CHAPTER IV
RESULTS AND DISCUSSION

4.1 Rice Bran Lipase Extraction, Purification and Activity

The purification of Thai RBL was carried out in three steps, i.e., extraction (Step I), fractionation with ammonium sulfate (Step II), and DEAE-cellulose column chromatography (Step III). As summarized in Table 4.1, the "crude extract" with specific activity 21.9 mU/mg was obtained from extraction step. The crude extract was further purified by fractionation with ammonium sulfate to obtain "crude RBL" with the specific activity 232.1 mU/mg. In order to obtain "crude RBL solid", the lyophilization was applied. Generally, the activity of enzyme is significantly decreased because of the enzyme denaturation during lyophilization. In the present case, the specific activity was decreased for a half to be 122.6 mU/mg.

Table 4.1 Typical data in RBL preparation process, using 1 000 g of rice bran as a starting material

Step of purification / Item	Crude extract (Step I)	Crude RBL (Step II)	Purified RBL (Step III)
Volume (mL)	1 010.0	62.0	128.0
Activity (mU/mL)	169.1	1 111.6	400.0
Total activity (U)	170.7	68.8	51.2
Protein (mg/mL)	7.7	4.8	0.6
Specific activity (mU/mg)	21.9	232.1	667.8
Overall yield (%)	100.0	40.3	30.0

The crude RBL was purified by DEAE-cellulose column chromatography. Figure 4.1 shows the presence of enzyme and the hydrolytic activity belonging to each fraction after purification. The preferred lipase was detected from hydrolytic activity and found at the fraction number 2 to 40 among various enzyme fractions. After collecting these fractions together, the specific activity of purified RBL was determined to be 667.8 mU/mg. The activity was found to be thirty times higher than that of crude extract and three times higher than that of crude RBL.

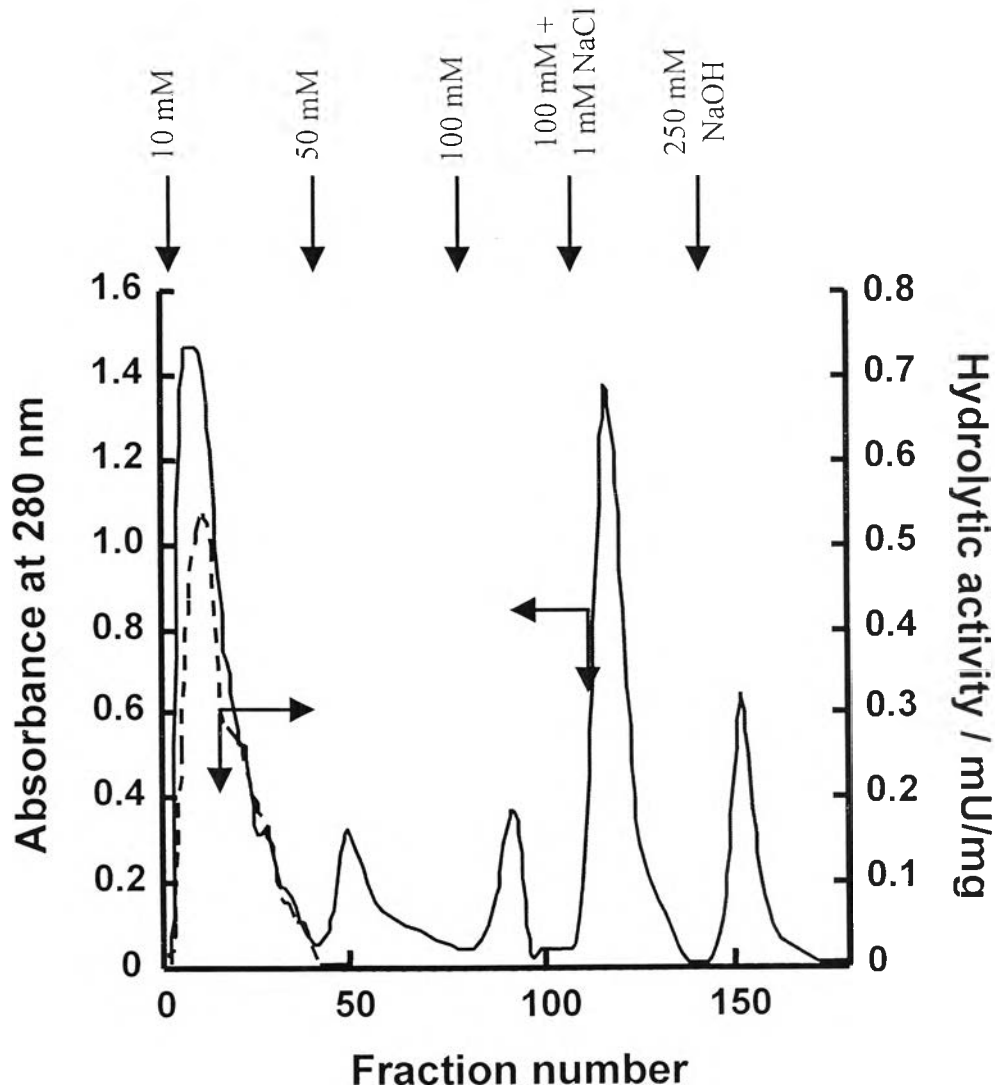

Figure 4.1 Presence of RBL and hydrolytic activity of the fractions purified from DEAE-cellulose column chromatography.

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4.2 Rice Bran Lipase Immobilization

4.2.1 Rice Bran Lipase Immobilization via Physisorption

In order to obtain immobilized RBL with high activity, two factors which are the ratio of fumed silica to RBL and types of carrier were studied.

4.2.1.1 *Effect of Fumed Silica:RBL Ratio*

The ratio of fumed silica to RBL on physisorption immobilization was studied in terms of % immobilization and immobilized RBL activity (Figure 4.2). The ratio was varied from 1:30 to 1:50 g/mL. The % immobilization was increased when increasing the amount of enzyme. At the ratio of 1:40, the immobilization was achieved for 37 %, which was close to 39 % in the case of 1:50. This implied that the immobilization by physisorption method might be saturated at the ratio 1:40.

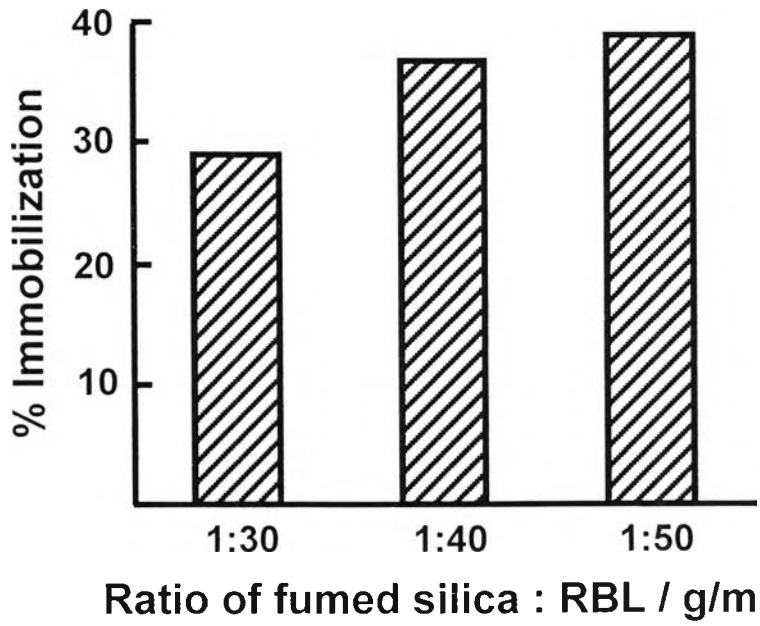


Figure 4.2 Percent immobilization of RBL of fumed silica and crude RBL at various ratios.

4.2.1.2 *Effect of Carrier Types*

Three carrier types, i.e., fumed silica, alumina, and celite on % immobilization and activity were studied. As shown in Figure 4.3a) and 4.3b), fumed silica provides physisorption at 38 % immobilization while the activity is 21 mU/mg, which are higher than those of other carriers. This might be explained in terms of the properties of each carrier, i.e., surface area, pore size, and pH.

