completely resuspended

the cell pellet by vortexing well or pipetting. It was essential to thoroughly resuspend the cells. The 250 µl of Wizard®*Plus* SV Cell Lysis Solution was added and mixed by inverting the tube 4 times (do not vortex). It was incubated until the cell suspension clear (approximately 1-5 minutes). The 350 µl of Wizard®*Plus* SV Neutralization Solution was added and immediately mixed by inverting the tube 4 times (did not vortex). The bacterial lysate was centrifuged at 14,000 ×g in a microcentrifuge for 10 minutes at room temperature.

2.3.1.2) Plasmid DNA Purification

The clear lysate (Part 2.3.1.1; approximately 850 µl) was transferred to the prepared Wizard®*Plus* SV Minipreps Spin Column, by decanting. Avoid disturbing or transferring any of the white precipitate with the supernatant. If the white precipitate is accidentally transferred to the Spin Column, the spin Column contents should be poured back into a steriled 1.5 ml microcentrifuge tube and centrifuged for another 5-10 minutes at 14,000 ×g. The resulting supernatant was transferred into the same Spin Column that was used initially for this sample. The supernatant was centrifuged at 14,000 ×g in a microcentrifuge for 1 minute at room temperature. The Wizard®*Plus* SV Minipreps Spin Column was removed from the tube and discarded the flow

through from the Collection Tube. The Spin Column was reinserted into the Collection Tube. The 750 µl of Wizard®*Plus* SV Minipreps Spin Column Wash Solution was added and it was centrifuged at 14,000 ×g in a microcentrifuge for 1 minute at room temperature. The Wizard®*Plus* SV Minipreps Spin Column was removed from the tube and discarded the flow through. The Spin Column was reinserted into the Collection Tube. The 250 µl of Wizard®*Plus* SV Minipreps Spin Column Wash Solution was added and it was centrifuged at 14,000 ×g in a microcentrifuge for 2 minute at room temperature. The Wizard®*Plus* SV Minipreps Spin Column was carefully transferred to a new steriled 1.5 ml microcentrifuge tube without transferring any of the Wizard®*Plus* SV Minipreps Spin Column Wash Solution with the Spin Column. If the Spin Column had Column Wash Solution associated with it, it should be centrifuged again for 1 minute at 14,000 ×g before transferring to the new 1.5 ml tube. The plasmid DNA was eluted by adding 100 µl of steriled deionized water to the Wizard®*Plus* SV Minipreps Spin Column and centrifuged at 14,000 ×g for 1 minute at room temperature in a microcentrifuge. After eluting the DNA, it was removed from the 1.5 ml microcentrifuge tube and discarded the Wizard®*Plus* SV Minipreps Spin Column. DNA was stable in water without addition of a buffer such as TE buffer if stored at -20°C or lower. DNA was stable at

4°C in TE buffer. To store the DNA in TE buffer, 10 µl of 10× TE buffer was added to the 100 µl of eluted DNA. It was important to consider the consequences of DNA storage in TE buffer. The microcentrifuge tube was capped and the purified plasmid DNA was stored at -20°C or below.

2.3.2) PCR Clean up

Cleaning up the PCR was done using PCR clean up system kit. The PCR product or contaminated DNA (10-100 µl) was pipetted into a new 1.5 ml tube and 0.5 ml Buffer PX was added and mixed. A membrane-spin column was placed on a 1.5 ml tube and all (about 0.6 ml) of the extract from step 1 was pipetted into the column, centrifuged at 10,000 ×g for 30 seconds and discarded the flow-through. This was washed once with 0.5 ml of Wash I buffer by centrifuging at 10,000 ×g for 30 seconds and discarded the flow-through. It was washed once with 0.7 ml of Wash II buffer (add 30 ml of 98% ethanol to the bottle when first open) by centrifuging at 10,000 ×g for 30 seconds and discarded the flow-through. Then, it was centrifuged at 10,000 ×g for 1 minute to remove ethanol residue. It was placed in a new 1.5 ml tube. The 30 µl of dd. water was added into the center of the column, stood for 30 seconds and centrifuged at 10,000 ×g for 1 minute to elute DNA.

