CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Chemical	Molecular weight	Company
N-(2-Acetamido)-2-imidoacetic acid (ADA)	190.2	Sigma
N-(2-Acetamido)-2-imidoacetic acid, sodium salt (ADANa)	212.1	Sigma
Boric acid	61.83	Fluka
Boron trifluoride in methanol (BF_3-methanol)	-	Fluka
Butanol	74.12	Merck
Butyl stearate	340.6	Fluka
Calcium chloride	111.0	Fluka
Chloroform	119.3	Merck
Citric acid	210.1	Sigma
Citric acid, monosodium salt	232.1	Fluka
Cupric acetate	119.6	Merck
3-(Cyclohexylamino)-2-hydroxyl-1-propanesulphonic acid (CAPSO)	237.3	Sigma
3-(Cyclohexylamino)-2-hydroxyl-1-propanesulphonic acid, sodium salt (CAPSONa)	259.3	Sigma
Dichloromethane	84.93	Merck
Diethyl ether	74.12	Merck
Diglyceride mixtures	-	Sigma
3-[(1,1-Dimethyl-2-hydroxyethyl)amino]-2-hydroxy propanesulphonic acid (AMPSO)	227.3	Sigma

Chemical	Molecular weight	Company
3-[(1,1-Dimethyl-2-hydroxyethyl)amino]-2-hydroxy propanesulphonic acid, sodium salt (AMPSONa)	249.3	Sigma
Disodium hydrogen phosphate	141.9	Sigma
Ethanol	46.07	Merck
Ethyl myristate	256.4	Sigma
Ethyl oleate	310.5	Sigma
Ethyl palmitate	284.4	Sigma
Ethyl stearate	312.5	Sigma
Glutamic acid (Glu)	147.1	Sigma
Glutamic acid, monosodium salt (GluNa)	169.1	Aldrich
Glycine (Gly)	75.07	Sigma
Glycine sodium salt (GlyNa)	97.05	Sigma
Hexane	86.18	Carlo Erba
Hydrochloric acid, 36.5-38.0%	36.46	J.T.Baker
N-(2-Hydroxyethyl) piperazine-N^{I}-(2-Hydroxypropanesulphonic acid) (HEPPSO)	268.3	Sigma
N-(2-Hydroxyethyl) piperazine- N^{I}-(2-hydroxy propanesulphonic acid, sodium salt) (HEPPSONa)	290.3	Sigma
i-octane	114.2	BDH
i-propanol	60.10	Merck
Magnesium sulfate anhydrous	120.3	Fluka
Maleate disodium salt	160.0	Sigma
Maleate monosodium salt	138.1	Sigma
Methanol	32.04	BDH
Methyl myristate	242.4	Sigma
Methyl oleate	296.5	Sigma
Methyl palmitate	270.5	Sigma
Methyl stearate	298.5	Sigma
Molecular sieve 4 $\mathring{A}$	-	Fluka

Chemical	Molecular weight	Company
Monoglyceride mixtures	-	Sigma
2-(*N*-Morpholino) ethanesulphonic acid (MES)	195.2	Sigma
2-(*N*-Morpholino) ethanesulphonic acid, sodium salt (MESNa)	217.2	Sigma
Oleic acid	282.5	BDH
Olive oil	-	Fluka
Palm oil (olein)	-	Kesorn
Phenolphthalein	318.3	Fluka
Piperazine-*N*- N^{I}-bis (2-ethanesulphonic acid) (PIPES)	302.4	Sigma
Piperazine-*N*- N^{I}-bis (2-ethanesulphonic acid), sodium salt (PIPESNa)	324.2	Sigma
Potassium hydrogen phthalate	204.2	Fluka
Pyridine	79.10	BDH
Sodium acetate anhydrous	82.00	Fluka
Sodium chloride	58.44	Fluka
Sodium dihydrogen phosphate	119.9	Sigma
Sodium hydroxide	40.00	Fluka
Tributyrin	302.3	Fluka
Trimyristin	723.2	Sigma
Triolein	885.4	Fluka
Tripalmitin	807.3	Fluka
Tris (hydroxymethyl) aminomethane	121.1	Merck
N-Tris (hydroxymethyl) methy1-2-aminoethane sulphonic acid (TES)	229.2	Sigma
N-Tris (hydroxymethy1) methy1-2-aminoethane sulphonic acid, sodium salt (TESNa)	251.2	Sigma
Tristearin	891.5	Fluka