Fumed silica has the highest surface area, 320 m^2/g, with very small pore size, 7 Å, comparing to that of alumina, 150 m^2/g, and celite, 3 m^2/g, which has the pore size 50 Å and 17 μm, respectively. Thus, the immobilization via physisorption on the surface could form effectively in the case of fumed silica. It should be noted that high % immobilization does not always give high activity. Celite gave 28 % immobilization while the activity was only 7 mU/mg. In this case, the appropriate pH of carrier surface for lipase was considered. Aizono *et al* (1973) reported that the pH stability of *japonica* RBL was in the range of 4 to 9. Although our work was dealt with Thai RBL, we assumed that the stability of our Thai RBL be in the same range as that of *japonica* RBL. The pH of fumed silica, alumina, and celite surface are 5, 6, and 10, respectively. The low activity of immobilized RBL on celite might come from the high pH of the celite surface, which induced the enzyme denaturation after RBL was introduced to the surface.

4.2.2 Rice Bran Lipase Immobilization via Covalent Bonding

For practical use, the reuse of catalyst is required for the process due to the economic reason. Although physisorption immobilization is a simple and easy method, the main problem is the ease of enzyme desorption. The covalent immobilization is an alternative method. Thus, it is our interest to study the immobilization via covalent bonding of Thai RBL on the carrier.

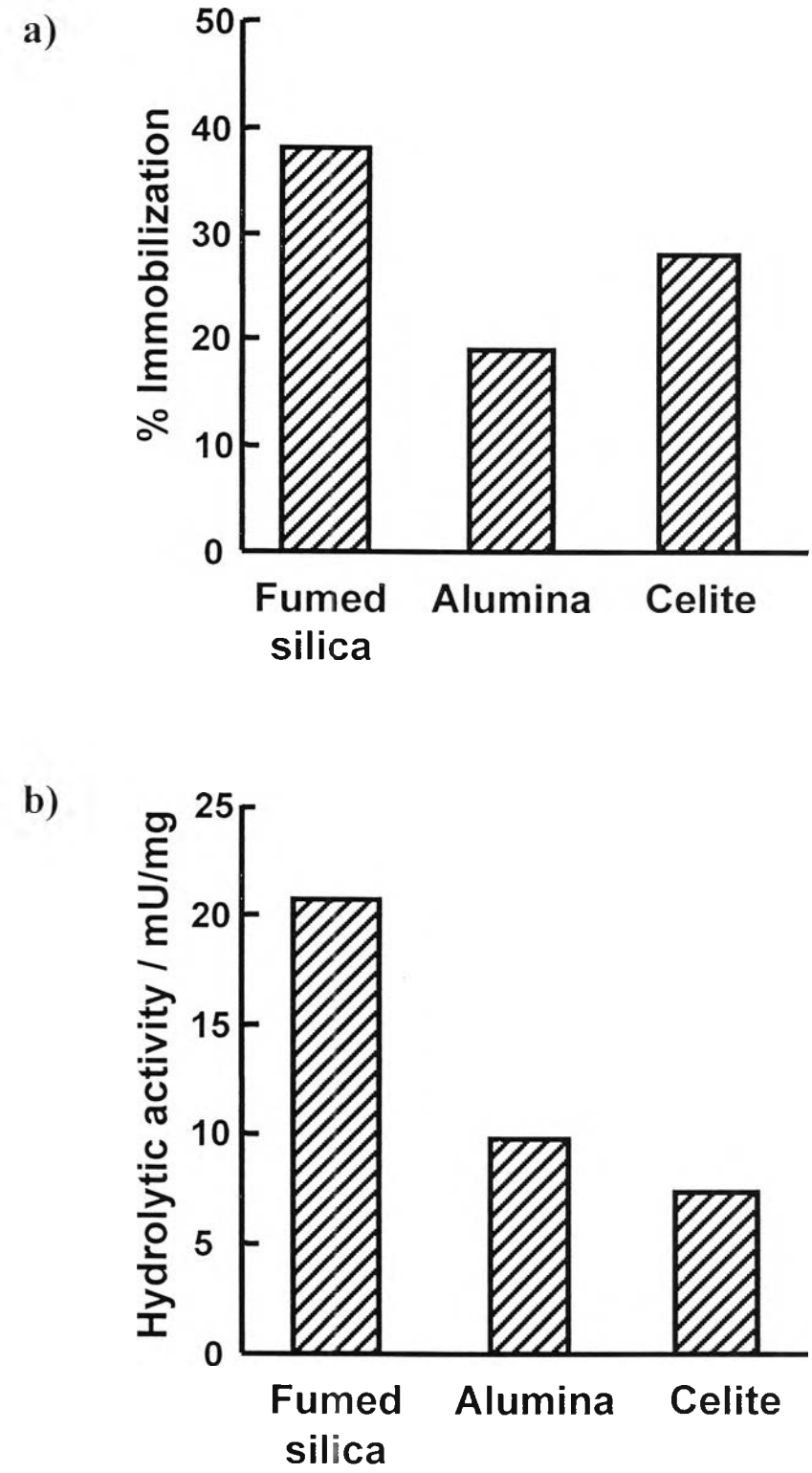
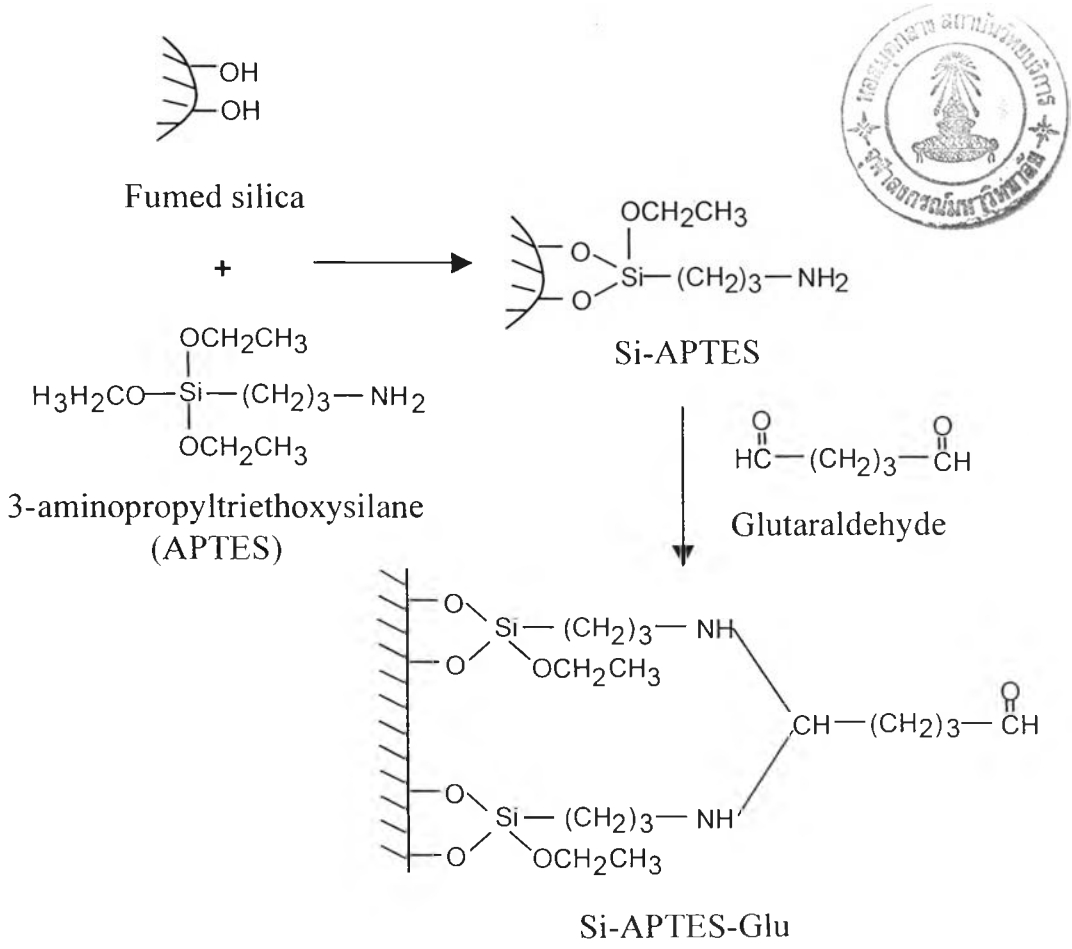
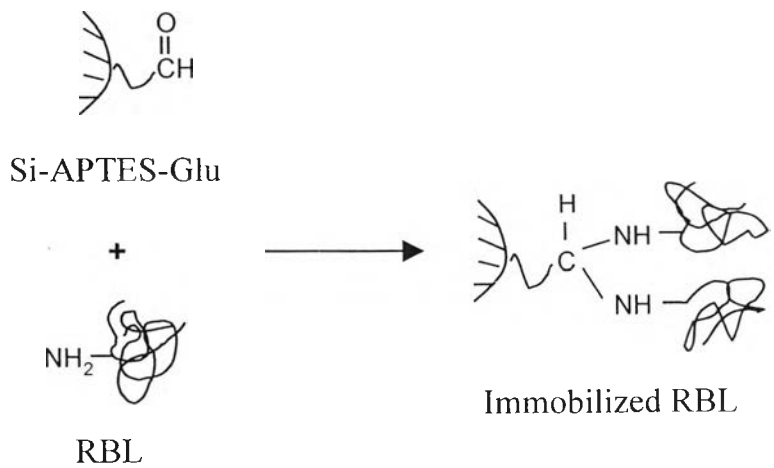


