CHAPTER IV
RESULTS AND DISCUSSION

In order to consider rice bran lipase as a catalyst for esterification system, the specific activity of rice bran lipase was investigated. However, specific activity of rice bran lipase depends on the optimum condition in lipase extraction step. In the present work, the extraction conditions, i.e., the method, solvent used in extraction, ratio of rice bran to solvent, extraction time including the re-extraction procedure, were studied to determine a suitable method for obtaining the lipase with high specific activity and high yield.

4.1 Rice Bran Lipase Extraction, Purification and Activity

4.1.1 Lipase Extraction Method

Extraction methods were studied in two systems, i.e., the blender system and mixer system. Table 4.1 shows the specific activity of the obtained rice bran lipase from each system in different extraction solvents, i.e., phosphate buffer pH 7 and 10 mM $CaCl_2$.

These two solvents were chosen because at pH 7 almost lipase shows the high specific activity. Ca^{2+} is known to activate lipase to show high activity. Therefore, phosphate buffer pH 7 and 10 mM were used to study in this work.

Table 4.1 Specific activity of rice bran lipase in blender and stirrer systems in phosphate buffer pH 7 and 10 mM calcium chloride solvents

Method / Solvent	Specific activity (mU/mg)	
	Blender	Stirrer
Phosphate buffer pH 7	2.05	138
10 mM $CaCl_2$	71.19	145.47

The stirrer system gives higher specific activity for rice bran lipase than that of blender system. This may be due to the low mechanical shear of the stirrer system provides preferable environment to rice bran lipase. Generally, protein is denatured by heat and organic solvents. Mechanical shear in blender can be considered for heat generation to denature the enzyme. Therefore, rice bran lipase in stirrer system is not much denatured and shows high specific activity.

4.1.2 Solvent System in Extraction

To optimize the solvent system in the extraction, two solvents were considered, 50 mM phosphate buffer pH 7 and 10 mM calcium chloride solution. It is found that the rice bran lipase extracted from 10 mM calcium chloride solution shows higher specific activity in either blender or stirrer than that of rice bran lipase in 50 mM phosphate buffer pH 7 (Table 1).

Although, many researchers proposed that rice bran lipase shows high specific activity in phosphate buffer pH 7 (Funatsu *et al.*), the present study found that, specific activity of rice bran lipase extracted from 10 mM calcium chloride solution is significant. This may be due to the pH of 10 mM calcium chloride which is close to 7 combining with Ca^{2+}, as rice bran lipase activator leading to the high specific activity.

4.1.3 Ratio of Rice Bran Amount to 10 mM Calcium chloride Solution

The weight of rice bran was varied to the volume of 10 mM calcium chloride solution in order to optimize the extraction system. As shown in Figure 4.1, at the high concentration of rice bran lipase, i.e., the ratio of weight of rice bran to volume of 10 mM calcium chloride solution at 1:3, the specific activity of the obtained lipase becomes significant to obtain nearly 150 mU/mg. The value of crude rice bran lipase specific activity is acceptable as compared to the purified lipase catalysis specific activity such as porcine pancreatic lipase for 100 mU/mg suggesting the potential for using rice bran lipase as a catalyst.