2.3.3) Polymerase Chain Reaction (PCR) amplification

The PCR mixture consisted of 1 µl of 0.5 µg/µl DNA template of lipase plasmid, 1 µl of 0.5 µg/µl lipase-F, 1 µl of 0.5 µg/µl lipase-R, 3 µl of 10× PCR buffer + Mg^{2+}, 1 µl of 2.5 mM dNTP, 1 µl of Taq DNA polymerase, and 17 µl of dd. water was put them into a PCR tube. The PCR was run using condition: 1. 94°C for 5 min; 2. Cycle: denature: 94°C for 30 sec, annealing: 55°C for 30 sec, elongation: 72°C for 2 min (30 cycles); 72°C for 7 min. PCR product was cleaned up with PCR clean up kit and run on 1.5% agarose gel electrophoresis with 100 V for 50 min.

2.3.4) Digestion DNA with restriction enzymes

The DNA solution from PCR product (30 µl) was mixed with 6 µl of steriled water in a steriled Eppendorf tube. The approximately 6 µl of 1× buffer K as digestion buffer containing 0.1% BSA was added and mixed by tapping tube. The 1 unit of restriction enzyme was added and mixed by tapping tube. (Ten unit of each enzyme completed digest 1 µg of DNA at 37°C in 1 hour in 50 µl reaction mixture.) For this research, 4 µl of each *Nco* I and *Hind* III were used. The DNA and restriction enzymes mixture was incubated at 37°C for more than 2 hours. The reaction was then done the PCR clean up and checked the digestion with agarose gel electrophoresis. If

the DNA was to be analyzed directly on a gel, 6 µl of gel-loading dye was added, mixed by vortexing briefly and loaded the digest into the gel slot. If the restricted DNA is to be purified, extracted once with phenol/ chloroform, once with chloroform, and precipitated the DNA with ethanol.

2.3.5) Ligation

The DNA fragment of the lipase gene (0.01-0.1 pmol) and pQE-60 as an expression vector that were cut with the same restriction enzymes (*Nco* I and *Hind* III) were mixed in the volumes of 5 and 2 µl, respectively. They were added with 10× ligation buffer, ligase and ATP each 1 µl and then incubated at 16°C for at least 4 h.

2.3.6) Transformation of competent *E. coli* M15[pREP4] and JM109

2.3.6.1) Preparation of competent *E. coli* M15[pREP4] and JM109

A trace of M15[pREP4] cells was removed from the vial with a steriled toothpick or inoculating loop, and streaked it out on LB agar containing 25 µg/ml kanamycin. It was incubated at 37°C overnight. A single colony was picked and inoculated into 10 ml of LB-kanamycin (25 µg/ml) and grown overnight at 37°C. One of overnight culture was added into 100 ml prewarmed LB broth containing 25 µg/ml

kanamycin in a 250-ml flask and shaken at 37°C until OD_{600} of 0.5 was reached (approximately 90-120 min). The culture was cooled on ice for 5 min and transferred into a steriled round-bottom tube. The cells were collected by centrifugation at low speed (5 min, 4,000 ×g, 4°C). The supernatant was carefully discarded and the cells were kept on ice all the times. The cells were gently resuspended in cold (4°C) TFB1 buffer (30 ml for a 100 ml culture) and the suspension was kept on ice for an additional 90 min. The cells were collected by centrifugation (5 min at 4,000 ×g, 4°C). The supernatant was carefully discarded and the cells were kept on ice all the times. The cells were carefully resuspended in 4 ml ice-cold TFB2 buffer. The aliquots of 100-200 µl in steriled microfuge tubes were prepared and frozen in liquid nitrogen or dry-ice-ethanol. The component cells were stored at –70°C). The preparation of competent cells of *E. coli* JM109 was done the same as *E. coli* M15[pREP4] but it was grown in LB medium without antibiotic.