Figure 4.3 a) Percent immobilization of RBL and b) hydrolytic activity of immobilized RBL, onto various types of carriers.

Scheme 4.1 Preparation of Si-APTES-Glu



Scheme 4.2 Schematic draw of RBL immobilization via covalent bonding



Silica is known for the reactive surface and can be coupled with organic species easily. In the present work, the covalent bonding between Thai RBL and silica was attempted. First, the reactive silica (Si-APTES-Glu) was prepared by modifying the surface of the fumed silica with APTES and glutaraldehyde consequently (Scheme 4.1). 3-aminopropyltriethoxysilane (APTES) was used as coupling agent, owing to its good leaving group, the ethoxy group, which can be removed easily by reacting with hydroxy group of the silica surface. Glutaraldehyde was used as a spacer to connect RBL to the modified silica (Si-APTES) via covalent bonding between the amino groups of enzyme and modified silica (Scheme 4.2). The successful of surface modification was evaluated by DRIFT-FTIR and elemental analysis (EA)techniques. By comparing the FTIR spectrum of the modified silica (Si-APTES) to the starting materials (Figure 4.4), i.e., fumed silica and APTES, the characteristic peaks at 2984 cm^{-1} and 2888 cm^{-1} of C-H stretching and at 3290 cm^{-1} of N-H stretching are identified. This implied that Si-APTES was obtained.

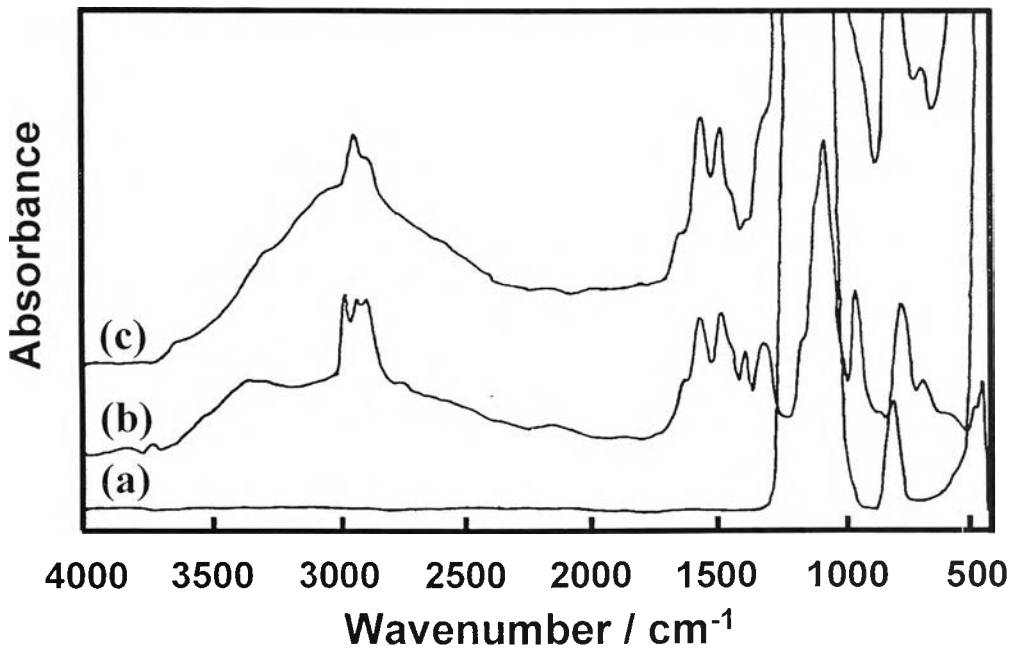


Figure 4.4 FTIR spectra of (a) fumed silica, (b) APTES, (c) Si-APTES by DRIFT-FTIR.

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After that, Si-APTES was further reacted with glutaraldehyde, as shown in Figure 4.5, the peak of C=O stretching of aldehyde group is observed at 1720 cm^{-1}. This suggested the successful coupling of aldehyde group on the surface of Si-APTES. The EA result (Scheme 4.3) informed the efficiency of the surface modification. By varying the amount of SiO_2 (X value) to be coupled on APTES-Glu, it was found that the calculated C, H, and N values are close to the found values when X is equal to 20. In another words, every 20 molecules of SiO_2 was bonded with APTES-Glu 1 molecule. The reactive silica, then, was used as a carrier in covalent immobilization. Because the aldehyde group at the surface of reactive silica is reactive, it can be reacted with amino group of the enzyme even at room temperature. In order to prevent the enzyme denaturation and compare the immobilization efficiency to physisorption method, reaction time was done in the same condition, i.e., 1 h. It was found that the immobilization was achieved 50% and the activity was 9 mU/mg.

4.2.3 Comparison of Rice Bran Lipase Immobilization via Physisorption and Covalent Bonding

The immobilized RBL from physisorption and covalent bonding were compared in terms of % immobilization and activity. By considering the % immobilization, as shown in Figure 4.6a), covalent bonding give 50 % immobilization, which is higher than physisorption for 12 %. This might be because reactive silica had the abundant reactive groups on the surface, which reacted and formed the covalent bonds with enzyme molecules. In the case of physisorption, the desorption easily occurred, which leaded to low % immobilization.

It is known that enzyme shows the activity only at a certain conformation. Here, the covalent bonding of the enzyme occurred at amino group might make the conformation changed.