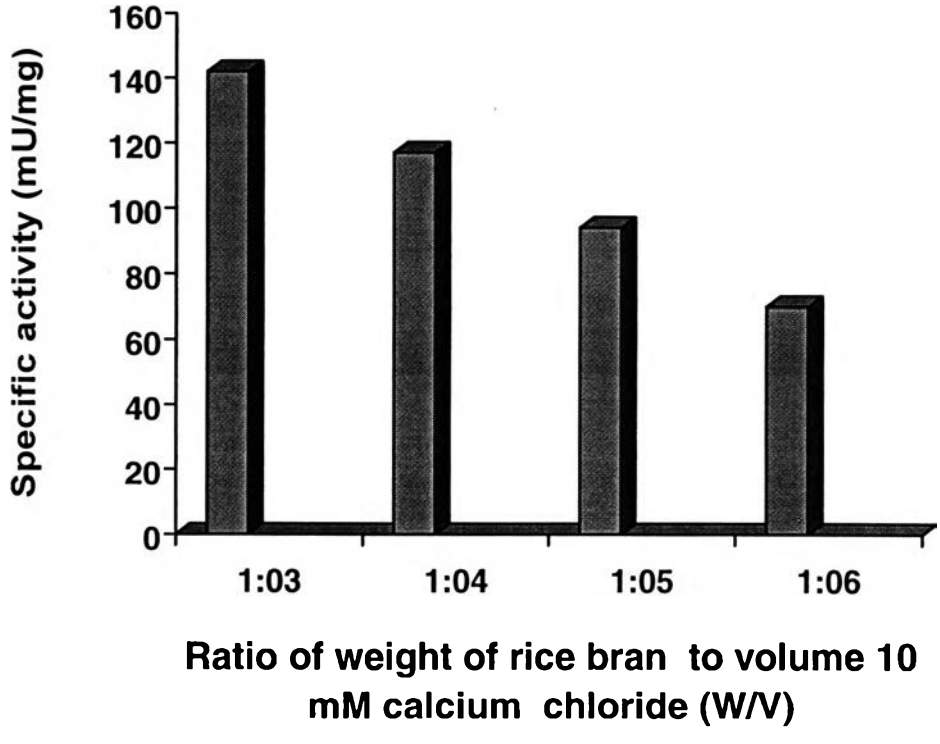


Figure 4.1 Specific activity of rice bran lipase solution in various ratio of rice bran amount to 10 mM calcium chloride.

4.1.4 Effect of Extraction Time

Extraction time was found to effect the activity of the extracted lipase activity. The optimum time for rice bran lipase extraction was studied by varying the time from 1 to 8 hours. Figure 4.2 shows the specific activity becomes constant when the extraction time is more than 3 hours. The highest rice bran lipase specific activity is reached at 3 hours.

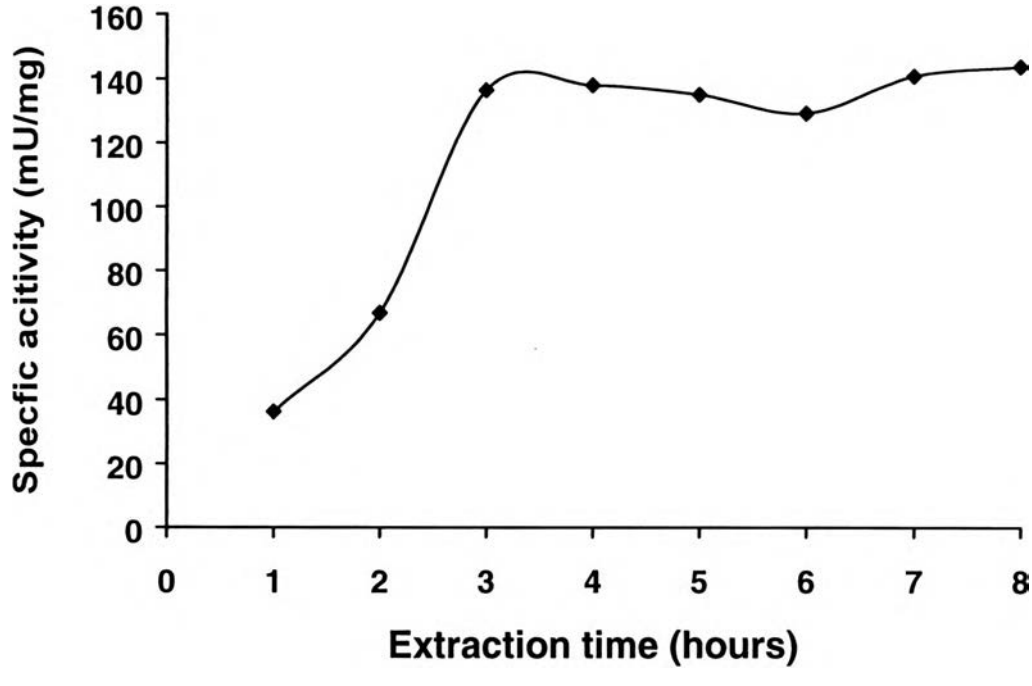


Figure 4.2 Specific activity of the extracted rice bran lipase solution in various extraction time.

