CHAPTER IV
RESULTS AND DISSCUSSION

4.1 Preparation of NaDEHP Microemulsions

4.1.1 NaDEHP Microemulsion Formation in the Absence of Cosurfactant

NaDEHP is formed during the mixing of the organic and the aqueous solutions. The reaction can be represented as:

$$HDEHP + NaOH \longrightarrow NaDEHP + H_2O$$

The NaDEHP/isooctane system was prepared by mixing 10 ml of 0.1 M HDEHP/isooctane with 10 ml of aqueous phase (0.1 M NaOH and NaCl). Without adding NaCl to the aqueous phase, NaDEHP formed Winsor type I and after adding NaCl into the system the phase transition is shown in Figure 4.1. At salt concentration 0.1 M in the aqueous phase, the system was still Winsor type I. When increasing the salt concentration from 0.1 to 0.5 and 1.0 M, the system was separated into three phases or Winsor type III where the transparent phase was formed in the middle between the oil and the aqueous phases. When NaCl was further increased from 1.0 to 4.0 M, the system was transformed into two phases or Winsor type II with the organic as the upper phase.

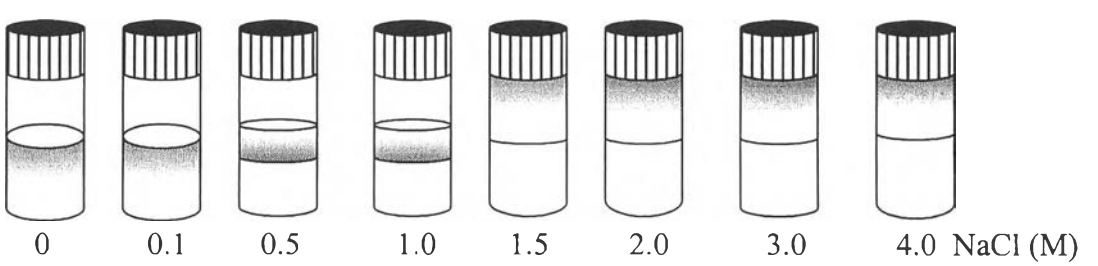

Figure 4.1 Phase transition of the 0.1 M NaDEHP/isooctane/NaCl/0.1 M NaOH.

4.1.2 NaDEHP Microemulsion Formation with Tributyl Phosphate (TBP) as a Cosurfactant

The NaDEHP/TBP/isooctane system was prepared by mixing 10 ml of 0.1 M HDEHP/0.1 M TBP/isooctane with equal volume of 0.1 M NaOH at various NaCl concentrations (0.1-3.0 M). At NaCl concentration of 0.1 M the system was Winsor type I and after increasing salt concentration from 0.1 M to 0.2 M and higher, the system was separated into two phases with the organic in the upper phase or Winsor type II as shown in Figure 4.2.

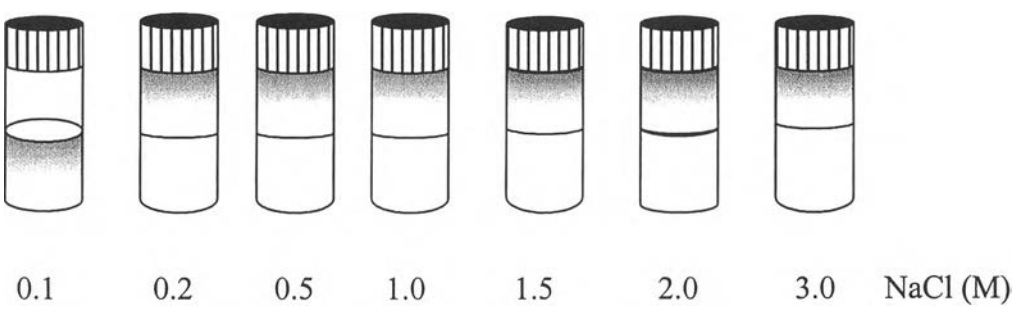

Figure 4.2 Phase transition of the 0.1 M NaDEHP/0.1 M TBP/isooctane/NaCl/0.1 M NaOH.

4.1.3 NaDEHP Microemulsion Formation with 2-Ethyl-1-Hexanol as a Cosurfactant

The NaDEHP/2-ethyl-1-hexanol/isooctane system was prepared by mixing 10 ml of 0.1 M HDEHP/0.1 M 2-ethyl-1-hexanol/isooctane with equal volume of 0.1 M NaOH at various NaCl concentrations (0.1- 3 M). For all NaCl concentrations, the system was separated into two transparent phases or Winsor type II as shown in the Figure 4.3.