2.3.6.2) Transformation of competent *E. coli* M15 cells and JM109

An aliquot of the ligation mixture (10 µl or less) was transferred into a cold steriled 1.5 ml microcentrifuge tube and kept on ice. An aliquot of frozen competent M15[pREP4] cells was thawed on ice. The cells were gently resuspended and 100 µl

of the cell suspension was transferred into the microcentrifuge tube with the ligation mixture. It was mixed carefully and kept on ice for 20 min. The tube was incubated at 42°C for 90 sec. The 500 µl Psi broth was added to the cells and incubated for 60-90 min at 37°C. (Shaking increases transformation efficiency.) The aliquots of 50-, 100-, and 200-µl were plated on LB-agar plates containing 100 µg/ml ampicillin and 25 µg/ml kanamycin. The plates were incubated at 37°C overnight. For transformation in *E. coli* JM109, the transformant DNA was plated on LB-agar plate containing 100 µg/µl ampicillin.

2.3.7) Checking the inserted DNA

For the cloned lipase in *E. coli* M15[pREP4], the single colony on the LB agar plate containing 100 µg/ml ampicillin and 25 µg/ml kanamycin after incubated at 37°C overnight was picked and transferred into the new LB broth containing 100 µg/ml ampicillin and 25 µg/ml kanamycin and incubated at 37°C with shaking at 200 rpm for 16-18 h. The cultures medium was taken for the PCR as the DNA templatc. The PCR condition was done by using the method as described above and the primers used were lipase-F and lipase-R, P1 and P2, and pQE-F and pQE-R. Checking of the inserted DNA of the cloned lipase in *E. coli* JM109 was done the same as *E. coli*

M15[pREP4] but it was grown in LB agar plate and/or LB broth containing 100 μg/ml ampicillin. In addition, they were also determined the nucleotide sequences of the lipase gene by nucleotide sequence analysis.

2.3.8) Agarose gel electrophoresis

The 1.5%(w/v) agarose gel was prepared by addition of the powdered agarose to the 0.5× TBE buffer and then the slurry was heated in a microwave oven until the agarose dissolved (about 5 min). The agarose gel solution is cooled to 50°C. The agarose gel setting equipment was set and the agarose gel solution was poured into the equipment about 0.5-1.0 mm of agarose between the bottom of the teeth and the base of the gel. The comb was immediately clamped into position near one end of the gel. After the gel was completely set (30-45 minutes at room temperature), the comb was removed carefully and the gel was mounted in the electrophoresis tank. The 0.5× TBE buffer was added into the electrophoresis tank to cover the gel to a depth of about 1 mm and 1-2 drops of 0.5 μg/ml ethidium bromide was added. The samples were mixed with 10× loading buffer containing 5-10% glycerol, 7% sucrose and 0.025% bromophenol blue and loaded into the wells of the submerged gel. Usually the loading buffer was made up as a 6-fold to 10-fold concentrated solution which was mixed

with the sample and then slowly applied to the gel using a dispensable micropipette. It was made sure to include appropriate DNA molecular weight markers. The agarose gel was run on the voltage of 100 V for 50 min and then photographed directly on UV transilluminator or first staining with 0.5 μg/ml ethidium bromide 10 to 30 min and destaining 30 min in water if necessary.

2.3.9) Expression of a recombinant protein

The recombinant lipase P1 in *E. coli* M15[pREP4] was tested for expression by preparing a cell lysate as follows: cells were grown to late log phase in 100 ml of LB broth containing 100 μg/ml ampicillin and 25 μg/ml kanamycin at 37°C with a vigorous shaking until OD_{600} was 0.5-0.7 and then the IPTG (isopropyl-β-D-thiogalactopyranoside) was then various concentrations of 0.2, 0.4, 0.6, 0.8, 1, 2, 3, and 5 mM were added. After culturing for 1,2,3,4 and 5 h, the cell lysates of each concentration were each collected by centrifugation at 6,500 ×g for 20 min. The expression of the lipase was checked by using SDS-PAGE and assayed the lipase activity.

