CHAPTER 4

DISCUSSION AND CONCLUSIONS

CPL was separated from the whole latex from papaya fruits. Besides the verification of the optimum pH and optimum temperature of hydrolysis reaction, the specificity on TAGs and alcohols in alcoholysis reactions were also determined. Methanolysis of triolein was used as a model reaction for synthesis of biodiesel fuel from palm oil. Although a number of parameters of methanolysis were optimized, the maximum yield of methyl ester obtained was less than 4%. Therefore, inactivated protection of CPL was applied by washing the enzyme with alcohols having three or four of carbon atoms. Isopropanol washed CPL (iPCPL) increased its catalytic activity on methanolysis, 21.2% of methyl ester was produced. The specificities of iPCPL on TAGs in methanolysis were examined. The optimum methanolysis of triolein catalyzed by iPCPL was verified for synthesis of methyl ester. Biodiesel fuel (methyl esters of fatty acids) was synthesised by iPCPL catalyzed methanolysis of palm oil by using the same condition as that of triolein.

4.1 Production of CPL from whole latex

The fresh *C. papaya* latex obtained from incision of unripe papaya fruits (70-100 days) was freezed at -20^{o}C to maintain the activity of lipase. The particulate part which suspended in latex was separated by centrifugation at high speed (10,000xg). The wet particulate was lyophilized to preserve the activity of enzyme for a long period, 5% yield of lyophilized CPL was obtained. The separation of particulate part from a homogenate of spray-dried latex from *C. papaya* [86] strongly confirmed the presence of lipase activity in the particulate fraction of fresh latex. No effect after treatment of CPL with either proteolytic enzymes or detergents (cationic or neutral) was found which indicated a tight association of lipase with the particulate fraction [86]. Consequently, CPL was used as a biocatalyst without further purification in subsequent experiments.

The hydrolysis activity of lipase from *C. papaya* latex using tributyrin and olive oil demonstrated that CPL exhibited 1,941±94 and 1,159±108 u/g, respectively.

This was indicated that CPL preferred to hydrolyse tributyrin than olive oil. The lyophilized lipase possessed the water content and water activity of 4.32% and 0.448, respectively.

The study of temperature showed that CPL exhibited the maximal activity of 1,100 u/g on hydrolysis of olive oil at 55°C. It corresponded to that of the hydrolysis activity on tributyrin of CPL from spray-dried *C. papaya* latex [86]. For optimum pH, the CPL demonstrated the highest activity (1,080 u/g of lipase) in the presence of phosphate buffer pH 7.0 at 55°C (**Table 4.1**).

Table 4.1 Production yield and some properties of lyophilized CPL from papaya latex.

Subjects	Papaya latex	Lyophilized CPL
Weight (g)	100*	-
Production yield (%)	-	5.0**
Activity (u/g of CPL)		
Hydrolysis of tributyrin	-	1,941±94
Hydrolysis of olive oil	220±73	1,159±108
Water content (%)	-	4.32
Water activity	-	0.448
Optimum temperature (°C)	-	55
Optimum pH	-	7

*wet weight **dry weight

4.2 Specificity of CPL on methanolysis and ethanolysis of TAGs

Various amounts of CPL (2-24% w/w of TAG) were used as biocatalysts in methanolysis of triolein. The highest content of ME (methyl oleate), 3.60% was obtained at 18% (w/w) of enzyme. Then, the methanolysis and ethanolysis of TAG were performed in the presence of 18% (w/w) of CPL.

