CHAPTER IV
RESULTS AND DISSCUSSION

4.1 Formation of NaDEHP Microemulsions

4.1.1 NaDEHP Microemulsion Formation in the Absence of Cosurfactant

NaDEHP was formed during the mixing of the organic phase containing HDEHP and the NaOH aqueous solution. The reaction can be represented as:

$$HDEHP + NaOH \longrightarrow NaDEHP + H_2O \qquad (4.1)$$

The NaDEHP/isooctane system was prepared by mixing 10 ml of 0.1 M HDEHP/isooctane with 10 ml of aqueous phase of 0.1 M NaOH and NaCl. The pH of the aqueous phase was adjusted to 7.4 by using NaOH solution. Without adding NaCl, NaDEHP formed two phases with excess water at the lower transparent phase or Winsor type I. After adding NaCl into the system, the phase transitions are shown in Figure 4.1. The volume of lower phase of Winsor type I increased with increasing salt concentration. At salt concentration of 0.1 M in the aqueous phase, the system was still Winsor type I. When the salt concentration was increased to 0.5, 1.0, 2.0 and 3.0 M, the system was separated into 3 phases or Winsor type III with the transparent phases being formed between the excess oil and excess aqueous phases. When NaCl concentration was further increased to 4.0 M, the system was transformed into two phases or Winsor type II with the microemulsion phase presented in the upper phase.

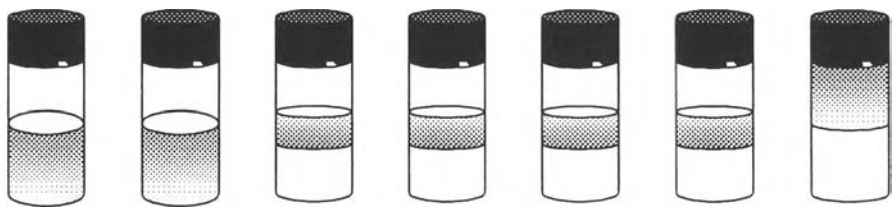

Figure 4.1 Phase transition of the 0.1 M NaDEHP/isooctane/brine.

4.1.2 NaDEHP Microemulsion Formation with Tributylphosphate (TBP) as a Cosurfactant

The NaDEHP microemulsion using TBP as a cosurfactant was performed by adding TBP into 0.1 M HDEHP/isooctane solution before mixing with equal volume of 0.1 M NaOH at various NaCl concentration (0.1-0.4 M). TBP concentration was fixed at 0.1 M which was used in previous study and presented high extraction efficiency (Sooksomsin, 2002). The pH of aqueous phase was adjusted in a similar manner as described in section 4.1.1. After adding NaCl into the system, the phase transitions are shown in Figure 4.2. At 0.1 M NaCl the system was Winsor type III and after increasing the salt concentration to 0.2 and higher the system was transform into two separated phases with the excess organic in the upper phase or Winsor type II.

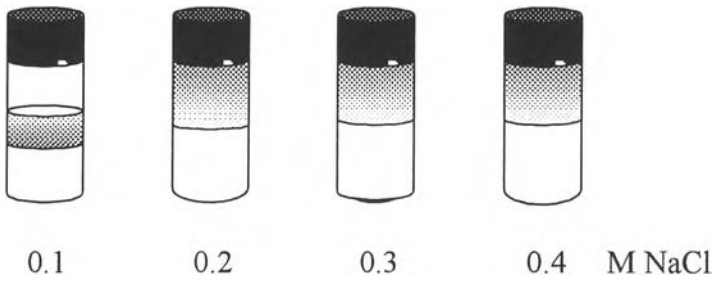

Figure 4.2 Phase transition of the 0.1 M NaDEHP/0.1 M TBP/isooctane/brine.

4.1.3 NaDEHP Microemulsion Formation with 3-[(3-cholamidyl-propyl) dimethylammonio]-1-propanesulfonate (CHAPS) as a Cosurfactant

The NaDEHP/CHAPS/isooctane system was performed by adding 10 ml of NaCl stock solution/0.1 M NaOH/aqueous solution to a vial containing solid CHAPS, stirring until the solution was clear and then mixing with 10 ml of 0.1 M HDEHP/isooctane solution. In contrast to TBP, CHAPS has never been studied in this system and thus both salinity scan and varying cosurfactant concentration were done. The pH of aqueous phase was adjusted in a similar manner as described in section 4.1.1. After mixing the organic solution with the aqueous solution, the phase transitions by salinity scan are shown in Figure 4.3. Between 0.00 and 0.05 M NaCl the white haze gel was observed the upper phase. At 0.2 M NaCl there was no gel

observed and the system was separated into two phases with the excess water in the lower phase or Winsor type I. After increasing the salt concentration to 2 M the system became three phases with the transparent middle phase or Winsor type III. At 3 M NaCl and higher, the system was transformed into two separated phases with the microemulsion in the upper phase or Winsor type II.