2.4) Structural modeling

2.4.1) 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 [75] and Garnier *et* al. [76]. The surface hydrophobicity profile based on the empirical Kyte-Doolittle hydropathy scale [77] 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 had been reversed in order to plot the hydrophilicity instead of hydrophobicity scale. A window of size N=7 was run along the length of the protein; for each window, the hydropathy values of 7 amino acids were summed and divided by 7 to obtain the average hydrophilicity per residue for the window. The values above the axis denoted hydrophilic regions which might be exposed on the outside of the analyzed molecule whereas those below the axis indicated the hydrophobic regions which tended to be buried inside the lipase structure molecule.

2.4.2) Homology modeling and computer graphic study of the lipase structure

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 University, New York) [78-81]. MAXHOM alignment of the lipase sequences was based on published multiple sequence alignment which in turn on the secondary structure correspondences and confirmed completely with the WU-blastp program (Washington University, Saint Louis, Missouri, USA) using EMBL database for studying the reference structure models [82-84]. 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) [85]. 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 searched a set of the 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 were estimated in order to assess the validity and relative accuracy of the homology modeling and the computerized simulating graphics.

2.4.3) Site-directed mutagenesis

The mutant lipase genes were constructed by site-directed mutagenesis with the PCR overlap extension method [86] using a 5′ and 3′ mutagenic primers containing the desired mutations at Ser-113, Asp-317 and His-358 to Ala as non-polar amino acid residue. After the digestion by *Nco* I and *Hind* III, 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. The PCR overlap extension method was done as follows: a) The oligonucleotide primers lipase 1F, 1R, 2F, 2R, 3F and 3R that were changed the amino acid residues at Ser-113, Asp-317 and His-358 were designed and synthesized. b) In a steriled 0.5-ml microfuge tube or amplification tube, PCR 1 was set up by mixing the following reagents: template DNA ~ 100 ng, 10× amplification

buffer 10 µl, 20 mM mixture of four dNTPs 1 µl, 5 µM primer pQE-F (30 pmoles) 6.0 µl, 5 µM primer lipase 1R (30 pmoles) 6.0 µl, thermostable DNA polymerase 1-2 units and H_2O to 100 µl. c) In a second steriled 0.5-ml microfuge tube or amplification tube, PCR 2 was set up by mixing the following reagents: template DNA ~ 100 ng, 10× amplification buffer 10 µl, 20 mM mixture of four dNTPs 1 µl, 5 µM primer pQE-R (30 pmoles) 6.0 µl, 5 µM primer lipase 1F (30 pmoles) 6.0 µl, thermostable DNA polymerase 1-2 units and H_2O to 100 µl. d) The nucleic acids were amplified using the denaturation, annealing, and polymerization times and temperatures listed in Table 2.2.

Table 2.2. Amplification step for site-directed mutagenesis by using PCR.

Cycle Number	**Denaturation**	**Annealing**	**Polymerization**
Previous cycle	5 min at 94°C		
30 cycles	1 min at 94°C	1 min at 50°C	1-3 min at 72°C
Last cycle	1 min at 94°C	10 min at 72°C	

e) 5% of each of the two PCRs were analyzed on an agarose gel and estimated the concentrations of the amplified target DNAs. f) In a steriled 0.5-ml microfuge tube or

amplification tube, the following reagents were mixed in an amplification reaction to join the 5′ and 3′ ends of the target gene: amplification product PCR 1 (step 2) ~ 50 ng, amplification product PCR 2 (step 3) ~ 50 ng, 10× amplification buffer 10 µl, 5 µM primer pQE-F (30 pmoles) 6.0 µl, 5 µM primer pQE-R (30 pmoles) 6.0 µl, thermostable DNA polymerase 1-2 units and H_2O to 100 µl. g) The nucleic acids were amplified using the denaturation, annealing, and polymerization times and temperatures listed above in step d. h) 5% of the PCR was analyzed on an agarose gel and estimated the concentration of the amplified target DNA. i) The complete sequence of the amplified DNA fragment was verified after cloning to ensure that no mutations other than those in primers and were introduced during these manipulations.

