CHAPTER 3

RESULTS

As known that *C. papaya* latex contains lipase activity in the particulate part. The enzyme was separated from the latex by centrifugation and dried by lyophilization. The specificity of CPL on the methanolysis of TAG was investigated and the lyophilized lipase was used as a biocatalyst. However, it was found that low yield of methyl esters was obtained. Therefore, washing lipase with alcohols having three or four carbon atoms was introduced for increasing lipase activity on methanolysis of triolein. Among the alcohol-treated CPLs, the *i*-propanol and *t*-butanol washing enzymes provided higher ester yields than that produced from untreated lipase. The *i*-propanol washed lipase (iPCPL) exhibited the highest yield. Therefore, the specificity of iPCPL on TAG in methanolysis was investigated for further applications of biodiesel fuel from palm oil. Several parameters affecting the methanolysis of triolein by iPCPL were determined for obtaining the good yield of methyl ester. The optimum triolein methanolysis was used for synthesis of biodiesel fuel from iPCPL-catalyzed methanolysis of palm oil.

3.1 Separation of lipase from papaya latex

One hundred grams of fresh papaya latex were collected from approximately 30 fruits of unripe papaya. High speed (10,000xg) was needed for separation of the particulate part from the latex. Clear solution of latex possessed no lipase activity, in opposite to the particulate which contained activity of lipase. Lyophilized lipase (particulate) of 5% by weight of latex was obtained as detailed in **Table 3.1** and solid powder of CPL was shown in **Figure 3.1**.

Figure 3.1 CPL was separated from whole latex by centrifugation and dried by lyophilization.

Table 3.1 Weight of lyophilized lipase obtained from papaya latex.

Step		Weight (g)
1. Fresh papaya latex		100*
2. Centrifugation	: supernatant	65.0*
	: particulate	35.0*
3. Lyophilization	: lipase powder	5.0**

*wet weight **dry weight

3.1.1 Hydrolase activity of CPL

CPL obtained by incision of unripe fruit from several sources of papaya trees was used as a biocatalyst in hydrolysis reaction to determine lipase activity. The hydrolysis activities of CPL on two types of TAGs, tributyrin and olive oil were examined in order to compare its specificity. The hydrolysis reactions were carried out in phosphate buffer pH 7 at 55^{o}C for 1 h. The free fatty acids released from the hydrolysis reactions of tributyrin and olive oil were determined by titrimetric and colorimetric methods, respectively.

3.1.1.1 Hydrolase activity on tributyrin

The hydrolysis of tributyrin which catalyzed by CPL clearly showed the specificity of enzyme on short-chain fatty acid of TAG. The determination of butyric acid that released from TAG was performed by titrimetric method in comparison with the earlier report [86]. The activity of CPL (u) was defined as µmol of butyric acid released in 1 min per g of lyophilized CPL and calculated by the equation as described in **Appendix A**. The results were shown in **Table 3.2**. The average of CPL activity was 1,941±94 u/g of lyophilized lipase.

3.1.1.2 Hydrolase activity on olive oil

Samples of lyophilized CPL collected from unripe papaya fruit were used as catalysts in hydrolysis of olive oil. To compare reproducibility between titrimetric and colorimetric methods, oleic acid from the reaction was quantified by measuring

Table 3.2 Hydrolysis activity on tributyrin of CPL which was determined by titrimetric method.

Sample of CPL No.	0.0495 M NaOH (ml)	Free fatty acid (µmol)	Hydrolysis activity (u/g)
1	4.58	226.92	1,891
2	4.65	230.40	1,920
3	4.82	238.68	1,989
4	4.85	240.00	2,000
5	4.64	229.44	1,912
6	4.41	218.52	1,821
7	4.36	216.00	1,800
8	4.86	240.60	2,005
9	5.14	254.40	2,120
10	4.73	234.36	1,953
Average	4.70	232.93	1,941± 94

the absorbance of upper layer which was 2-fold diluted with *i*-octane at 715 nm and by titration with NaOH. The lipase activity was calculated as described in **Appendix A** and **Appendix B** for titrimetry and colorimetry, respectively. The results were shown in **Table 3.3**. The average of CPL activities determination were 1,159±108 and 1,083±53 u/g of lyophilized lipase for titrimetric and colorimetric method, respectively.

Table 3.3 Hydrolysis activity of CPL on olive oil by titrimetric and colorimetric methods.

Sample of CPL No.	Titrimetric method			Colorimetric method		
	0.0495 M NaOH (ml)	Free fatty acid (μmol)	Hydrolysis activity (u/g)	A_{715}	Conc. of fatty acid (μmol/ml)	Hydrolysis activity (u/g)
1	3.19	158.28	1,319	0.471	4.31	1,083
2	3.16	156.60	1,305	0.447	4.10	1,028
3	2.54	124.42	1,048	0.486	4.45	1,117
4	2.70	134.04	1,117	0.457	4.19	1,051
5	2.68	133.32	1,111	0.446	4.08	1,024
6	3.10	153.84	1,282	0.500	4.58	1,149
7	2.82	139.80	1,165	0.447	4.10	1,028
8	2.59	128.40	1,070	0.511	4.68	1,174
9	2.77	137.40	1,145	0.477	4.37	1,096
10	2.48	123.00	1,025	0.467	4.28	1,073
Average	2.80	138.91	1,159±108	0.471	4.32	1,083±53

3.1.2 Recovery yield of CPL

Papaya latex contained 88±38 u of lipase/g of latex. The wet particulate (35 g) was separated from the latex (100 g) by centrifugation and it contained 220±73 u of lipase/g of latex (**Table 3.4**). After lyophilization, 61% yield of lipase was recovered in dried particulates.

Table 3.4 Recovery yield of CPL obtained from papaya latex.

Step	Hydrolysis activity (u/g)	Total activity (u)	Recovery activity (%)
1. Fresh papaya latex	88±38	8,800	100
2. Centrifugation : supernatant	0	0	0
particulate	220±73	7,700	87
3. Lyophilization : lipase powder	1,083 ±53	5,415	61

3.1.3 Optimum temperature and optimum pH of CPL

The hydrolase activity on olive oil of papaya latex lipase in phosphate buffer pH 7.0 was performed at a number of temperatures for 1 h. The absorption at 715 nm of cupric acetate-fatty acid complex in *i*-octane was shown in **Table 3.5** and the corresponding fatty acid concentrations were obtained from the standard curves as illustrated in **Appendix B**. The activity of CPL was then calculated and the results were exhibited in **Table 3.5**. The activity increased as temperatures were increased from 25 to 55^{o}C (**Figure 3.2**). The maximum activity was observed at 55^{o}C and further increased in temperature from 55 to 65^{o}C resulted in rapidly decreased activities. The enzyme had poor activity at 65^{o}C (approximately 55% compared to that of the optimum temperature, 55^{o}C).

Table 3.5 Hydrolysis activity of CPL at pH 7 on olive oil at different temperatures.

Temperature (°C)	A_{715}	Conc. of fatty acid (µmol/ml)	Hydrolysis activity (u/g)
25	0.114	1.04	263
37	0.301	2.76	692
45	0.400	3.66	920
50	0.435	3.98	1,000
55	0.479	4.39	1,100
60	0.348	3.19	799
65	0.261	2.39	601

The hydrolysis activity on olive oil at 55^{o}C of CPL in different buffers were determined by colorimetric method. The results were exhibited in **Table 3.6**. The maximum activity was obtained at pH 7 (**Figure 3.3**). At pH 6 and 8, the activities remained only 74 and 42% when compared to that at pH 7, respectively.

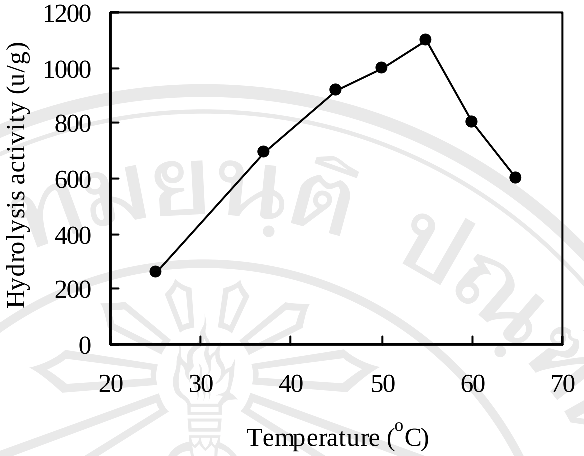

Figure 3.2 Effect of temperature on hydrolysis of olive oil by CPL at pH 7.