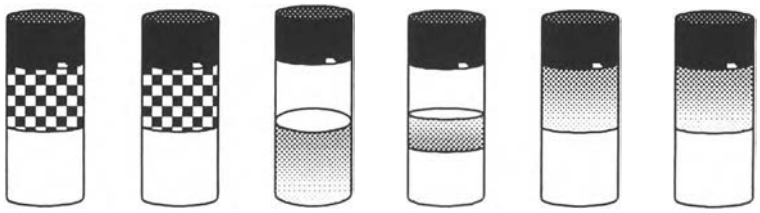

Figure 4.3 Phase transition of the 0.1 M NaDEHP/15 mM CHAPS/isooctane/brine.

In order to investigate the effect of CHAPS concentration, the NaDEHP microemulsion systems were formed with various CHAPS concentrations. From 0.0 to 15.0 mM CHAPS, the system having two separated phases was observed. When CHAPS concentration was increased to 20 mM and higher the white haze gel was formed in the upper phase as illustrated in Figure 4.4. This limits the study of the extraction in next part to only 15.0 mM CHAPS as the highest concentration.

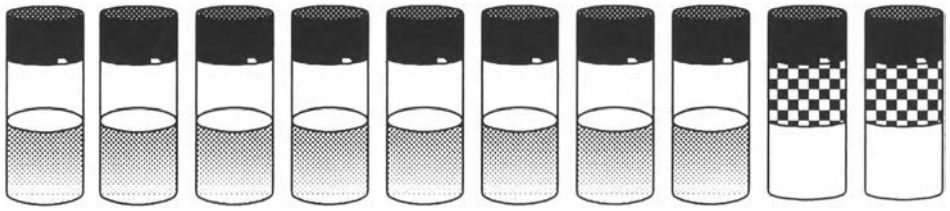

Figure 4.4 Phase transition of the 0.1 M NaDEHP/CHAPS/isooctane/0.2 M NaCl/0.1 M NaOH/aqueous solution.

4.2 Water Content in the NaDEHP Microemulsion

Water content in the upper phase of microemulsion was measured by coulometer (Karl Fischer titration technique) and the concentration ratio of water to surfactant (ω_o) could be calculated by the following equation:

$$\omega_o = \frac{[\text{water}]}{[\text{surfac tan t}]} \qquad (4.2)$$

Where:

[water] = water concentration in oil phase
[surfactant] = surfactant concentration in oil phase

4.2.1 ω_o of NaDEHP System with Tributylphosphate as a Cosurfactant

In the NaDEHP microemulsion system using TBP as a cosurfactant, when the salt concentration was increased to 0.2 M and higher the reverse micelles were formed. From this point onwards, the water content, ω_o, of the NaDEHP reverse micelles could be measured at various salt concentrations. Figure 4.5 shows the effect of salt concentration on the water content in the reverse micelle of NaDEHP/TBP/isooctane. It can be seen that the water to surfactant ratio (ω_o) decreased with increasing salt concentration. This can be explained by the effect of electrolyte that causes a decrease in the repulsion between headgroups of the surfactant forming reverse micelles resulting in smaller aggregates and less water solubilized inside the reverse micelles.