4.1.5 Re-extraction Procedure

To increase the yield of lipase, we investigated the effect of multiple extractions. The first extracted rice bran was re-extracted for a second and a third time. Figure 4.3 compares the activities of the protein obtained after each extraction. The specific activity of the second and third extraction decrease rapidly indicating that after the first extraction, only small amount of rice bran lipase is left in rice bran. Considering with the price of rice bran, it can be concluded that the re-extraction is not beneficial.

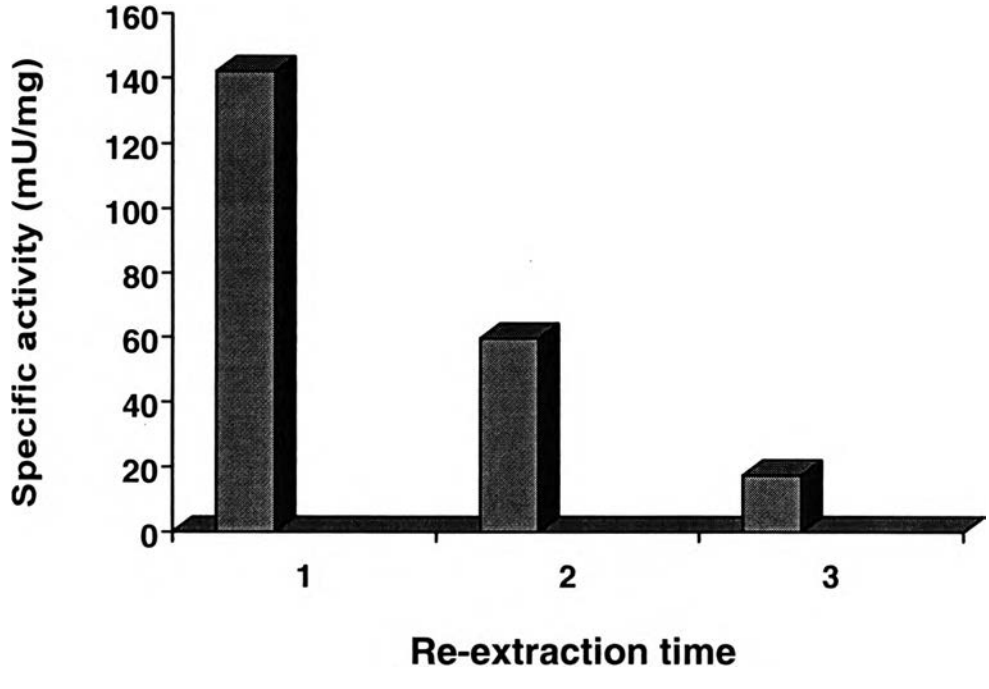


Figure 4.3 Specific activity of rice bran lipase solution in various re-extraction time.