Table 3.6 Hydrolysis activity of CPL on olive oil in different buffer solutions at 55°C.

pH of reaction mixture	A_{715}	Conc. of fatty acid (μmol/ml)	Hydrolysis activity (u/g)
3	0.044	0.40	100
4	0.087	0.80	200
5	0.131	1.20	300
6	0.348	3.19	800
7	0.471	4.32	1,080
8	0.196	1.79	450
9	0.109	1.00	250
10	0.022	0.20	50

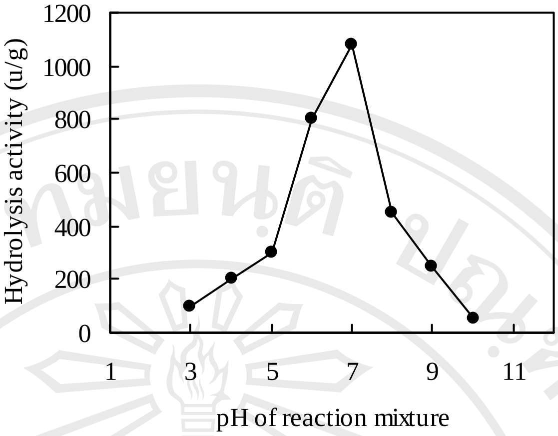

Figure 3.3 Effect of pH on hydrolysis of olive oil catalylyzed by CPL at 55^{o}C.

3.2 Optimum enzyme amount for methanolysis of triolein

Methanolysis of triolein was catalyzed by various amounts of CPL for 24 h. ME forming was analyzed by GLC and its peak area was shown in **Table 3.7**. The total weight of ME was calculated by comparing peak area with standard curve of methyl oleate as demonstrated in **Appendix D**, with an injection volume of 1 µl and total volumes of reaction mixture of 0.5 ml. The content of ME was calculated as described in **Section 2.2.9**. The effect of lipase quantity on methanolysis of triolein was presented in **Table 3.7**. ME content was increased by increasing lipase quantity up to 18%. The highest methyl oleate content (3.60%) was observed at 18% lipase by weight of triolein (**Figure 3.4**). When amount of enzyme was increased from 18 to 24%, ME content decreased rapidly. This is possibly due to the water quantity of enzyme increased which induced the hydrolysis reaction more than methanolysis reaction.

3.3 CPL specificity for methanolysis and ethanolysis

Methanolysis and ethanolysis of tripalmitin, tristearin and triolein catalyzed by CPL (18% by weight of TAGs) were investigated at 37^{o}C for 24 h. The reaction mixture was 10-fold diluted with chloroform before analyzing by GLC.

Table 3.7 Effect of enzyme amounts on methanolysis of triolein at 37°C for 24 h.

Enzyme amount (% w/w)	ME Peak area	 Total (mg)	 Content (%)
2	20	0.09	0.14
4	38	0.18	0.27
6	61	0.29	0.43
8	99	0.47	0.70
10	158	0.76	1.12
12	217	1.05	1.53
14	240	1.16	1.70
16	375	1.81	2.65
18	509	2.46	3.60
20	339	1.64	2.40
22	240	1.16	1.70
24	141	0.68	1.00

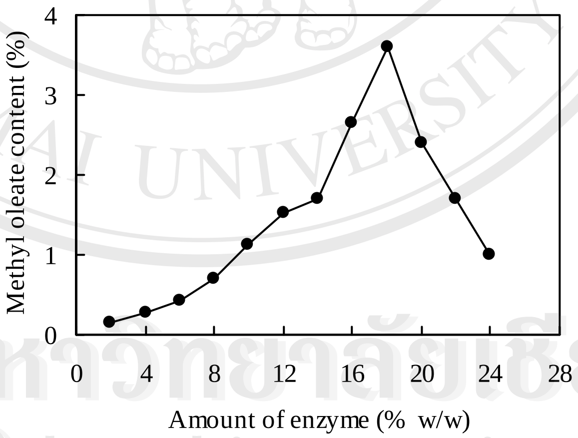

Figure 3.4 Effect of enzyme amounts on methanolysis of triolein catalyzed by CPL.

Chromatograms of each reaction product were shown in **Appendix E** to **Appendix H**. The percentages of ME, DAG and MAG contents were calculated as methods described in **Section 2.2.9**. For fatty acid content, it was expressed as the percentage proportion of mol of converted free fatty acid to initial TAG multiplied by 3.

3.3.1 Methanolysis and ethanolysis of tripalmitin

The contents of products (esters, palmitic acid and diacylglycerol) from methanolysis and ethanolysis of tripalmitin catalyzed by CPL were exhibited in **Table 3.8**. The ester forming from ethanolysis was higher than that of methanolysis (**Figure 3.5**). This result indicated that CPL was deactivated by MeOH > EtOH.

Table 3.8 Product contents from methanolysis and ethanolysis of tripalmitin catalyzed by CPL.

Product	Methanolysis			Ethanolysis		
	Peak area	Total (mg)	Content (%)	Peak area	Total (mg)	Content (%)
Esters	244	1.10	1.77	1430	2.38	3.62
Palmitic acid	23	0.48	0.82	56	1.18	1.99
Dipalmitoylglycerol	454	4.60	3.50	542	5.51	4.20

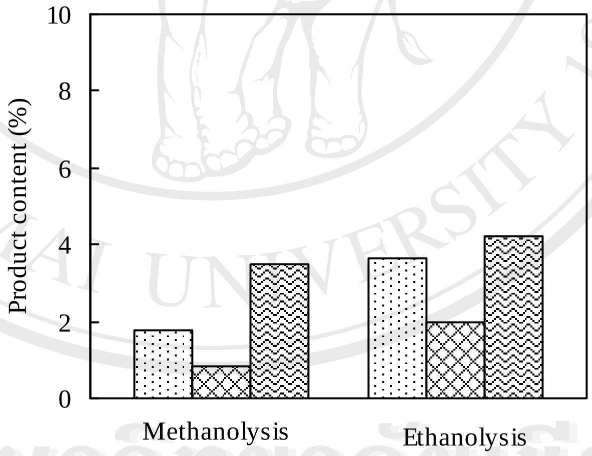

Figure 3.5 Contents of (⬚) esters, (⊠) palmitic acid and (▧) dipalmitoylglycerol from methanolysis and ethanolysis of tripalmitin catalyzed by CPL.

3.3.2 Methanolysis and ethanolysis of tristearin

The products including esters, stearic acid and diacylglycerol from both methanolysis and ethanolysis of tristearin catalyzed by CPL were quantified by GLC. The results were shown in **Table 3.9**. The contents of esters, distearoylglycerol and stearic acid from methanolysis was less than those from ethanolysis (**Figure 3.6**).

Table 3.9 Product contents from methanolysis and ethanolysis of tristearin catalyzed by CPL.

Product	Methanolysis			Ethanolysis		
	Peak area	Total (mg)	Content (%)	Peak area	Total (mg)	Content (%)
Esters	11	0.23	0.34	1120	2.15	2.98
Stearic acid	6	0.12	0.19	17	0.35	0.54
Distearoylglycerol	142	1.44	1.00	501	5.10	3.53

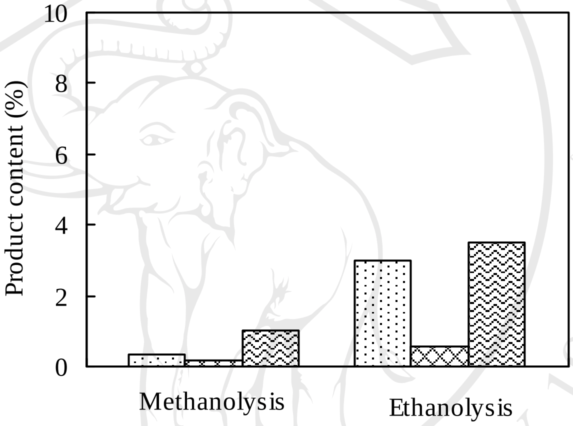

Figure 3.6 Contents of (⬚) esters, (▩) stearic acid and (▨) distearoylglycerol from methanolysis and ethanolysis of tristearin catalyzed by CPL.

3.3.3 Methanolysis and ethanolysis of triolein

The contents of products (esters and diacylglycerol) from methanolysis and ethanolysis of triolein catalyzed by CPL were shown in **Table 3.10**. The esters and dioleoylglycerol forming from methanolysis were 3.60 and 6.97%, respectively, and were less than those from ethanolysis (**Figure 3.7**).

Table 3.10 Product contents from methanolysis and ethanolysis of triolein catalyzed by CPL.

Product	Methanolysis			Ethanolysis		
	Peak area	Total (mg)	Content (%)	Peak area	Total (mg)	Content (%)
Esters	509	2.46	3.60	8250	4.83	6.74
Dioleoylglycerol	982	10	6.97	1339	13.6	9.5

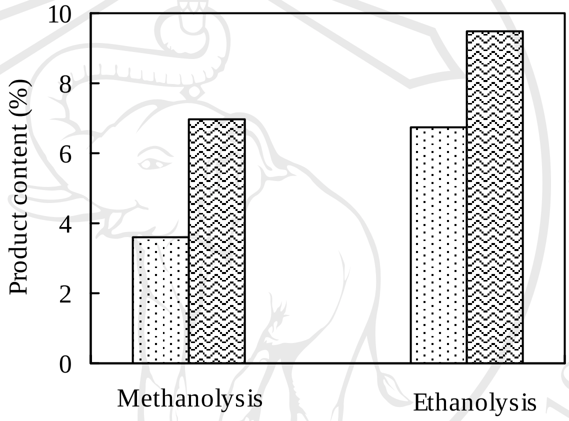

Figure 3.7 Contents of (⬚) esters and (▨) dioleoylglycerol resulted from methanolysis and ethanolysis of triolein catalyzed by CPL.