The specificity of CPL in catalysis of methanolysis and ethanolysis of tripalmitin, triolein and tristearin were investigated due to the esters of long-chain fatty acids possess the property of diesel-like. CPL (activity = 13.4 u (or 18% w/w) and water content = 4.32%) catalyzed methanolysis and ethanolysis of triolein >> tripalmitin > tristearin as shown in **Figure 4.1**. The methanolysis produced 3.60, 1.77 and 0.34% contents of methyl oleate, methyl palmitate and methyl stearate, respectively. In case of ethanolysis of TAGs, contents of ethyl oleate, ethyl palmitate and ethyl stearate were 6.74, 3.62 and 2.98%, respectively. These results indicated that CPL had the specificity on unsaturated fatty acids in TAG more than saturated fatty acids and preferred to catalyzed ethanolysis >> methanolysis. This is in accordance with the specificity of CPL on unsaturated fatty acids in esterification with 1-butanol [97]. Unfortunately, contents of methyl and ethyl esters from both reactions were still rather low. The main reason for obtaining low activity of methanolysis and ethanolysis was immiscibility between methanol or ethanol and TAGs. Consequently, when methanol or ethanol was absorbed into the active site of CPL, the entry of TAGs into the active site would possibly be blocked and the reaction was then stopped [76]. The other reason would be water content of CPL (4.32%) which led to hydrolysis reaction. This result showed that both of hydrolysis and alcoholysis reactions had occurred.

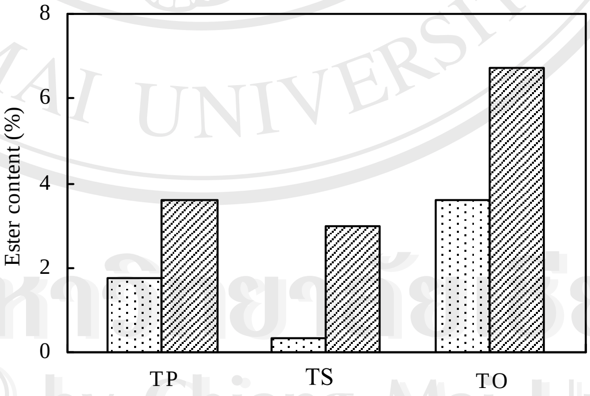

Figure 4.1 Contents of (░) methyl esters and (▨) ethyl esters obtained from methanolysis and ethanolysis, respectively, of tripalmitin (TP), tristearin (TS) and triolein (TO).

4.3 Improvement of CPL efficiency on methanolysis

It has been discovered that washing immobilized lipase with alcohol having three or four carbon atoms can effectively activate enzyme such as Novozyme 435 [77]. Therefore, *n*-propanol, *i*-propanol, *n*-butanol and *t*-butanol were tested for activating the catalytic activity of CPL in methanolysis reaction. The methanolysis of triolein at 37^{o}C using triolein:methanol molar ratio of 1:3 was used as a model reaction. The results of ME content were shown in **Table 4.2**. The CPL washed with *i*-propanol produced methyl ester from methanolysis of triolein > CPL washed with *t*-butanol >> CPL washed with *n*-propanol and *n*-butanol. Native CPL produced only 3.59% of methyl oleate content. Washing CPL with *i*-propanol could effectively increase the change of triolein to 21.2% of ME, 8.87% of diolein and 3.27% of monoolein. The more effective catalysis of CPL with *t*-butanol comparing to native CPL was also found. However, it was less effective than iPCPL according to formation of product, 18.7% of methyl oleate. Washing CPL with *n*-propanol and *n*-butanol could not improve the efficiency of CPL based on their methyl oleate contents which was found as those of native CPL.

Table 4.2 ME contents obtained from methanolysis of triolein catalyzed by native and alcohol washed CPLs.

Type of CPL	ME content (%)
Native	3.59
n-propanol	3.27
i-propanol	21.20
n-butanol	3.10
t-butanol	18.70

A possible explanation on low production of methyl ester by CPL as previously shown is that TAG is less soluble in methanol. Since methanol is easier to be absorbed by lipase than TAG [30], then TAG is difficult to enter into active site of CPL. Therefore, the methanolysis reaction is less active. On the other hand, when