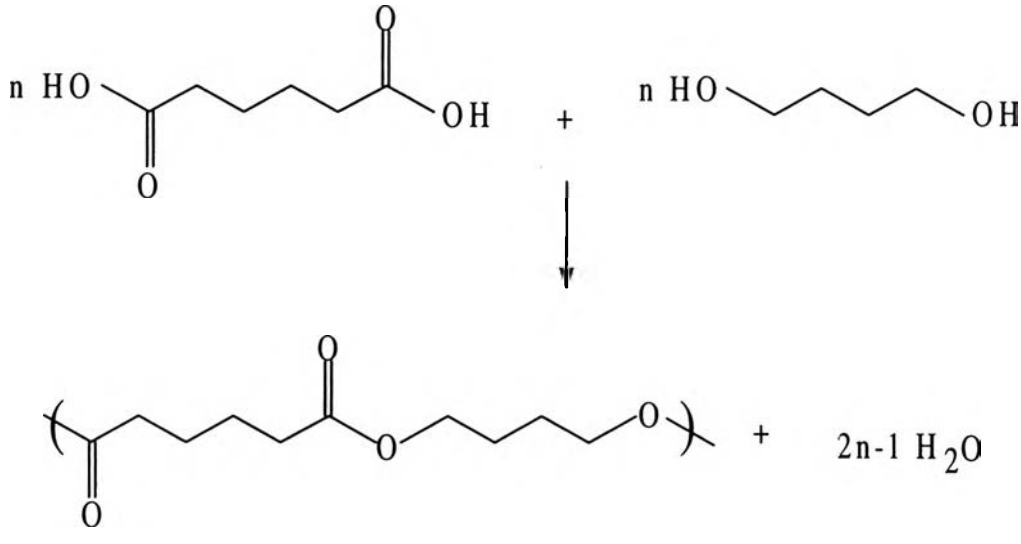
After lyophilizing, the rice bran lipase is obtained as a flake solid. This optimum condition gives the specific activity of rice bran solid at about 70 mU/mg. The specific activity of lipase using in esterification is reported in the range of 4 mU/mg to 4,200 mU/mg, i.e., *Asperillus niger* for 4 mU/mg,

porcine pancreatic lipase for 100 mU/mg, and *Pseudomonas fluorescens* for 4,200 mU/mg. Thus rice bran lipase is in the range of activities observed with other lipases found in nature and can be used as an esterification catalyst.

4.2 Activity of Rice Bran Lipase as an Esterification and Polyesterification

4.2.1) Qualitative Analysis

The polyesterification of adipic acid and 1,4-butanediol (Scheme 4.1) were studied under mild conditions (at 40°C).



Scheme 4.1 Polyesterification of diacids and diols

In order to determine qualitatively that esterification was catalyzed by the rice bran lipase, FT-IR of the starting material and the product were compared. Here, it is expected that when esterification between adipic acid and 1,4-butanediol is achieved, the product will show carbonyl of ester group at above 1720 cm^{-1}. As shown in the Figures 4.4-4.6, the carbonyl of aliphatic

hydrocarbon appears significantly at 1730 cm^{-1} for the product in the presence of rice bran lipase. In the absence of rice bran lipase adipic acid and 1.4-butanediol, the product gives C=O stretching of starting materials. Thus it can be concluded that the rice bran lipase provides a potential catalytic system for the reaction of diacid and diol to give an ester compound.