3.4 Improvement of CPL activity in methanolysis of triolein by alcohol washing

The physical factors such as immisibility between methanol and TAG and the easier absorption of methanol by lipase rather than TAG were considered as the main causes of lowering of lipase activity in methanolysis [77,80]. It has been reported that washing lipase with alcohol having three or four of carbon atoms could improve the activity of the enzyme in methanolysis [30]. Therefore, *i*-propanol, *n*-propanol, *n*-butanol and *t*-butanol were investigated for the improvement of CPL activity in the methanolysis of triolein. The analysis results of products (ME, MAG and DAG) were shown in **Table 3.11** and **Figure 3.8**. The unwashed CPL possessed very low activity and only 3.59% (w/w) of ME was obtained. MEs forming by *i*-propanol and *t*-butanol washed CPLs were increased about 6 and 5 times whereas those from the *n*-propanol

Table 3.11 Product contents from methanolysis of triolein catalyzed by native and alcohol washed CPLs.

Type of CPL	Product					
	Peak area			Content (%)		
	ME	DAG	MAG	ME	DAG	MAG
native	508	982	0	3.59	6.97	0
i-propanol washed	3,000	1,250	733	21.2	8.87	3.27
n-propanol washed	462	948	0	3.27	6.73	0
n-butanol washed	438	578	74	3.10	4.10	0.35
t-butanol washed	2,640	714	958	18.7	5.07	4.27

DAG = diacylglycerol, MAG = monoacylglycerol

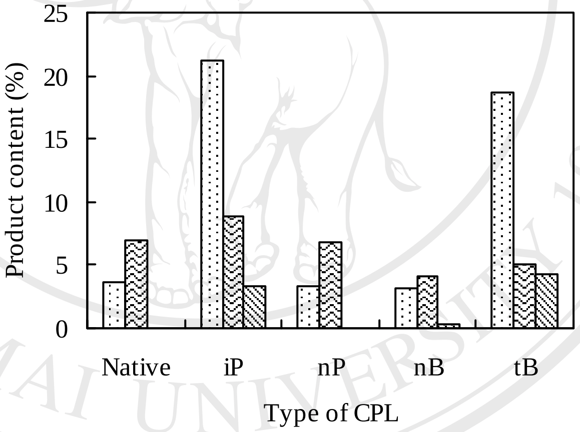

Figure 3.8 Contents of (⬚) methyl ester, (▩) diacylglycerol and (▧) monoacylglycerol which produced from the methanolysis of triolein catalyzed by native CPL and CPL washing with *i*-propanol (iP), *n*-propanol (nP), *n*-butanol (nB) and *t*-butanol (tB).

and *n*-butanol washed enzymes had the activity close to that of the native enzyme. Consequently, in all subsequent experiments, the CPL was treated with *i*-propanol before using as a biocatalyst.

3.5 Specificity of iPCPL on alcoholysis of TAG

The specificity of iPCPL on TAGs (trimyristin, tripalmitin, tristearin and triolein) was examined in methanolysis and ethanolysis. The butanolysis of tristearin was also investigated due to the lower formation of methyl and ethyl esters. The reaction was carried out in the presence of chloroform to dissolve the solid TAG at 37^{o}C for 24 h. All of the reaction mixtures were 10-fold diluted before GLC-analysis. The content of products was defined as described in **Section 2.2.9**.

3.5.1 Methanolysis and ethanolysis of trimyristin

Table 3.12 showed the content of products from methanolysis and ethanolysis of trimyristin. Ethyl ester contents from ethanolysis were higher than methyl esters produced from methanolysis whereas dimyristoylglycerol forming from ethanolysis was less than methanolysis (**Figure 3.9**). A few of monomyristoylglycerols were obtained from ethanolysis but none of them was found in methanolysis. No myristic acid was formed in both reactions.

Table 3.12 Product contents from alcoholysis of trimyristin catalyzed by iPCPL.

Alcoholysis	Product					
	Peak area			Content (%)		
	Esters	DAG	MAG	Esters	DAG	MAG
Methanolysis	650	997	0	6.08	8.57	0
Ethanolysis	2057	601	18	11.2	5.17	0.09

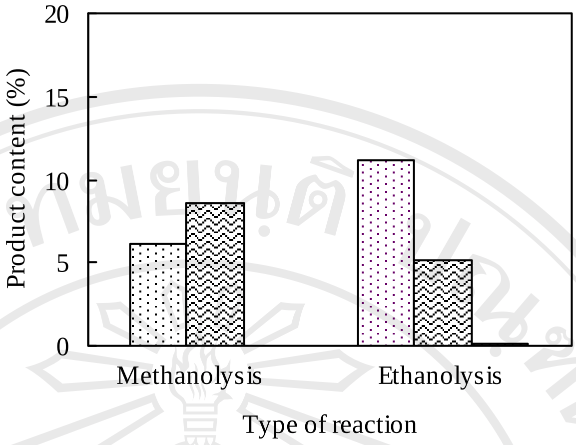

Figure 3.9 Contents of (░) esters , (▨) dimyristoylglycerol, (▧) monomyristoylglycerol from both reaction types catalyzed by iPCPL.

3.5.2 Methanolysis and ethanolysis of tripalmitin

The results obtained from GLC-analysis of products from methanolysis and ethanolysis of tripalmitin were illustrated in **Table 3.13**. In ethanolysis reaction, tripalmitin was converted into 7.36% of ethyl palmitate, 12.1% of dipalmitoylglycerol and traces of monopalmitoylglycerols (**Figure 3.10**). The lower contents of methyl palmitate (2.9%) when compared to that from ethyl palmitate (7.36%) revealed that CPL is specified to ethanol rather than methanol.

Table 3.13 Product contents from alcoholysis of tripalmitin catalyzed by iPCPL.

Alcoholysis	Product					
	Peak area			Content (%)		
	Esters	DAG	MAG	Esters	DAG	MAG
Methanolysis	395	508	0	2.90	3.93	0
Ethanolysis	2845	1566	83	7.36	12.1	0.39

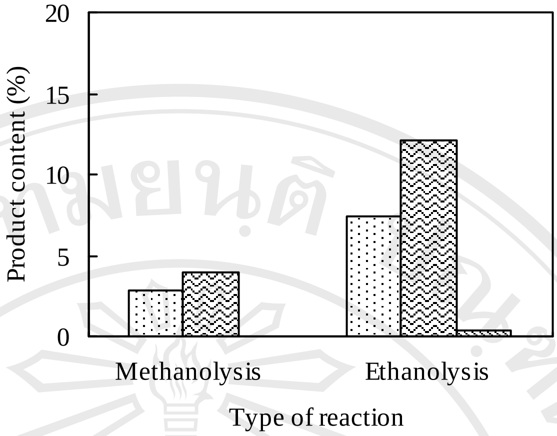

Figure 3.10 Contents of (⬚) esters, (▩) dipalmitoylglycerol, (▧) monopalmitoylglycerol from both reaction types catalyzed by iPCPL.

3.5.3 Methanolysis and ethanolysis of triolein

Table 3.14 showed peak area and the results of products from GLC-analysis. In methanolysis, triolein was converted into methyl oleate (15.1%), dioleoylglycerol (11.6%) and monooleoylglycerol (6.6%) (**Figure 3.11**). In ethanolysis reaction, it gave the same high yields of ethyl oleate, dioleoylglycerol and monooleoylglycerol. The content of esters was less than dioleoylglycerol content in both reactions. Monooleoylglycerol content in methanolysis and ethanolysis were 6.6 and 3.77%, respectively.

Table 3.14 Product contents from alcoholysis of triolein catalyzed by iPCPL.

Alcoholysis	Product					
	Peak area			Content (%)		
	Esters	DAG	MAG	Esters	DAG	MAG
Methanolysis	2125	1630	733	15.1	11.6	6.60
Ethanolysis	19377	1423	853	16.0	10.1	3.77

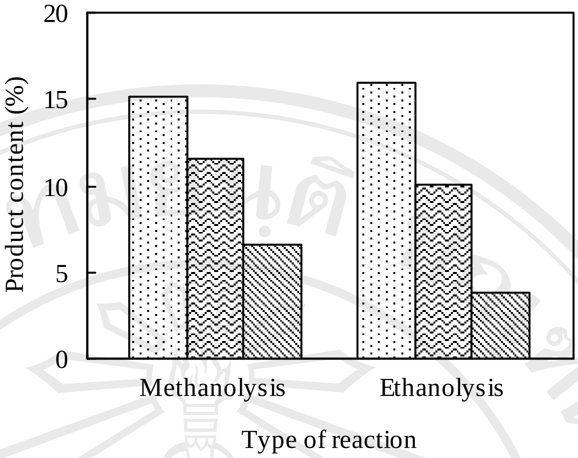

Figure 3.11 Contents of (⬚) esters, (▩) dioleoylglycerol, (▧) monooleoylglycerol from both reaction types catalyzed by iPCPL.