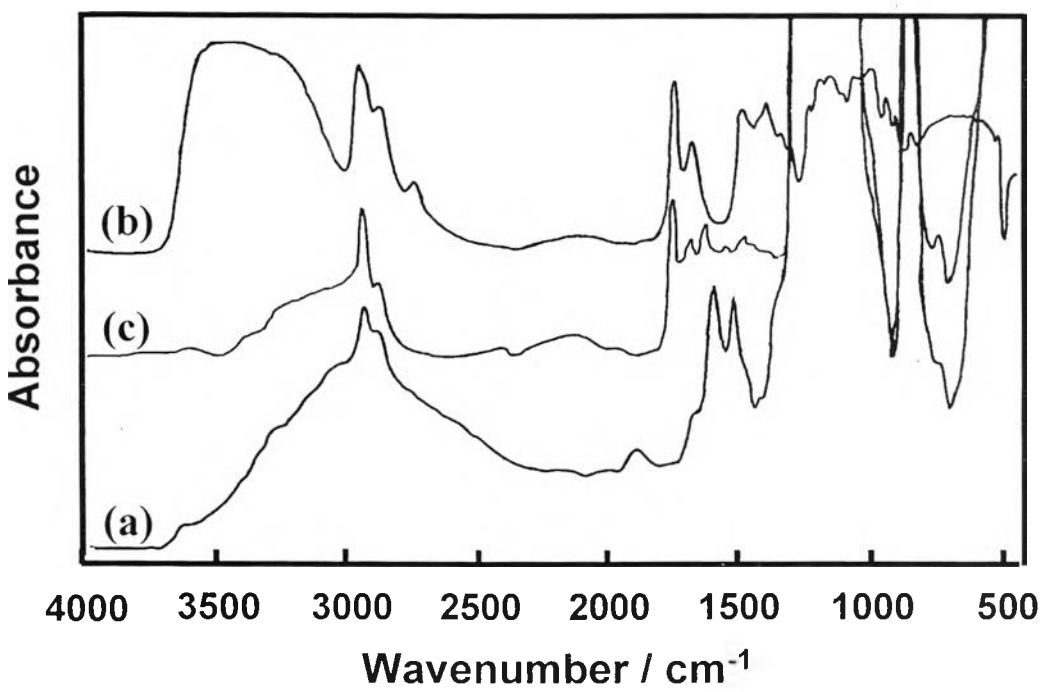
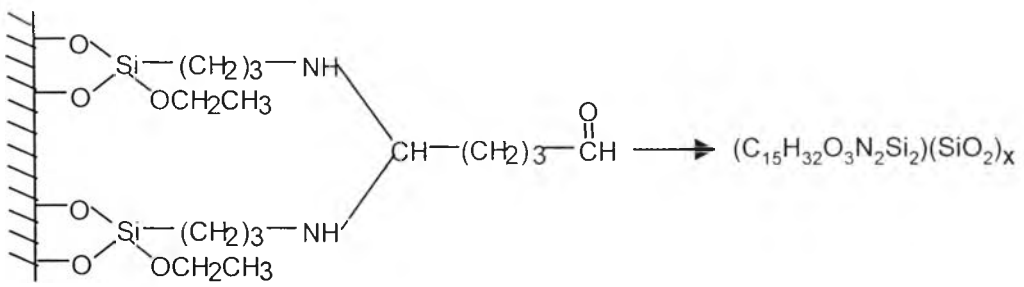


Figure 4.5 FTIR spectra of (a) Si-APTES, (b) glutaraldehyde, and (c) Si-APTES-Glu by DRIFT-FTIR.

Scheme 4.3 Elemental analysis of Si-APTES-Glu



Si-APTES-Glu

X = 20	% C	% H	% N
Calcd.	11.66	2.07	1.81
Found	11.80	2.05	1.89

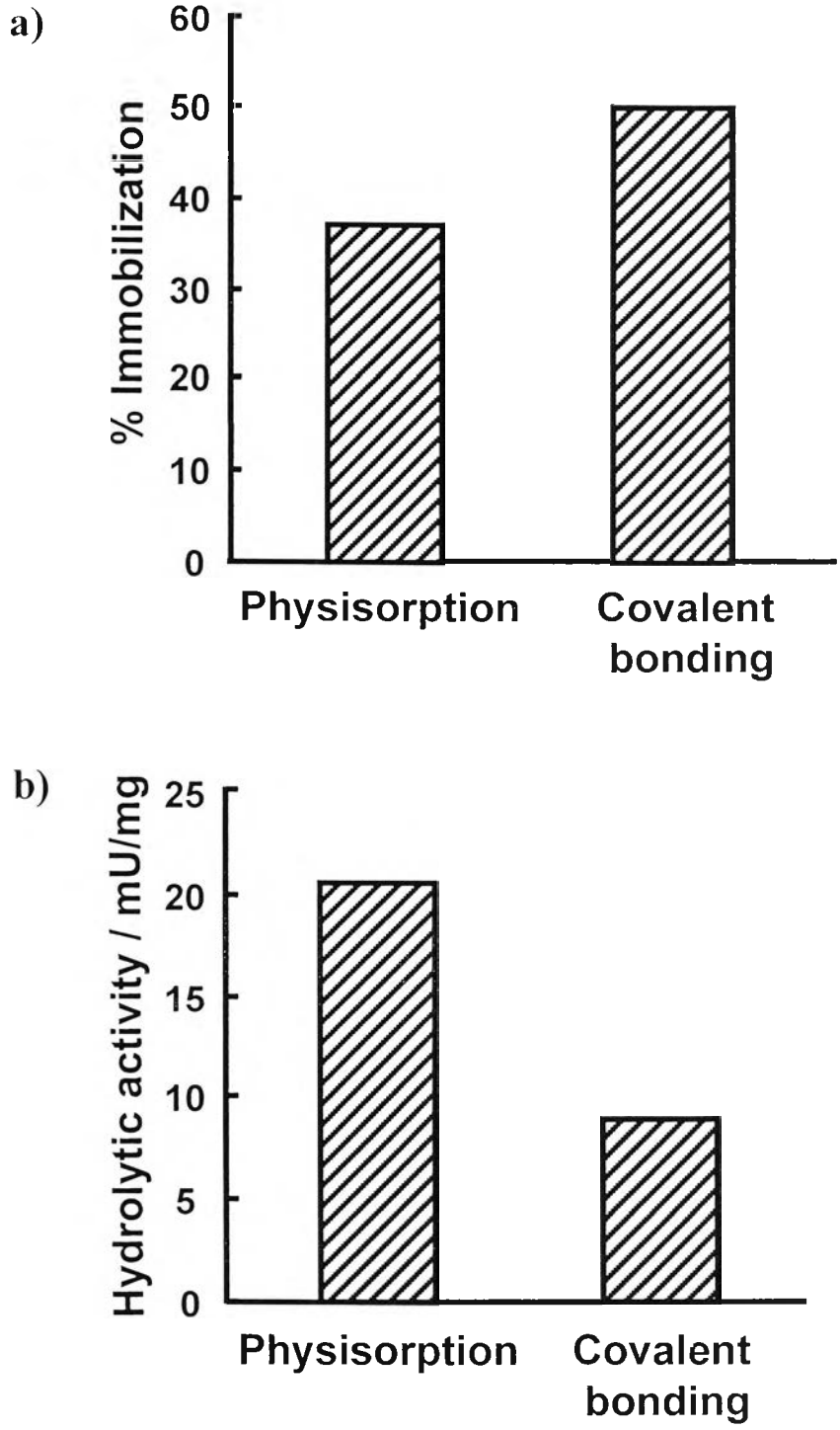


Figure 4.6 a) Percent immobilization of RBL and hydrolytic activity of immobilized RBL, onto fumed silica via physisorption and covalent bonding.

As a result, the activity of immobilized RBL via covalent bonding (9 mU/mg) was lower than that via physisorption (21 mU/mg) (Figure 4.6b)).

4.2.4 Effect of Rice Bran Lipase Purity on Immobilization

Crude RBL was applied to both physisorption and covalent bonding immobilization onto fumed silica. In addition to RBL, crude RBL consists of another proteins and materials as impurities, which are able to bind onto the carrier. If the amount of impurities binded onto carrier is higher than that of RBL, the immobilized RBL will give low activity.

In order to improve the activity of immobilized RBL, the enzyme purity was considered. By using purified enzyme, the possibility of enzyme binding onto carrier is increased, since in this case, most proteins binded onto carrier were RBL. The activity of immobilized silica was improved.

As shown in Figure 4.7a) and 4.7b), % immobilization of purified RBL from physisorption and covalent bonding are still maintained, whereas, the activity of immobilized RBL with more purity from both immobilization methods are improved about two times (Figure 4.8a) and 4.8b)), as compared to the case of crude enzyme. This inferred that the activity improvement of immobilized RBL was achieved by using the purified enzyme.

4.3 Thermal Stability of Rice Bran Lipase Solid in Toluene

Zak and Klibanov (1984) found that dry PPL performed catalytic activity for many hours at 100°C in organic media. It is an important issue to use enzyme at elevated temperature because the reaction rate is increased due to kinetic effect. Thus, it is our interest to find the range of temperature which Thai RBL still maintained the activity.

The thermal stability of crude RBL solid in toluene was investigated by suspending RBL in toluene at various incubated temperature for 24 h.

