CHAPTER IV
RESULTS AND DISCUSSION

4.1 Microemulsion Studies for Esterification Reaction

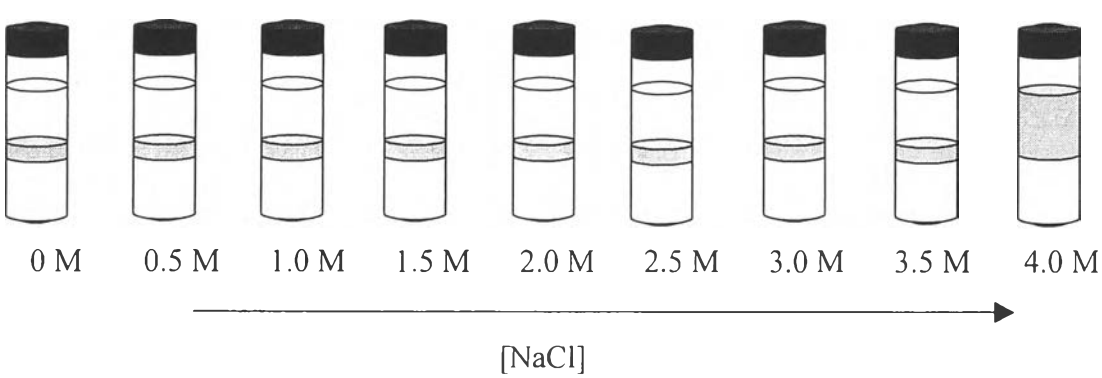
The NaDEHP/isooctane/water microemulsion system was prepared by mixing appropriate amount of hexanol as a cosurfactant in isooctane containing 100 mM HDEHP with 100 mM NaOH in the aqueous phosphate buffer (250 mM) with varying concentrations of NaCl from 0 to 4.0 M. NaDEHP was formed during the mixing of organic and brine phases. The reaction can be represented as:

$$\textbf{HDEHP} + \textbf{NaOH} \longrightarrow \textbf{NaDEHP} + \textbf{H}_2\textbf{O} \qquad (4.1)$$

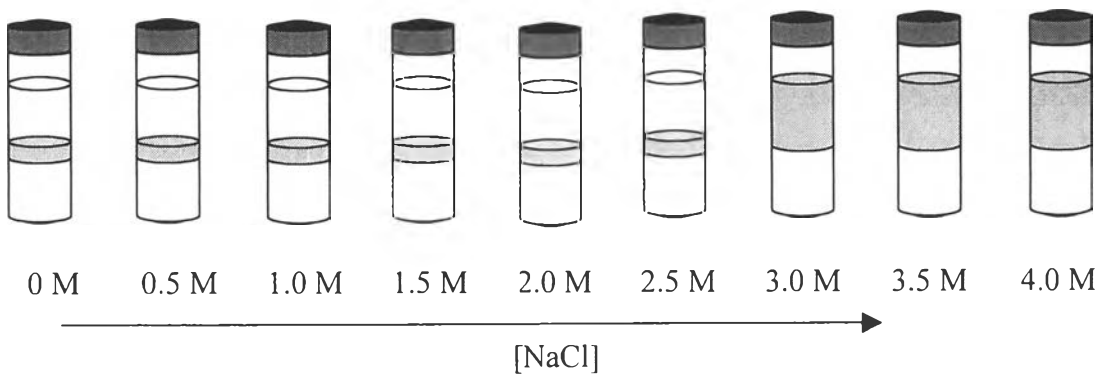
4.1.1 Phase Transition: Effect of Salt and Cosurfactant

The effects of salt and cosurfactant concentration on NaDEHP *W/O* microemulsion formation are shown in Figure 4.1. It was found that NaDEHP reverse micelles were not formed without adding NaCl or cosurfactant to the microemulsion system. Increasing NaCl and cosurfactant concentration was found to facilitate the formation of the reverse micelles. When NaCl concentration was further increased, the system changed from Winsor III microemulsion (bicontinuous structure) to Winsor II (water in oil) microemulsion. When hexanol as a cosurfactant was added, *W/O* microemulsion was formed at lower NaCl concentration.