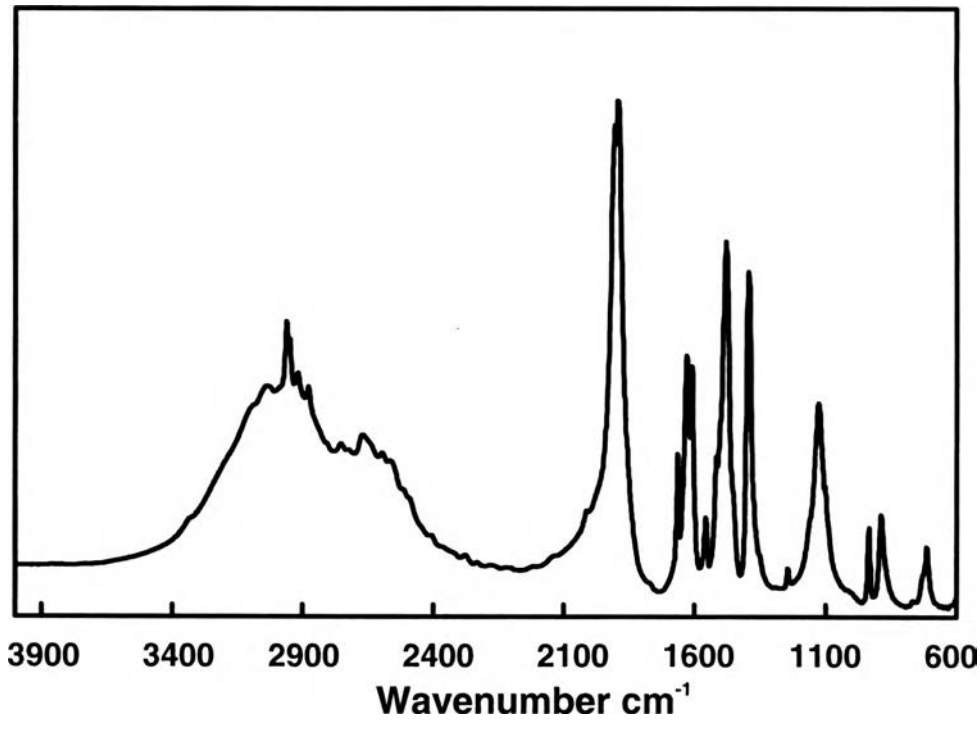


Figure 4.4 FT-IR Spectrum of adipic acid.

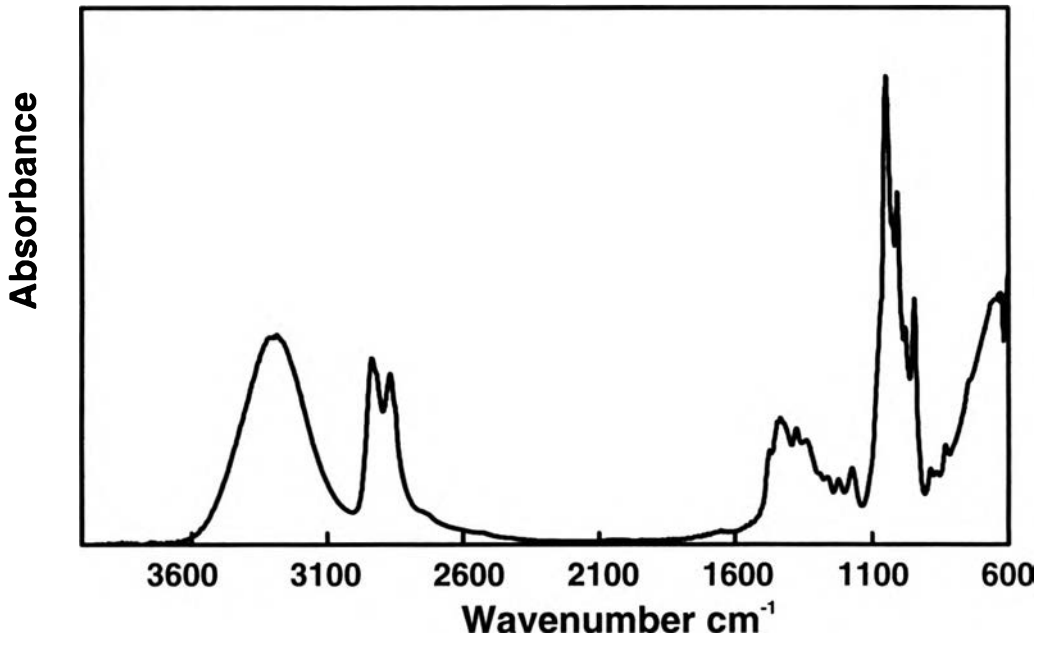


Figure 4.5 FT-IR spectrum of 1,4-butanediol.