3.5.4 Alcoholysis of tristearin

The contents of products from methanolysis, ethanolysis and butanolysis of tristearin that obtained from GLC-analysis were shown in **Table 3.15**. The results indicated that iPCPL preferred butanolysis to ethanolysis and methanolysis, respectively (**Figure 3.12**). The contents of butyl ester (11.7%) was also higher than those of the ethyl (4.33%) and methyl esters (2.38%). In all reactions, the contents of esters were lesser than distearoylglycerol. Traces of monostearoylglycerol were formed in both ethanolysis and butanolysis but not in methanolysis.

Table 3.15 Product contents from alcoholysis of tristearin catalyzed by iPCPL.

Alcoholysis	Product					
	Peak area			Content (%)		
	Esters	DAG	MAG	Esters	DAG	MAG
Methanolysis	78	284	0	2.38	2.00	0
Ethanolysis	1653	582	98	4.33	3.90	0.43
Butanolysis	4366	1634	53	11.7	11.2	0.23

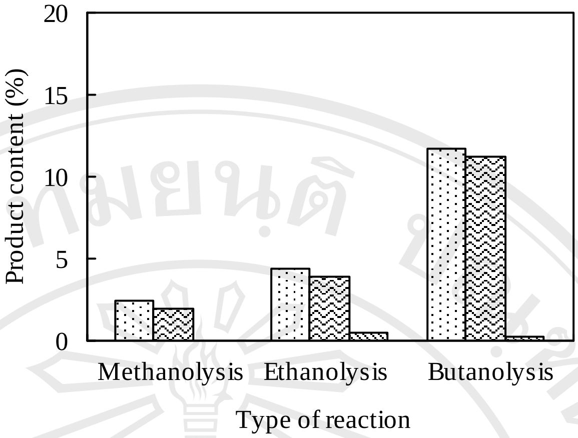

Figure 3.12 Contents of (⬚) esters, (▩) distearoylglycerol, (▧) monostearoylglycerol from methanolysis, ethanolysis and butanolysis of tristearin catalyzed by iPCPL.

3.6 Methanolysis of triolein catalyzed by iPCPL

The methanolysis of triolein catalyzed by iPCPL gave the highest content of ME among the methanolysis of used TAGs. Therefore, it was used as a model of biodiesel fuel production. In order to obtain the good production, the conditions (temperature, enzyme quantity, substrate ratio, solid buffer, water and organic solvent) were investigated. The solutions from all reactions were 10-fold diluted with chloroform before analysis by GLC.

3.6.1 Effect of temperature

Methanolysis of triolein catalyzed by iPCPL was performed at various temperatures for 10 h. The activity of iPCPL was determined on the basis of ME production. The analysis of products by GLC provided the area of ME peaks as shown in **Table 3.16**. The production of ME from methanolysis of triolein at different temperatures was demonstrated in **Figure 3.13**. When the temperature of the reaction increased, the activity of iPCPL increased significantly. At 37°C the iPCPL also produced the highest content of ME (10.8%) at 10 h. Thus, methanolysis reaction in the subsequent experiment was carried out at 37°C.

Table 3.16 The formation of products from methanolysis of triolein catalyzed by iPCPL at different temperatures.

Temperature (^{o}C)	Reaction time (h)	ME	
		Peak area	Content (%)
25	0	0	0
	2	88	0.61
	4	198	1.38
	6	308	2.21
	8	384	2.77
	10	461	3.20
30	0	0	0
	2	784	5.63
	4	859	6.06
	6	934	6.49
	8	1,008	6.93
	10	1,083	7.79
37	0	0	0
	2	837	6.06
	4	907	6.49
	6	976	6.93
	8	1,119	7.79
	10	1,519	10.8
40	0	0	0
	2	353	2.47
	4	525	3.81
	6	697	4.98
	8	742	5.19
	10	788	5.63
45	0	0	0
	2	113	0.78
	4	152	1.08
	6	190	1.30
	8	217	1.52
	10	244	1.73
50	0	0	0
	2	89.3	0.61
	4	97	0.69
	6	105	0.74
	8	117	0.84
	10	128	0.91

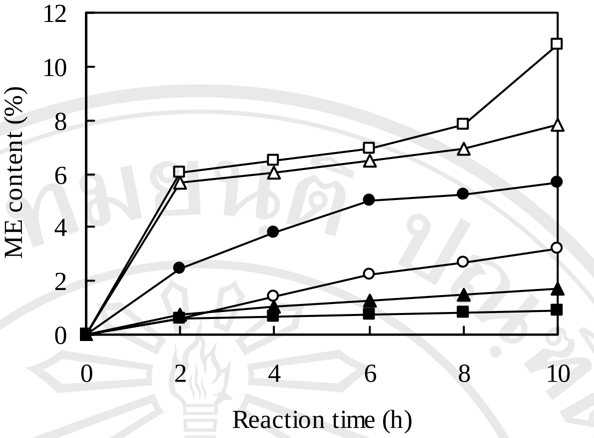

Figure 3.13 Contents of ME from methanolysis of triolein catalyzed by iPCPL at temperatures of (○) 25, (△) 30, (□) 37, (●) 40, (▲) 45 and (■) 50^{o}C.

3.6.2 Effect of lipase quantity

The effect of lipase quantities on the methanolysis of triolein was examined at 37^{o}C for 24 h. The contents of methyl esters that resulted from GLC-analysis were presented in **Table 3.17**. Methyl ester contents from methanolysis were increased by increasing lipase quantity. The highest methyl ester formation (21.2%) was observed in reaction using 0.50 g of iPCPL (**Figure 3.14**).

Table 3.17 Percentage of ME content from methanolysis of triolein catalyzed by different quantities of iPCPL.

iPCPL		ME	
Amount (g)	Activity (u)	Peak area	Content (%)
0.10	108	438	3.10
0.20	217	613	4.33
0.30	325	853	6.03
0.40	433	1,791	12.7
0.50	542	2,997	21.2
0.60	650	2,309	16.3
0.70	758	1,602	11.3
0.80	866	1,037	7.30

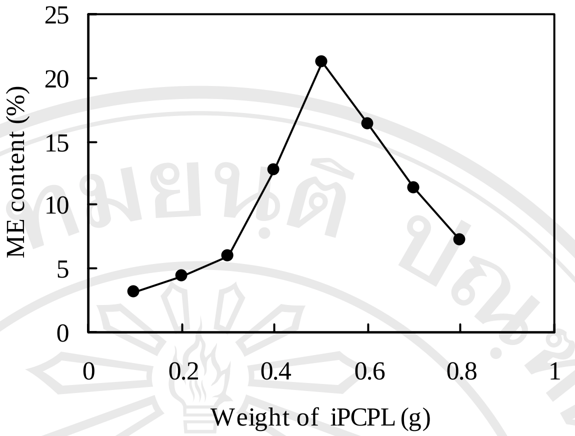

Figure 3.14 Contents of ME obtained from methanolysis of triolein catalyzed by different amounts of iPCPL.

3.6.3 Effect of substrate ratio

The effect of different molar ratios of triolein:methanol on the catalytic activity of iPCPL was investigated in order to avoid the inactivation of lipase by the excess methanol. The results were shown in **Table 3.18** and **Figure 3.15.** The contents of ME gradually increased by increasing the molar ratios of triolein to methanol from 1:1 to 1:4 and then decreased when the molar ratio was 1:5. The formation of ME from the reaction that consisted of triolein:methanol molar ratios at 1:5 and 1:6 were greater than molar ratios of triolein:methanol at 1:1 and 1:2. The content of ME obtained with a triolein:methanol molar ratio of 1:4 was approximately the same as that produced by a molar ratio of 1:3.

Table 3.18 Product contents from methanolysis of triolein catalyzed by iPCPL at different concentrations of methanol.