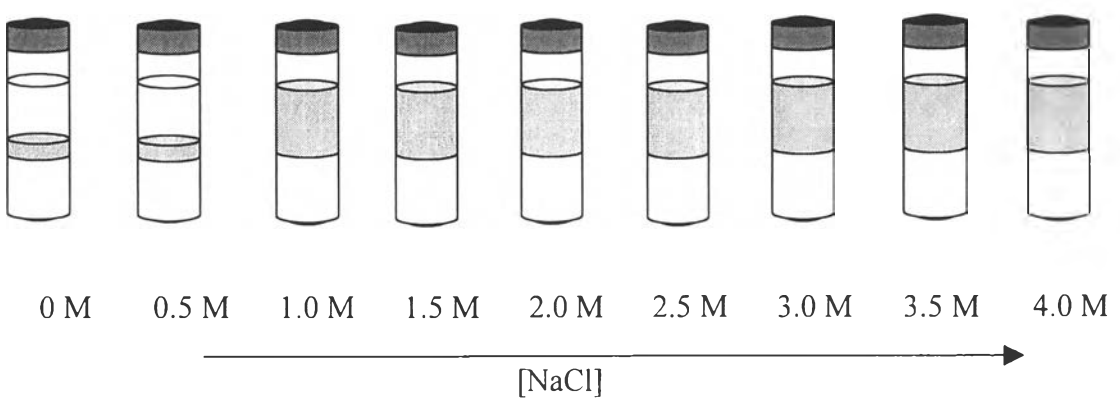
0 mM Hexanol as a cosurfactant



20 mM Hexanol as a cosurfactant



40 mM Hexanol as a cosurfactant



50 mM Hexanol as a cosurfactant

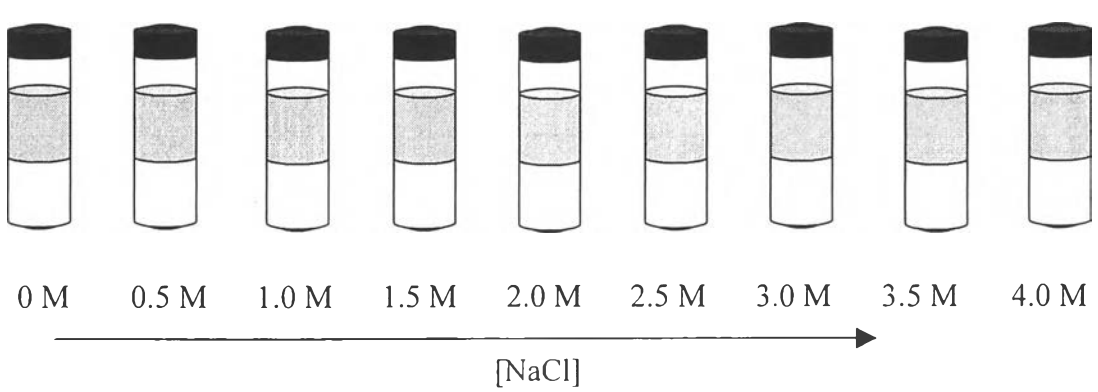


Figure 4.1 Phase behaviors of NaDEHP/isooctane/NaCl aqueous solution with hexanol as a cosurfactant at various concentrations (35°C and pH 7.4).

4.1.2 Water content and micellar size in NaDEHP *W/O* microemulsion

After reverse micelle was formed, the water content (expressed in terms of the water to surfactant molar ratio, W_0) and reverse micellar size (Rh) in *W/O* microemulsion (the upper phase) system of NaDEHP/isooctane were measured by coulometer and dynamic light scattering (DLS), respectively, at various NaCl and substrate concentrations (see Appendix A, Table A-1, A-2). Figure 4.2 presents the relationship between the water content and micellar size.

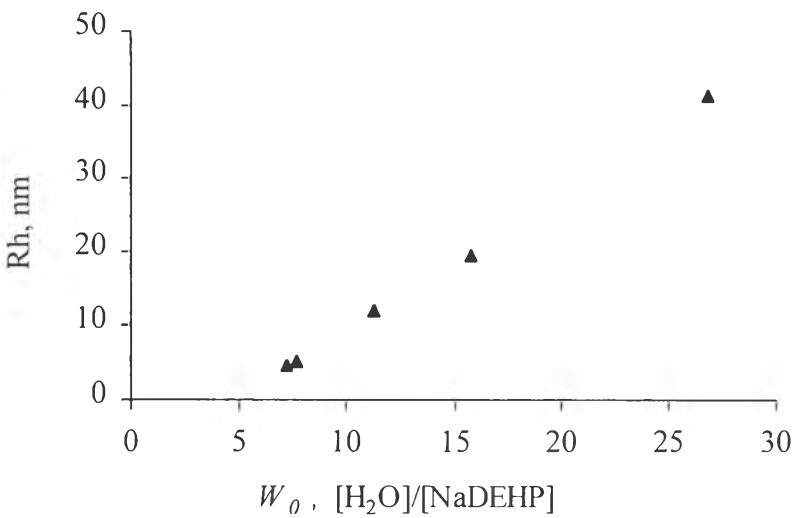


Figure 4.2 The relation between W_0 and the hydrodynamic radius, Rh for 100 mM NaDEHP/isooctane reverse micelles at 35^0C.

The results showed that the water/NaDEHP molar ratio (W_0), is directly proportional to the size (radius) of dispersed water droplets as also observed by Hayes and Gulari (1990). In this study the reverse micellar size (Rh) during the reaction was varied in the range of 4.6-19.5 nm (Appendix A).

4.1.3 Determination of Water content

4.1.3.1 *Effect of salt and cosurfactant concentration on water content*

The microemulsion systems of 100 mM NaDEHP/isooctane/ 20, 40, 50 mM hexanol as a cosurfactant prepared at various concentrations of NaCl from 0.5 to 4.0 M, were studied for water content and the results are presented in Figure 4.3.

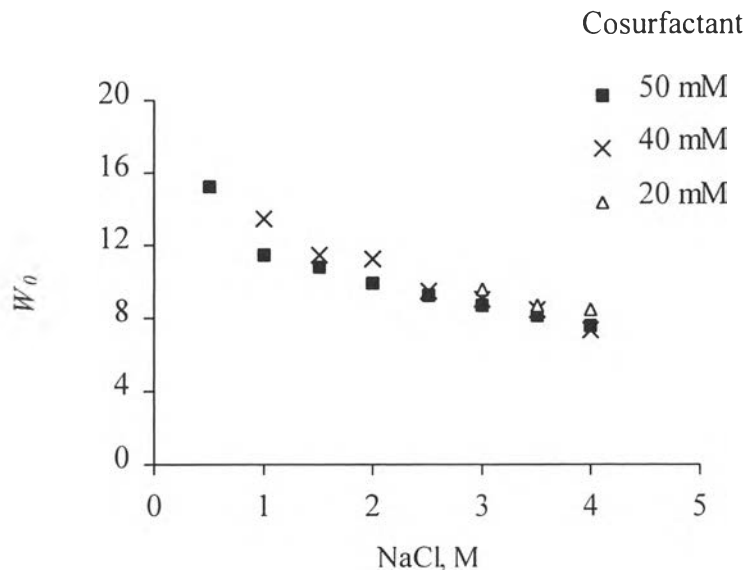


Figure 4.3 Effects of NaCl and hexanol as a cosurfactant on water content of 100 mM NaDEHP/isooctane/0.5-4.0 M NaCl (aq).