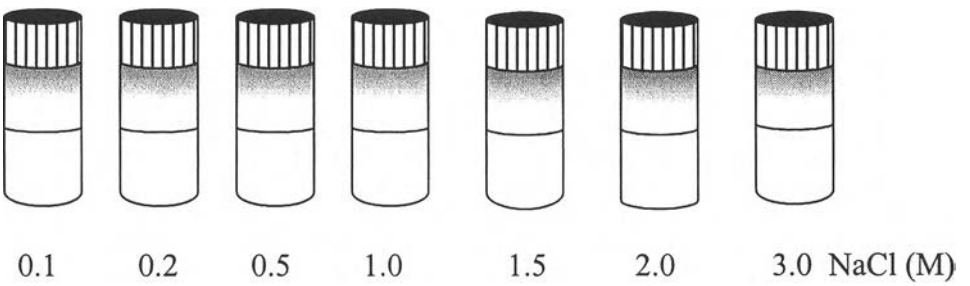

Figure 4.3 Phase transition of the 0.1 M NaDEHP/2-ethyl-1-hexanol/isooctane/ NaCl/0.1M NaOH.

4.1.4 NaDEHP Microemulsion Formation with 1-Heptanol as a Cosurfactant

The NaDEHP/1-hepanol/isooctane solution system was prepared by mixing 10 ml of 0.1 M HDEHP/0.1 M 1-hepanol/isooctane with equal volume of 0.1 M NaOH at various NaCl concentrations (0.1- 3.0 M). For all of NaCl concentrations, the system is separated into two transparent phases or Winsor type II as shown in the Figure 4.4.

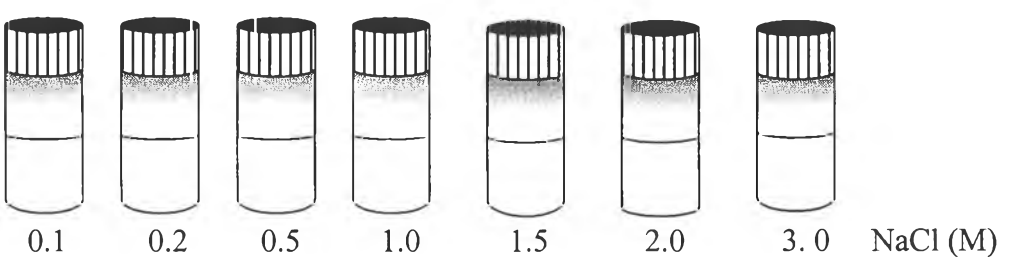

Figure 4.4 Phase transition of the 0.1 M NaDEHP/1-heptanol/isooctane/NaCl /0.1M NaOH.

The results suggest that all three cosurfactants can shift the Hydrophile-Lipophile Balance (HLB) of surfactant NaDEHP in the system toward lipophilic, the hydrophile-lipophile property of anioninic surfactants could be balanced to form w/o microemulsion easier than without adding cosurafctant.

4.2 Water Content in the NaDEHP Microemulsions

Water content of microemulsion the oil phase was measured by coulometer (Karl Fischer titration technique) and represented by ω_o as described by this equation:

$$\omega_o = \frac{[\text{Water}]}{[\text{Surfactant}]}$$

[Water] = Water concentration in oil phase (M)

[Surfactant] = Surfactant concentration in oil phase (M)

4.2.1 NaDEHP System with Tributyl Phosphate (TBP) as a Cosurfactant

Figure 4.5 shows the effect of salt concentration on the water content in reverse micelle of NaDEHP/TBP/isooctane. At 0.1 M NaCl, the system exhibited two phases but the upper phase was slightly cloudy and at this salt concentration the upper phase appeared to be macroemulsion. When salt concentration in the system was increased to 0.2 M, the water content in the upper organic phase dramatically decreased as the system became Winsor type II. Further increasing in salt concentration to 4 M caused little decrease in the water content in reverse micelles.

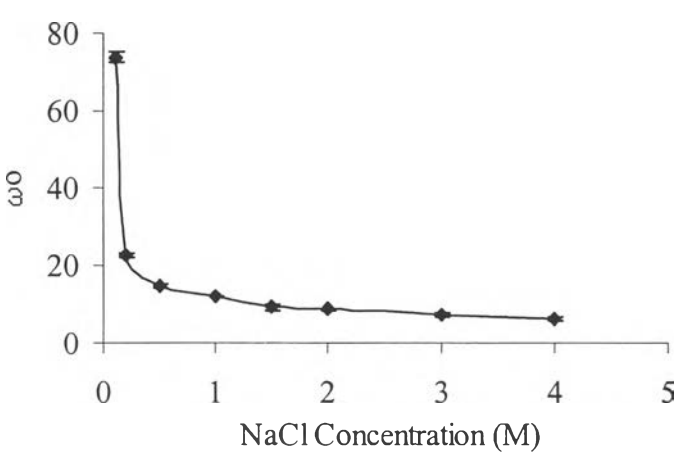

Figure 4.5 Effect of salt concentration on water content in reverse micelle of NaDEHP/TBP/isooctane.