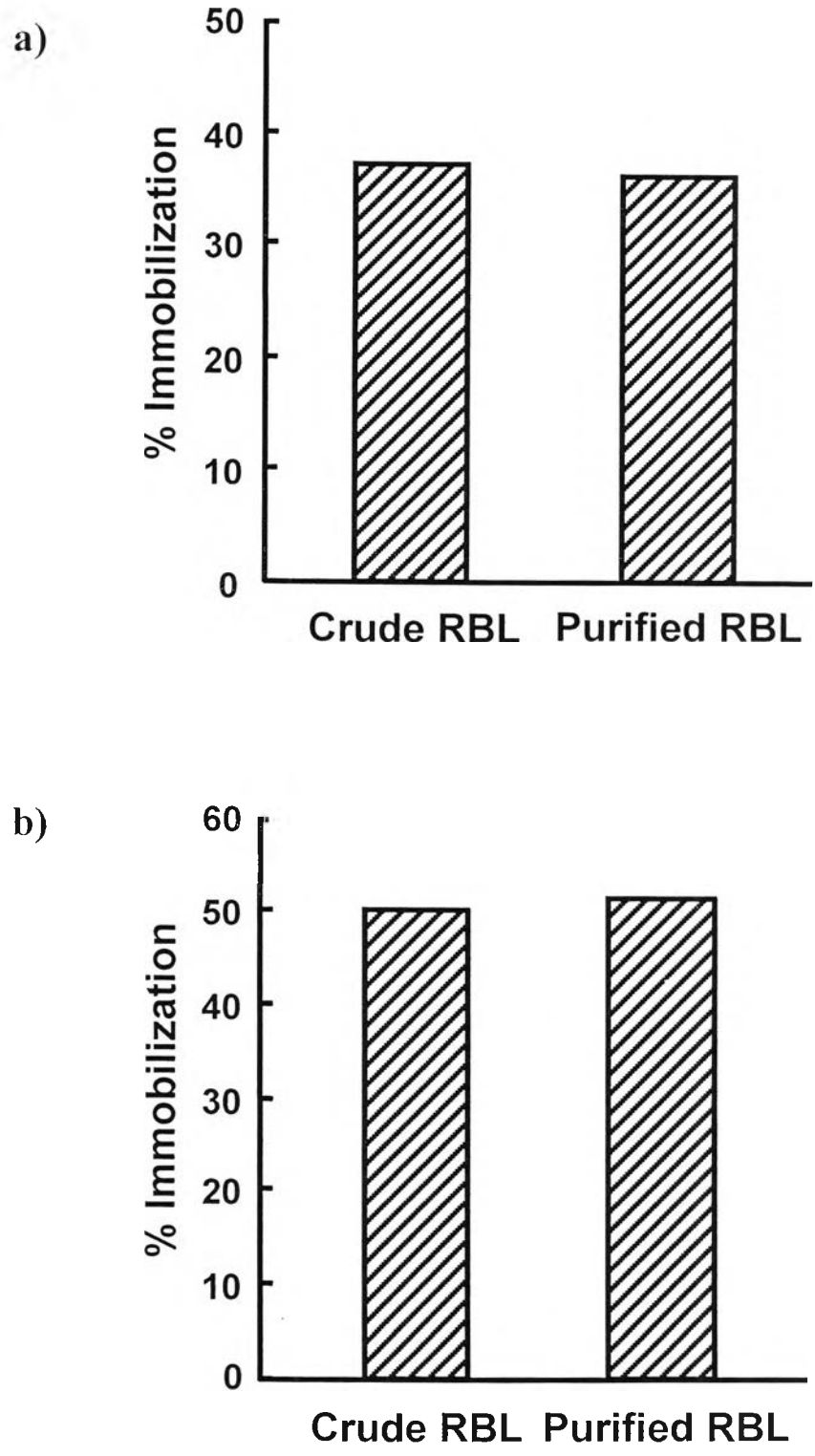


Figure 4.7 Percent immobilization of crude and purified RBL onto fumed silica via a) physisorption and b) covalent bonding.

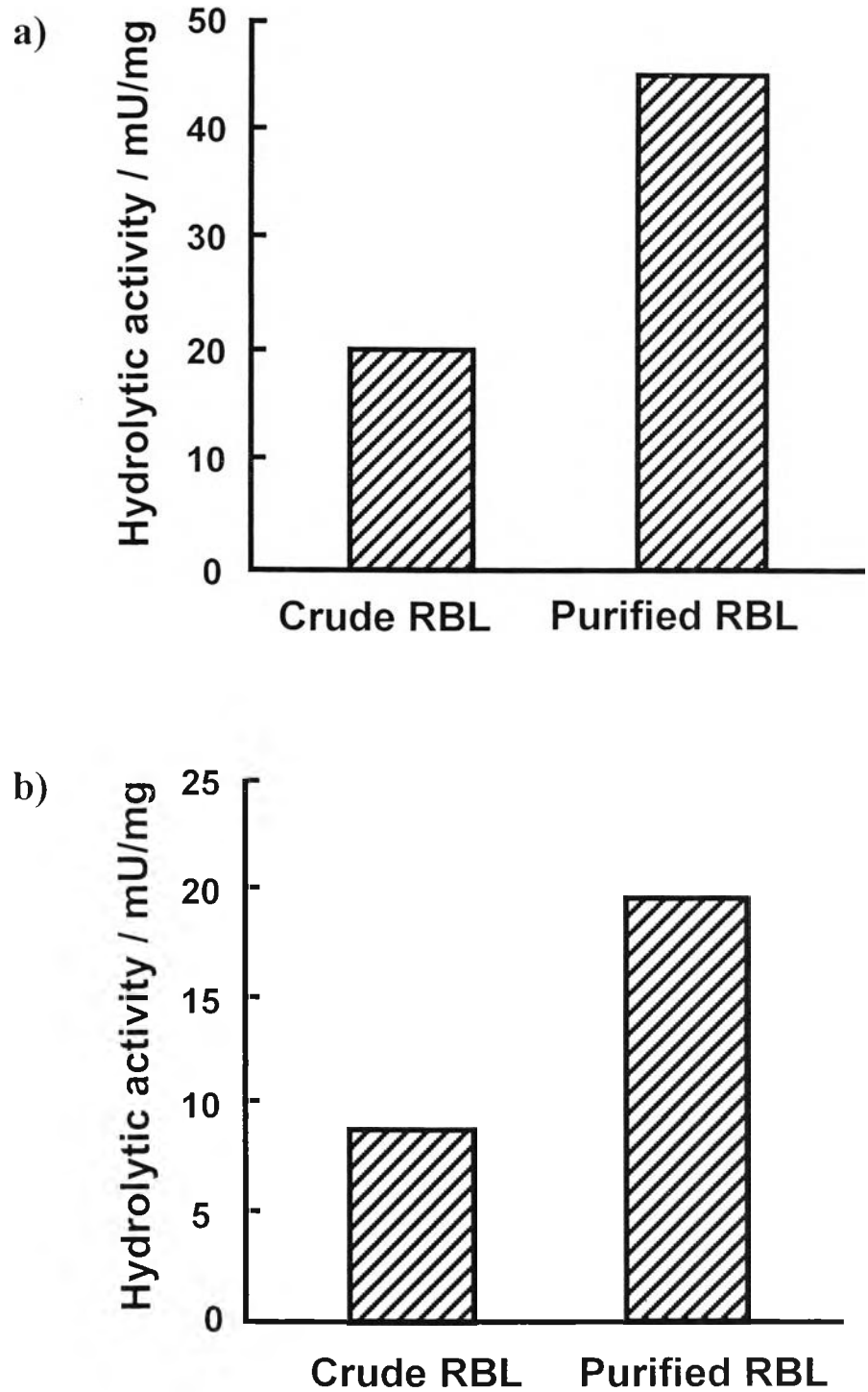


Figure 4.8 Hydrolytic activity of immobilized RBL obtained from immobilization when using crude and purified RBL in the cases of a) physisorption and b) covalent bonding.

The thermal stability of crude RBL solid and that of commercial crude PPL are shown in Figure 4.9. Only 22 % of the RBL activity was decreased when increasing temperature up to 100°C, whereas activity of PPL was decreased up to 28 %. This suggested that the thermal stability of Thai RBL be as high as commercial lipase. Aizono *et al.* (1973) reported that, in aqueous solution, the RBL activity decreased dramatically above 40°C and denatured completely at 60°C. In our case, Thai RBL was treated in organic media and found the activity was maintained even at 100°C. This implied that Thai RBL could be used as a catalyst in organic media for polyesterification at high temperature with a considerable activity.