Molar ratio of triolein : MeOH	Reaction time (h)	Product					
		Peak area			Content (%)		
		ME	MAG	DAG	ME	MAG	DAG
1:1	0	0	0	0	0	0	0
	2	1,546	0	1,400	10.9	0	9.9
	4	1,687	0	1,540	11.9	0	10.9
	6	1,696	0	1,550	12.0	0	11.0
	24	1,791	0	1,644	12.7	0	11.7
	48	1,871	0	1,724	13.2	0	12.2
1:2	0	0	0	0	0	0	0
	2	2,008	0	1,766	14.2	0	12.5
	4	2,149	0	1,907	15.2	0	13.5
	6	2,168	0	1,926	15.3	0	13.7
	24	2,375	0	2,132	16.8	0	15.1
	48	2,502	202	2,076	17.7	0.9	14.7
1:3	0	0	0	0	0	0	0
	2	2,380	0	1,912	16.8	0	13.6
	4	2,596	0	2,109	18.4	0	15.0
	6	2,662	172	1,935	18.8	0.8	13.7
	24	2,997	0	1,710	21.2	0	12.1
	48	3,242	0	1,465	22.9	0	10.4
1:4	0	0	0	0	0	0	0
	2	2,832	0	1,874	20.0	0	13.3
	4	2,988	247	1,564	21.1	1.1	11.1
	6	3,035	322	1,470	21.5	1.4	10.4
	24	3,228	628	1,085	22.8	2.8	7.7
	48	3,289	726	963	23.3	3.2	6.8
1:5	0	0	0	0	0	0	0
	2	2,611	0	2,095	18.5	0	14.9
	4	2,700	142	1,916	19.1	0.6	13.6
	6	2,757	232	1,804	19.5	1.0	12.8
	24	3,040	681	1,240	21.5	3.0	8.8
	48	3,091	763	1,137	21.9	3.4	8.1
1:6	0	0	0	0	0	0	0
	2	2,121	0	1,879	15.0	0	13.3
	4	2,356	0	2,114	16.7	0	15.0
	6	2,498	0	2,208	17.7	0	15.7
	24	2,592	150	2,020	18.3	0.7	14.3
	48	2,639	224	1,926	18.7	1.0	13.7

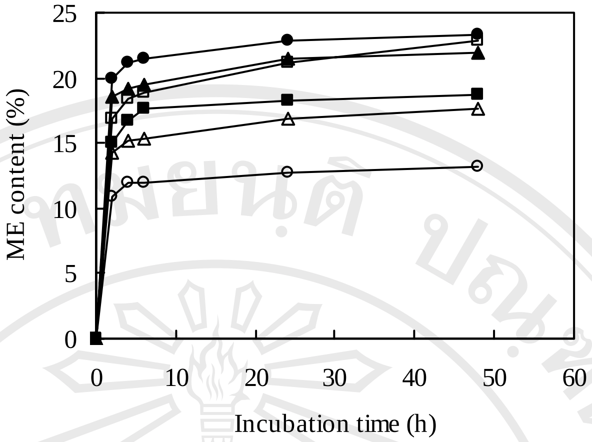

Figure 3.15 Influence of methanol concentration on methanolysis of triolein catalyzed by iPCPL using molar ratios of triolein:methanol at (O) 1:1, (△) 1:2, (□) 1:3, (●) 1:4, (▲) 1:5 and (■) 1:6.

3.6.4 Effect of solid buffer (acid and their Na salts)

As the addition of buffer solutions (pH 4 and pH 7) into CPL before washing the enzyme with *i*-propanol provided the higher ME formation from methanolysis of triolein than the control (**Table 3.19**). It appeared that added buffers might increase the methanolysis activity of iPCPL. Since solid-state buffers are able to increase yields of transesterification of *N*-acetyl-L-tyrosine ethyl ester with *n*-propanol catalyzed by subtilisin [107], therefore, it is interesting to investigate the effect of methanolysis of triolein catalyzed by iPCPL. ME formations from iPCPL catalyze methanolysis of triolein in the presence of various solid buffers at 37^{o}C for 24 h were shown in **Table 3.20** and **Figure 3.16**. Adding solid buffers ($\mathrm{pK_a}$ 3.1-9.6) did not significantly improve the amount of ME compared to the control reaction. A solid buffer having a low $\mathrm{pK_a}$ (3.1) gave more ME than the solid buffer having a high $\mathrm{pK_a}$ (9.6). Only solid buffers having $\mathrm{pK_a}$ values of 3.1 and 4.3 gave ME contents close to that from the control. None of free fatty acids or monoacylglycerol was formed in all reactions containing solid buffer. This indicated that hydrolysis reaction was not occurred apart from methanolysis.

Table 3.19 Contents of ME from methanolysis of triolein catalyzed by iPCPL which contained various buffer solutions.

iPCPL	ME	
	Peak area	Content (%)
Control	2,997	21.2
With buffer solution pH 4	3,294	23.3
With buffer solution pH 7	3,237	22.9
With buffer solution pH 10	2,615	18.5

Table 3.20 Product contents from methanolysis of triolein catalyzed by iPCPL in the presence of different solid buffers.

Solid buffer	pK_a	Product			
		Peak area		Content (%)	
		ME	DAG	ME	DAG
Control	-	2,997	1,710	21.2	12.1
Citric/CitrateNa	3.1	2,893	1,813	20.5	12.9
Glu/GluNa	4.3	2,931	1,775	20.7	12.6
MES/MESNa	6.1	2,417	1,428	17.1	10.1
MaleateNa/MaleateNa$_2$	6.2	2,417	1,602	17.1	11.4
ADA/ADANa	6.6	2,196	1,531	15.5	10.9
PIPES/PIPESNa	6.8	2,064	1,278	14.6	9.1
NaH_2PO_4/Na_2HPO_4	7.2	1,664	1,334	11.8	9.5
TES/TESNa	7.5	1,466	1,226	10.4	8.7
HEPPSO/HEPPSONa	7.8	1,324	1,179	9.4	8.4
AMPSO/AMPSONa	9.0	1,197	1,052	8.5	7.5
CAPSO/CAPSONa	9.6	763	634	5.4	4.5
Gly/GlyNa	9.6	900	474	6.4	3.4

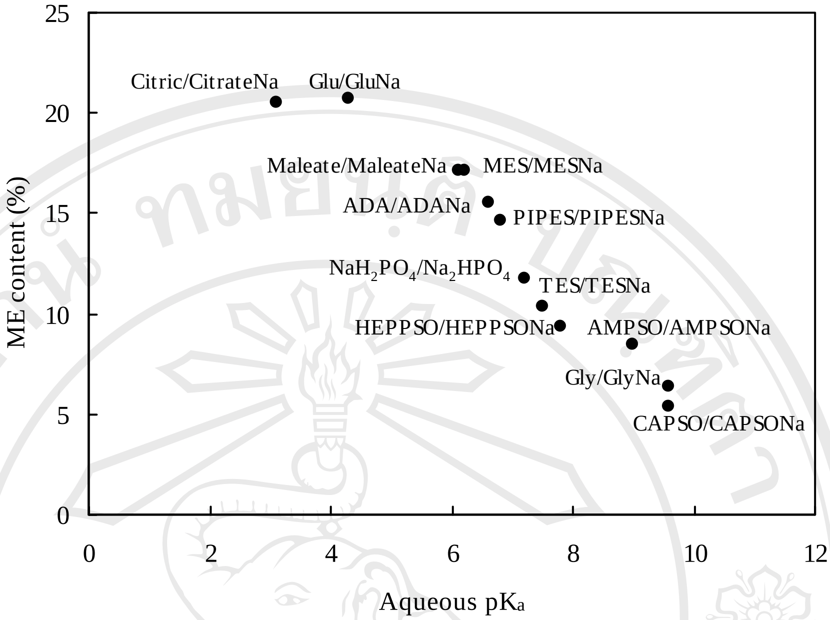

Figure 3.16 Effect of solid buffers on production of ME from methanolysis of triolein catalyzed by iPCPL.

3.6.5 Effect of water

The influence of water on catalytic activity of iPCPL to methanolysis of triolein was studied by adding water into either iPCPL or mixture of substrates. The reaction was performed at 37^{o}C for 24 h. The cleared solution from reaction mixture was 10-fold diluted with chloroform before GLC-analysis.

3.6.5.1 Water added to enzyme

Water contents of 5-20% (w/w) were mixed with iPCPL before adding to substrate solution in methanolysis of triolein at 37^{o}C for 24 h. The products at 0, 2, 6, 8 and 24 h were analyzed by GLC. The results (**Table 3.21**) revealed that all of water added iPCPL retarded both the initial reaction rate and ME contents **(Figure 3.17)**. The addition of 5% water provided the ME content closed to that produced by iPCPL without added water. The more water added into iPCPL, the more triolein remained in reaction mixture. It should be noted that no oleic acid was found in the reaction catalyzed by iPCPL added water. This seemed to indicate that the water added into enzyme did not induce hydrolysis reaction.

Table 3.21 Effect of water added to iPCPL on product contents from methanolysis of triolein.