4.2.2 NaDEHP System with 2-Ethyl-1-Hexanol as a Cosurfactant

Figure 4.6 shows the effect of salt concentration on the water content (ω_o) in reverse micelles of NaDEHP/2-ethyl-1-hexanol/isoctane. Similar results to the system when TBP was used as a cosurfactant were observed. ω_o was highest (~ 26) at the lowest salt concentration used in this study (0.2 M). When salt concentration in the system was increased from 0.2 to 0.5 M, the water content in reverse micelles dramatically decreased. Further increasing salt concentration from 0.5 to 4 M caused little decrease in the water content in reverse micelles.

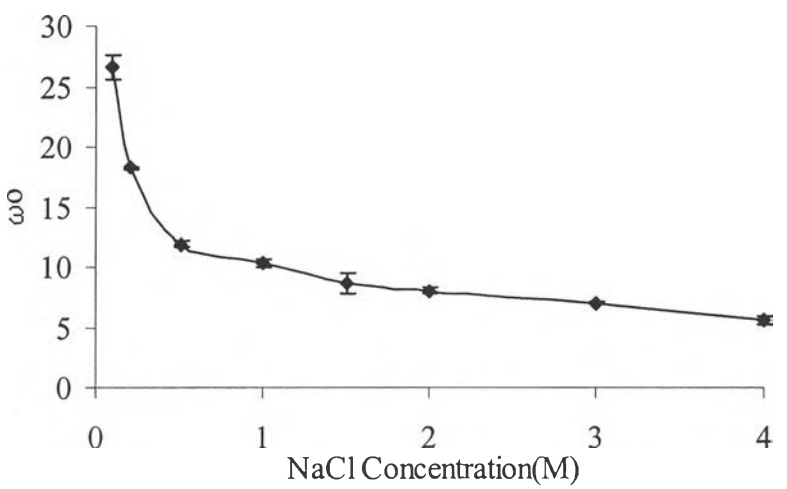

Figure 4.6 Effect of salt concentration on water content in reverse micelle of NaDEHP/2-ethyl-1-hexanol/isooctane.

4.2.3 NaDEHP System with 1-Heptanol as a Cosurfactant

Figure 4.7 shows the effect of salt concentration on water content (ω_o) in reverse micelles of NaDEHP/1-heptanol/isoctane. Similar results to the system when TBP was used as a cosurfactant was observed. ω_o was highest (~ 22) at the lowest salt concentration used in this study (0.2 M). When salt concentration in the system was increased from 0.2 to 0.5 M, the water content in reverse micellse sharply decreased and at higher salt concentrations the water content of reverse micelle slightly decreased even up to the salt concentration of 2 M.

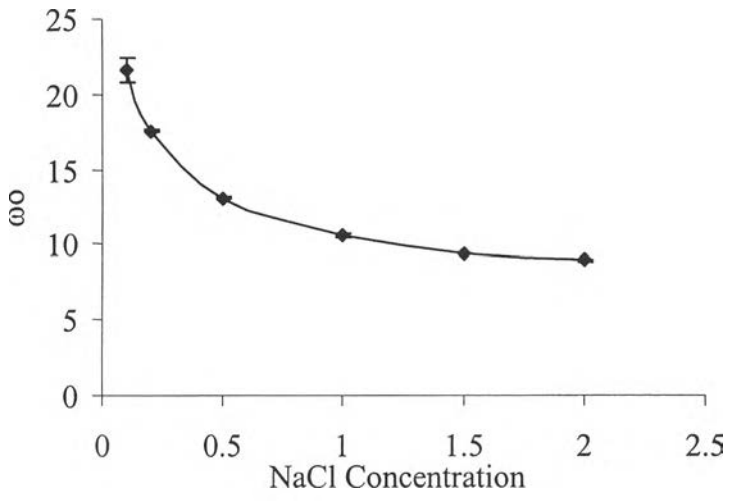

Figure 4.7 Effect of salt concentration on water content in reverse micelle of NaDEHP/1-heptanol/isooctane.

It is worth noting that the water content (ω_o) in the reverse micelle of NaDEHP/isooctane brine system with TBP as a cosurfactant was higher than the systems with the other two cosurfactants at any salt concentration. For example, at salt concentration of 0.5 M the water contents in the reverse micelles are 15, 12, and 12 for NaDEHP systems with TBP, 2-ethyl-1-hexanol, and 1-heptanol, respectively.

4.3 Extraction of α-Chymotrypsin using NaDEHP Reverse Micellar Systems

The NaDEHP reverse micellar system with TBP as a cosurfactant was used as a base case in this part of the study. Using this base case, effects of various system parameters on the extraction efficiency and the activity of the recovered protein were first examined. Subsequently, effect of type of cosurfactants was then studied using 2-ethyl-1-hexanol or 1-heptanol as a cosurfactant instead of TBP.

