CHAPTER 4

DISCUSSION AND CONCLUSION

4.1) Cloning and overexpression of the lipase gene from *B. stearothermophilus* P1

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 and the low expression of the cloned lipase in *E. coli* DH5α using pUC-19 as a vector had no promoter and the host *E. coli* DH5α had more glycoprotein that was difficult to isolate the plasmid, we markedly increased production of this enzyme by changing the cloning system that cloned the lipase gene into *E. coli* M15[pREP4] and/or JM109 using the QIAexpress system. The goal of this strategy using pQE 60 and based on the T5 promoter transcription-translation system was to put the gene under the control of IPTG induction. The expression of recombinant proteins could be rapidly induced by the addition of IPTG which bound to the *lac* repressor protein and inactivated it. Expression of the recombinant protein was rapidly induced by IPTG addition to the 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. Due to the highest expression of the recombinant lipase in *E. coli* M15[pREP4] showing the lipase activity higher than the recombinant lipase in *E. coli* JM109 and DH5α, so it was chosen to study the expression of the cloned lipase P1 gene. Although the lipase gene was cloned using the same vector as pQE-60 but different host cells of *E. coli* M15[pREP4] and JM109,

the expression of *E. coli* JM109 was less efficient than M15[pREP4] because its expression was regulated less tightly than in strains harboring the pREP4 plasmid. In addition, the expression of the recombinant lipase P1 in *E. coli* M15[pREP4] was studied by determination of the condition for IPTG induction and the result showed that it was overexpressed in the IPTG concentration range of 0.4-0.6 mM that could give the larger protein bands of lipase on SDS-PAGE. Therefore, the determination of condition for IPTG induction could get the high level expression of the cloned lipase P1 and it would be easily to use for the purification and applications of the enzyme.

The NH_2-terminal amino acid sequence of the lipase from *B. stearothermophilus* P1 was similar to that of other lipases cloned in *E. coli* [40,97-99] which also had a 29 amino acid signal sequence and a cleavage site between Ala-29 and Ala-30. Its deduced mature sequence was very similar indeed to the lipases isolated from the thermophilic bacteria *B. thermoleovorans* ID-1, *B. stearothermophilus* L1 and *B. thermocatenulatus* which showed 96, 93, and 91% identity of residues, respectively. The availability of the detailed structure of the *B. stearothermophilus* P1 lipase will thus enable work on the other three enzymes to be placed in a structural context. Although the amino acid sequence of the recombinant lipase in *E. coli* DH5α had a signal sequence that had a function in refolding and secretion in *Bacillus* system but it did not help in secretion in the *E. coli* system. Therefore, the amino acid sequences of the new recombinant lipases in *E. coli* M15[pREP4] and JM109 were no signal sequence. However, the overexpression of this lipase can be obtained by cloning in *Bacillus* system that will be obtained the higher expression and production of the lipase.

4.2) Secondary structure prediction and structural modeling of a thermostable lipase

The secondary structure prediction and solvent accessibility of the lipase gene showed that the hydrophilicity of the lipase was slightly more than its hydrophobicity and the non-polar amino acids were slightly less than the polar amino acids in the amino acid composition of the lipase gene structure and the surface probability and transmembrane of the lipase gene structure showed the low level. Therefore, it indicated that the lipase molecular structure turned the non-polar group of amino acid into its core structure and turned the polar group of the amino acid outside the molecule.

For the structural modeling of this lipase, the other lipases of known three dimensional structure were used to be the reference proteins such as 3LIP and 1TAH. The modeled lipase structure included both α-helix and β-sheet secondary structures in the folded protein and the β-sheet was in the core region surrounding with α-helix. Based on the similarity in amino acid sequences of several lipases in the region of the catalytic triad His, Ser, Asp [100] and on the modeled structures of several lipases, it was suggested that Ser-113, Asp-317 and His-358 form the catalytic triad of the lipase from *B. stearothermophilus* P1. The catalytic site of the modeled lipase was confirmed by site-directed mutagenesis and the result showed that Ser-113, Asp-317 and His-358 were the active site residues in the structural molecule of this lipase. More detail structure was also found from the preliminary analysis of X-ray diffraction on lipase crystal. A more extensive kinetic and structural analysis of the mutant is currently under way.