CPL was washed with alcohol that consisted of three or four of carbon atoms, the enzyme was surrounded by those alcohols. So that alcohol can act as an intermediate solvent for better solubility of the two substrates. Interestingly, washing CPL with *i*-propanol and *t*-butanol produced a higher amount of ME, it may be the fact that these alcohols would solubilize TAG better than *n*-propanol and *n*-butanol. Thus, iPCPL and tBCPL catalyzed more methanolysis than nPCPL and nBCPL. The improvement of lipase activity was also successful for washing of immobilized lipase Novozyme 435 with *i*-propanol and *t*-butanol. The enzyme washed with *i*-propanol, 2-butanol and *t*-butanol produced methyl esters from methanolysis of soybean oil about 7 to 10 times higher than that for immobilized lipase without washing [77]. These results obviously confirmed that washing with alcohols having three or four of carbon atoms could improve the activity of enzyme in methanolysis. So the lyophilized lipase from *C. papaya* latex was required the washing with *i*-propanol for the further study of alcoholysis.

4.4 Specificity of iPCPL on alcoholysis of TAGs

The examination of the specificity of iPCPL on TAGs (trimyristin, tripalmitin, tristearin and triolein) in formation of fatty acid ester from methanolysis, ethanolysis and butanolysis was exhibited in **Figure 4.2**. All alcoholysis reactions were performed in the presence of chloroform in order to dissolve all substrates, especially tristearin.

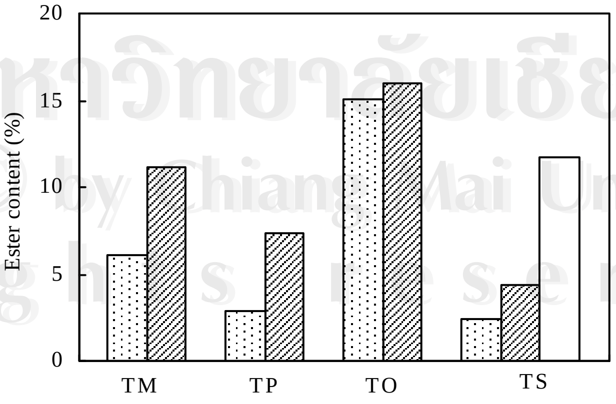

Figure 4.2 Contents of (⬚) methyl esters, (▨) ethyl esters and (□) butyl ester obtained from alcoholysis of the corresponding TAGs in chloroform at 37°C.

Methanolysis of trimyristin, tripalmitin, triolein and tristearin catalyzed by iPCPL produced ME contents of 6.08, 2.90, 15.1 and 2.38%, respectively. Additionally, 8.57% dimyristoylglycerol, 3.93% dipalmitoylglycerol, 11.6% dioleoylglycerol and 2% distearoylglycerol were obtained from that reaction. These results demonstrated that iPCPL preferred to catalyze methanolysis of triolein > trimyristin > tripalmitin > tristearin. Therefore, TAG having unsaturated (C=C) bond was a suitable substrate for iPCPL. This is contrary with interesterification of tricaprylin and various TAGs [81] and with esterification of 1-butanol and fatty acids [97] that CPL preferred to catalyze myristic acid more than oleic acid. The reason is that the washing of CPL with *i*-propanol permitted the solubilization of triolein more than trimyristin.

The iPCPL could also catalyze ethanolysis of triolein > trimyristin > tripalmitin > tristearin. The content at 24 h of ethyl oleate, ethyl myristate, ethyl palmitate and ethyl stearate were 16.0, 11.2, 7.36, and 4.33%, respectively. Also diacylglycerols and monoacylglycerols were produced from that ethanolysis reaction. The formation yield of ME indicated that iPCPL preference for ethanolysis than methanolysis as previously found from alcoholysis catalyzed by other lipases [9,36-37].

For butanolysis of tristearin, it was transformed to 11.7% of butyl stearate, 11.3% of distearoylglycerol and 0.23% of monostearoylglycerol. The result denoted that iPCPL preference for butanolysis of tristearin than ethanolysis and methanolysis. As the case of esterification of octanoic acid with several alcohols, CPL prefers to catalyze alcohol having more than three carbon atoms [98].