2.5) Purification

2.5.1) Ammonium sulfate precipitation

Aliquots of the extract, ideally 10-20 ml, were placed in beakers and pre-chilled to 4°C. The amount of 20% ammonium sulphate saturation (1.33 g/11.75 ml solution) was added to aliquot whilst stirring (use a magnetic follower and stirrer). The mixture was left stirring at 4°C for 30 min and then centrifuged at 10,000 ×g for

20 min. The supernatant was precipitated to 20-40% saturated ammonium sulphate (1.42 g/11.75 ml). The aliquot was left stirring at 4°C for 1 h and centrifuged at 10,000 ×g for 20 min. The supernatant was removed and the pellet was collected. The pellet was dissolved in 20 mM Tris-HCl buffer pH 8.5 and dialyzed overnight with the same buffer for desalting.

2.5.2) Ultrafiltration

The enzyme solution was added into ultrafiltration unit using the membrane that had the MW cut-off 10,000. The nitrogen gas with the pressure 2 lb/cm^2 was blown into the ultrafiltration unit and the flow rate of eluted solution was 0.2 ml/min at 4°C. The concentrated enzyme solution was assayed for the lipase activity and protein concentration, respectively.

2.5.3) Dialysis

2.5.3.1) Preparation of dialysis tube

Dialysis tubing of suitable diameter was selected and cut into suitable lengths to contain the required volume. The dialysis tubes were submerged in a solution of 2% sodium bicarbonate and 0.05% EDTA and made sure that sufficient volume was

used to amply cover the dialysis tubing. The solution was boiled for 10 minutes. The solution was discarded and boiled for 10 minutes in distilled water and repeated this step once more. Finally, the solution was cooled and the dialysis bag was placed into a suitable solution to prevent microorganism growth (such as 20% ethanol or 0.1% sodium azide). In the suitable solution, dialysis bag could be stored at 4°C for up to 3-6 months.

2.5.3.2) Dialysis procedure

The dialysis tubing inside and outside was rinsed with dd. water or buffer before use. The tubing was sealed one end with a double knot or dialysis clip. The enzyme solution was poured into the tubing and the air from the bag was expelled. The top end of tubing was sealed with a double knot or dialysis clip. The bag was placed in a large volume of buffer. The solution was agitated gently with a magnetic bar and stirrer motor and made sure that the bag was not knocked to the magnetic bar, to prevent rupture. The solution was stood preferably at 4°C, the buffer was changed every 5-8 hours and left to reach equilibrium, usually about 24 hours. The sample was concentrated by lyophilization and kept dry at 4°C.

2.5.4) Strong anion exchange chromatography

2.5.4.1) Preparation of Q-Hyper D column

Gel Q HyperD F (Biosepra Co.) was washed to remove any particles from the matrix with 5-6 times of the basal buffer (20 mM Tris-HCl buffer pH 8.5) and the gel was allowed to settle and the cloudy supernatant was decanted off. The step of washing gel was repeated 3-5 times before the slurry was degassed under suction.

The chromatography column (3×15 cm) was cleaned and clamped in vertical direction. The matrix suspension was slowly poured until the gel hight about 15 cm, a column inlet was fit and the solvent was pumped by using FPLC pump with a flow rate of 2 ml/min through with basal buffer about 500 ml.

For POROS®HQ Perfusion chromatography, the POROS®HQ column could be connected to M-6 metric fitting systems (such as the Pharmacia FPLC system) by using the adapt kit provides. The short tubing section was connected to the column using the red Finger tight fittings. The black metric nuts were slipped over the end of the tubing and followed by a blue ferrule with the conical end pointing toward the nut. Then, the nut was connected to a female M-6 fitting. The POROS®HQ column was shipped in 0.1 M Na_2SO_4/30% methanol. Before using the column for the first time, the column was eluted with 5 to 10 column volumes of water to remove the methanol

and washed with 5 to 10 column volumes of the high salt eluent buffer and equilibrated with 1 to 15 column volumes of the starting buffer.