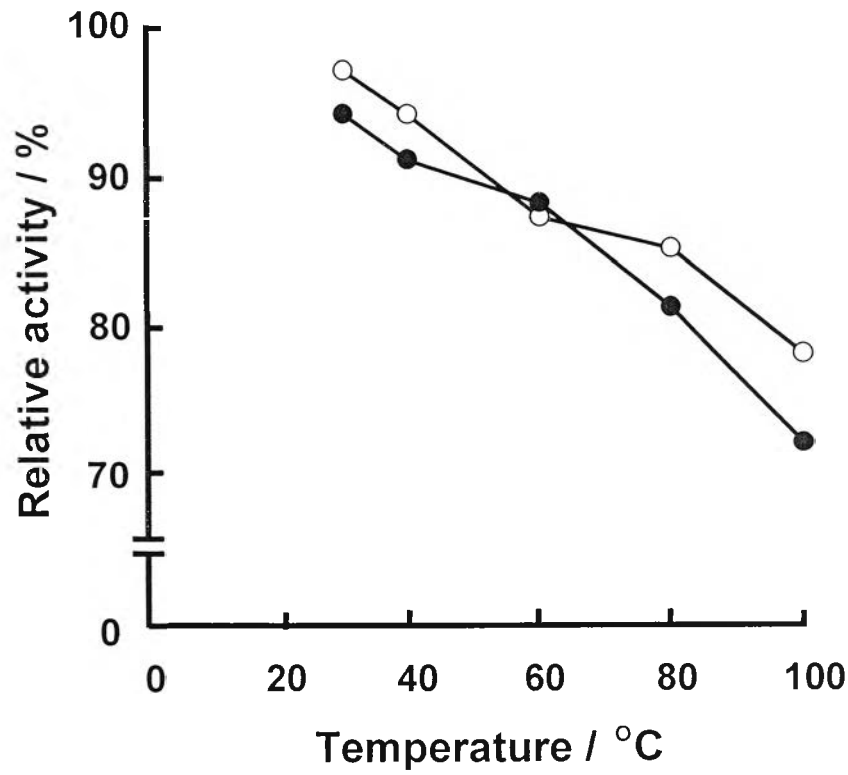


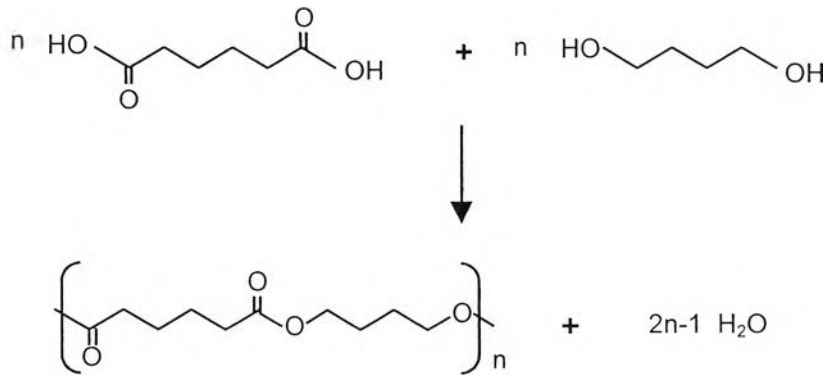
Figure 4. 9 Thermal stability of two types of lipase in toluene: ○) free RBL; and ●) PPL.

4.4 Rice Bran Lipase-Catalysed Polyesterification

4.4.1 Rice Bran Lipase-Catalysed Polyesterification

The polymerization of adipic acid and 1,4-butanediol (Scheme 4.4) with RBL catalyst was performed under various reaction temperatures and times.

Scheme 4.4 Polymerization of adipic acid and 1,4-butanediol



The FTIR of starting monomers and the obtained product are compared, as shown in Figure 4.10. The obtained product shows the characteristic peak at 1745 cm^{-1} belonging to C=O stretching of ester compound. Figure 4.11 shows the proton resonances at δ 2.31 and 4.16 ppm, assigned to methylene adjacent to esterified oxygen (-CH_2OCO-) and methylene adjacent to esterified carbonyl (-CH_2COO-), respectively. In addition to these 2 peaks, the multiplet lying between δ 1.6-1.8 ppm referred to the methylene (-CH_2-) of 1,4-butyl and adipate moieties. The results from FTIR and ^{1}H-NMR implied that poly(1,4-butyl adipate) was obtained.

The molecular weight of the obtained polyester was determined by gel permeation chromatography (GPC) based on polystyrene standards. As shown in Figure 4.12, the molecular weight obtained from all of reactions are

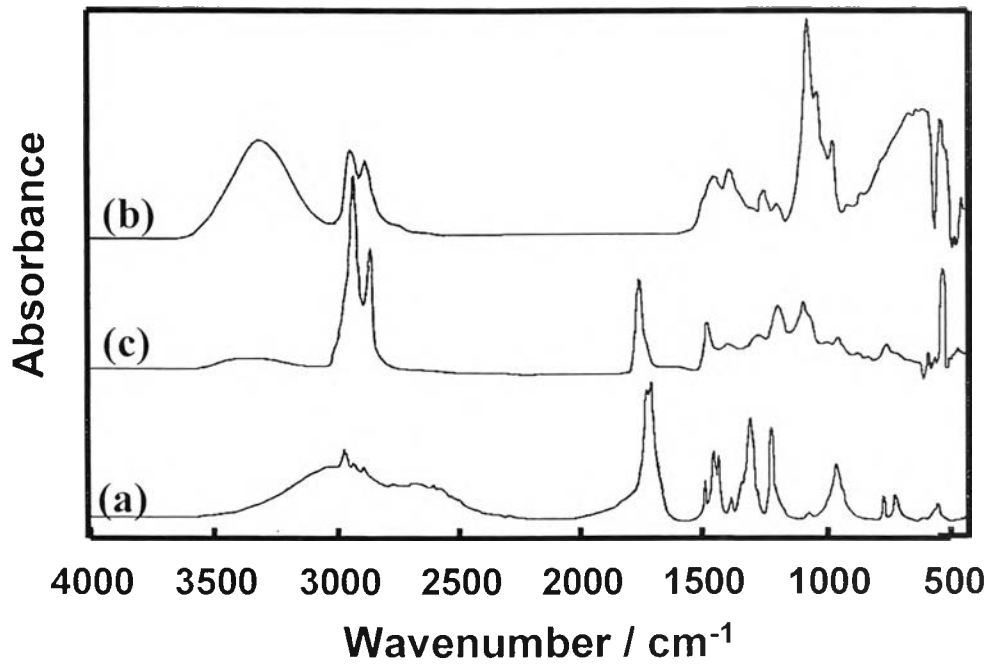


Figure 4.10 FTIR spectra of (a) adipic acid, (b) 1,4-butanediol, and (c) the obtained product.

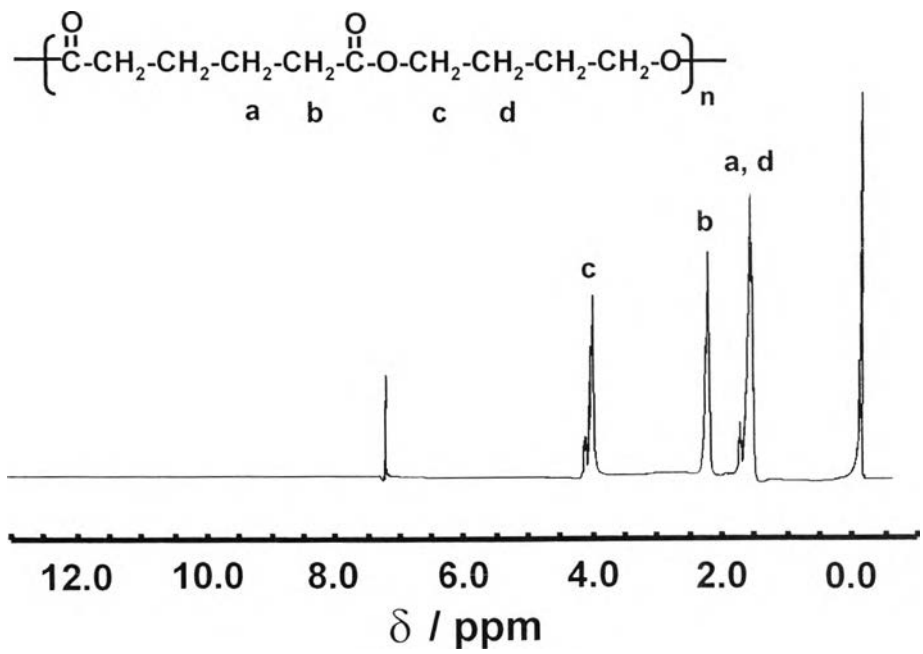


Figure 4.11 ^{1}H-NMR spectrum of poly(1,4-butyl adipate).