The results showed that the water to surfactant molar ratio (W_0) decreased as NaCl concentration increased. In the same way, varying concentration of cosurfactant from 20 to 50 mM resulted in decreasing water content (Figure 4.3). The decrease in water content might be due to the screening effect of NaCl, which decreases ionic repulsion between the surfactant head groups, leading to closer packing of hydrophilic head group of the surfactant molecules forming the reverse micelle. To compare the effects of salt and cosurfactant, the decrease in water content due to increasing the amount of NaCl was more pronounced than increasing the amount of cosurfactant. Varying salt and cosurfactant concentrations can be used to control a hydrodynamic radius (Figure 4.2) and amount of water which, in turn,has

significant effect the esterification catalyzed by lipase in reverse micelle (Anunukulprasert, 2001).

As salts can affect on the reaction rate by decreasing enzyme activity, low concentrations of salt in the range of 0.2-1.0 M were used to form reverse micelles for the subsequent esterification reaction in this study. Consequently, an appropriate amount of cosurfactant used was set at 50 mM (Figure 4.1).

Water content was also found to depend on the substrate concentration, especially fatty acid, as shown in Tables 4.1 and 4.2.

Table 4.1 Effects of caprylic acid and oleic acid on water content of *W/O* microemulsion formed by 100 mM NaDEHP/50 mM hexanol as a cosurfactant in isooctane/0.5 M NaCl (aq)

[fatty acid], mM with 75mM hexanol	W_0
no substrate	15.2
50 mM caprylic acid	9.1
50 mM oleic acid	8.3
75 mM oleic acid	7.6
150 mM oleic acid	4.6

Table 4.2 Effect of hexanol on water content of *W/O* microemulsion formed by 100 mM NaDEHP/50 mM hexanol as a cosurfactant in isooctane/0.75 M NaCl (aq)

hexanol], mM with 50 mM oleic acid	W_o
75 mM	7.4
105 mM	7.2
135 mM	7.0
165 mM	7.1
195 mM	7.0
225 mM	7.1

Upon adding substrates, the size of reverse micelle was smaller as the water to surfactant molar ratios (W_0) decreased at the same NaDEHP concentration. The results showed that oleic acid has much stronger effect on W_0 than caprylic acid. It might be due to the closer packing of surfactant head groups of the longer chain one (oleic acid). In contrast, when hexanol was increased from 75 mM to 225 mM, the effect on water content was not as significant as observed in Figure 4.3 and Table 4.1.

mM to 225 mM, the effect on water content was not as significant as observed in Figure 4.3 and Table 4.1.

All the results shown above were obtained from the system without the presence of lipase. Table 4.3 presents the water content of *W/O* microemulsion system before and after the addition of lipase. The results showed that in the presence of lipase in the reverse micelle, the water content increased and so did the reverse micellar size (Rh). These results confirmed that lipase added to the aqueous solution (lower phase) was transferred and encapsulated or hosted in the reverse micelle in the upper phase. When lipase was transferred from the aqueous phase to the reverse micelle, they brought some solubilized water molecules along so the increase in the micellar size was observed.

Table 4.3 Water content (W_0) of *W/O* microemulsion formed by 100 mM NaDEHP/50 mM hexanol as a cosurfactant in isooctane/0.5 M NaCl (aq) before and after injecting lipase

Substrate	W_0	
	Without lipase	With lipase
No substrate	15.8	16.5
50 mM caprylic acid/ 75 mM hexanol	10.7	11.1
50 mM oleic acid/ 75 mM hexanol	7.7	7.8
75 mM oleic acid/ 100 mM hexanol	7.2	7.5

The results obtained in this part of study clearly showed that the amount of water which controls a rate of esterification catalyzed by encapsulated enzyme in the reverse micelle can be modified by changing the system parameters such as NaCl concentration, type and concentration of fatty acid, and cosurfactant concentration.

4.2 Stability of Thai Rice Bran Lipase

4.2.1 Storage Stability of the Extract Lipase at 4°C

After lipase was extracted with phosphate buffer (Figure 3.1), the extract obtained was stored in refrigerator. The activity of the lipase in the extract solution (aqueous buffer) was determined as a function of storage time using esterification reaction of 75 mM hexanol with 50 mM oleic acid in the microemulsion system containing 100 mM NaDEHP/50 mM cosurfactant in isooctane/0.5 M NaCl, and lipase concentration of 0.025 mg/ml. It was found that rice bran lipase activity remained almost 100% for approximately 24 h and thereafter a sharp decrease in the activity was observed. Until the fourth day, the enzyme activity was found to be less than 10% of the initial activity as seen in Figure 4.4.

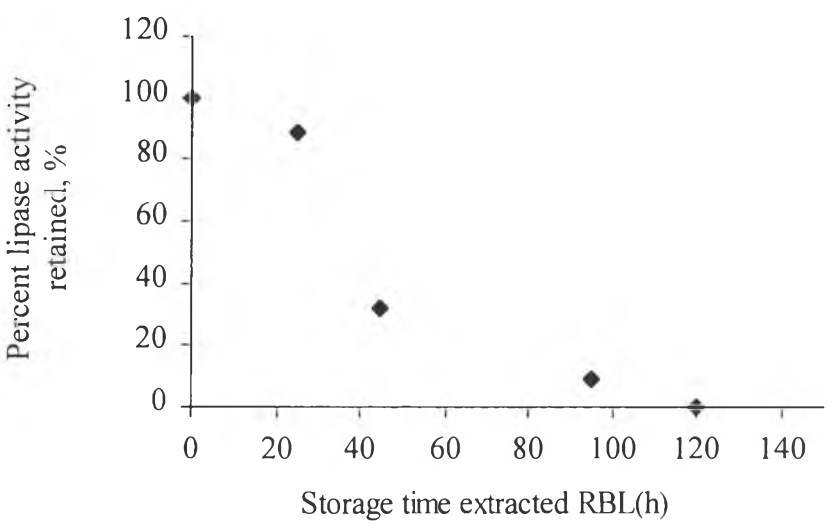


Figure 4.4 Effect of storage time on the activity of the extracted rice bran lipase kept at 4°C assayed in reverse micelle.