2.5.4.2) Procedure

The column was connected with the FPLC machine and equilibrated with 20 mM Tris-HCl buffer pH 8.5. The column was eluted with a linear gradient of 0-1.0 M NaCl in the same buffer at a flow rate of 1 ml/min and the detection at 280 nm. One ml/fraction was collected and assayed for lipase activity.

For the purification by perfusion chromatography, 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 × 100 mm) using a BioCAD workstation (Applied Biosystems). Elution was with 20 mM 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}. Each fraction eluted from HQ column was assayed for the lipase activity and checked the purity by SDS-PAGE.

2.5.5) Gel filtration chromatography (Sephacryl HR column)

2.5.5.1) Preparation of gel

The Sephacryl S-200 HR gels were supplied swollen in 20 mM Tris-HCl

buffer pH 8.5 and ready to use as a suspension. It was washed twice with the same buffer before use.

2.5.5.2) General procedure for column packing

The packing reservoir was attached tightly and the column (1.6×100 cm, Pharmacia Biotech) was mounted vertically on the stand. The adaptor was wet through by drawing water (or 20% ethanol) through it. (It was make sure that no air bubbles were trapped under the net.) This was best done by submerging the plunger into a beaker of water (or 20% ethanol) and attaching the tubing to a pump or a syringe. The tubing was closed with a stopper when all air bubbles had been removed. The adaptor was inserted at the bottom of the column far enough to give the desired bed height. The column glass tube was washed with 20 mM Tris-HCl buffer pH 8.5 as an eluent leaving a few centimeters of fluid in the bottom making sure that the net was completely free from air bubbles. The well-mixed gel suspension was carefully poured down the wall of the column using a glass rod. It was poured in a single operation and filled the reservoir to the top with 20 mM Tris-HCl buffer pH 8.5. The reservoir cap was screwed tightly and connected to the pump with opening the outlet. The gel was packed into the column using the flow rates of 2.5 ml/min for 2 hours or

until the gel bed had reached a constant height, then the flow rate was increased to the value listed for STEP 2 and packed for 60 minutes. The pump was stopped, the column outlet was closed, and the packing reservoir was removed.

2.5.5.3) Sample application

The sample was pumped onto the equilibrated column at a flow rate of 0.25 ml/min and the column was then eluted with 20 mM Tris-HCl buffer pH 8.5. The samples were collected with a fraction collector approximately 2.5 ml/fraction. Each fraction was determined A_{280}, assayed the lipase activity and checked the purity by SDS-PAGE. The fractions containing the lipase activity and the lipase band were pooled. The activity of lipase was reassayed and the protein was determined by dye-binding method. In addition, the purity was checked again by SDS-PAGE.

2.5.6) SDS-PAGE

2.5.6.1) Sample preparation

The samples were added with an equal volume of 1×loading buffer (50 mM Tris-HCl buffer pH 6.8, 0.1 M DTT, 10% glycerol, 2% SDS and 0.1% bromophenol blue) or 1/3 volume of 3×loading buffer with maximum volume approximately 25 µl

and placed in boiling water bath for 5 min. The samples were centrifuged at 12,000 ×g for 10 min before loading. The molecular weight calibration kit (Amersham Pharmacia Biotech, Sweden) was used as standard marker proteins 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).

2.5.6.2) Electrophoresis procedure

The electrophoresis of the samples was done using NuPAGE electrophoresis system with 12.5% polyacrylamide slab gel. The separating gel was prepared and contained 5.21 ml of 30% Acrylamide:Bis (29:1), 3.13 ml of 1.5 M Tris-HCl buffer pH 8.5, 0.13 ml of 10% SDS, 3.97 ml of dd. water, 125 µl of 10% ammonium persulfate and 15 µl of TEMED. The gel solution was introduced into the NuPAGE gel cassette and overlay with absolute ethanol. After approximately 30 minutes for the separating gel to polymerize, it was washed twice with water and blotted with a tissue to remove the water. The stacking gel was prepared and contained 0.67 ml of 30% Acrylamide:Bis (29:1), 1.25 ml of 0.5 M Tris-HCl buffer pH 6.8, 50 µl of 10% SDS, 3 ml of dd. water, 25 µl of 10% ammonium persulfate and 5 µl of TEMED. The gel