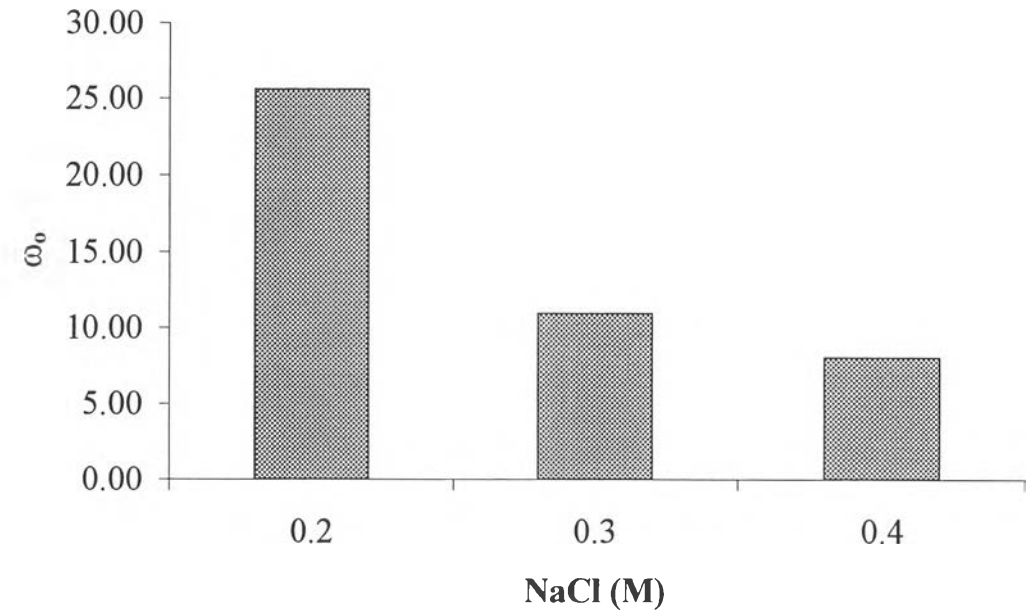

Figure 4.5 Effect of salt concentration on water content in the reverse micelles of NaDEHP/TBP/isooctane.

4.2.2 Effect of CHAPS as a Cosurfactant on ω_0 of NaDEHP System

From the study of NaDEHP microemulsion formation at 4 M NaCl with and without 15 mM CHAPS as a cosurfactant (Figure 4.1 and 4.3, respectively), both experiments formed two separated phases with the upper phase microemulsion or Winsor type II. On this account the ω_0 of the upper phase of both experiments were measured to assess the effect of CHAPS as shown in Figure 4.6. The significant increase of ω_0 was observed when CHAPS was added to NaDEHP microemulsion. This can be explained that when CHAPS was transferred into the oil phase the hydrophilic moiety of sulfonate group in CHAPS brought along water molecules. As the results, the ω_0 of the microemulsion then increased in the presence of CHAPS (Freeman *et al.*, 1998).

In the systems that the NaCl concentration was fixed at 0.2 M and the CHAPS concentration was varied from 0.0 to 50.0 mM, the amounts of water solubilized in the upper phase were analyzed as shown in Figure 4.7.

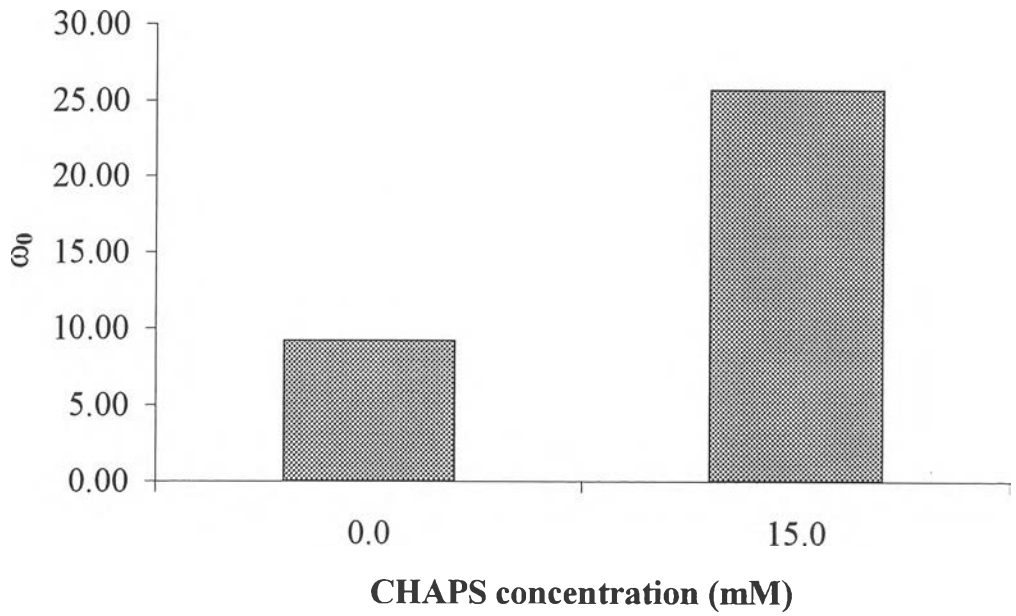

Figure 4.6 Effect of CHAPS as a cosurfactant on water content in the microemulsion of 0.1 M NaDEHP/isooctane/4 M NaCl/0.1 M NaOH/aqueous solution system.