Therefore, the esterification reactions catalyzed by Thai rice bran lipase were always carried out using freshly extracted enzyme within 1 day after the extraction. Furthermore, the activity and stability of extracted lipase have been shown to be very sensitive to the extraction conditions. To avoid any variation that might occur, the

extraction was carefully conducted at fixed conditions and lipase obtained from the same batch was used for the same set of experiments.

4.2.2 Stability of Lipase Encapsulated in Reverse Micelle

After the enzymes were transferred from the aqueous phase of microemulsion into the reverse micellar in the upper phase, the stability of lipase encapsulated in reverse micelles was examined as a function of time. As shown in Figure 4.5, the deactivation of lipase from Thai rice bran was observed after 10 min of incubation and there was no activity remained after 5 h. At time ~ 0, low activity was observed which is expected to be due to the fact that most of the enzymes were still in aqueous phase, it took a few minutes for them to be transferred to the upper phase where the reaction occurred.

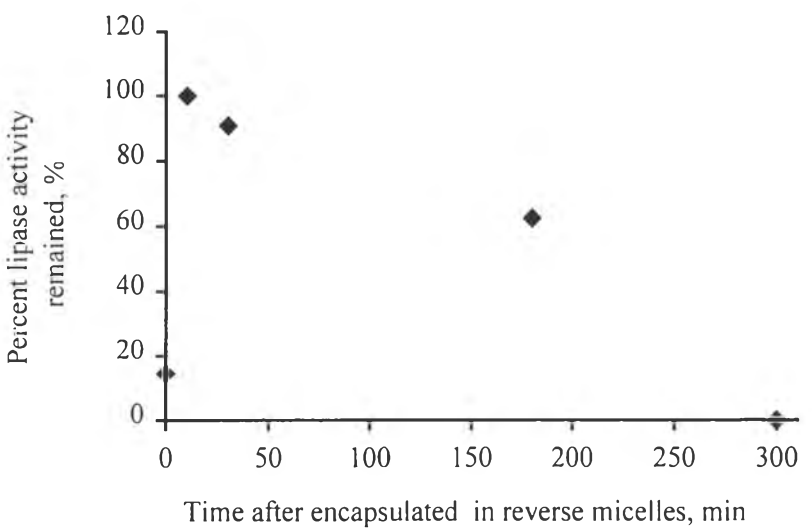


Figure 4.5 Effect of incubation time of lipase in reverse micelles on lipase activity in the esterification of 75 mM hexanol with 50 mM oleic acid in 100 mM NaDEHP/50 mM cosurfactant in isooctane/0.5 M NaCl microemulsion; lipase concentration 0.010mg/ml, W_0=8.3, pH 7.4, T=35°C.

4.2.3 Effect of Temperature on Stability of Lipase

In this section, rice bran lipase was kept at three different temperatures (4, 25, 35 °C) in the aqueous solution and in the reverse micelle for comparison. The

activity of lipase was assayed in reverse micelle at the selected time; reaction, reaction conditions, and substrate concentrations were the same as in Figure 4.5.

From the results shown in Figure 4.6, it was clearly seen that enzyme activity decreased more rapidly at higher temperature. The medium in which was stored; aqueous or reverse micellar media had shown to have little effect on the activity of lipase, although lipase encapsulated in reverse micelle showed slightly higher activity than that kept in the aqueous solution at higher temperature.

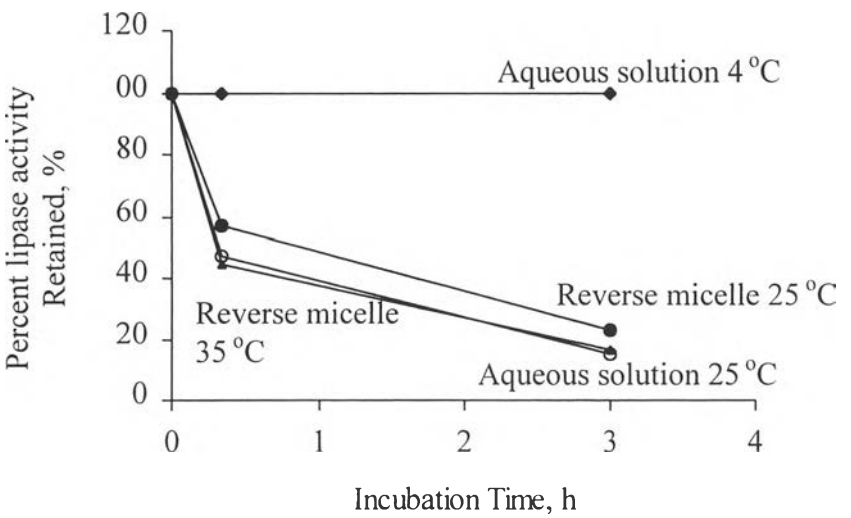


Figure 4.6 The effect of temperature on the stability of lipase; (◆,○) the stability of lipase in aqueous solution assayed in reverse micelle, (●,▲) the stability in reverse micelle.

4.3 Lipase-Catalyzed Esterification

The enzymatic activity of Thai rice bran lipase encapsulated in NaDEHP reverse micelles was determined from the initial rate of the esterification reaction. The reverse micellar system of 100 mM NaDEHP/50 mM cosurfactant/NaCl in isooctane was used in this part of the study. The substrates used in this study were hexanol and two fatty acids; caprylic and oleic acids. Salt concentration was varied to control the water content of the system. The effect of type and concentration of

substrates on enzyme activity were also investigated. All reactions were conducted at 35°C and pH 7.4.

4.3.1 Effect of W_0

Figure 4.7 presents the effect of the water content (expressed as W_0) on the activity of lipase in esterification reaction using both types of the substrates. W_0 was adjusted by varying salt concentration.