solution was introduced into the NuPAGE gel cassette and the comb was inserted on the slab gel. After approximately 30 minutes for stacking gel to polymerize, the comb was pull out of the cassette and the tape from the bottom of the cassette was peeled. The gel cassette was washed twice with dd. water and orient in the Mini-Cell so that the notched "well" side of the cassette faces inwards toward the buffer core. The cassette was seated on the bottom of the Mini-cell and sealed into place with the set of wedges. The running buffer or 1×TG-SDS buffer was filled into the inner and outer buffer chamber so that the buffer level exceeds the level of the wells. The prepared sample was loaded into each well. The cover was attached and run the gel at 25 mA for approximately 1 h. When the run was complete, the power was shut off and the gel was removed from the Mini-Cell by inserting the gel knife's beveled edge into the gap between the cassette's two plates and separating the plates.

2.5.6.3) Staining and destaining procedure

The gel was shaken in the staining solution containing 0.1% coomassie brilliant blue R-250 in a mixture solution of 5:4:1 of methanol: dd. water: acetic acid for 30-60 min. After that, it was destained with the destaining solution containing a mixture of 6:3:1 of methanol: dd. water: acetic acid with orbital shaker slowly. The

destaining solution was changed 2-3 times until the protein band appeared.

2.5.7) Western blotting

2.5.7.1) Preparation of PVDF membrane

The PVDF membrane and filter papers were cut to the same size as the gel. Then, the PVDF membrane was wet with a brief immersion in 100% methanol (2-3 sec), rinsed with the water (2-3 min) and equilibrated in CAPS transfer buffer (10 mM CAPS, 10% methanol, pH 11.0) for at least 15 min.

2.5.7.2) Procedure of western transfer

After electrophoresis was completed, the gel was incubated in transferring buffer for 5 min. The filter paper was soaked in the transferring buffer. This step was optional. For optimal results with this method, however, the gel was equilibrated with transferring buffer prior to transfer. The 6 sheets of filter papers were placed on the cathode (negative, usually black), followed by the gel, the PVDF membrane, 6 sheets of filter papers, and finally the anode (positive, usually red). In this research, the current of 400 mA and the transferring time for 2 h were used according to the manufacturer's instructions.

2.5.7.3) Procedure for staining proteins transferred to membrane

After the protein transferring was completed, the orientation of the membrane on the gel was marked and immerse in staining solution containing 0.1%(w/v) solution of Naphthol Blue Black in 10%(v/v) methanol and 2%(v/v) acetic acid with gentle shaking for 2 min. The membrane was rapidly destained in 50%(v/v) methanol, 7%(v/v) acetic acid for 5-10 minutes or until the protein bands can be seen clearly. The membrane was cut at the desired protein and then determined the NH_2-terminal amino acid sequence by amino acid analyzer.

2.6) Lipase assay

The lipase assay was examined by the spectrophotometric assay using triacylglycerols and *p*-nitrophenyl (*p*-NP) esters as the substrates. For the triacylglycerols, 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 [87]. The substrate solution consisted of triglycerides (100 mM) emulsified with homogenizer in distilled water with 0.2 mM gum arabic at maximum speed of 2 min. Copper (II)-acetate-1-hydrate aqueous solution (90 mM) was adjusted to pH 6.1 with pyridine and used as copper reagent.

The chromogenic reagent contained 5.8 mM diethyldithiocarbamic acid was 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 [88,89]. 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 was $\varepsilon = 1.85$ l.mmol^{-i}.mm^{-1}.

2.7) Protein determination

The protein concentration was measured spectrophotometrically at 280 nm and by using the dye-binding assay based on the method of Bradford [90] with BSA as a standard. For the calibration curve, the triplicate volumes of 10-100 µl of 10 µg/ml BSA solution were pipetted into 1.5 ml polyethylene microfuge tubes and the volumes was adjusted to 100 µl with dd. water. The 100 µl of dd. water was used for the reagent blank. Then, 100 µl of sample solution containing between 1-10 mg/ml protein was pipetted into microfuged tubes. The range of dilutions (1, 1/10, 1/100, 1/1000) should be assayed if the sample concentration was unknown. The 1 ml of dye reagent was added to each tube, mixed thoroughly and incubated at room temperature for at least 5 min. The absorbance was measured at 595 nm.