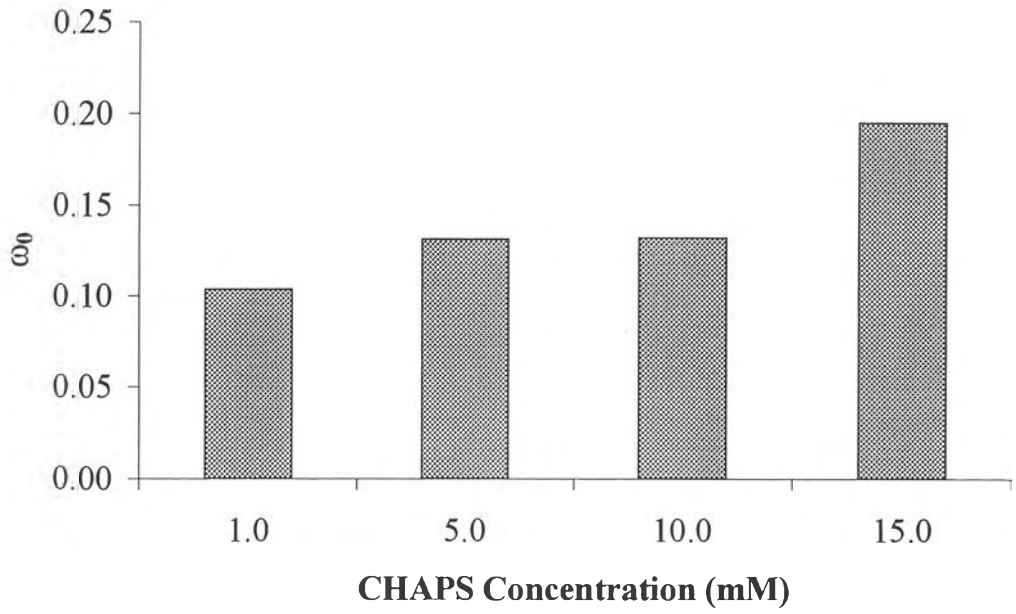

Figure 4.7 Effect of CHAPS concentration on water content in the microemulsion system of NaDEHP/CHAPS/isooctane/0.2 M NaCl/0.1 M NaOH/aqueous solution.

An increase in the concentration of CHAPS resulted in the increase of water solubilized in the organic phase. This is probably due to the capability of binding water molecules of the sulfonate group in CHAPS as discussed previously. As CHAPS concentration increased, more water molecules were bound and solubilized in the upper phase (Freeman *et al.*, 1998).

4.3 Size of the NaDEHP Microemulsion Systems

4.3.1 Effect of NaCl concentration on the size of NaDEHP microemulsion with TBP as a cosurfactant

In the presence of TBP, the NaCl concentration did not show significant effect on microemulsion size. The hydrodynamic radius (Rh) of the NaDEHP microemulsion with TBP as a cosurfactant at various NaCl concentrations (0.2-0.4 M) was in a range of 10-13 nm.

4.3.2 Effect of CHAPS on the size of the NaDEHP microemulsion

The hydrodynammic radius (Rh) of the microemulsion of NaDEHP using 4 M NaCl with and without 15 mM CHAPS as a cosurfactant were determined. It was found that Rh of the system that used CHAPS was in the range of 22 to 23 nm and Rh of the other system was in the range of 16 to 18 nm. As expected, this is directly related to the water content (ω_0) of the microemulsion. Same explanation can be offered here as the addition of CHAPS increases water binding in the organic phase, leading to the increase in the microemulsion size.

In the systems that the NaCl concentration was fixed at 0.2 M and the CHAPS concentration was varied from 1.0 to 15.0 mM, Rh was found to be relatively constant, indicating that CHAPS had a little effect on the size of microemulsion.

4.4 Extraction of α-Chymotrypsin using NaDEHP Microemulsion Systems with Different Cosurfactants

From several systems studied in section 4.1, a few systems were systematically selected for the study on the extraction of α-chymotrypsin. They are listed in Table 4.1. System 1 was the microemulsion containing 4 M NaCl without cosurfactant. System 2 with 0.1 M TBP and 0.2 M NaCl was selected since it showed best extraction efficiency and activity of α-chymotrypsin in previous study (Sooksomsin, 2002). System 1 and System 3 were used to determine the effect of CHAPS, two systems using same NaCl concentration at 4.0 M with and without 15.0 mM CHAPS. The effect of CHAPS concentration was investigated by varying concentration of CHAPS with fixed NaCl concentration at 0.2 M. These systems were used in the whole extraction process, which includes forward extraction, backward extraction, and activity test of recovered protein.