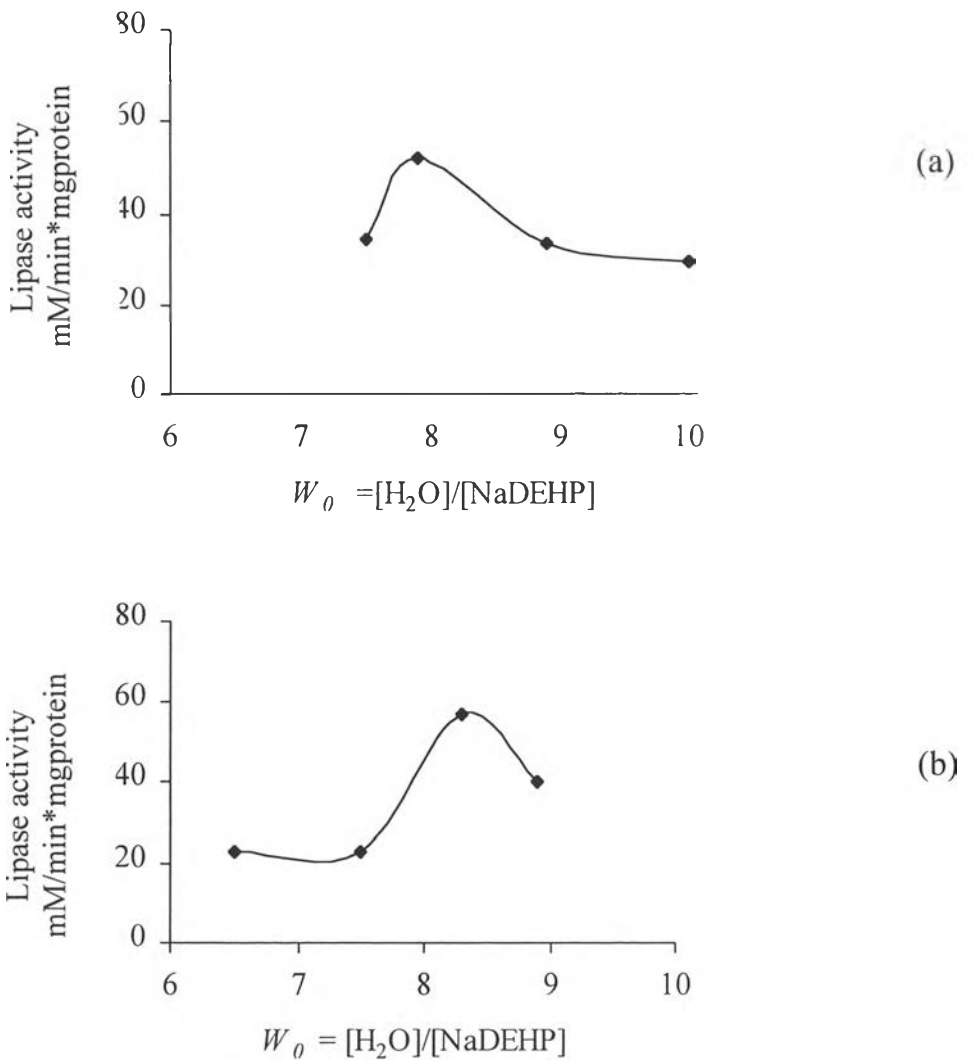


Figure 4.7 The effect of W_0 on the activity of lipase; 0.025 mg/ml in the esterification of 75 mM hexanol with (a) 50 mM caprylic acid; (b) 50 mM oleic acid.

The results showed that lipase activity followed a rather sharp bell-shaped profile as reported in other studies using different micellar-encapsulated enzymes (Hayes and Gulari, 1990; Stamatis *et al.*, 1994). The maximum activity was observed at $W_0 = 8$ for both reactions, above this value a slight decrease of enzyme activity was observed. This optimum value appears to be specific for particular type of lipase and microemulsion system. For this study, at $W_0 = 8$, rice bran lipase in NaDEHP reverse micellar system might be located in close contact with lipophilic substrates, thus resulting in a maximum activity.

4.3.2 Effect of Fatty Acid Chain Length

The role of substrate chain length in esterification reaction catalyzed by rice bran lipase was investigated using caprylic acid (C8) and oleic acid (C18), representing a short chain and long chain substrate, respectively. The reactions were carried out at the same water content of reverse micelle. Figure 4.8 demonstrates high activity of the rice bran lipase for esterification of hexanol with long chain fatty acid, indicating that rice bran lipase has a preference for the long chain fatty acid. This selectivity might be related to the localization of the lipase molecules within the reverse micellar microstructure due to the hydrophobic/hydrophilic characters of the enzyme and the availability of substrate.

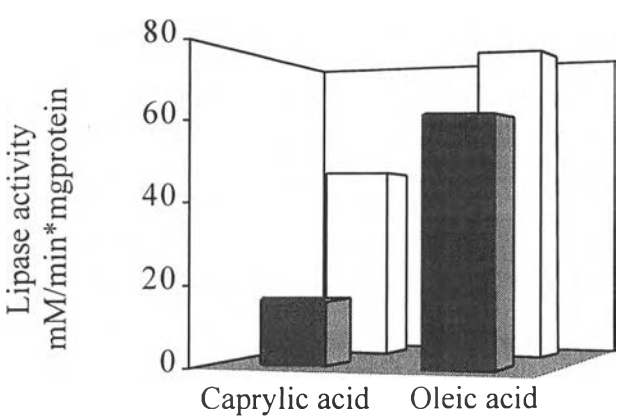


Figure 4.8 Effect of fatty acid chain length on the activity of lipase in the esterification of 75 mM hexanol with 25 mM fatty acid; enzyme concentration 0.010 mg/ml (■), 50 mM fatty acid; enzyme concentration 0.017 mg/ml (□).

4.3.3 Effect of Fatty Acid Concentration

The effect of fatty acid concentration was studied by using different concentrations of oleic acid and caprylic acid, ranging from 15 to 65 mM, and hexanol at a fixed concentration (75 mM). Since fatty acid can also act as a cosurfactant, increasing fatty acid concetration causes a decrease in micellar size which is proportional to the value of W_0 as shown in Table 4.4.

