CHAPTER II
BACKGROUND AND LITERATURE SURVEY

2.1 The Function of Lipase as an Enzyme

2.1.1 Enzyme

Enzymes are the tools of biocatalysis, controlling biochemical reaction at the molecular level. They are divided into six classes according to the reaction they catalyze: 1) oxido-reductases, 2) transferases, 3) hydrolases, 4) lyases, 5) isomerases, and 6) ligases. Hydrolases or hydrolytic enzymes (lipase, protease, and nuclease) are the most commonly used enzymes in organic chemistry.

2.1.2 Lipase

Among several types of enzymes, lipase which hydrolyzes ester group of lipids, is one of the most advantage because it is stable, inexpensive and has high potential for biochemical applications in fat and oil, pharmaceuticals and other industries. Lipases can be obtained from plant, animal, and microbial sources.

2.2 Rice Bran Lipase (RBL)

RBL is obtained from plant source, rice bran, which is an important and abundant by-product gained during milling of the rice. Bran is known to be rich in a number of important components such as edible oil, vitamins, sugars, and proteins. An important protein present in the rice bran is lipase (Prabhu *et al.*, 1999).

There are many studies on rice bran lipase that deal with the extraction from rice bran, and the elucidation of its characteristics and biochemical properties. In 1993, Munshi and his group extracted lipase from rice bran by using 0.1M potassium phosphate buffer (pH 7) and purified the enzyme by ammonium sulphate fractionation followed by DEAE-cellulose ion-exchange chromatography. Then in 1995, Rajeshwara and Prakash found that high enzyme activity and quantity could be obtained by defatting the rice bran before the extraction step. They also characterized their extracted and purified RBL and found that the enzyme has an

ultraviolet absorption maximum at 276 nm with extinction coefficient value of 15.25, a pH optimum is 7.5 and a temperature optimum is 30°C, and the molecular weight of enzyme is 30000. The stability of RBL has been observed by Prabhu *et al.* (1999) who stated that RBL extraction should be carried out and stored at low temperature around 10°C in order to keep their characteristics. They found that for extractions carried out at 10°C, 100% activity of RBL can be retained for 24 h and also discovered that a 50% glycerol addition enabled the enhancement of the half-life of the lipase at 30°C from less than 1 day to 3 days.
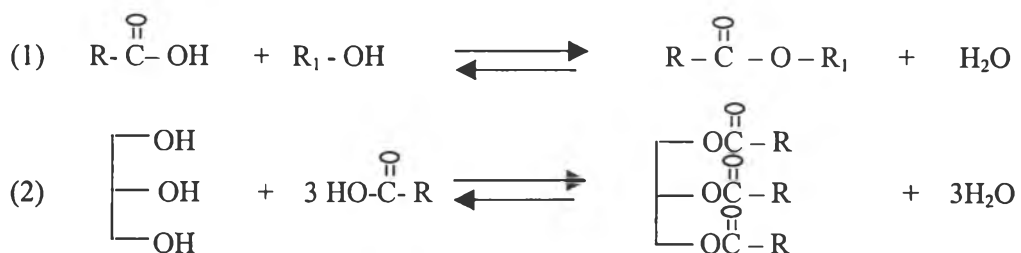
Thai rice bran lipase has also been studied, Sirisaneeyakul and his co-worker (1997) extracted RBL from Thai rice bran by using 0.01M $CaCl_2$, and purified by ammonium sulphate precipitation followed by dialysis with 0.01M $CaCl_2$. They reported the optimum conditions for Thai scented rice bran lipase are pH~7.4 and T~35°C. Phraephrewngarm (1999) investigated activity of Thai rice bran in esterification and polyesterification. RBL was extracted from Thai rice bran at the condition of 1:3 W/V of rice bran to 10 mM calcium chloride in the stirrer system for 3 hours at 4°C, the obtained solid RBL shows specific activity about 70 mU/mg. Then, it was concluded that the activity of enzyme is significantly decreased due to the enzyme denatures during solidification.