Table 4.1 The NaDEHP microemulsion systems used to study the extraction of α-chymotrypsin

System	Cosurfactant	NaCl concentration
1	-	4.0 M
2	0.1 M TBP	0.2 M
3	15 mM CHAPS	4.0 M
4	1 mM CHAPS	0.2 M
5	5 mM CHAPS	0.2 M
6	10 mM CHAPS	0.2 M
7	15 mM CHAPS	0.2 M

4.4.1 Forward Extraction

Forward extraction experiments were carried out by contacting 8 ml of the upper phase of NaDEHP microemulsion with equal volume of the aqueous phase of 0.5 mg/ml of α-chymotrypsin in 0.1 M NaCl/0.025 M Tris-HCl. After forward extraction, the protein was transferred into the excess organic phase. The remaining concentration of protein in aqueous phase was determined by using UV/VIS spectrophotometer at 281 nm and used for the calculation of the forward extraction efficiency (%) by the following equation. The extraction efficiency was presented in Table 4.2.

$$\%\,Forward = \frac{[protein]_i - [protein]_f}{[protein]_i} * 100 \qquad (4.3)$$

Where:

% Forward = Amount of protein being extracted into reverse micellar phase

$[protein]_i$ = Protein concentration in aqueous phase before forward extraction

$[protein]_f$ = Protein concentration in aqueous phase after forward extraction

Table 4.2 The %Forward Extraction by Using NaDEHP Microemulsion Systems

System	Cosurfactant	NaCl	Forward Extraction (%)
1	-	4.0 M	0.00
2	0.1M TBP	0.2 M	71.05
3	15mM CHAPS	4.0 M	8.71
4	1mM CHAPS	0.2 M	9.38
5	5mM CHAPS	0.2 M	28.39
6	10mM CHAPS	0.2 M	31.58
7	15mM CHAPS	0.2 M	2.88

The NaDEHP microemulsion system using TBP as a cosurfactant gave the highest efficiency of forward extraction whereas the microemulsion system at high NaCl concentration (4.0 M) with no addition of cosurfactant presented the lowest extraction efficiency (0%). This is due to the effect of salt, which causes the shielding of electrostatic interaction between micellar wall and protein, resulting in low extraction as seen in System 1 and System 3. However, the system containing 4 M NaCl with 15 mM CHAPS yielded approximately 9% extraction, which was better than the system without cosurfactant. With various CHAPS concentrations used in the microemulsion systems containing 0.2 M NaCl, the system using 10.0 mM CHAPS gave the best extraction efficiency (approximately 32%).

4.4.2 Backward Extraction

The backward extraction was performed to recover and concentrate the protein by contacting α-chymotrypsin loaded reverse micellar organic phase with 0.1 M $CaCl_2$ aqueous solution. This method is based on intrinsic properties of NaDEHP, the alkali salts of HDEHP that are surface active and easily form reverse micelles. Nevertheless, the divalent metal, Ca^{2+} can react with two NaDEHP to form a stable complex of $Ca(DEHP)_2$ which are not surface active as shown below (Hu and Gularli, 1996):

$$Ca^{2+} + 2\ NaDEHP \longrightarrow Ca(DEHP)_2 + 2\ Na^{+} \qquad (4.4)$$

Therefore, the reverse micelles of NaDEHP can readily be broken by converting the surfactant, NaDEHP, to non-surface active divalent salt, $Ca(DEHP)_2$. Consequencely, the protein in the reverse micellar phase is released back to the aqueous phase.

From 7 systems in Table 4.2, only 2 systems were found to be able to release the extracted protein to the aqueous phase. These systems are System 2 and System 6, which gave 74.4% and 3.5% extraction of α-chymotrpysin, respectively. The recovery of α-chymotrypsin obtained from the system of NaDEHP microemulsion with TBP cosurfactant was quite similar to that reported in the

previous work (Sooksomsin, 2002). On the other hand, the recovery obtained from the system with CHAPS was only 3.5%. This small recovery might be due to the attractive interaction between the sulfonate group in CHAPS which presents its negatively charge and the positively charge at the protein interface. Consequently, once protein is extracted into the microemulsion, it is difficult to release it back to aqueous phase by contacting with solution containing divalent cation (Ca^{2+}).

The evidence that protein was transferred to the upper phase of the microemulsion in forward extraction and recovered back to the second aqueous phase in backward extraction can be supported by the measurement of water in the upper phase during the extraction, represented by the water to surfactant ratio, ω_0, of the system. Figures 4.8 and 4.9 show the ω_0 during the extraction of protein in System 2 and System 6, respectively.