4.3) Purification and characterization of a thermostable lipase

In the first purification, the recombinant lipase P1 in *E. coli* M15[pREP4] was purified by using ultrafiltration and strong anion-exchange chromatography with Q HyperD^{TM}10 prepacked column but it had not obtained the high purity of the lipase. Therefore, the purification step was developed to get the better method that obtained a higher-specific activity and purity. The lipase was purified by ammonium sulphate fractionation, strong anion-exchange chromatography with Poros 20HQ column and gel filtration with Sephacryl S-200HR. The specific activity of purified lipase was higher than by using the last method about 3.2 fold. Otherwise, this enzyme appeared a single band on SDS-PAGE with a molecular mass of 43 kDa that was similar to the high molecular mass of thermostable lipase from *B. thermocatenulatus* [39].

The characterizations of the purified lipase were 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 30-65°C. Its half-life at 55°C as the optimal temperature was about 7.6 h showing the stability of this enzyme was high at this high temperature and somewhat higher than that of other lipases from *Bacillus* sp. [97,40,99]. Moreover, the lipase P1 was 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. thermoleovorans* 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 found that there were no metal ions that could 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 the lipase P1 was inactivated by 1 mM $ZnCl_2$ and $FeSO_4$ and remained the activity of 35 and 53%, respectively whereas the lipase from *B. stearothermophilus* L1 was inactivated and remained the activity 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.

For the positional specificity of this lipase testing the hydrolysis of mono-, di- and triolein, all of mono-, di- and triolein were hydrolyzed by the lipase P1 but the TLC result was not clear and seemed to be the non-specificity because it could not determined the product that was hydrolyzed except that it will have the control spot on TLC of the mixture of mono-, di- and triolein using the buffer instead of the lipase in the hydrolysis. The data of the lipase activities of the hydrolysis of the lipase on each substrate showed the lipase P1 preferred to hydrolyze at the first ester bond. On the other hand, when the activity was compared between the hydrolysis of 1,2-diolein and 1,3-diolein, the activity from the hydrolysis of 1,2-diolein was slightly higher than 1,3-diolein indicating that this lipase preferred 1,2-specificity than 1,3-specificity. The determination of the positional specificity will be clarified if the standard of 2-monoolein is also used for assaying the lipase activity.

Most lipases have a catalytic triad consisting of Ser-His-Asp/Glu [101,102], 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 [103,104]. 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* [97], *B. subtilis* [98] and *B. thermoleovorans* ID-1 [99]. The catalytic serine was also confirmed in the lipase molecule by testing with 1-dodecanesulfonyl chloride or 1-hexadecanesulfonyl chloride which had an unbranched alkyl sulfonyl chloride structure similar to monoglyceride and a high affinity for the active site. The result showed that the lipase was strongly inhibited by 10 mM 1-hexadecanesulfonyl chloride and by 10 mM PMSF. Therefore, a serine residue plays a key role in the catalytic mechanism.

For the crystallization of this lipase, the crystals were shown to diffract well, and an attempt was made to find a solution through molecular replacement. The soaking crystal with Cu or Zn showed that the residues surrounding that ion are His81 and His87, Asp61 and Asp238. The calcium ion is surrounded by Gly286 O, Glu360 Oe2, Asp365 Od1, Pro366 O and a water. It had been suggested that some mutagenesis studies could be carried out on the metal ion site to test for stability. In addition, the lipase structure was 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.

4.4) Application of a thermostable lipase

In this report, the acyclic racemic 3-phenoxy-1,2-propanediol as substrate was chosen for the 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. Otherwise, (R,S)-1,2-propanediol is used extensively as antifreeze, food preservative and drug solvent. Its R isomer is an intermediate in the mammalian metabolism of acetone [105]. 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 and this property intends to use in the industrial applications. Moreover, when the lipase P1 was compared with the other lipases from the commercial trade in the enantioselectivity on 3-phenoxy1,2-propanediol, the lipase P1 has the preference on S(−) configuration more than the other lipases in the same condition of dichloromethane, 55°C and 1 h except the lipase from *Candida rugosa* and *Geotrichum candidum*. Therefore, this lipase had the higher specificity on S(−) configuration of (R,S) 3-phenoxy-1,2-propanediol and could separate the R(+) configuration to apply in the industries.