4.3.1 Forward Extraction

Forward extraction experiments were carried out by contacting 8 ml of the reverse micellar phase of 0.1 M NaDEHP/0.1 M TBP/isooctane/0.2 M NaCl/0.1 M NaOH with equal volume of aqueous phase of 0.5 mg/ml of α-chymotrypsin in

0.1 M NaCl/0.025 M Tris-HCl. After forward extraction protein concentration in an aqueous phase was measured the remaining concentration by using UV/VIS spectrophotometer at 281 nm and calculated the percentage of forward extraction as shown in appendix C1.

4.3.1.1 Effect of pH on forward extraction

Using the system mentioned above, effect of pH on forward extraction of α-chymotrypsin was studied by varying pH of the aqueous phase using HCl or NaOH. The results are shown in Figure 4.8. It can be seen that the extraction efficiency as represented by the percentage of protein transferred into the reverse micelles (or %extraction) is a strong function of pH of the aqueous phase. This can be explained that the electrostatic interactions between α-chymotrypsin and the NaDEHP head groups can favor the transfer of protein into organic phase. The pH of the aqueous solution actually determines the net charge of α-chymotrypsin. The isoelectric point (pI) of α-chymotrypsin is 8.5 and thus at pH above this pI value, the surface of protein becomes negatively charged. Therefore the transfer of protein dramatically decreases at pH above 8.5 due to the reduced electrostatic attraction.

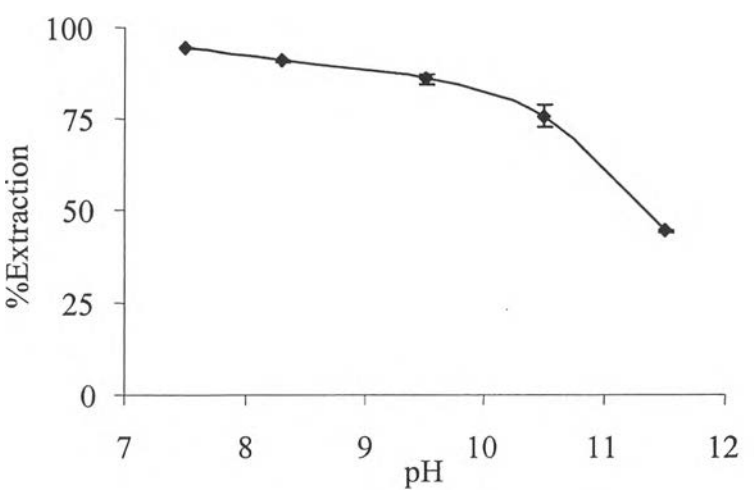

Figure 4.8 Effect of pH of the aqueous phase on forward extraction.

Figure 4.9 shows the comparison of the hydrodynamic radii (Rh) of reverse micelles before and after forward extraction. The hydrodynamic radius (Rh)

of reverse micelles in the organic phase was measured by using dynamic light scattering technique. After forward extraction, protein was transferred from aqueous phase to oil phase and stabilized in water-pool of reverse micelles resulting in an increase in size of the reverse micelles. Consequently, the results shown in Fig. 4.9 confirm the transfer of α-chymotrypsin into the reverse micelles.

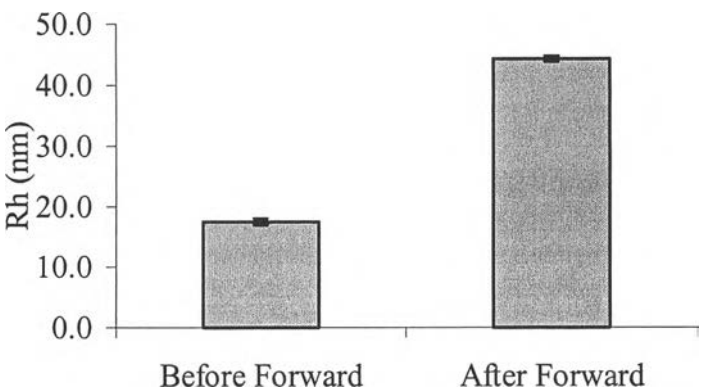

Figure 4.9 Hydrodynamic radius (Rh) of reverse micelle before and after forward extraction.

4.3.1.2 Effect of salt concentration in aqueous phase

Figure 4.10 shows the % extraction of the protein as a function of salt concentration in the aqueous phase. In this study, the pH of the aqueous solution was kept constant at 7.5 which is the pH value that gives the highest efficiency in the forward extraction (Fig. 4.8). It is obvious that the transfer of the protein into the reverse micelles is strongly dependent on the salt concentration. Increasing salt concentration in the aqueous phase causes a significant reduction in the extraction efficiency. It is attributed to the primary effect of salt which is through shielding of electrostatic attraction (or Debye screening effect) between micellar wall of surfactant and protein. At higher ionic strengths, the interactions between molecules of protein and the NaDEHP polar headgroups were reduced. As a consequence, the amount of water contained in the reverse micelles was decreased, leading to a decrease in the amount of protein being transferred into the reverse

micelles. In other words, the transfer of the protein was limited by the water content or the water pool in reverse micelles and size of the reverse micelles.