Added water (%)	Reaction time (h)	Product					
		Peak area			Content (%)		
		ME	DAG	MAG	ME	DAG	MAG
0	0	0	0	0	0	0	0
	2	2,380	1,912	0	16.8	13.6	0
	6	2,662	1,935	172	18.8	13.7	0.8
	8	2,677	1,921	194	18.9	13.6	0.9
	24	2,997	1,710	0	21.2	12.1	0
5	0	0	0	0	0	0	0
	2	1,974	1,658	0	14.0	11.8	0
	6	2,568	2,137	0	18.2	15.2	0
	8	2,662	1,949	150	18.8	13.8	0.7
	24	2,837	1,696	277	20.1	12.0	1.2
10	0	0	0	0	0	0	0
	2	1,569	1,207	0	11.1	8.6	0
	6	2,125	1,761	0	15.0	12.5	0
	8	2,309	1,944	0	16.3	13.8	0
	24	2,757	1,202	591	19.5	8.5	2.6
15	0	0	0	0	0	0	0
	2	1,032	794	0	7.3	5.6	0
	6	1,890	1,649	0	13.4	11.7	0
	8	2,073	1,832	0	14.7	13.0	0
	24	2,097	1,921	0	14.8	13.6	0
20	0	0	0	0	0	0	0
	2	796	559	0	5.6	4.0	0
	6	1,654	1,414	0	11.7	10.0	0
	8	1,838	1,710	0	13.0	12.1	0
	24	1,861	1,733	0	13.2	12.3	0

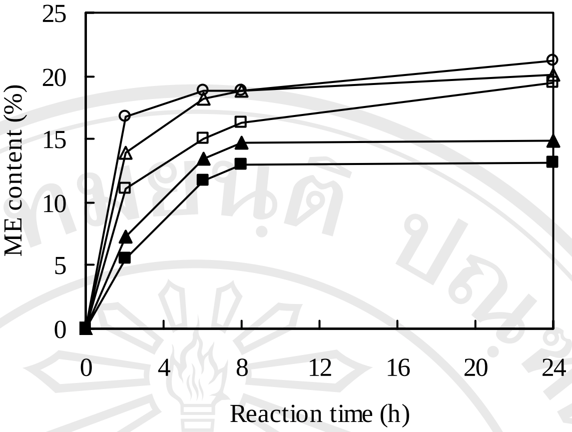

Figure 3.17 Effect of water at (△) 5%, (□) 10%, (▲) 15% and (■) 20% added to iPCPL on methanolysis of triolein compared with the (O) control.

3.6.5.2 Water added to reaction

It has been reported that lipase from *R. oryzae* which its specificity was the same as iPCPL could efficiently catalyze methanolysis of plant oil in the presence of water [11]. Therefore, the addition of water (5-20% by volume) into the substrates solution of the methanolysis reaction of triolein was investigated. The GLC analysis of products such as esters, monoacylglycerol, diacylglycerol and free fatty acids were shown in **Table 3.22**. The formation rate of ME decreased with increasing water and the ME content also dropped with respect to the amounts of added water (**Figure 3.18**). Two products, fatty acid (oleic acid) and monoacylglycerol, appeared only from methanolysis with the incorporation of water. The formation of diacylglycerol decreased when increasing added water into the methanolysis reaction. These results clearly showed that added water induced hydrolysis apart from the methanolysis reaction.

Table 3.22 Effect of water adding into substrate mixtures on the product contents from methanolysis of triolein catalyzed by iPCPL.

Added Water (%)	Reaction time (h)	Product							
		Peak area				Content (%)			
		ME	DAG	MAG	FFA	ME	DAG	MAG	FFA
0	0	0	0	0	0	0	0	0	0
	2	2,380	1,912	0	0	16.8	13.6	0	0
	6	2,662	1,935	172	0	18.8	13.7	0.8	0
	8	2,677	1,921	194	0	18.9	13.6	0.9	0
	24	2,997	1,710	0	0	21.2	12.1	0	0
5	0	0	0	0	0	0	0	0	0
	2	1,135	704	201	61	8.0	5.0	0.9	3.0
	6	1,836	844	350	68	13.2	6.0	1.6	3.3
	8	2,071	984	498	74	14.6	7.0	2.2	3.7
	24	2,602	1,124	646	81	18.4	8.0	2.9	4.0
10	0	0	0	0	0	0	0	0	0
	2	1,044	468	371	101	7.4	3.3	1.7	5.0
	6	1,693	608	519	108	12.0	4.3	2.3	5.3
	8	1,975	748	667	115	14.0	5.3	3.0	5.7
	24	2,257	935	889	135	16.0	6.6	4.0	6.7
15	0	0	0	0	0	0	0	0	0
	2	808	280	519	133	5.7	2.0	2.3	6.6
	6	1,620	466	667	169	11.5	3.3	3.0	8.3
	8	1,831	605	815	176	13.0	4.3	3.7	8.7
	24	2,065	744	963	183	14.7	5.3	4.3	9.0
20	0	0	0	0	0	0	0	0	0
	2	765	93	704	136	5.4	0.6	3.2	6.7
	6	1,553	186	1,037	170	11.0	1.3	4.7	8.3
	8	1,782	325	1,185	204	12.7	2.3	5.3	10.0
	24	1,922	464	1,333	210	13.7	3.3	6.0	10.3

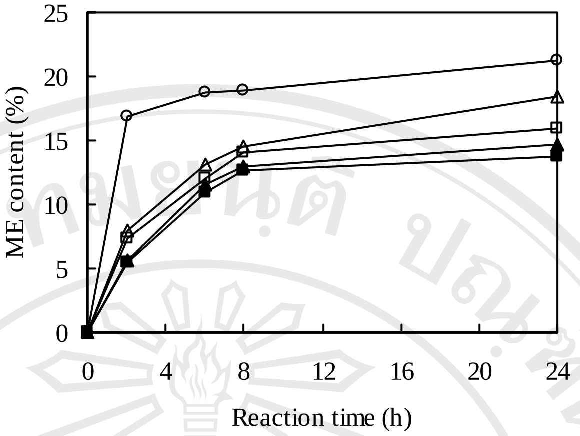

Figure 3.18 Effect of water (△) 5%, (□) 10%, (▲) 15% and (■) 20% added to reaction mixture on methanolysis of triolein compared with the (O) control.

3.6.6 Effect of organic solvent

Methanolysis activities of iPCPL in several organic solvents were investigated at 37°C for 24 h. The results were presented in **Table 3.23** and **Figure 3.19**. The enzyme demonstrated similar formation of ME in non-polar solvents (hexane, diethyl ether and dichloromethane). Monoacylglycerols were formed only from the reaction containing organic solvents. However, when slightly non-polar solvent (chloroform) was used, lower ME formation was appeared. These results indicated that non-polar solvent had lesser influence on iPCPL activity than a slightly non-polar solvent.

Table 3.23 Product contents from methanolysis of triolein catalyzed by iPCPL in several organic solvents.

Solvent	Product					
	Peak area			Content (%)		
	ME	MAG	DAG	ME	MAG	DAG
No solvent	2,997	0	1,710	21.2	0	12.1
Hexane	2,922	823	1,268	20.7	3.67	9.00
Diethyl ether	2,893	1,257	1,024	20.5	5.60	7.27
Dichloromethane	2,865	1,332	1,005	20.3	5.93	7.13
Chloroform	1,861	1,871	1,667	13.2	8.33	11.8

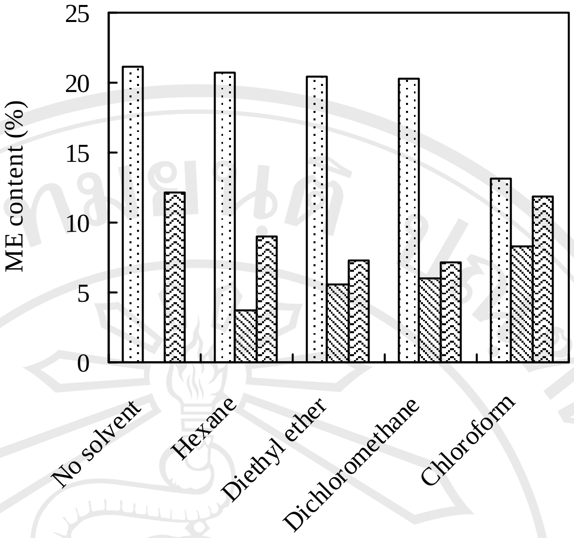

Figure 3.19 Influence of organic solvents on production of (⬚) methyl ester, (▧) mono- and (▨) diacylglycerols from methanolysis of triolein catalyzed by iPCPL.

3.7 Biodiesel fuel from methanolysis of palm oil

Palm oil is a form of edible vegetable oil obtained from the fruit of the oil palm tree. It is the second-most widely produced edible oil, after soybean oil [108]. In synthesis of biodiesel fuel from methanolysis of palm oil, the reaction was performed at an optimum condition of methanolysis of triolein using different molar ratios of palm oil:methanol. In this experiment, palm oil compositions were investigated to calculate the average molecular weight of palm oil. To determine molecular weight of TAG in palm oil, ester bond of fatty acids in TAGs was hydrolyzed and then converted into its methyl ester. The free fatty acids were identified by GC-MS and the contents of fatty acids in palm oil were evaluated by GLC.

3.7.1 Fatty acid composition of palm oil

Fatty acid composition in palm oil was determined by transesterification with BF_3-methanol. Methyl esters forming were extracted and analyzed by GC-MS. The chromatogram of reaction mixture from transesterification of palm oil and methanol catalyzed by BF_3 was presented in **Figure 3.20**. **Figures 3.21-a** to **3.21-e** represented the mass spectra of peak a-e from chromatogram in **Figure 3.20**. The identification of mass spectra of methyl esters of fatty acids was performed by comparison these spectra to standard MS spectra library in mass spectrometer. Mass spectra as shown in **Figures 3.21-a**, **3.21-b**, **3.21-c**, **3.21-d** and **3.21-e** have the fragmentation patterns nearly identical to the standard MS spectrum of methyl myristate, methyl palmitate, methyl linoleate, methyl oleate and methyl stearate, respectively (**Figures 7** (a), (b), (c), (d) and (e) in **Appendix I**, respectively). The molecular ions of these esters from their spectra and molecular weight of their fatty acids were shown in **Table 3.24**.