2.1.2 Instruments

Name	Model	Company
Capillary columm	7483 WCOT	Chrompack
Coulometer	684 KF	Metrohm
Centrifuge, refrigerated	6800 (Roter RA-1500)	Kubota
Gas chromatograph	6890 HP	Hewllet Packard
Gas chromatograph-Mass spectrometer	5973 (EI)	Hewllet Packard
Incubator shaker	1083	GFL
Lyophilizer	Flexi-Dry MP	FTS systems
Spectrophotometer UV-VIS	PU 8625	Philips
Steam bath	KG D-91126	Memmert
Thermoconstanter	TH 2/RTD 33	Novasia

2.2 Methods

2.2.1 Preparation of lipase from fresh papaya latex

Fresh papaya (*Carica papaya* L.*)* latex was collected from a number of sources of Thai papaya trees. The insoluble part of the latex was separated and the activity of lipase was determined after drying by lyophilization. The lyophilized CPL was used as a biocatalyst in hydrolysis of olive oil at differing temperatures and pHs.

2.2.1.1 Collection of latex

The collection method of papaya latex was carried out by the method described in Theppakorn [87]. The papaya latex was collected by making 4-6 longitudinal incision on the surface of the unripe fruit (70-100 days) in the morning, with a stainless steel knife. The liquid latex dripped onto the collecting tray attached to the trunk with wire supports [93] and some latex coagulated on the fruits. Whole latex was scraped into the plastic container. Impurities were removed by agitation and then filtered with a screen (50 mesh). The liquid latex was stored at -20^{o}C in sealed plastic containers until experiment was performed.

2.2.1.2 Separation of lipase

The frozen latex was defrosted at room temperature. The latex was centrifuged at 10,000xg for 15 min. The insoluble fraction was washed three times with distillated water and then dried by lyophilization. The lipase powder was kept at -20^{o}C in sealed plastic containers.

2.2.1.3 Assay of lipase activity

A. Activity on tributyrin

Hydrolysis activity on tributyrin was determined by using reaction mixture contained 0.50 g of tributyrin, 4.0 ml of 0.15 M phosphate buffer pH 7 and 300 μl of 10 mM $CaCl_2$ in the presence of 2 mg of lyophilized lipase. The reaction mixture without enzyme was used as blank. The reaction was performed in shaking water bath at 55^{o}C for 1 h. Reaction was stopped by adding 20 ml of acetone:methanol mixture (1:1, v/v). Blank was added the enzyme after the reaction was stopped. The reaction mixture was titrated with 0.0495 M NaOH (standardized by 0.0500 M potassium hydrogen phthalate) by using phenolphthalein as an indicator for determination of the amount of free fatty acid [102]. The activity of lipase was defined as μmol of free fatty acid released in 1 min per g of enzyme and can be calculated by the equation as demonstrated in **Appendix A.**

B. Activity on olive oil

Substrate mixture contained 0.50 g of olive oil, 4.0 ml of 0.15 M phosphate buffer pH 7.0 and 300 μl of 10 mM $CaCl_2$. To start the reaction, 2 mg of lipase powder was added and reaction was carried out at 55^{o}C for 1 h in shaking water bath at 200 rpm. The reaction was stopped by adding 6 M HCl to pH 1-2 and 5 ml of *i*-octane, followed by mixing and boiling the reaction mixture for 5 min. The released free fatty acid from reaction was determined by colorimetry [103]. The 5 ml of upper *i*-octane layer was drawn off to a test tube. Then 1 ml of cupric acetate-pyridine reagent which was prepared from a 5% (w/v) aqueous solution of cupric acetate and adjusted pH to 6.1 using pyridine, was added and vigorously mixed for 90 sec. The mixture was allowed to stand until the aqueous phase was sedimented clearly from the solution of *i*-octane. The absorbance of *i*-octane solution was measured at 715 nm

against the blank which contained only *i*-octane. Calculation of lipase activity was shown in **Appendix B**.

For determination of lipase activity on olive oil by titrimetry, the reaction was performed as described in **Section 2.2.1.3A** in which tributyrin was substituted with olive oil.