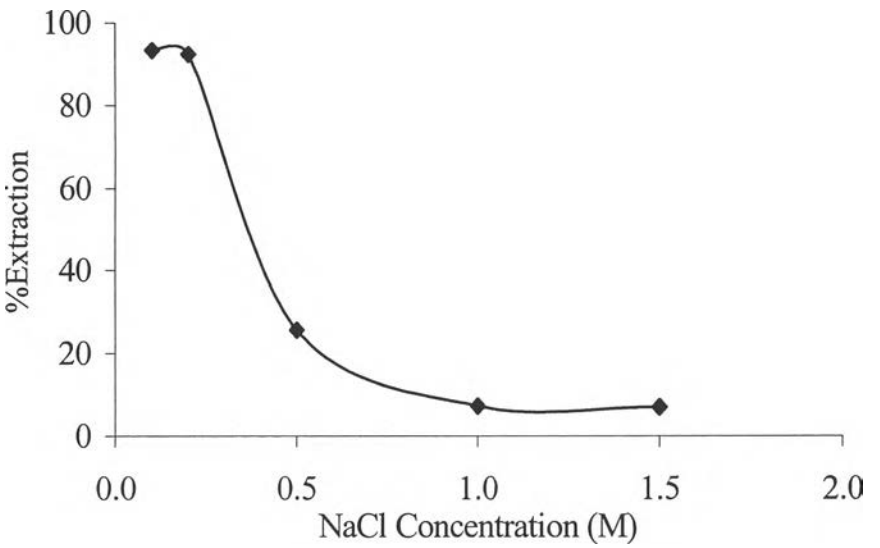

Figure 4.10 Effect of salt concentration in the aqueous phase on the forward extraction.

4.3.1.3 Effect of protein concentration on forward extraction

In order to examine whether protein concentration has any effect on the extraction efficiency, two more protein concentrations (0.1 and 1 mg/ml) were studied in comparison to the protein concentration previously used (0.5 mg/ml). The experiments were carried out in the same manner but with different protein concentrations in the aqueous phase. Figure 4.11 shows the % extraction of α-chymotrypsin as a function of protein concentration. When the initial protein concentration in the aqueous phase was 0.5 mg/ml, the extraction percentage was highest. Approximately 93% of the protein were transferred to the reverse micelles in the oil phase. When the initial protein concentration was decreased to 0.1 mg/ml, the %extraction was slightly lower than that of at 0.5 mg/ml. Similar trend was observed when the protein concentration was increased to 1.0 mg/ml where only 88% extraction was observed. These results were also supported by the results obtained from the hydrodynamic radius measurements as shown in Table 4.1. The largest size of the reverse micelles (~44 nm) among these three cases was observed

when 0.5 mg/ml protein concentration was used, confirming the higher amount of protein transferred into the reverse micelles.

Actually, increasing the initial protein concentration should increase the driving force between charge polar heads of the surfactant and opposite of protein molecules. The results observed here indicated that, besides the driving force resulting from the electrostatic interaction, the ratio of the surfactant to the amount of transferred protein may also be the factor affecting the extraction of protein into the reverse micelles (Pires *et al.*, 1996).

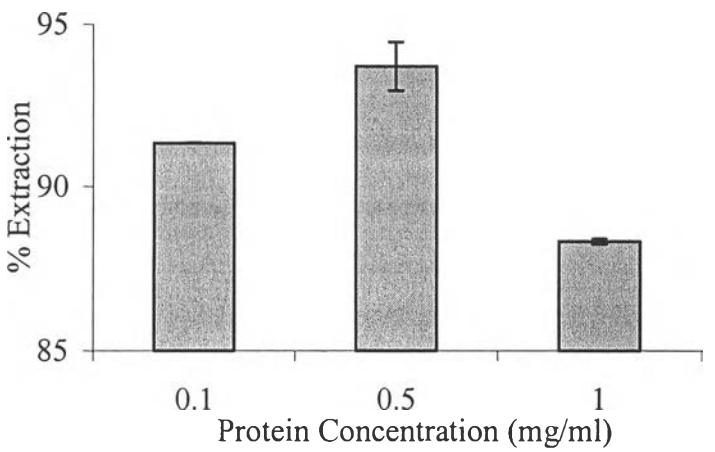

Figure 4.11 Effect of protein concentration in the aqueous phase to the forward extraction.

Table 4.1 Effect of protein concentration in the aqueous phase on hydrodynamic radius of reverse micelles.