Table 4.4 Effect of fatty acid concentration on water content

[oleic acid], mM	W_0	[Caprylic acid], mM	W_0
15	9.37	20	9.15
30	8.40	35	7.67
45	7.90	50	7.40
60	6.90	65	6.73

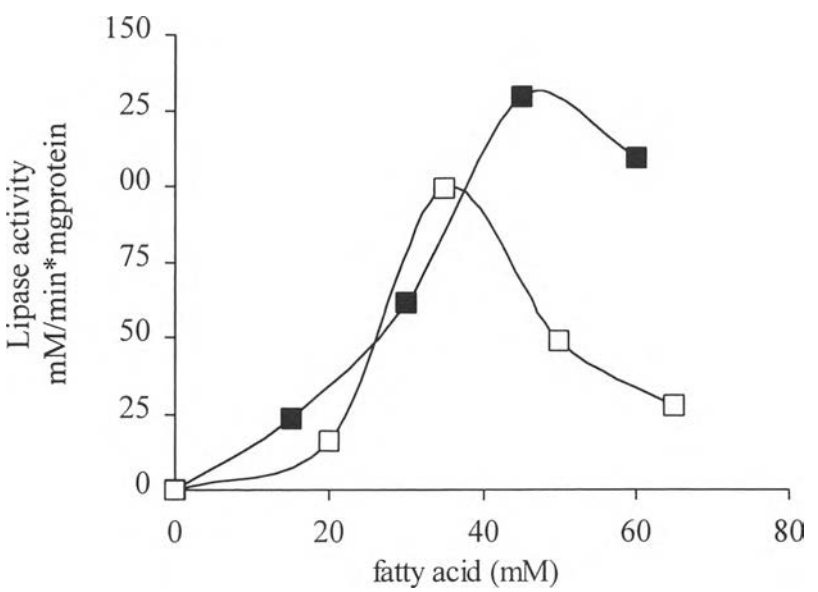


Figure 4.9 Effect of fatty acid concentration on the activity of lipase in the esterification of 75 mM hexanol with 15-60 mM oleic acid; W_0 9.37-6.9, enzyme concentration 0.010 mg/ml, (□) and 20-65 mM caprylic acid; W_0 9.15-6.73, enzyme concentration 0.017 mg/ml (■).

Figure 4.9 shows the enzymatic activity of lipase for the esterification reaction as a function of fatty acid concentration. It can be seen that, when the concentration of fatty acid increased, the lipase activity increased up to a maximum value. However, the decrease of enzyme activity was observed when fatty acid concentration was further increased. Moreover, it was found that the maximum lipase activity was obtained when W_0 was about 8 (Table 4.5), which agreed well with the results observed in the previous section. This might be due to the combined effect of fatty acid concentration and the change in W_0. The presence of high amount of fatty acid within micellar interface could start to cause an inhibitory effect. Furthermore, it could also cause a decrease in a micellar size which leads to a lower water content in the system (W_0), resulting in a decrease in the enzyme activity.

4.3.4 Effect of Hexanol Concentration

As hexanol concentration was increased from 20 to 120 mM in the esterification of oleic acid at a constant fatty acid concentration (40 mM), a decrease in the water content (W_0) of the system was not significant (Apendix D4).

Figure 4.10 shows the lipase activity of the esterification reaction as a function of hexanol concentration. The results showed that when the concentration of hexanol increased, the activity of lipase increased up to an alcohol concentration of 75 mM. At higher hexanol concentrations, the lipase activity was quite constant. The results showed that lipase activity follows a hyperbolic shape as reported by Stamatis *et.al* (1993) for the activity of *P. simplicissimum* lipase encapsulated in AOT reverse micelle.

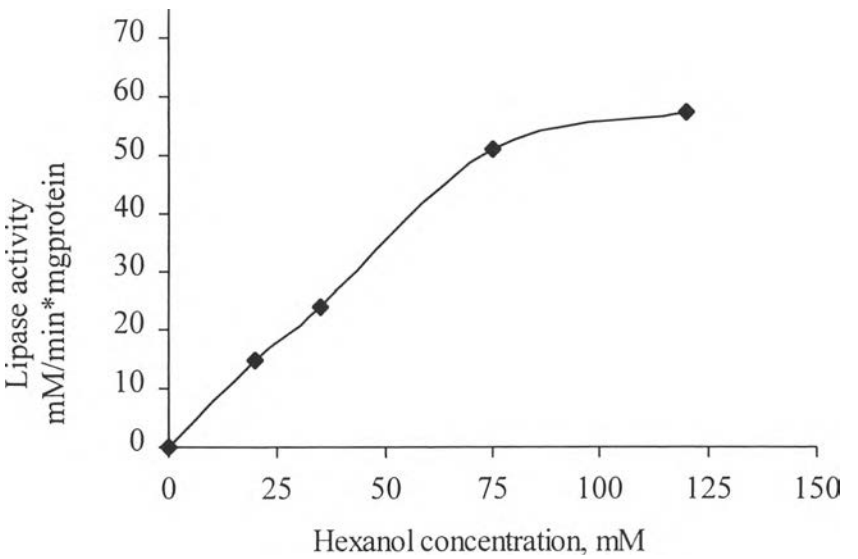


Figure 4.10 Effect of hexanol concentration on the activity of lipase in the esterification of 40 mM fatty acid with 20-120 mM hexanol; W_0 7.80-7.29, enzyme concentration 0.020 mg/ml.

4.3.5 Water Content after Reaction

According to the esterification reaction shown in Scheme 2.1, water is one of the products formed during the reaction. As the result, the water content in the reverse micellar system after the reaction should increase. Table 4.5 shows the water contents before and after reaction at the various NaCl concentrations. All the results showed an increase in the water content after the reaction. Moreover, it was found that the amount of water produced (observed as a difference of the water content before and after the reaction) depended on the reaction conversion. The higher conversion, the higher amount of water produced.