2.8) Characterization of the recombinant lipase

2.8.1) Effect of pH on lipase activity and stability

To determine the optimal pH, the 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. For pH stability, the lipase was incubated at 55°C for 1 h in various buffers and assayed for the remaining activity at pH 8.5. The pH stabilities were measured in the pH ranges 4.0-11.0.

2.8.2) 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 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 in a period of times up to 15 h, then 0.1 ml sample was assayed for the lipase activity at 55°C.

2.8.3) 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 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.

2.8.4) Kinetic study of lipase

The reactions were carry out at 55°C by using various *p*-NP caprate concentrations of 0.2, 0.4, 0.6, 0.8, 1, 2, 3 and 5 mMand 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.

2.8.5) Positional specificity

The positional specificity of the lipase was determined by use of a method modified from that described by Lesuisse *et* al. [88]. 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 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.

2.8.6) 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 pre-incubated at room temperature for 5 min, then assayed for lipase activity. The lipase activity of the enzyme without added metal ion was defined as the 100% level.

2.8.7) 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.

2.8.8) Effect of detergents on lipase 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.

2.8.9) 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. [91]. The activity was determined at the beginning and the end of incubation time.

2.8.10) Crystallization

2.8.10.1) Crystallization in Linbro box

The rims were greased with silicon grease. The reservoir was then filled up with one ml of filtered (0.22 μm) crystallizing agent. The 2-10 μl drops of filtered (0.22 μm) biological macromolecule solution were mixed with an equivalent volume of reservoir. The drop was layered on the 22 mm diameter cover slip (do not touch the cover slip with the extremity of the tip of the pipetor or it will spread) so that a nearby hemispherical drop was formed. Cover slip was returned with a pair of brussel (or fingers). The grease rim was set on by gently pressing the grease to seal the well. Inspecting the rim in an azimuthal way checked the sealing. Crystallization would occur eventually, but would be very difficult to reproduce. The glass cover slips were adjusted tangent to each other and otherwise, they overlapped. The platicene was put in the corners to avoid the contact between cover and grease. Otherwise, the cover slips were gotten stuck at the cover. A Linbro box was placed at 16°C.

2.8.10.2) Varying the condition for crystallization

The experiment was done by the hanging drop method under the wild range of experimental conditions [92]. One ml of reservoirs at various buffer and pH values

such as acetate buffer pH 4.6, imidazole buffer pH 6.5, Hepes buffer pH 6.8-8.0 and bicine buffer pH 9.0 and precipitant agents such as ammonium sulphate and PEG 4000 were put in 24-well tissue culture plates (Falcon). Drops consisted of the protein solution at various concentrations and reservoirs. The experimental plates were settled at 16°C. The growth of crystals was observed by using a microscope.

2.9) Chiral separation of lipase

A solution of racemic 3-phenoxy-1,2-propanediol (168.2 mg, 0.1 mmol) in various organic 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. In addition, the enantioselectivities of the lipase P1 between at the room temperature and at 55°C in acetone, dichloromethane and chloroform for 0.5, 1, 3 and 6 h were compared. The comparison of the enantioselectivities between the lipase P1

and the other lipases such as lipase AP6 (*Aspergillus niger*, Amano), lipase AY-30 (*Candida cylindracea*, Amano), lipase from *Candida cylindracea* (Meito-Sangyo), lipase GC4 (*Geotrichum candidum*, Amano), lipase MAP (*Mucor meihei*, Amano), lipase N (*Rhizopus niveus*, Amano), lipase R (*Humicola* sp., Amano), lipase R-10 (*Humicola lanuginose*, Amano) and lipase L1754 (*Candida rugosa*, Sigma) in dichloromethane was also done at the room temperature for 1 h.