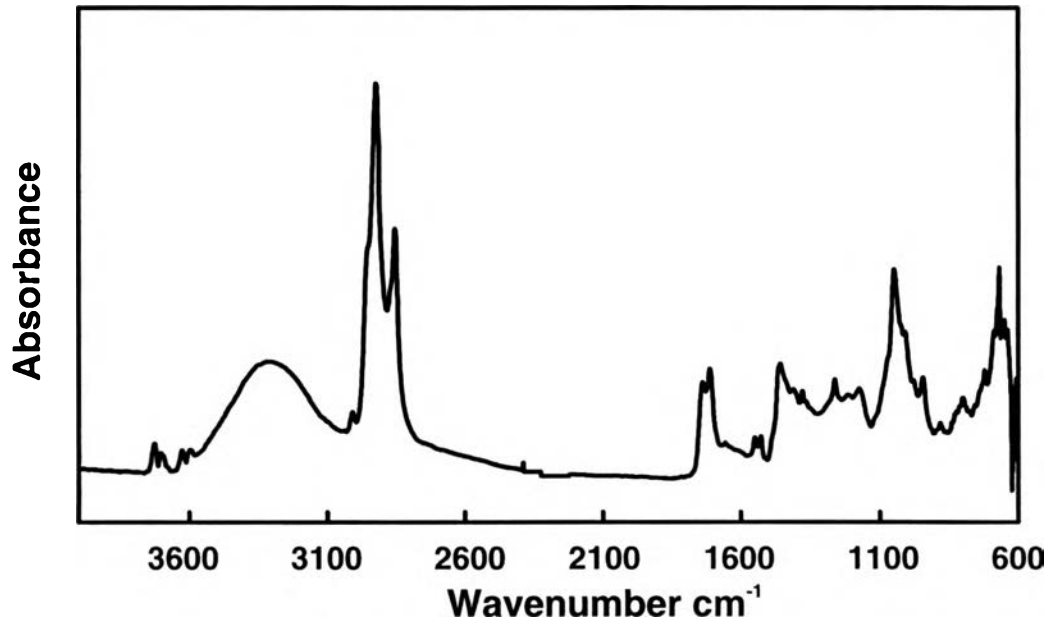


Figure 4.6 FT-IR spectrum of the product from the reaction of adipic acid and 1,4-butanediol in the presence of rice bran lipase.

4.2.2 Quantitative Analysis

The molecular weights of the ester products were determined by gel permeation chromatography (GPC). In case of the reaction time for 3 days, the products from the reaction were found to be the range from dimer (M.W. = 233) to tetramer (M.W. = 437) with a narrow polydispersity (1.05) (Figure 4.7). The chromatogram of the product without rice bran lipase catalyst shows only the molecular of adipic acid (M.W. = 144) and 1,4-butanediol (M.W. = 90).

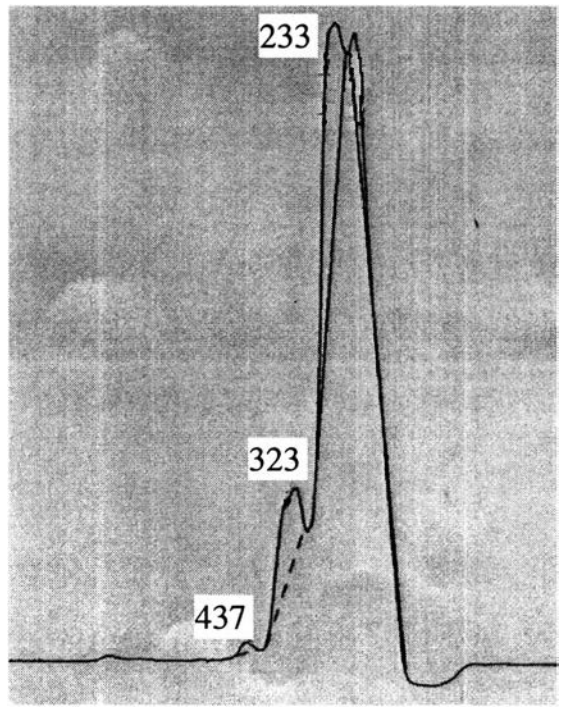


Figure 4.7 Chromatogram of the product from the reaction of adipic acid and 1,4-butanediol in the presence of rice bran lipase for 3 days.

The FT-IR also shows OH peak indicating that there is OH group in the compound (Figure 4.6). The most abundant species formed by the esterification was found to be the hydroxy-terminated oligomer, BAB, where A for the adipic acid moiety and B for the 1,4-butanediol residue. The peaks

referred to molecular weight of the product is close to that of calculated molecular weight in the combination of BAB type.