4.5) Conclusion

The thermophilic bacterium *B. stearothermophilus* isolated from a hot spring in Chiang Mai, Thailand could produce the highly thermostable lipase. Due to the limited production, it had been cloned into *E. coli* DH5α using pUC-19 vector but the expression of this recombinant lipase was still low level. Therefore, the lipase gene was optimized by changing the cloning system that was cloned into *E. coli* M15[pREP4] or JM109 using a pQE-60 as an expression vector for markedly increased production. The lipase gene from *B. stearothermophilus* P1 cloned in *E. coli* DH5α using pUC-19 vector that had the nucleotide revealed an open reading frame of 1,254 bp encoding a 417 amino acid polypeptide consisting of a 29 amino acid signal sequence and a mature lipase of 388 amino acid residues was cut the mature sequence on a 1.2 kb *Nco I*/*Hind* III fragment into pQE-60 vector and transformed to *E. coli* M15[pREP4]. 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.

The secondary structure of this lipase was predicted and aligned with the other lipases with known the 3-D structure. The amino acids belonging to the catalytic triad, Ser-113, Asp-317 and His-358 were predicted correctly that was 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.

The recombinant lipase in *E. coli* M15[pREP4] was overexpressed better than the recombinant lipase in *E. coli* DH5α and JM109 including the native lipase. The expression could be rapidly induced by IPTG addition, especially the best expression was achieved with 0.4 mM IPTG for 3 h at 37°C. The recombinant lipase P1 in *E. coli*

M15[pREP4] was chosen for the lipase production and purification. It was purified by 20-40% saturated ammonium sulphate fractionation, anion-exchange perfusion chromatography on Poros 20 HQ column and gel filtration on Sephacryl S-200. The lipase was purified 39-fold over the crude extract with a specific activity of 2,601 U/mg and a yield of 19%. The purified lipase showed a single band by SDS-PAGE with the molecular mass approximately 43kDa. It was also confirmed by mass spectrometry with the molecular mass approximately 43,209 Da and the purity of the purified lipase showed a single peak eluting from the HPLC using C18 column. The characterizations of the purified lipase 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 as the optimal temperature was about 7.6 h. It showed the high activity toward tricaprylin (C8) and *p*-NP caprate (C10) as the substrates and the Michaelis constant, K_m and V_{max}, toward the *p*-NP caprate at 55°C were found to be 0.286mM and 88 $U.ml^{-1}.min^{-1}$, respectively. For the positional specificity, this lipase had a preference either for digestion at 1,2- or 1- or 2-positional ester bond. The lipase activity was inhibited by 10 mM $ZnCl_2$ and $FeSO_4$ and strongly inhibited by 10 mM 1-hexadecanesulfonyl chloride and PMSF indicating that a serine residue plays an important role in the enzymatic mechanism. In addition, it was stable at 37°C for 1 h in the presence of 0.1% detergents such as CHAPS and Triton X-100 and also highly stable in the presence of organic solvents such as n-heptane, toluene, benzene and chloroform. For the crystallization of the purified lipase, the hanging-drop vapour-diffusion method was used to grow crystals in 24-well Linbro plates. Tetragonal crystals grew rapidly after 24 h at 16°C with 20% saturated ammonium sulphate as precipitant in 0.1 M

Hepes buffer pH 6.8-7.0 and the protein concentration used was 15 mg ml^{-1} in 20 mM Tris-HCl buffer pH 8.5. The crystal of this lipase was diffracted to 2.5 Å. 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 such as Cu or Zn indicate that this strategy will allow the structure to be solved. However, the lipase structure from the X-ray crystallographic study was similar to the modeled lipase structure that was predicted using the other lipases as the references and the catalytic triad was Ser-113, Asp-317 and His-358. In addition, this lipase could catalyzed the acylation of 3-phenoxy-1,2-propanediol that its pure forms were of interest as pharmaceuticals and the enantioselectivity of this lipase was preferentially catalyzed the S(−) configuration in dichloromethane better than in acetone and chloroform. This lipase also catalyzed the acylation on S(−) configuration better than the other lipases in dichloromethane at 55°C for 1 h except the lipase from *Candida rugosa* and *Geotrichum candidum*.