2.3 Lipase-Catalyzed Reaction

The lipase-catalytic reaction normally occurs at water/oil interfaces which are necessary for lipases to display their activity due to the existence of hydrophobic lid in their structure, which covers the catalytic site (Carlile *et al.*, 1996). These enzymes can be used in reactions involving a wide variety of fatty acids, alcohols or esters. The most well known use of lipase-catalyzed reaction lies in esterification, hydrolysis, interesterification, and transesterification.

2.3.1 Lipase-Catalyzed Esterification and Hydrolysis

The lipase reaction is freely reversible to have synthesis and hydrolysis occurring together, Scheme 2.1 shows two examples of esterification with lipase.

(1) $R-\overset{\overset{O}{\|}}{C}-OH \;+\; R_1-OH \;\rightleftharpoons\; R-\overset{\overset{O}{\|}}{C}-O-R_1 \;+\; H_2O$

(2) $\left[\begin{array}{l} -OH \\ -OH \\ -OH \end{array}\right. \;+\; 3\,HO\text{-}\overset{\overset{O}{\|}}{C}\text{-}R \;\rightleftharpoons\; \left[\begin{array}{l} -O\overset{\overset{O}{\|}}{C}-R \\ -O\overset{\overset{O}{\|}}{C}-R \\ -O\overset{\overset{O}{\|}}{C}-R \end{array}\right. \;+\; 3H_2O$
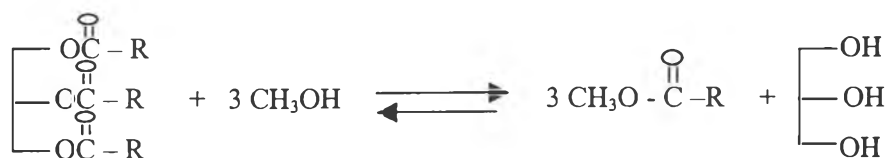
Scheme 2.1 (1) Fatty-acid-alcohol esterification, (2) Glyceride synthesis.

Lipase-catalyzed esterification occurs in good yield in very low water content system such as microemulsion. Hayes and Gulari (1990) showed that the products from glyceride synthesis in reverse micelles are mono and di-glycerides, no production of triglyceride. It is believed that triglyceride does not form in microemulsion because the intermediate di-glyceride is too lipophilic. Once formed, it rapidly partitions into continuous hydrocarbon domain leaving behind the enzyme (Holmberg *et al.*, 1994).

The formation of water presents a problem in the reaction as it favors the reverse (hydrolysis) reaction. To facilitate the reversal of the hydrolytic action of lipase, several approaches have been explored including the use of immobilized enzyme on support, anhydrous organic solvents, supercritical gases and reverse micelle (Stamatis *et al.*, 1993)

2.3.2 Lipase-Catalyzed Transesterification

Lipase-catalyzed transesterification, the exchange of carboxyl group between ester (i.e., replacement of one acyl group in triglyceride), is a commercial reaction. The production of biodiesel or methyl ester is a well-known example, as shown in Scheme 2.2.

$\left[\begin{array}{l} -O\overset{\overset{O}{\|}}{C}-R \\ -C\overset{\overset{O}{\|}}{C}-R \\ -O\overset{\overset{O}{\|}}{C}-R \end{array}\right. \;+\; 3\,CH_3OH \;\rightleftharpoons\; 3\,CH_3O\text{-}\overset{\overset{O}{\|}}{C}-R \;+\; \left[\begin{array}{l} -OH \\ -OH \\ -OH \end{array}\right.$
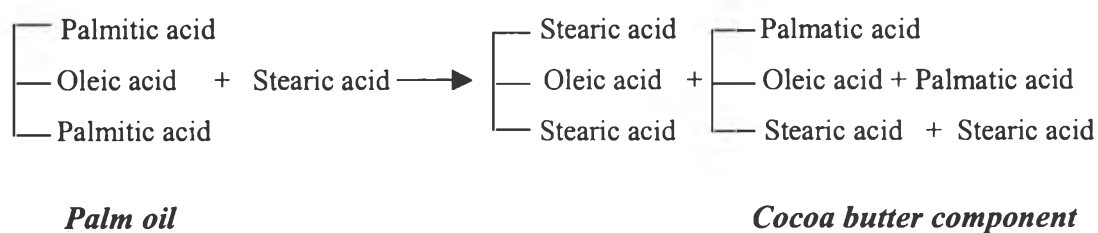
Scheme 2.2 The production of biodiesel or methyl ester by transesterification.