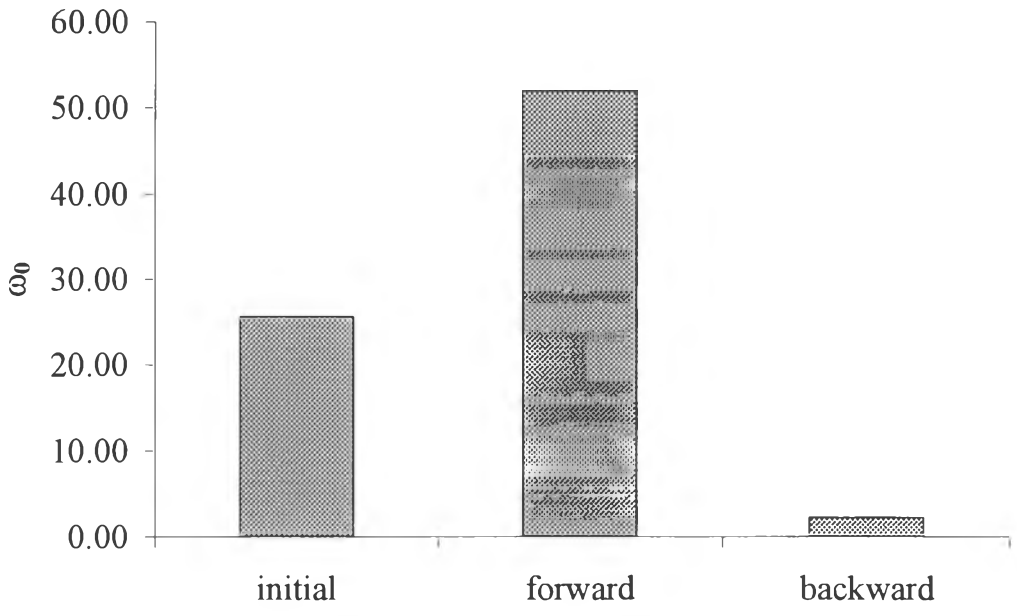

Figure 4.8 ω_0 during the enzyme extraction using the microemulsion of 0.1 M NaDEHP/0.1 M TBP/isooctane/0.2 M NaCl/0.1 M NaOH/aqueous solution.

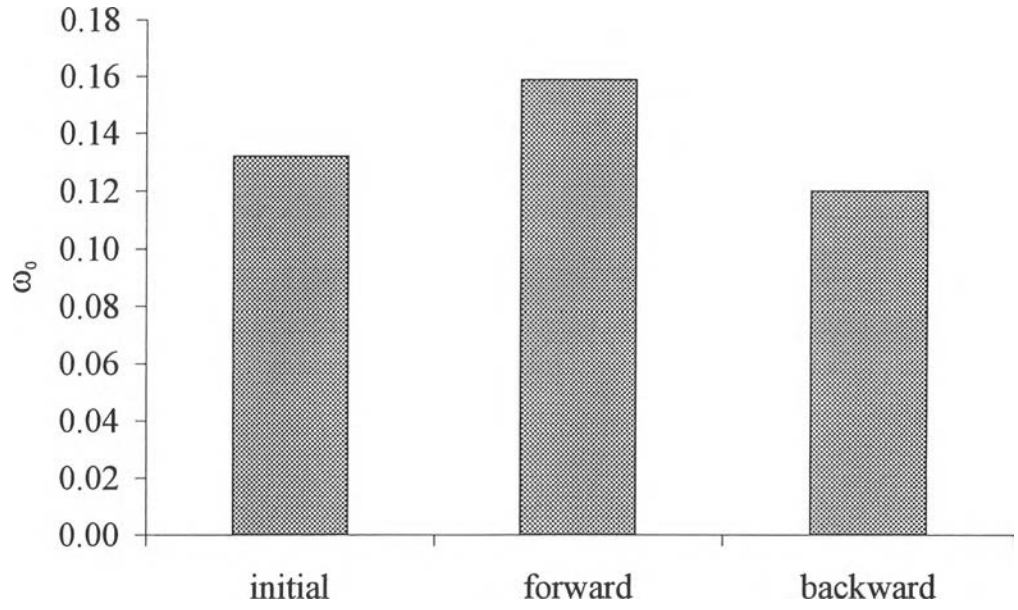

Figure 4.9 ω_0 during the enzyme extraction using the microemulsion of 0.1 M NaDEHP/10 mM CHAPS/isooctane/0.2 M NaCl/0.1 M NaOH/aqueous solution.

According to Figure 4.8, after forward extraction the protein was transferred to the upper phase, resulting in the increase in ω_0. Upon adding $CaCl_2$ solution to perform the backward extraction, the extracted protein was transferred back to the aqueous phase, leading to the decrease in ω_0. The microemulsion size was also measured and used to support the release of protein. The hydrodynamic radius (Rh) of the microemulsion with TBP cosurfactant was increased from 13.4 to 47.5 nm after forward extraction and decreased significantly to 2.3 nm after backward extraction. The decrease in ω_0 and Rh confirmed that the microemulsion with TBP cosurfactant were broken. In the extraction using the microemulsion system with 10 mM CHAPS as a cosurfactant (Figure 4.9), the Rh was increased from 0.4 to 1.4 nm after the transfer of protein into the upper phase and after backward extraction ω_0 of this system was relatively unchanged. Moreover, the low recovery and the small change in ω_0 were observed after backward extraction of this system. Therefore, the backward technique using divalent metal ion appeared to be not suitable for the microemulsion with CHAPS in this work.