Table 4.5 Water content (W_0) before and after reaction in the esterification of 50 mM oleic acid with 75 mM hexanol

[NaCl], M	W_0 = [H2O]/ [NaDEHP]		% conversion
	Before reaction	After reaction	
0.2	8.9	9.6	87.63
0.5	8.3	8.9	81.45
0.8	7.5	7.9	77.63
1.0	6.5	7.3	77.09

In this study, it was found that the activity of rice bran lipase that defined as the rate of esterification of fatty acids with hexanol in NaDEHP reverse micelle strongly depended on the reverse micellar structure, water content, type and concentration of substrate. The results obtained in this study agreed well with those reported in other studies using similar reverse micellar system (Hayes and Gulari, 1990; Stamatis *et al.*, 1993). Thai rice bran lipase has a preference for long chain fatty acid. This selectivity appears to be related to localization of enzyme molecule in micellar microstructure, due to the hydrophobic/hydrophilic character of protein as well as the optimum water content that is specific for particular type of enzymes. Rice bran lipase showed a strong dependence on the water content in microemulsion and the optimum was about 8 while Anukulprasert (2001) reported $W_0 \sim 6$ for *R. delemar* lipase in the same NaDEHP reverse micellar system.

Moreover the lipase activity as represented by the rate of esterification was studied and the reaction conversion was also determined. The conversion was shown to vary from 27 to 88% but the conversion was not clearly depenence on the system parameters, i.e. salt, type and substrate concentration. The highest conversion obtained was 87.63 % from the esterification of 50 mM oleic acid with 75 mM hexanol (Table D1-1). All reaction conditions employed in this study yielded high conversion because the rice bran used was always fresh and the extraction conditions were well controlled.

4.4 Chemical Analyses

4.4.1 Determination of Fatty Acid Concentration by Spectrophotometric Method

Fatty acid concentration was determined using the spectrophotometric assay developed by Lowry and Tinsley (1976) as follows: 0.1 ml of organic solution (upper phase) was added to screw-cap vial containing 4.9 ml of isooctane and 1 ml of cupric acetate-pyridine (5% w/v, pH 6.0). After centrifugation at 3000 rpm for 1 min, free fatty acid was determined in the upper organic phase. The absorbance was measured at 715 nm using UV-VIS spectrophotomete. Investigation of other components in the reverse micelle (upper phase) and the aqueous phase was also verified by UV-VIS spectrophotometer. It was found that the absorbance of phosphate buffer, hexanol, and lipase did not interfere with the absorbance of fatty acid except NaDEHP. Thus, in order to calculate the actual rate of reaction, the absorbance of microemulsion without fatty acid was used as a background to subtract from the absorbance measured during the reaction.

4.4.2 Identification of Ester Product by FT-IR

The samples from upper phase of microemulsion were taken before and after the reaction to identify the production of ester by FT-IR. The absorbance peaks of carbonyl of ester band was at 1740 cm^{-1} and fatty acid band at 1700 cm^{-1} (Appendix E). In the esterification of 50 mM caprylic acid and 50, 100 mM oleic acid with hexanol (Figures 4.11-4.12), the peaks of ester after the reaction did not appear. It might be due to the concentration of ester product from the esterification was too low to be observed. The intensity of fatty acid peak was obviously decreased in all experiments. In Figures 4.13-4.14, higher substrate concentration and the optimum conditions (i.e., low salt concentration, $W_0 \sim 8$) were used to get a higher amount of ester product. The IR spectra observed from the esterification clearly showed an ester peak at 1740 cm^{-1}.

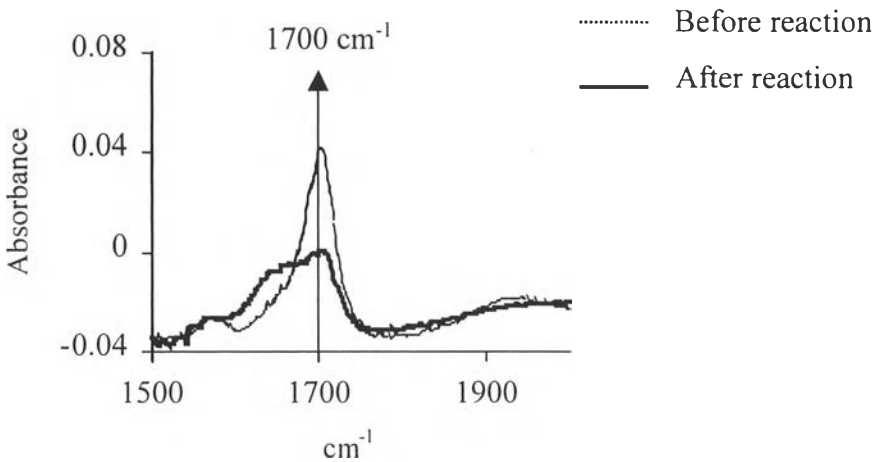


Figure 4.11 Spectra of before and after esterification of 50 mM caprylic acid with 75 mM hexanol in microemulsion system of 100 mM NDEHP/isooctane/50 mM cosurfactant/0.5 M NaCl.