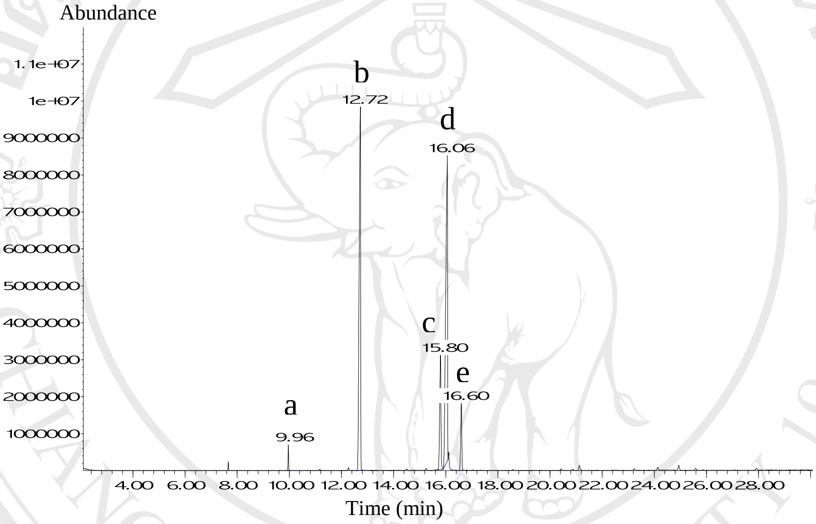

Figure 3.20 GC-MS chromatogram of reaction mixture from methanolysis of palm oil catalyzed by BF_3.

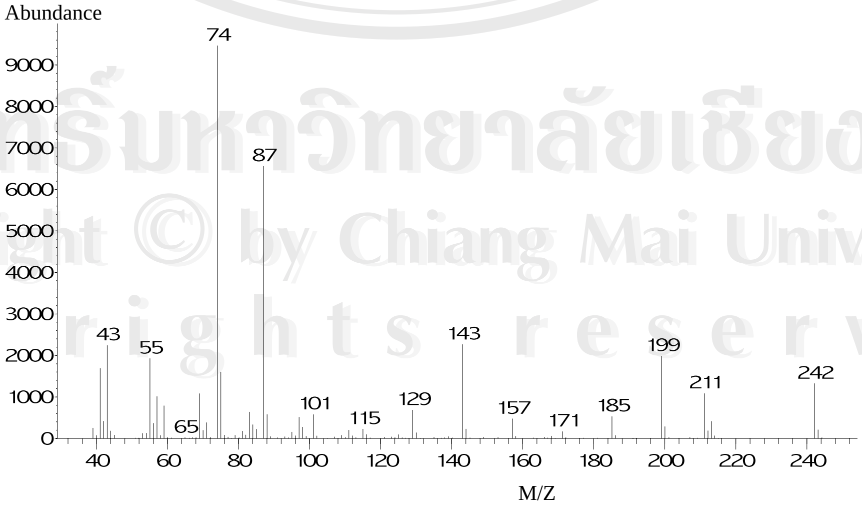

Figure 3.21a Mass spectrum of peak a in **Figure 3.20** which was corresponded to methyl myristate.

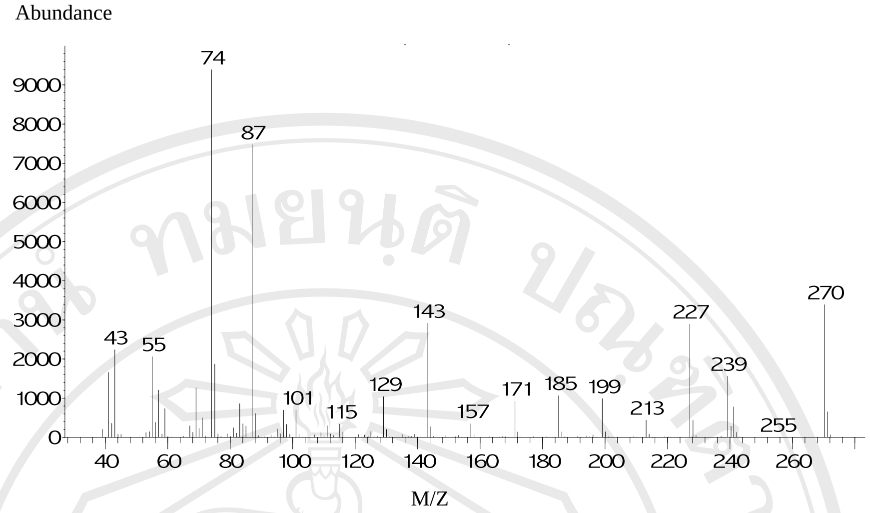

Figure 3.21b Mass spectrum of peak b in **Figure 3.20** which was corresponded to methyl palmitate.

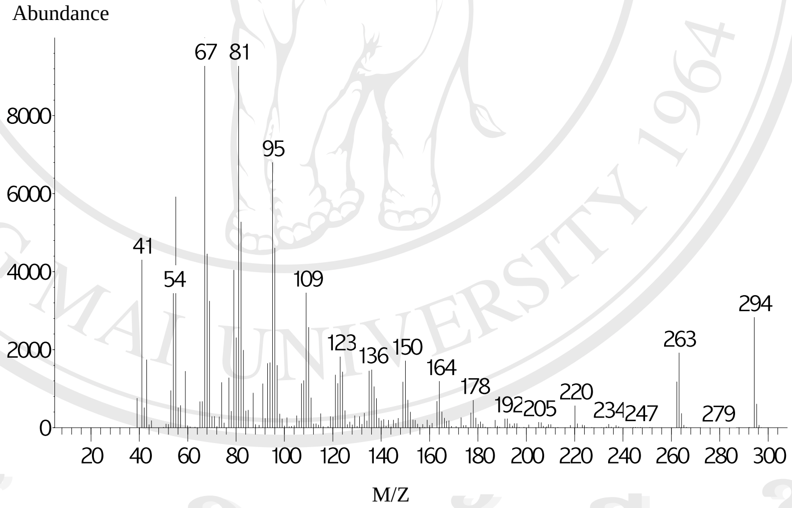

Figure 3.21c Mass spectrum of peak c in **Figure 3.20** which was corresponded to methyl linoleate.

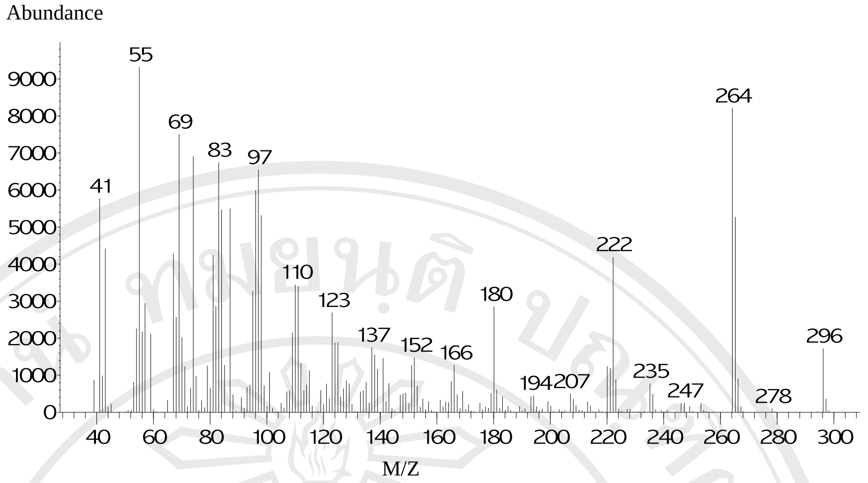

Figure 3.21d Mass spectrum of peak d in **Figure 3.20** which was corresponded to methyl oleate.

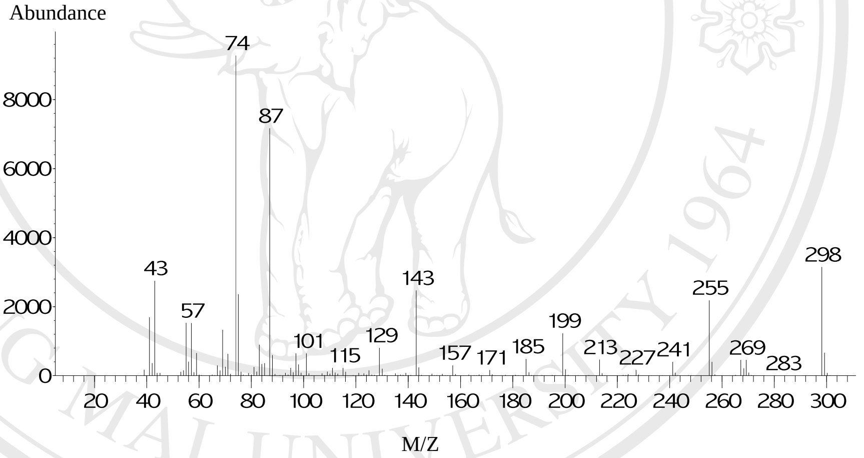

Figure 3.21e Mass spectrum of peak e in **Figure 3.20** which was corresponded to methyl stearate.