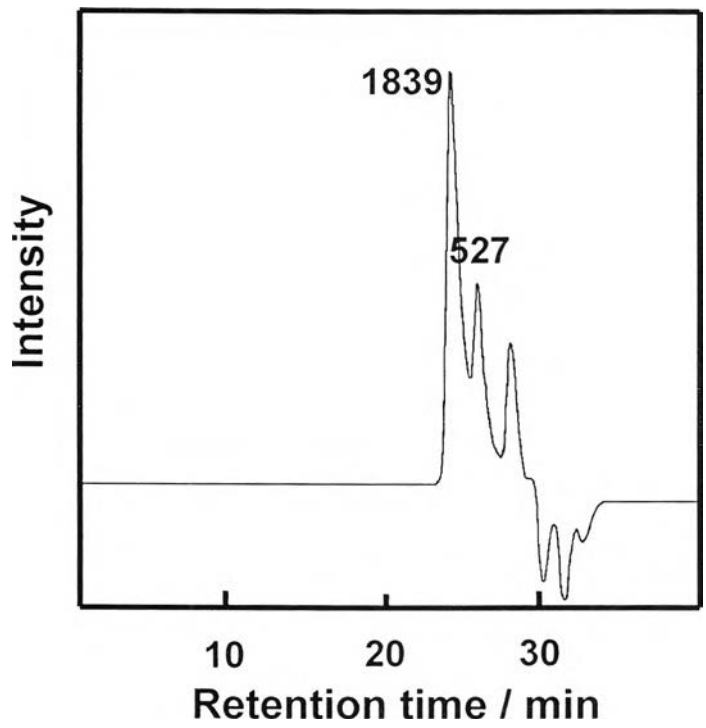


Figure 4.12 GPC chromatogram of poly(1,4-butyl adipate).

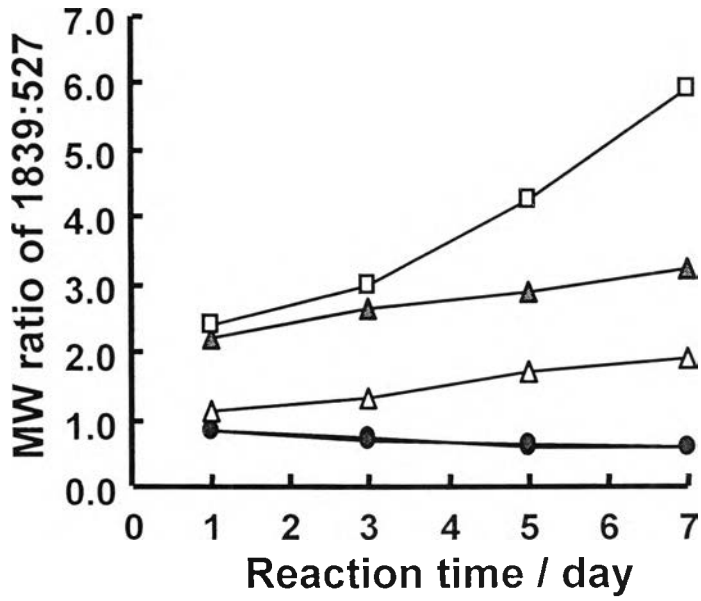


Figure 4.13 Molecular weight ratio of the obtained product when the reaction proceeded at various reaction temperatures: ○) 30°C; ●) 40°C; △) 60°C; ▲) 80°C; and □) 100°C.

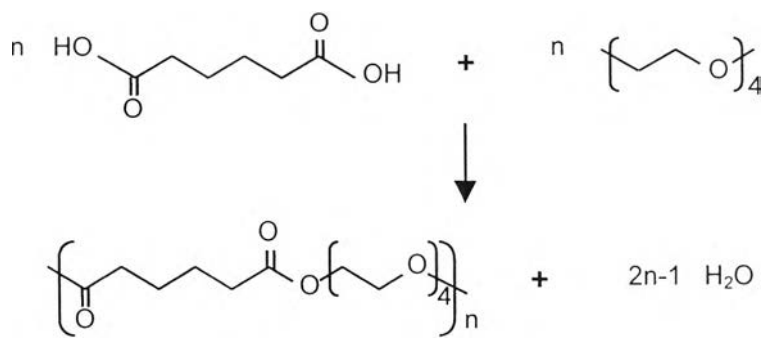
found in two groups of molecular weight, i.e., 527 (n=3) and 1 839 (n=9), with a narrow polydispersity (1.3-1.4). The effects of reaction temperature and time on the molecular weight of the obtained product were studied. Figure 4.13 shows that at 30° and 40°C, the molecular weight ratio decreases along the reaction time but increases when the reaction was operated at 60°, 80°, and 100°C. This might be because, at low temperature, the water by-product was remained or/and increased continuously in the reactor. The reverse reaction or hydrolysis, thus, was significant. However, at high temperature, the kinetic rate of reaction increased as well as water was evaporated from the solution. As a result, the equilibrium of hydrolysis and esterification was shifted to esterification direction, leading to the high molecular weight.

4.4.2 Rice Bran Lipase-Catalysed Polyesterification via polyol

Up to now, the most reported polyester syntheses with lipase catalytic system have been carried out by using two monomers. Polyesterification from monomer and polymer, thus, is an interesting point to study the lipase reactivity and to obtain long chain polymer.

Polyesterification via polymer chain was carried out by using adipic acid and poly(ethylene glycol) MW 200 as starting materials in RBL catalyst system (Scheme 4.5).

Scheme 4.5 Polymerization of adipic acid and poly(ethylene glycol)



The success of the reaction was observed by the C=O peak of ester compound at above 1720 cm^{-1}. Comparing the FTIR spectrum of the obtained product to the spectra of adipic acid and PEG (Figure 4.14), the obtained product shows the peak at 1747 cm^{-1}.

Figure 4.15 shows ^{1}H-NMR spectrum of the obtained product displaying the peak at δ 4.15 ppm assigned to methylene adjacent to esterified oxygen (-C$\underline{H_2}$OCO-) and δ 2.28 ppm assigned to methylene adjacent to esterified cabonyl (-C$\underline{H_2}$COO-). Additionally, the proton resonance of methylene (-C$\underline{H_2}$-) from adipate moiety was appeared at δ 1.59 ppm while that of methylene adjacent to oxygen (-C$\underline{H_2}$O-) from PEG moiety was observed at δ 3.62. Thus, it was clarified that poly(poly(ethylene glycol) adipate) was obtained.

The molecular weight of the obtained polyester was determined by gel permeation chromatography (GPC), using polystyrene standards curve. Figure 16 shows two groups of molecular weight identified, i.e., 1 795 (n=6) and 508 (n=2). Considering Figure 4.12 and 4.16, it can be concluded that Thai RBL has a limit of polyester chain production to produce polyester at MW ~ 1 800, which may be due to the specific characteristic active site of Thai RBL. Figure 4.17 suggests that the reaction depend on the time and temperature. The ratio of hexamer:dimer increased along the reaction time when the reaction was operated at high temperature (60°, 80°, and 100°C). This result showed the same trend to polyesterification of adipic acid and 1,4-butanediol.