The formation of diacylglycerol and monoacylglycerol from alcoholysis catalyzed by iPCPL markedly confirmed the 1,3-specificity of enzyme as determined by Villeneuve *et al*. [99].

4.5 Optimum condition of methyl ester synthesis from triolein

The best condition for methanolysis of triolein catalyzed by iPCPL and maximum ME content was concluded in **Table 4.3**. The effect of amount of added iPCPL on production of ME from methanolysis of triolein showed that the content of ME increased proportionally with increasing lipase amount until 542 u (or 0.5 g) and

Table 4.3 Condition and maximum ME content obtained from methanolysis of triolein by iPCPL.

Subject	Concluded result
Condition	
iPCPL	542 u or 0.5 g/reaction
Optimum temperature	37^{o}C
Water added	
To iPCPL	Lower yield
To substrate mixture	Lower yield
Organic solvent	Lower yield
Substrate molar ratio (triolein:MeOH)	1:3-1:5
Solid buffer	Lower yield
Maximum ME content	21.2-23.3%

decreased at higher amounts of lipase (**Figure 3.14**). Due to an increase amount of lipase which led into an increased amount of water in reaction mixture and then hydrolysis reaction has occurred better than that of methanolysis reaction. The highest content of methyl oleate was observed at 37^{o}C, whereas it was rapidly decreased at 40-50^{o}C (**Figure 3.13**).

The most suitable molar ratio of triolein:methanol was investigated in the methanolysis of triolein catalyzed by iPCPL at 37^{o}C. The results indicated that the ME production increased when the molar ratio of triolein:methanol was increased from 1:1 to 1:3 and the highest content of ME was obtained at a molar ratio of 1:4. This is in accordance with the methanolysis of sunflower oil catalyzed by lipase from *P. fluorescens*, a methanol resistant strain [109], that high production of ME was obtained using a 1:4.5 molar ratio of sunflower oil to methanol [110]. Increasing the molar ratio of triolein:methanol up to 1:5 and 1:6 in methanolysis of triolein catalyzed by iPCPL produced nearly the same ME content as the molar ratio at 1:4 (only 1-4% lower). This may be explained that increasing of methanol led to lesser dissolution of TAG and then the entering of TAG into active site of iPCPL would decrease. This phenomenon was also found in methanolysis of cotton seed oil by *C. antarctica* [111] and in methanolysis of sunflower oil by *R. miehei* and *Thermomyces lanuginosa* [110]. However, the content of ME only slightly decreased in the case of iPCPL when compared to the methanolysis by these microbial lipases. A possible explanation is that *i*-propanol surrounded iPCPL would retard the entering of methanol into the active site of iPCPL, therefore methanol could lesser enter into active site of the enzyme and yield of ME was a little decrease.

The effects of solid-state buffers (acid and their Na salts) on methanolysis of triolein catalyzed by iPCPL were studied. The result (**Figure 3.16**) demonstrated that all methanolysis reactions of triolein which performed in solid-state buffers gave the methyl ester contents lower than that of control. Among them, citric/citrateNa buffer (pK_a 3.1) and Glu/GluNa buffer (pK_a 4.3) are the best buffers and ME contents are approximately as the same as the control.

Incorporating water to the methanolysis of soybean oil catalyzed by *C. rugosa, P. cepacia* and *P. fluorescens* could prevent the inactivation of these lipases by methanol [41]. Therefore, the effects of water added in the enzyme and water added in the reaction mixture on methanolysis of triolein catalyzed by iPCPL were carried out in this study. Surprisingly, both the adding water (5-20% v/w) to the enzyme and the reaction mixture could decrease the content of ME when comparing to the control (**Figures 3.17** and **3.18**). When water was added into iPCPL and the substrate, the reaction might be shifted to hydrolysis reaction as found in methanolysis of soybean oil catalyzed by water added *P. cepacia* [41].