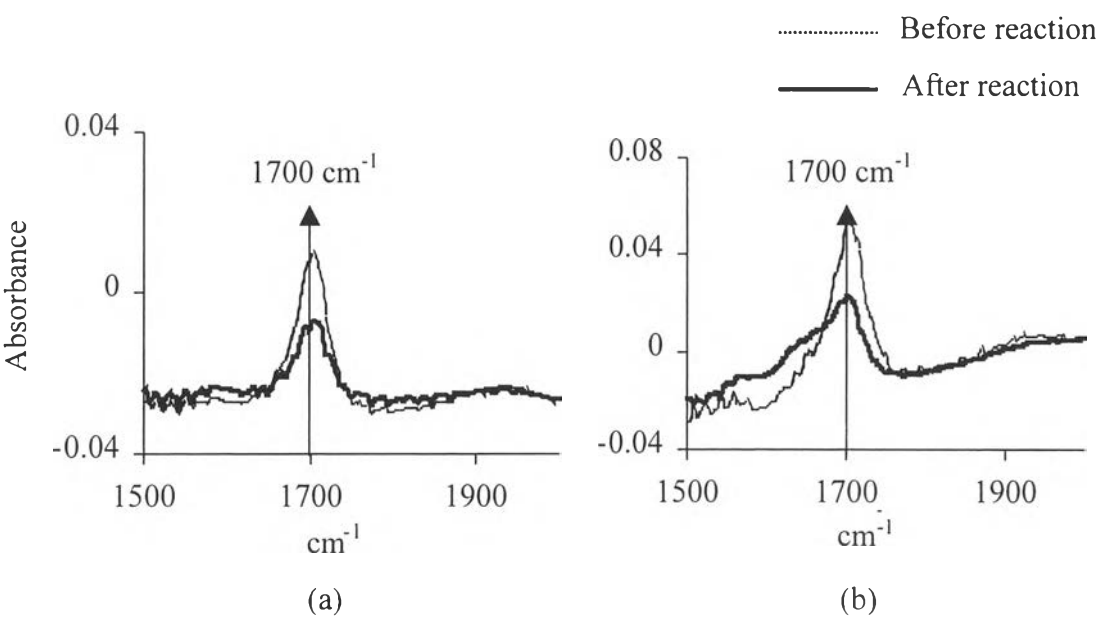


Figure 4.12 Spectra of before and after esterification of oleic acid with 75 mM hexanol in microemulsion system of 100 mM NDEHP/isooctane/ 50 mM cosurfactant/0.5 M NaCl: oleic acid (a) 50 mM and (b) 100 mM.

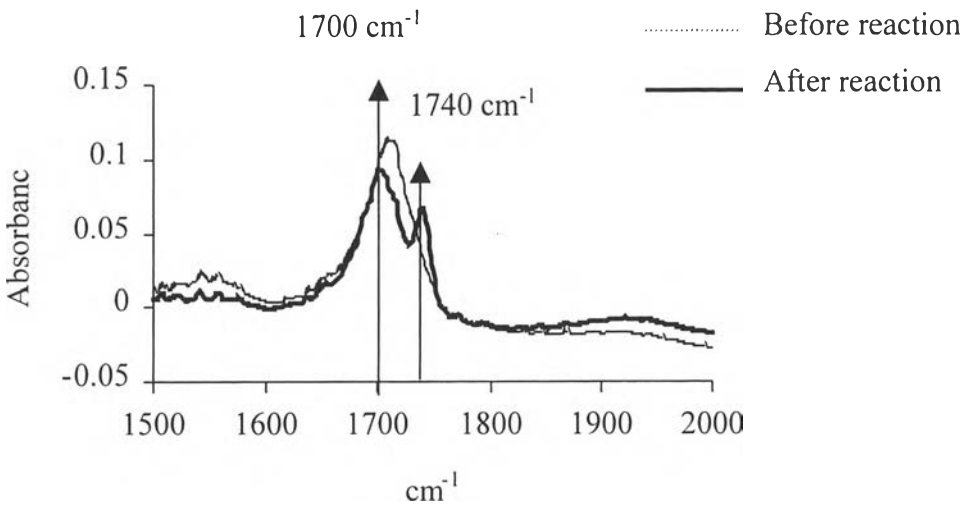


Figure 4.13 Spectra of before and after esterification of 150 mM oleic acid with 100 mM hexanol in microemulsion system of 150 mM NDEHP/isooctane/ 50 mM cosurfactant/0.25 M NaCl.

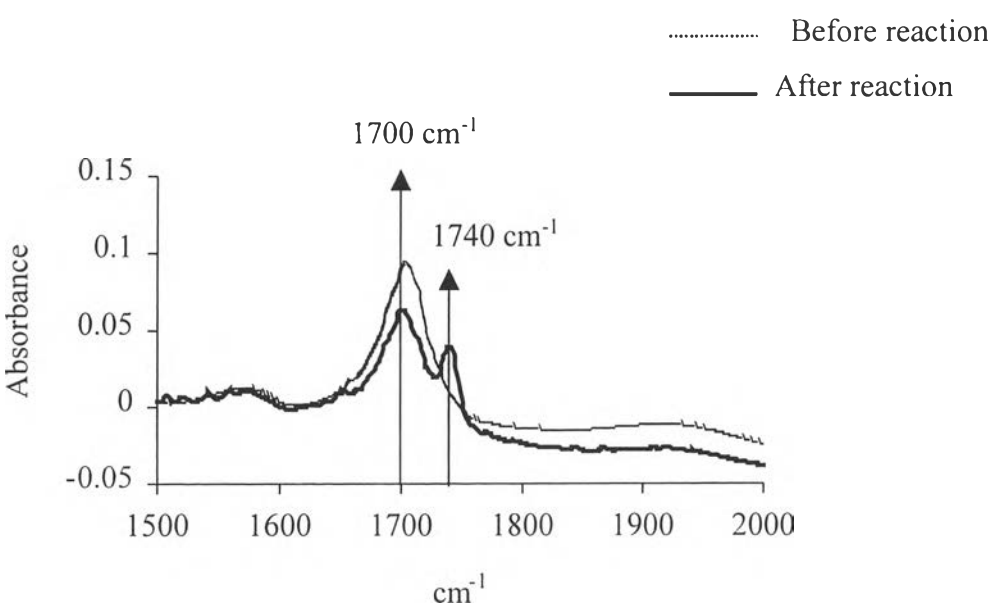


Figure 4.14 Spectra of before and after esterification of 200 mM oleic acid with 150 mM hexanol in microemulsion system of 200 mM NDEHP/isooctane/ 50 mM cosurfactant/0.25 M NaCl.