Protein concentration (mg/ml)	Size (nm)	
	Before Forward	After Forward
0.1	17.53±0.54	41.23±0.29
0.5	17.53±0.54	44.3±0.43
1	17.53±0.54	37.9±0.14

4.3.2 Backward Extraction

Upon the forward extraction, the organic phase containing α-chymotrypsin solubilized in the reverse micelles was obtained. The next step is to recover and concentrate the protein in a new aqueous phase which is called backward extraction. The backward extraction was carried out by contacting 0.1 M $CaCl_2$ with α-chymotrypsin loaded into reverse micelle. This method is based on the intrinsic properties of NaDEHP. The alkali salts of HDEHP are surface active and easily form reverse micelles. Nevertheless, the divalent metal salts, $M(DEHP)_2$ are not surface active and each calcium cation Ca^{2+} reacts with two NaDEHP molecules and form a stable complex of $Ca(DEHP)_2$ as shown in the equation.

$$Ca^{2+} + 2\ NaDEHP \longleftrightarrow Ca(DEHP)_2 + 2\ Na^{+}$$

Therefore, the reverse micelle of NaDEHP can readily be broken down by converting the surfactant, NaDEHP, to a non-surface active divalent salt, $M(DEHP)_2$. Consequently, the protein in the micellar phase is released back to aqueous phase (Hu and Gulari, 1996).

4.3.2.1 Effect of salt concentration on backward extraction

Figure 4.12 shows the effect of salt concentration on the percentage of backward extraction. The results reveal that the salt concentration in the aqueous phase during forward extraction has essentially no effect on the backward extraction. An average extraction percentage in the backward extraction step was found to be approximately 70% at any salt concentration. This can be expected since Na^+ ions from the aqueous phase in the forward extraction stay only in the water pool inside the reverse micelles and thus should have no effect on the recovery step according to the mechanism of the backward extraction described above. It can also be seen from Fig. 4.12 that the average recovered protein of 70% indicates that not all protein being extracted into the reverse micelles were recovered in the backward extraction using this technique. It is speculated that a portion of extracted protein was still bound to the surfactant molecules and stayed in the organic phase. In addition, some proteins may be precipitated with the surfactant

molecules at the interface, resulting in white precipitates as observed in this study and the previous study (Hu and Gulari, 1996).

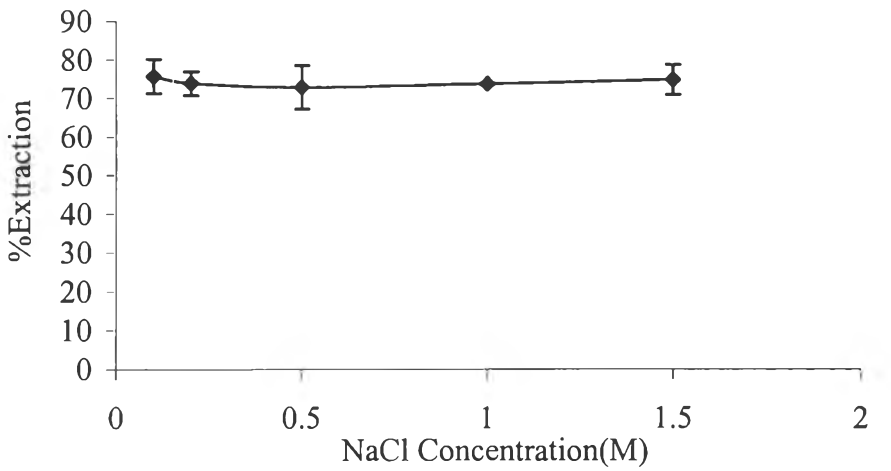

Figure 4.12 Effect of salt concentration on the backward extraction.

Figure 4.13 shows the comparison of the water contents in the reverse micelles before and after backward extraction. It can be seen that the water content in the upper phase dramatically decreased after backward extraction.

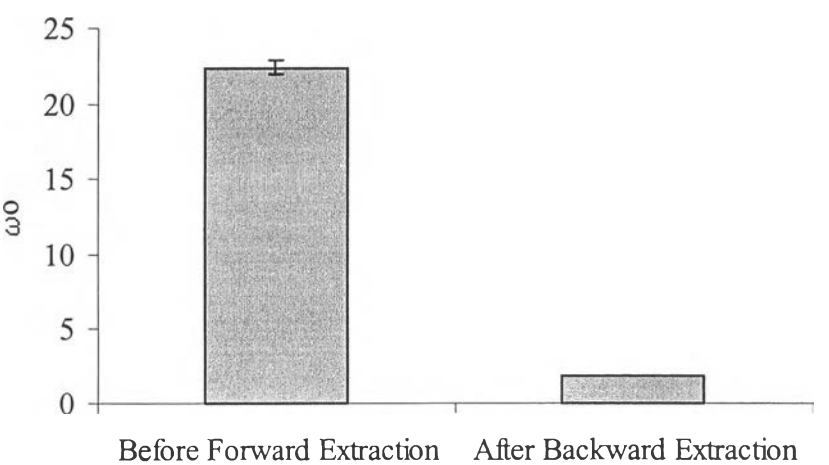

Figure 4.13 Water contents in the upper phase before and after backward extraction.