4.5 Enzymatic Activity Test

The enzymatic activity of α-chymotrypsin was determined by using a simple hydrolysis reaction of (GPNA) to *p*-nitroaniline as shown here:

$$\alpha\text{-chymotrypsin} + \text{GPNA} \longrightarrow p\text{-nitroaniline (yellow)} \qquad (4.5)$$

The reaction was carried out by mixing 1.0 ml of α-chymotrypsin with 5.0 ml of GPNA as a substrate and allowed to proceed for 10 minutes. Usually an acetic acid was added to stop reaction after 10 minutes. *p*-nitroaniline produced from the reaction was quantified by UV/VIS spectrophotometer at 365 nm. The activity of the enzyme was presented as the initial rate of hydrolysis reaction, which is denoted as the slope of the linearity. The activity of recovered α-chymotrypsin were compared with fresh protein and reported as %Activity by the equation shown below:

$$\%\, Activity = \frac{rate_{\,recovered}}{rate_{\,fresh}} * 100 \qquad (4.6)$$

Where:

% Activity = Activity of recovered protein compared with fresh protein

$rate_{recovered}$ = rate of *p*-nitroaniline produced by recovered protein

$rate_{fresh}$ = rate of *p*-nitroaniline produced by fresh protein

Figure 4.10 shows the activity of fresh protein as a function of time. The initial rate of hydrolysis reaction presented by fresh protein was illustrated as the slope at the beginning of linear plot between *p*-nitroaniline produced with the reaction time. The hydrolysis rate was highest at the beginning, and then decreased gradually and became relatively constant after approximately 4-5 minutes.

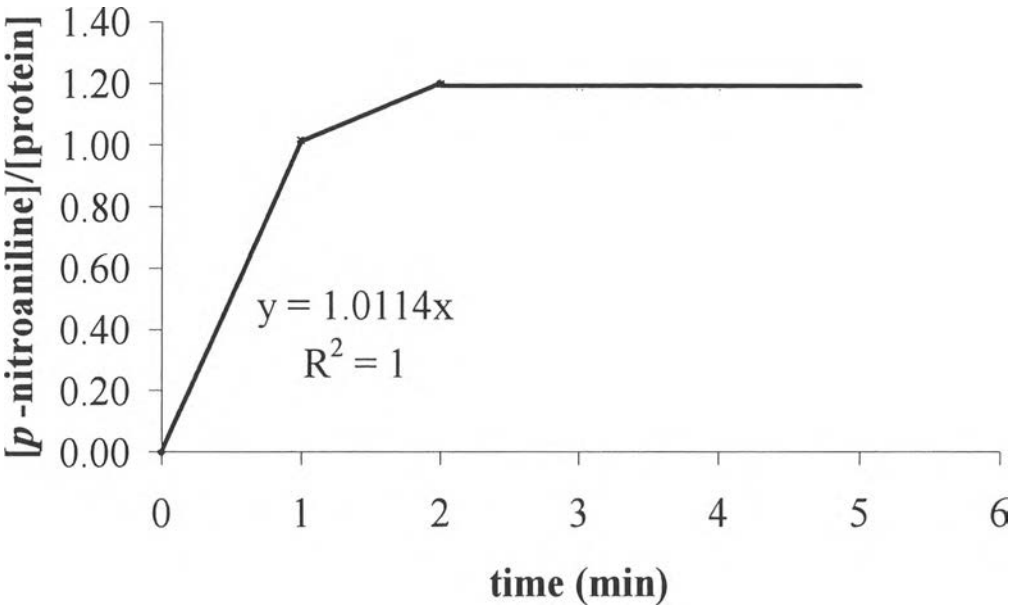

Figure 4.10 The activity of fresh α-chymotrypsin which is represented by the amount of *p*-nitroaniline (ppm) produced by hydrolysis reaction of GPNA.

The activity of recovered protein from the system with TBP and the system with CHAPS were measured in the same manner as the fresh protein. The results are shown in Figures 4.11 and 4.12, respectively.