The majority of the methyl ester produced today is done with lipase-catalyzed tranesterification because it is the most economic for several reasons such as low temperature and pressure system, high conversion and direct conversion to methyl ester with no intermediate steps.

2.3.3 Lipase-Catalyzed Interesterification

The lipase-catalyzed reaction allows production of various triacylglycerides by a process called interesterification. Enzymes act as acidolyases (carboxyl group exchange between esters and alcohols). Cocoa butter production, a commercial process to produce high value triglycerides from more plentiful and cheaper raw materials, is the outstanding example as shown in Scheme 2.3.

$$
\begin{bmatrix}
\text{Palmitic acid} \\
\text{Oleic acid} \\
\text{Palmitic acid}
\end{bmatrix}
+ \text{Stearic acid} \longrightarrow
\begin{bmatrix}
\text{Stearic acid} \\
\text{Oleic acid} \\
\text{Stearic acid}
\end{bmatrix}
+
\begin{bmatrix}
\text{Palmatic acid} \\
\text{Oleic acid} + \text{Palmatic acid} \\
\text{Stearic acid} + \text{Stearic acid}
\end{bmatrix}
$$

Palm oil   *Cocoa butter component*

Scheme 2.3 Conversion of palm oil into cocoa butter fat.

2.4. Encapsulated Enzyme Using Microemulsion

Microemulsion is a thermodynamically stable dispersion of one liquid phase into another (i.e., water & oil), stabilized by an interfacial film of surfactant. During the past decade there have been considerable properties being reported, namely ultralow interfacial tension, large surface area, spontaneous formation, and solubilization capacity for both water- and oil-soluble compound. These properties have been shown to provide suitable microenvironment for hosting enzymes.

2.4.1 Formation of Microemulsion

Microemulsion is generally prepared with more than one surfactant or with mixture of a surfactant and a co-surfactant. Hydrophilic and lypophilic properties of

combination have to be balanced in order to reduce the oil-water interfacial tension to the value that is low enough to be compensated by entropy of dispersion (Solans *et al.*, 1997).

2.4.2 Type of Microemulsion

There are three types of microemulsion discreted by the solubilization capacity for water relative to oil: 1) *O/W* microemulsion (oil droplets dispersed in water), 2) *W/O* microemulsion or reverse micelle (water dispersed in oil), and 3) bicontinuous structure. Changing the temperature of the system, and adding a co-surfactant or electrolyte can modify these types.
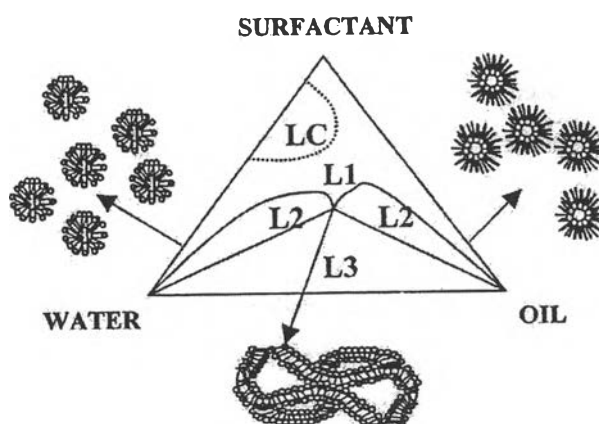
Figure 2.1 Schematic of ternary phase diagram for typical water/nonioic surfactant/oil system at HBL temperature. Microemulsion structure is shown in the normal regions of occurrence; left to right: *O/W* globular microemulsions (L1), bicontinuos microemulsion (L3), and *W/O* microemulsion (L2).