The results clearly show that after backward extraction the reverse micelles existing in the upper organic phase were broken down upon contacting with $CaCl_2$ solution as previously described. This leads to the breakage of the water-pool in the reverse micelles and thus releasing the extracted protein into the second aqueous phase.

4.3.2.2 Effect of protein concentration in aqueous phase

Table 4.2 shows the percentages of backward extraction with different initial concentrations of protein. From Fig. 4.11, the difference in the initial protein concentration resulted in different amount of protein transferred into the reverse micelles. It is interesting to learn that similar results were observed in the backward extraction step as seen in forward extraction step. Among three initial protein concentrations studied, optimal protein concentration was again found to be 0.5 mg/ml, which gave the highest percentage during backward extraction (74%).

Table 4.2 The effect of protein concentration on the backward extraction.

Protein Concentration (mg/ml)	Backward Extraction (%E)
0.1	39.12±8.82
0.5	74.16±4.44
1	61.39±7.84

4.3.3 Enzymatic Activity Test

4.3.3.1 Activity measurements

The enzymatic activity of α-chymotrypsin was determined by hydrolysis reaction of GPNA to *p*-nitroaniline as shown below:

α-chymotrypsin + GPNA ⟶ *p*-nitroaniline (yellow)

The reaction was carried out by mixing 0.5 ml of fresh α-chymotrypsin with 2.5 ml of the substrate GPNA. After 10 minutes, the reaction was put to its end by addition of acetic acid. *p*-nitroaniline produced from the reaction which has yellowish color, can be quantified by using UV/VIS spectrophotometer at 410 nm. Figure 4.14 presents the calibration curve of *p*-nitroaniline as measured by absorbance at 410 nm.

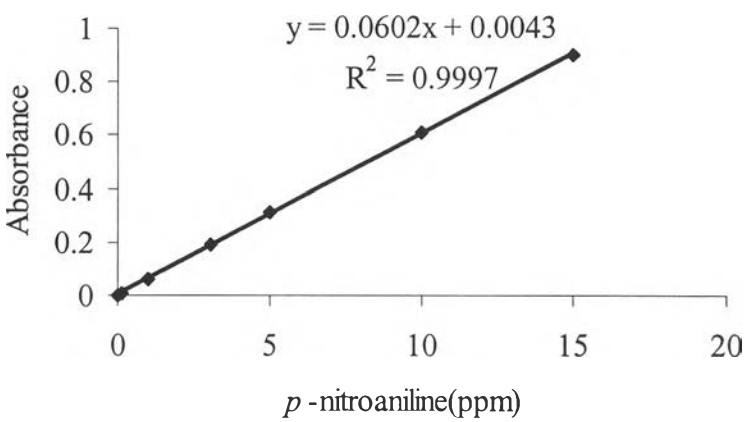

Figure 4.14 Calibration curve of *p*-nitroaniline at λ_{410} nm.

Once the product *p*-nitroaniline can be measured accurately, the direct correlation between protein concentration and *p*-nitroaniline formed by hydrolysis reaction at a fixed GPNA concentration can be generated. Figure 4.15 presents the relationship between the absorbance at 410 nm of *p*-nitroaniline produced in the reaction solution and a corresponding concentration of a fresh α-chymotrypsin. With a known α-chymotrypsin concentration, the enzymatic activity of the recovered protein from the extraction can be determined.

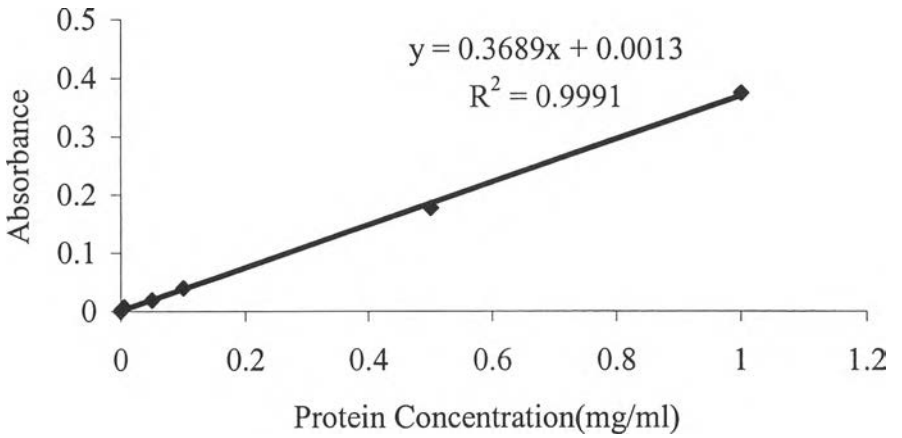

Figure 4.15 Calibration curve for the activity test of the fresh α-chymotrypsin.