4.3 Effect of the Reaction Time

In order to obtain the high molecular weight, the reaction time was varied from 1 day to 7 days and the progression of the reaction was detected by GPC. As shown in the Figure 4.8, the highest molecular weight of oligoester was obtained in the first day and decreased rapidly. This implies that under the present condition, the suitable reaction time is 1 day. While the decreasing of molecular weight should be related to the hydrolytic reaction of lipase. When the reaction proceeds, the water by-product is formed, which initiates the reverse reaction. As a result, the hydrolysis deforms the obtained oligoester chain to short chain ones.

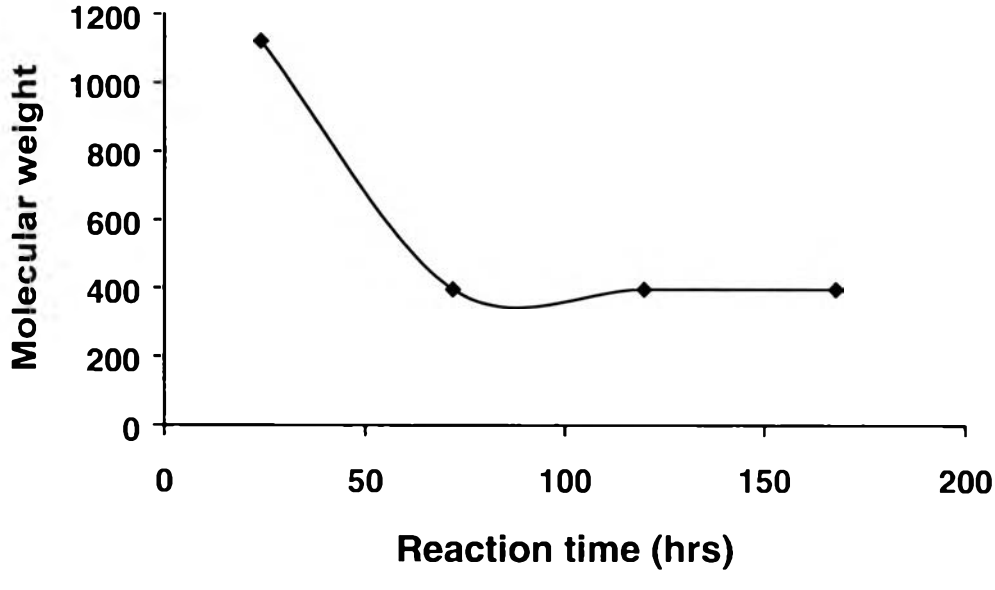


Figure 4.8 Molecular weight of the obtained product at various reaction time.

4.4 Effect of Solvent and Temperature

In order to improve the reaction, especially the exclusion of water by product which can lead the reverse reaction, the use of non polarity solvent was considered. Seppala *et al.* (1996) reported that diphenyl ether gives a high molecular weight polyester of 70,430. Here, diisopropyl ether was replaced by diphenyl ether. The qualitative analysis by FT-IR confirms the successful of the reaction. However, the product in liquid state can not be precipitated in methanol or in related solvent. However, according to the low molecular weight of the product, the precipitation was not succeeded. An attempt of product purification was done by vacuum distillation. It is found that the collection of the product can not be achieved which may be due to the close boiling point between the oligoester and diphenyl ether at around 80°C 2 mm Hg under pressure.

Temperature is an interesting parameter to consider the catalyzation. Funatsu *et al.* (1971) reported that the rice bran lipase denatures at temperature above 40°C denatures. The optimum temperature for specific activity at 37°C is obtained. In the present work, the reaction temperature was carried out at 35 ± 1°C to maintain the activity of rice bran lipase.

According to reaction of lipase for esterification, the equilibrium can be accelerated for the forward reaction by water exclusion. Falmai *et al.* (1993) proposed the utilization of molecular sieve to obtain the reaction in two chamber with a higher molecular weight for 100% of polyester product. Although the use of molecular sieve can be expected to improve the reaction, it should be noted in our one chamber system, the deformation of lipase may occur as a result of molecular sieve collision onto the lipase solid.