2.2.1.4 Determination of optimum temperature and optimum pH of CPL

A. Determination of optimum temperature

Each erlenmeyer flask consisted of 0.50 g of olive oil, 4.0 ml of 0.15 M phosphate buffer pH 7.0 and 300 μl of 10 mM $CaCl_2$. To start the reaction, 2 mg of CPL was added to the mixture. Reaction was carried out at certain temperatures (25, 37, 45, 50, 55, 60 and 65^{o}C) for 1 h in shaking water bath at 200 rpm. 6 M HCl was added into the reaction mixture until obtaining pH of around 1-2 and 5 ml of *i*-octane was then added to stop the reaction. The mixture was mixed and boiled for 5 min and the released free fatty acid was determined by colorimetry as described in **Section 2.2.1.3B**.

B. Determination of optimum pH

Olive oil (0.5 g) was mixed with 4.0 ml of 0.15 M of differing buffers (acetate buffer pH 3; phosphate buffer pH 4-7; Tris-HCl buffer pH 8-9 and borate buffer pH 10) in the presence of 300 μl of 10 mM $CaCl_2$. Each reaction was initiated by adding 2 mg of CPL to the mixture and carried out at 55^{o}C for 1 h in shaking water bath at 200 rpm. The reaction was stopped by adding 6 M HCl to pH 1-2 and 5 ml of *i*-octane. The mixture was mixed and boiled for 5 min and the released free fatty acid was determined by colorimetry as mentioned in **Section 2.2.1.3B**.

2.2.2 Determination of optimum enzyme amount for methanolysis of triolein

The mixture of substrates consisted of 0.077 mmol of triolein and 0.231 mmol of dried methanol (100 ml of methanol with 3 g of dried molecular sieve 4 Å) [104]. CPL (2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24% of triolein, w/w) was added to start the reaction of methanolysis. The reaction was shaken at 200 rpm and at 37^{o}C for 24 h. The mixture was then allowed to settle for 10 min. Clear solution (50 μl) was taken and 10-fold diluted with chloroform for analysis by GLC.

2.2.3 Determination of CPL specificity for alcoholysis

Substrate mixtures composed of 0.077 mmol of tripalmitin in 73 μl of chloroform and 0.231 mmol of dried methanol. The CPL (18% of TAG, w/w) was added to the mixture and the reaction was carried out at 37°C with shaking at 200 rpm for 24 h. The reaction mixture was allowed to settle out for 10 min and 50 μl of samples were taken and 10-fold diluted with chloroform for products analysis by GLC. For other methanolysis reactions, tripalmitin was substituted individually by tristearin and triolein. For ethanolysis, dried methanol was replaced by dried ethanol.

2.2.4 Improvement of CPL activity

2.2.4.1 Preparation of alcohol washed CPL

CPL (0.5 g) was mixed in 3 ml of numerous solvents (*n*-propanol, *i*-propanol, *n*-butanol and *t*-butanol) in screw-cap vial. The mixtures were agitated for a few minutes and allowed enzyme to settle out for 10 min. Clear supernatant was removed and then the precipitate was rewashed twice with *i*-propanol and 3 ml of dried methanol.

2.2.4.2 Methanolysis of triolein catalyzed by alcohol washed CPL

Native CPL (0.5 g) and alcohol washed CPL including *n*-propanol washed CPL (nP), *i*-propanol washed CPL (iP), *n*-butanol washed CPL (nB) and *t*-butanol washed CPL (tB) were added to the substrate mixture composed of 0.077 mmol of triolein and 0.231 mmol of dried methanol. The reactions were performed at 37°C with shaking at 200 rpm for 24 h. Sample (50 μl) was taken from the reaction mixture and 10-fold diluted with chloroform for GLC-analysis.

2.2.5 Investigation of specificity of iPCPL on alcoholysis of TAG

Each 0.5 g of lyophilized CPL was washed with *i*-propanol and used as a biocatalyst in the methanolysis and ethanolysis of trimyristin, tripalmitin, triolein and tristearin, and butanolysis of tristearin. The products formed from the reaction were analyzed by GLC.

2.2.5.1 Methanolysis of TAG

In screw-cap vial, 0.231 mmol of dried methanol was added to 0.077 mmol of trimyristin, dissolved in 73 µl of chloroform and started the reaction by adding iPCPL. The reaction mixture was shaken in water bath at 37^{o}C, 200 rpm for 24 h. Sample aliquot of 50 µl was withdrawn and 10-fold diluted with chloroform for product analysis by GLC. Methanolysis of tripalmitin, triolein and tristearin were carried out as that of trimyristin.