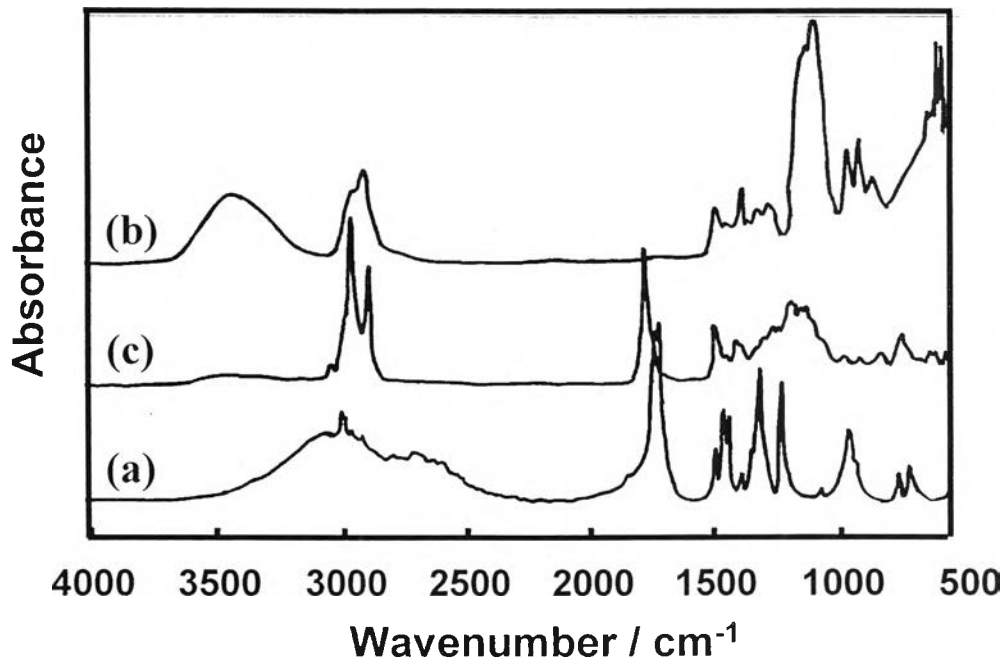


Figure 4.14 FTIR spectra of (a) adipic acid, (b) poly(ethylene glycol), and (c) the obtained product.

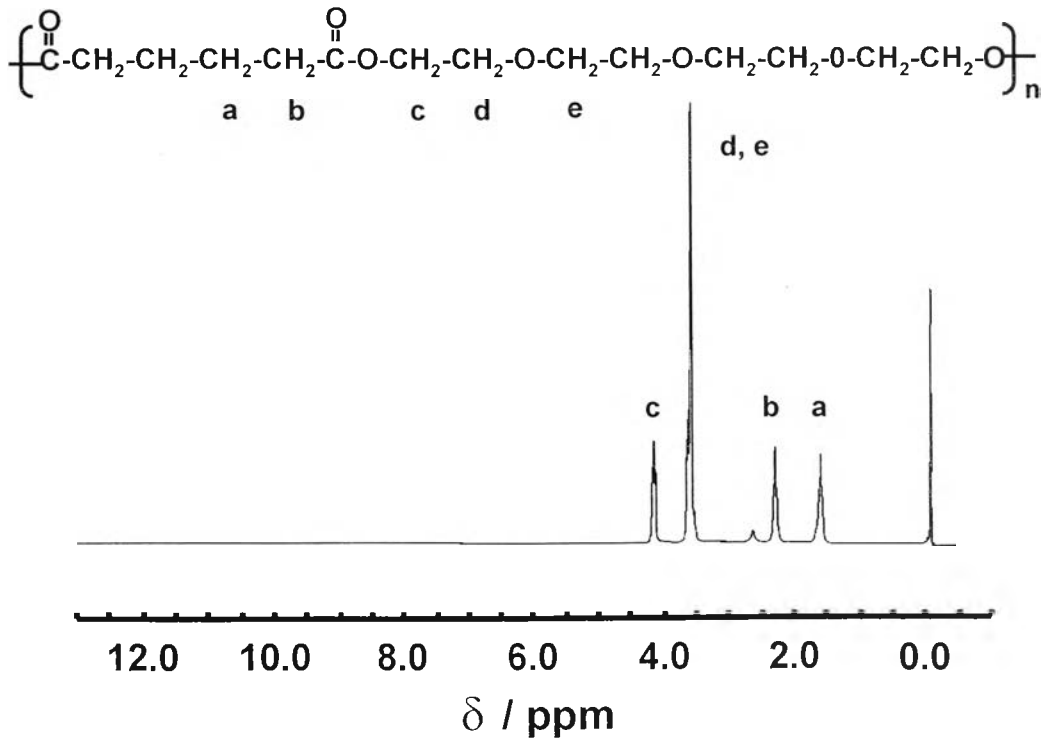


Figure 4.15 ^{1}H-NMR spectrum of poly(poly(ethylene glycol) adipate).

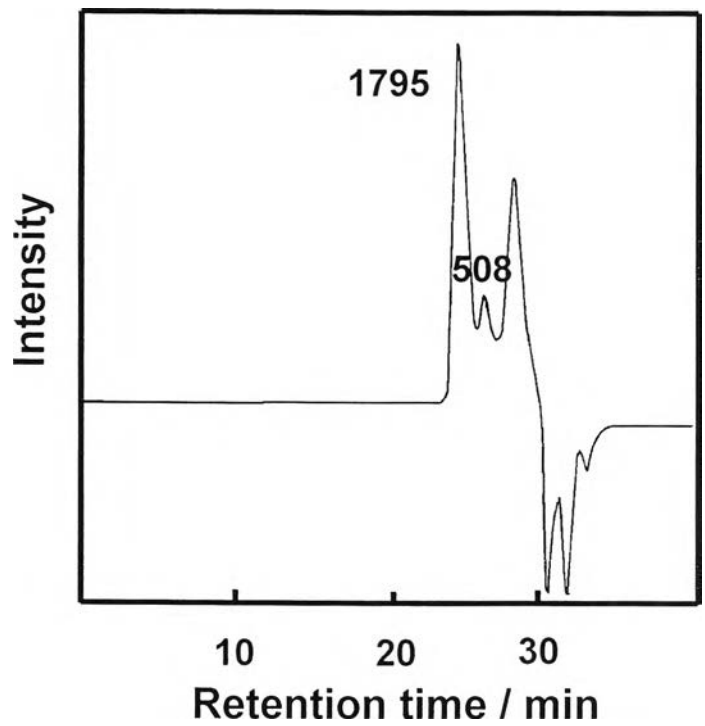


Figure 4.16 GPC chromatogram of poly(poly(ethylene glycol) adipate).

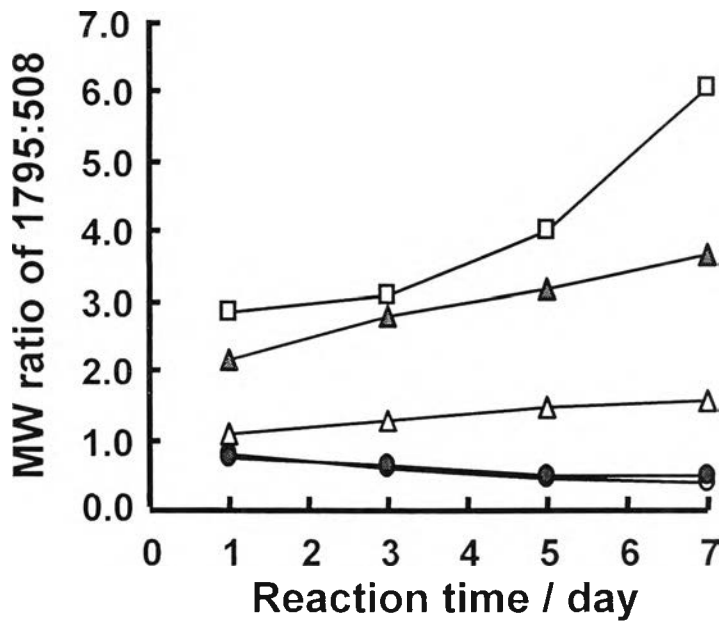


Figure 4.17 Molecular weight ratio of the obtained product when the reaction proceeded at various reaction temperatures: ○) 30°C; ●) 40°C; △) 60°C; ▲) 80°C; and □) 100°C.