The effect of organic solvents on methanolysis of triolein catalyzed by iPCPL at 37°C was evaluated. The results exhibited that methanolysis by iPCPL in the presence of non-polar organic solvents, i.e. hexane, diethyl ether, and dichloromethane provided the similar amount of ME as in the control. This is opposite to the lipases from *P. fluorescens, T. lanuginosa* and *R. miehei* which better catalyzed the methanolysis of sunflower oil in non-polar organic solvent media than the control [111]. For methanolysis in the presence of a less non-polar solvent, chloroform, a lower ME content was obtained (**Figure 3.19**). A possible explanation on the low methanolysis activity of iPCPL when the reaction mixture consisted of chloroform is that this solvent may act as both semi-polar and semi-non polar so it could dissolve some *i*-propanol and water that surround the enzyme molecules. As a result, the enzyme was then deactivated and lowered methanolysis when comparing to the control. Therefore, the methanolysis of triolein catalyzed by iPCPL should be performed without any adding organic solvents.

4.6 Biodiesel fuel synthesis from palm oil

The fatty acid compositions of palm oil as analyzed by GLC were: 9.52% linoleic acid, 0.99% myristic acid, 48.1% oleic acid, 40.8% palmitic acid and 0.57% stearic acid (**Table 3.34**). From this composition, an average molecular weight of 851.3 was calculated. When triolein was substituted with palm oil in methanolysis catalyzed by 0.50 g of iPCPL, the formation of ME was affected by the molar ratio of palm oil: methanol. ME yield increased with increasing oil:methanol molar ratio. At 48 h of reaction, the highest content of total ME (21%) was formed using palm oil: methanol molar ratio of 1:4 as found in case of methanolysis of triolein. Total ME from methanolysis of palm oil contained 11.4% methyl palmitate, 7.87% methyl oleate and 1.71% methyl linoleate.

In order to explain this result, the specificity of CPL and the composition of TAG in palm oil were then evaluated. Since CPL had specificity on 1- and 3-positions of TAG, the composition of palmitic, oleic and linoleic acids at 1- and 3-positions of TAG in palm oil were considered. TAG components of palm oil, i.e. LOP (24%), OOP (17%), LLP (8%), LOL (5%), and OOO (4%) [112] showed that 37% of linoleic acid and 21% of oleic acid were presented at 1-position, whereas 49% of palmitic acid, 4% of oleic acid, and 5% of linoleic acid presented at 3-position. If iPCPL had the same specificity on 1- and 3-positions, the content of methyl esters were obtained as following: methyl palmitate > methyl linoleate > methyl oleate. But iPCPL catalyzed the formation of methyl ester of palmitic acid > oleic acid > linoleic acid, which means this enzyme exhibited different specificities on 1- and 3-positions. The corresponding of the series of ME formation from methanolysis by iPCPL and the series of fatty acid presented at 3-position of TAG in palm oil indicated that iPCPL should have specificity on 3-position. This specificity of CPL has also been reported in hydrolysis of TAG [99] and in interesterification of BSP and MMM [81].

Accordingly, 83 mg of ME were produced from 1 g of palm oil at oil: methanol molar ratio of 1:4 catalyzed by 0.5 g of iPCPL and shaken at 37°C with 200 rpm for 48 h.

4.7 Conclusions

Lyophilized CPL, 5% (w/w) was obtained from the particulate of the fresh latex. It possessed lipase activity of 1,941±94 and 1,159±108 u/g of enzyme on hydrolysis of tributyrin and olive oil, respectively. Low ME content was produced by CPL catalyzed methanolysis of triolein. The iPCPL could provide 17.6% methyl ester higher than that of the catalysis by CPL. The optimum methanolysis of triolein catatyzed by iPCPL was performed the reaction at 37°C using molar ratio of triolein:methanol at 1:4 in the absence of organic solvent, solid buffer and added water. Methanolysis of palm oil using iPCPL at optimum condition of triolein produced biodiesel fuel of approximately 21% ME.