Table 3.24 The identification of fatty acid methyl esters obtained from palm oil.

Methyl ester			Fatty acid	
Type	Figure	M^+ ion	Type	MW
Myristate	3.21a	242	Myristic acid	228.4
Palmitate	3.21b	270	Palmitic acid	256.4
Linoleate	3.21c	294	Linoleic acid	280.5
Oleate	3.21d	296	Oleic acid	282.5
Stearate	3.21e	298	Stearic acid	284.5

The identification of fatty acid methyl esters was confirmed by comparison of their retention times with standard ME from GLC analysis (**Table 1** in **Appendix C**). Compositions of these fatty acid methyl esters from the transesterification of palm oil catalyzed by BF_3 were calculated from their peak area and their standard curves (**Figures 2** (a) to (f) in **Appendix D**). The results shown in **Table 3.25** indicated that palm oil composed of 0.99% myristic acid, 40.8% palmitic acid, 9.52% linoleic acid, 48.1% oleic acid, and 0.57% stearic acid.

Table 3.25 Composition of fatty acids in palm oil.

Fatty acid	Peak area	Composition (%)
Myristic acid	381	0.99
Palmitic acid	15,700	40.8
Linoleic acid	3,659	9.52
Oleic acid	18,493	48.1
Stearic acid	218	0.57

Therefore, the average molecular weight of TAGs in palm oil could be calculated by the following relationship.

Avererge MW of palm oil

= MW of glycerol + (average MW of fatty acids x 3) - $3H_2O$

MW of glycerol = 92

Average MW of fatty acid in palm oil :

= [Sum of (MW fatty acid composition x % content)] / 100

= [(228.4 x 0.99) + (256.4 x 40.8) + (280.5 x 9.52) + (282.5 x 48.1) + (284.5 x 0.57)] / 100

= [(226 + 10461 + 2670 + 13588 + 162)] / 100

= 271.1

Therefore average MW of palm

= 92 + (271.1 x 3) – (3 x 18)

= 851.3

3.7.2 Methyl esters from methanolysis of palm oil

Methanolysis of palm oil catalyzed by iPCPL was performed at 37^{o}C in various molar ratios of palm oil:methanol. The chromatogram of methanolysis using palm oil:methanol molar ratios of 1:4 at 24 h from GLC-analysis as shown in **Figure 3.22** revealed that the products were methyl palmitate (MP), methyl oleate (MO), methyl linoleate (ML) and diacylglycerols (DAG). The areas of these products were compared to those in standard curves of ME and diacylglycerols (**Figures 2** (a) to (f) in **Appendix D** and **Figures 10** (a) to (d) in **Appendix L**) and the results from calculation were shown in **Table 3.26**. The total methyl ester contents increased with increasing molar ratios of palm oil:methanol from 1:1 to 1:4 and decreased when molar ratios up to 1:5 (**Figure 3.23**). The highest content of total methyl ester occurred when using a palm oil:methanol molar ratios of 1:4. None of monoacylglycerol was detected in all reactions which possibly indicated that iPCPL catalyzed only 1- or 3-position of TAG molecules in palm oil.

Table 3.26 Composition of products from methanolysis of palm oil catalyzed by iPCPL.

Molar ratio of palm oil: methanol	Reaction time (h)	Product								
		Peak area				Content (%)				
		MP	MO	ML	DAG	MP	MO	ML	Total ME	DAG
1:1	0	0	0	0	0	0	0	0	0	0
	2	601	433	0	3,490	4.4	3.1	0	7.5	25.9
	4	615	430	0	3,841	4.5	3.0	0	7.5	25.8
	6	542	380	143	3,449	3.9	2.7	1.1	7.7	25.6
	24	601	422	128	3,369	4.4	3.0	1.0	8.4	25.0
	48	643	451	139	3,288	4.7	3.2	1.1	9.0	24.4
1:2	0	0	0	0	0	0	0	0	0	0
	2	620	441	0	3,463	4.5	3.1	0	7.6	25.7
	4	698	490	0	3,342	5.1	3.5	0	8.6	24.8
	6	702	490	0	3,337	5.1	3.5	0	8.6	24.8
	24	748	528	109	3,144	5.4	3.7	0.8	10.0	23.3
	48	794	547	109	3,081	5.8	3.9	0.8	10.5	22.9
1:3	0	0	0	0	0	0	0	0	0	0
	2	1,074	749	0	2,726	7.8	5.3	0	13.1	20.2
	4	1,143	792	183	2,430	8.3	5.6	1.4	15.3	18.0
	6	1,230	848	202	2,268	8.9	6.0	1.5	16.4	16.8
	24	1,308	928	277	2,044	9.5	6.6	2.1	18.2	15.2
	48	1,331	952	298	1,976	9.7	6.7	2.3	18.7	14.7
1:4	0	0	0	0	0	0	0	0	0	0
	2	1,157	796	0	2,600	8.4	5.6	0	14.0	19.3
	4	1,276	886	118	2,277	9.3	6.3	0.9	16.5	16.9
	6	1,317	1,371	121	2,214	9.6	9.7	0.9	20.2	16.4
	24	1,496	1,046	225	1,796	10.9	7.4	1.7	20.0	13.3
	48	1,565	1,112	223	1,671	11.4	7.9	1.7	21.0	12.4
1:5	0	0	0	0	0	0	0	0	0	0
	2	918	631	0	2,991	6.7	4.5	0	11.2	22.2
	4	1,051	721	0	2,776	7.6	5.1	0	12.7	20.6
	6	1,207	825	0	2,524	8.8	5.8	0	14.6	18.7
	24	1,322	924	0	2,318	9.6	6.5	0	16.1	17.2
	48	1,363	975	0	2,228	9.9	6.9	0	16.8	16.5

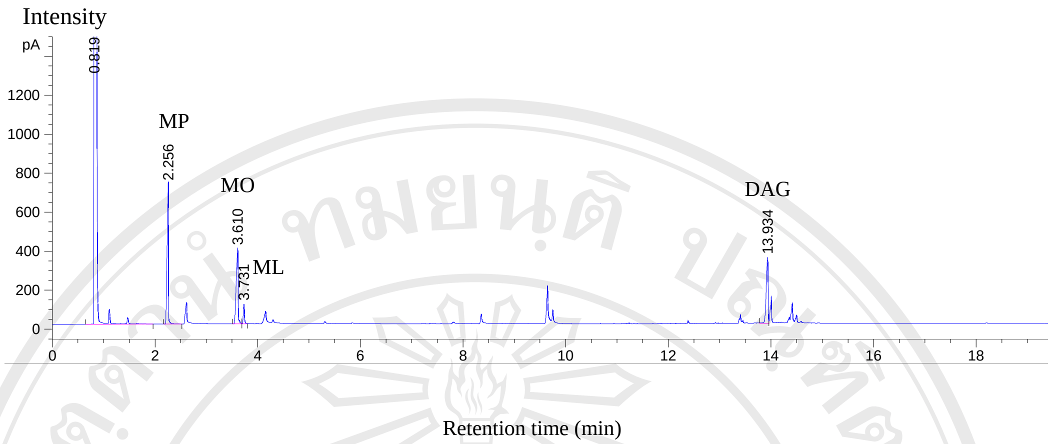

Figure 3.22 GLC chromatogram of reaction mixture from methanolysis of palm oil catalyzed by iPCPL using palm oil:methanol at molar ratio of 1:4 at 24 h of reaction.

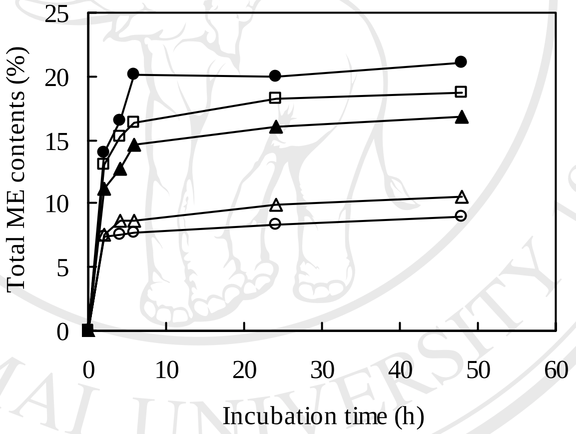

Figure 3.23 Influence of methanol concentration on methanolysis of palm oil. Reaction was performed at palm oil:methanol molar ratio of (○) 1:1, (△) 1:2, (□) 1:3, (●) 1:4 and (▲) 1:5.