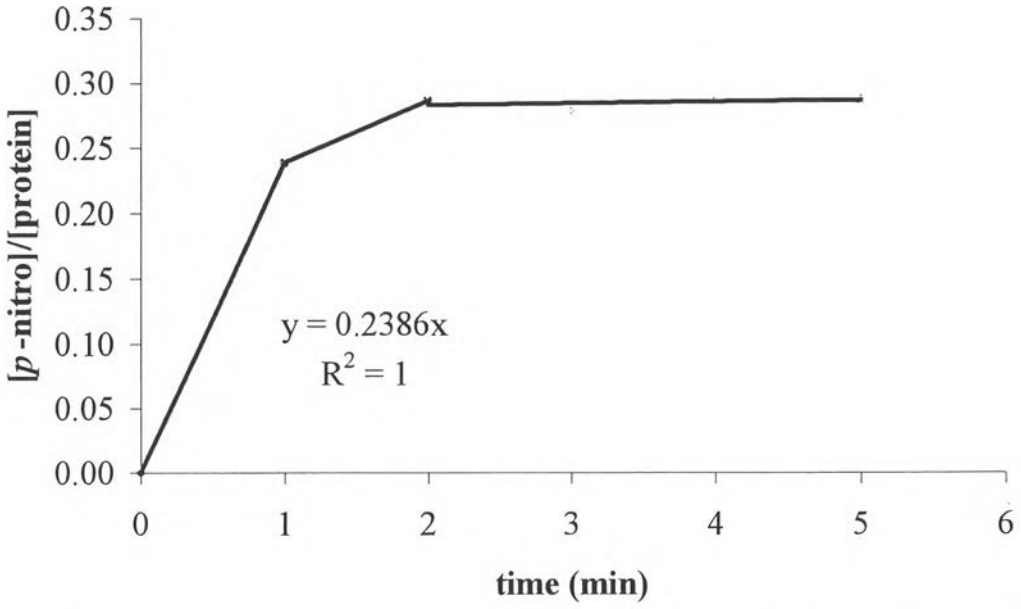

Figure 4.11 The activity of recovered α-chymotrypsin from System 2 (with 0.1 M TBP).

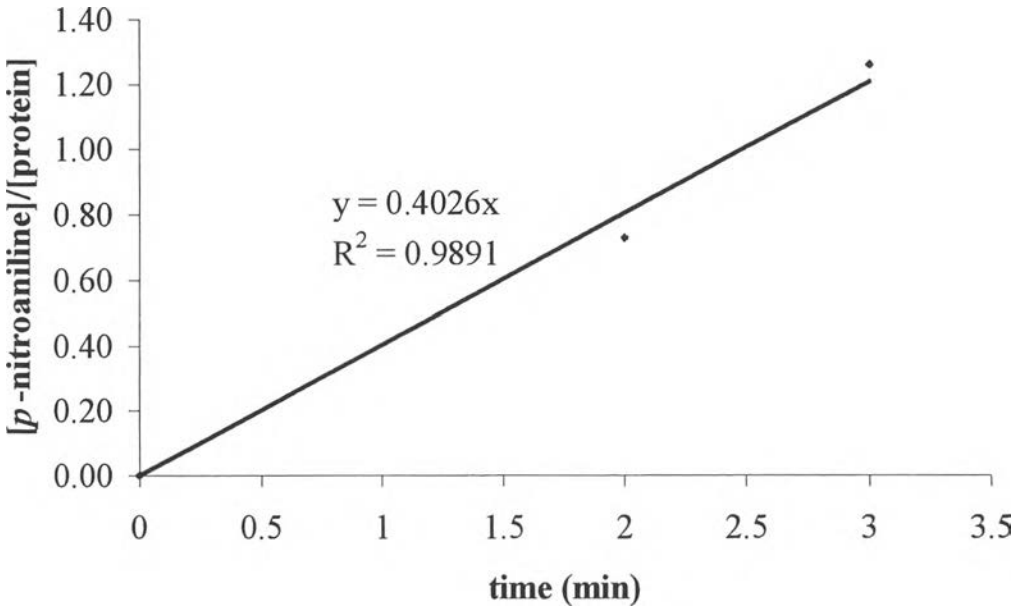

Figure 4.12 The activity of recovered α-chymotrypsin from System 6 (with 10.0 mM CHAPS).

From the calculation using equation 4.6 the % Activity of the recovered protein from the system with TBP was found to be approximately 24% as compared to the fresh protein whereas the recovered from the system with CHAPS presented approximately 40% as compared to the fresh protein. The high activity of that recovered from the system with CHAPS cosurfactant is expected to be due to the effect of bile salt which can modify the interfacial properties of microemulsion. This may result in more organized aggregate structure, the decreases of polarity experienced of the protein at the interface of microemulsion and more active enzyme conformation (Freeman *et al.*, 2000). In natural environment such as in living organism, bile salt always found in the same location as the α-chymotrypsin located, therefore, the enzyme can retain its function in CHAPS environment without denaturation.