2.4.3 Encapsulated Enzyme in Reverse Micelle

Reverse micelles are fine dispersions of water in an organic medium stabilized by amphiphilic molecules. The enzyme can be entrapped in a water-pool and sheltered from solvent detrimental effects, retaining their catalytic activity.

Reverse micellar media offers the advantage of high interfacial area, greater solubility of nonpolar substrates, ensconcement of enzyme in a water-pool, and shifting thermodynamic equilibria in favor of condensation. But lipase is reported to lose activity when incubated in this media, however the presence of substrates considerably improves its stability (Stamatis *et al.*, 1993). Moreover the stability of enzyme in reverse micelle is also related to enzyme source (Patel *et al.*, 1995).

2.5 Lipase-Catalyzed Esterification in Reverse Micelle

In the past decade, there had been many investigations on lipase-catalyzed esterification in reverse micelle. Hayes and Gulari (1990) observed the activity of lipase from *Canadida cylindracea* and *Rhizopus delemar* in water/AOT (sodium bis(2-ethyhexyl)sulfosuccinate)/iso-octane reverse micellar media. They demonstrated that the structure of the media or diameter of micelle (Rh), as dictated by the type and concentration of the substrates and products and by the water/AOT ratio (W_0) has a strong effect on enzyme activity. The activity of enzyme also depends on pH and temperature of the system. In addition, there were many studies that concentrated on the effect of water content (W_0) on lipase activity. It was commonly found that the rate of reaction as a function of W_0 gives rise to a bell-shape profile with a maximum in rate at W_0 5-10. The role of enzyme localization was also studied; lipases show a remarkable selectivity regarding the chain length and the structure of the substrates. This selectivity appears to be related to the micellar microstructure due to hydrophobic/hydrophilic character of protein (Stamatis *et al.*, 1993). Moreover, Stamatis and his group (1995) investigated the lipase kinetic behavior in fatty acid-alcohol esterification and suggested that the kinetic of this synthesis follows a Ping-Pong Bi-Bi mechanism.

Although lipase-catalyzed reaction in organic media (e.g., isooctane) has been an area of major research activity, supercritical fluid and liquefied gas have also been explored. The esterification by lipase has been examined in reverse micelles of anionic, AOT, (Sebastião *et al.*, 1993), cationic, CTAB, (Chaudhuri *et al.*, 1999) and nonionic, Marlipal 013-60, (Orlich *et al.*, 2001) surfactants. NaDEHP or sodium bis(2-ethyhexyl) phosphate, another anionic surfactant, has also been focused. For

example, in 2001, Zhou and his group studied the spectroscopic characterization of solubilized water in water /NaDEHP/n-heptane microemulsion. NaDEHP reverse micelles also have been used for protein extraction (Zi and Gulari, 1996). Recently, Anukulprasert (2001) investigated the esterification using a NaDEHP reverse micelle-encapsulated *R. delemar* lipase.

To date the number of reactions catalyzed by lipase in reverse micelles is large (Cavalho and Cabral, 2000). There are many studies that aim to improve the activity and stability of lipase such as Freeman and his group (2001) who discovered the effect of bile salt as a co-surfactant which was found to increase the reaction rate and stability of enzyme. Recently, it was demonstrated that the resulting synthetic activity in esterification is affected by choice of hydrocarbon/water interface and that there were differences in the effects of interfaces on the synthetic activity of difference lipases (Maruyama *et al.*, 2001).