2.2.5.2 Ethanolysis of TAG

Ethanolysis of 0.077 mmol of trimyristin in solution containing 73 µl of chloroform and 0.231 mmol of dried ethanol was performed using iPCPL at 37^{o}C in 200-rpm shaking water bath for 24 h. Sample of 50 µl was taken and 10-fold diluted with chloroform for GLC-analysis. Ethanolysis of other TAGs, trimyristin was replaced by tripalmitin, trolein and tristearin.

2.2.5.3 Butanolysis of tristearin

Butanolysis of 0.077 mmol of tristearin was investigated in solution containing 73 µl of chloroform and 0.231 mmol of *n*-butanol. The iPCPL was added to the mixture and incubated at 37^{o}C with shaking at 200 rpm for 24 h. Sample (50 µl) was withdrawn and 10-fold diluted with chloroform for analysis by GLC.

2.2.6 Methanolysis of triolein

The iPCPL catalyzed methanolysis of triolein > trimyristin > tripalmitin > tristearin. Therefore, methanolysis of triolein catalyzed by iPCPL at several conditions were investigated for obtaining maximum yield of methyl oleate. The enzyme using in each methanolysis reaction, 0.5 g iPCPL was prepared from 0.5 g of CPL (as in **Section 2.2.4.1**). The reaction was performed at 37^{o}C in shaking water bath at 200 rpm. The mixtures were then allowed to settle out for 10 min and clear solution (50 µl) of each mixture was withdrawn and 10-fold diluted with chloroform for product composition analysis by GLC.

2.2.6.1 Effect of temperature

Substrate mixtures composed of 0.077 mmol of triolein and 0.231 mmol of dried methanol were controlled at different temperatures (25, 30, 37, 40, 45 and 50^{o}C) for 10 min. Each of them was added to 0.5 g of iPCPL and shaken at designated temperature for 10 h in 200-rpm incubator shaker. The sample (50 µl) was withdrawn at interval times (2, 4, 6, 8 and 10 h).

2.2.6.2 Effect of lipase quantity

Several amounts of CPL including 0.10, 0.20, 0.30, 0.40, 0.50, 0.60, 0.70 and 0.80 g were washed with 3 ml of *i*-propanol three times in screw-cap vial. The substrate mixture consisted of 0.077 mmol of triolein and 0.231 mmol of dried methanol was added in each vial to start the reaction at 37^{o}C for 24 h.

2.2.6.3 Effect of substrate ratio

Methanolysis reaction was carried out in vials containing triolein and dried methanol with various molar ratios of 1:1, 1:2, 1:3, 1:4, 1:5, and 1:6. The reaction was started by adding 0.50 g of iPCPL into the substrate mixtures and incubated at 37^{o}C. The reaction mixtures at 0, 2, 4, 6, 24 and 48 h were taken for product content analysis by GLC.

2.2.6.4 Effect of solid buffer

The pairs of solid buffers (ADA/ADANa, AMPSO/AMPSONa, CAPSO/ CAPSONa, Citric/CitrateNa, Gly/GlyNa, Glu/GluNa, HEPPSO/HEPPSONa, NaH_2PO_4/Na_2HPO_4, MaleateNa/MaleateNa$_2$, MES/MESNa, PIPES/PIPESNa and TES/TESNa) were dried at 100^{o}C for 5 h and then kept to cool down to 25^{o}C in desiccator [105]. The substrate mixtures composed of 0.077 mmol of triolein and 0.231 mmol of dried methanol were prepared in vials. Each buffer pair (1 mg) was mixed with substrate mixture and added to 0.5 g of iPCPL to start the reaction. The substrate mixture without solid buffer pairs was used as a control. The reaction mixtures were incubated at 37^{o}C for 24 h.

2.2.6.5 Effect of water

A mixture of 0.077 mmol of triolein and 0.231 mmol of dried methanol was prepared in a screw-cap vial and used as a starting substrate. To study the effect of added water to enzyme, water (5, 10, 15 and 20% v/w) was mixed with 0.5 g of iPCPL and then added to the starting substrates to initiate the reaction. Another vial containing the iPCPL without added water was used as a control.

The effect of added water in the reaction mixture was also investigated. The starting substrate was mixed with water (5, 10, 15 and 20% v/w of triolein). The reaction was started by incorporating the substrate mixture into 0.5 g of iPCPL. The control was the reaction without addition of water to starting substrate. The reaction was carried out at 37^{o}C. The contents of methyl esters, monoacylglycerol, diacylglycerol and free fatty acid were analyzed from the reaction mixtures at 0, 2, 6, 8 and 24 h.

2.2.6.6 Effect of organic solvent

A number of organic solvents were studied including hexane, diethyl ether, dichloromethane and chloroform. Reactions were performed in 1 ml of organic solvent in screw-cap vial containing 0.077 mmol of triolein, 0.231 mmol of dried methanol and 0.5 g of iPCPL. The mixtures were incubated at 37^{o}C for 24 h.

2.2.7 Determination of fatty acid composition in palm oil

The fatty acid composition of palm oil were determined by acid-catalyzed methanolysis [106]. Palm oil (49.4 mg) was weighted into a small conical flask and 3 ml of 0.5 M methanolic sodium hydroxide was added. The mixture was heated over a steam bath until a homogeneous solution was obtained. BF_3-methanol (5 ml) was added to the mixture and boiled 2 to 3 min. Then, the solution was cooled and transferred into a separatory funnel containing 25 ml of hexane and 20 ml of saturated NaCl solution and shaked well, but gently, and allowed the layers to separate. The hexane layer, containing fatty acid-methyl esters, was dried with 1 g of anhydrous $MgSO_4$ and filtered into a vial. The solution was concentrated on the steam bath to a volume of about 0.5 ml. The solution of fatty acid methyl esters was analyzed by

GLC and gas chromatography-mass spectrometry (GC-MS) to determine fatty acid compositions of palm oil in order to calculate an average molecular weight of plam oil.

2.2.8 Methanolysis of palm oil catalyzed by iPCPL

A mixture of palm oil and dried methanol in the different molar ratios: 1:1, 1:2, 1:3, 1:4 and 1:5 was prepared in vials. The reactions were started by adding 0.5 g of iPCPL and performed at 37^{o}C in shaking water bath at 200 rpm for 48 h. The reaction mixtures were allowed to settle out for 10 min to remove the residual lipase. Clear solution of each reaction mixture was used for product analysis by GLC.

2.2.9 Analysis of composition of reaction mixture by GLC

The 50 μl of sample was added into 450 μl of chloroform. The solution was analyzed triplicate (each of 1 μl) by GLC equipped with a WCOT fused silica capillary column 25 m × 0.25 mm i.d.. The chromatographic conditions were: on column injection, flame ionization detector at 370^{o}C, helium as carrier gas at 16.05 psi, injector temperature at 360^{o}C. Separation was made using the following oven temperature profile; initial temperature 180^{o}C, programmed to 220^{o}C at 7^{o}C/min and to 350^{o}C at 20^{o}C/min, hold at final temperature 15 min. Retention times of products have been shown in **Appendix C.** Since alcoholysis of TAG catalyzed by CPL produced methyl ester (ME), diacylglycerol (DAG) and monoacylglycerol (MAG), the percentage content of each product was calculated by following equations.

$$\text{ME content (\%)} = \frac{\text{mol of converted ME}}{\text{mol of initial TAG} \times 3} \times 100$$

$$\text{DAG content (\%)} = \frac{\text{mol of converted DAG}}{\text{mol of initial TAG} \times 3} \times 100$$

$$\text{MAG content (\%)} = \frac{\text{mol of converted MAG}}{\text{mol of initial TAG} \times 3} \times 100$$

2.2.10 Analysis of free fatty acid composition in palm oil by GC-MS

The solution of fatty acid methyl esters from **Section 2.2.7** was characterized by GC-MS equipped with Alltech AT-1MS column (30 m × 0.25 mm i.d. × 0.25 μm film thickness), the analysis conditions were: on column injection, helium carrier gas at 1.0 ml/min, injector temperature at 250^{o}C. Separation of mixture was made using the following oven temperature profile; initial temperature 120^{o}C hold on 2 min, programmed to 200^{o}C at 10^{o}C/min hold on 3 min and to 245^{o}C at 3^{o}C/min hold at final temperature 12 min. The temperatures of MS Quadrupole and MS Source were 150 and 230^{o}C, respectively.