After backward extraction, protein loaded into reverse micelles was transferred back to the new aqueous phase and the activity of the recovered protein was measured using the method described above. The activity of the recovered protein was then compared with that of the fresh α-chymotrypsin and reported as % Activity as shown below:

$$\% \text{ Activity} = \frac{(\textit{p-nitroaniline}) \text{ after recovery}}{(\textit{p-nitroaniline}) \text{ of fresh protein}} \times 100$$

(*p-nitroaniline*) = concentration of *p-nitroaniline* (ppm) as a product after hydrolysis reaction

4.3.3.2 Effect of salt concentration on enzymatic activity

Figure 4.16 shows the activity of α-chymotrypsin after backward extraction at various salt concentrations in the aqueous phase. The highest activity of the recovered α-chymotrypsin was found to be approximately 60% at salt concentration of 0.5 M. The average enzymatic activity of the recovered protein was about 50% of the fresh one which indicates that some of recovered proteins had lost their activity in the extraction process. It may be a result of the interactions between

surfactant molecules in the reverse micelles and the protein molecules being extracted into the micelles. While protein molecules were being trapped in the reverse micelles, the surrounding surfactant molecules forming the reverse micelles may form hydrogen bonding with the protein molecules, thus disturbing the secondary structure of the protein. Consequently, this would cause a permanent denaturation of the protein. On the other hand, the surfactant may only adsorb onto the active sites of the protein molecule, leading to a non-permanent denaturation of the protein. The results also reveal that salt concentration in aqueous phase used in the forward extraction remarkably affects the enzymatic activity of α-chymotrypsin being extracted into the reverse micelles. Furthermore, it has been reported that the activity of recovered protein also depends on the condition of forward extraction, especially salt concentration in the aqueous phase. Na^+ ions in water-pool can bind to or block the active sites of the protein in the reverse micelles, causing a reduction in the activity of the protein after extraction (Marcozzi *et al.*, 1991).

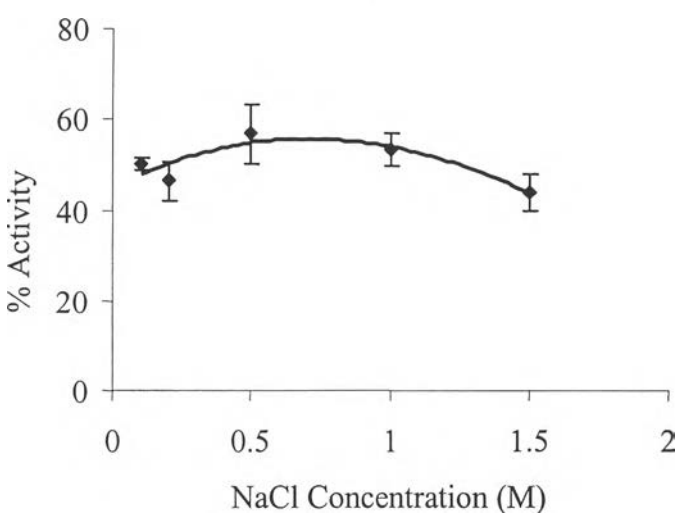

Figure 4.16 Effect of salt concentration in the aqueous phase on the activity test.

4.3.3.3 Effect of protein concentration on enzymatic activity

Figure 4.17 shows the effect protein concentration in aqueous phase on the enzymatic activity of α-chymotrypsin. It can be seen that the protein concentration in aqueous phase during the forward extraction has significant effect on the activity of the extracted protein. High enzymatic activity of the recovered

protein was observed at higher protein concentrations with the activity being highest at 1.0 mg/ml of initial protein concentration. It is not easy to explain these results without further experimentation studies.

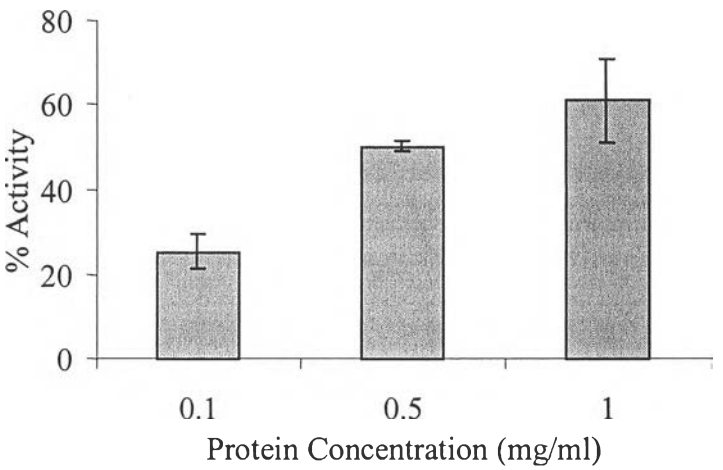

Figure 4.17 Effect of protein concentration in aqueous on activity test.

4.3.4 Effect of Type of Cosurfactants

The previous parts of the study deal with the NaDEHP systems with TBP as a cosurfactant. It is interesting to further examine whether type of cosurfactant has any effect on the extraction efficiency and activity of the protein. Therefore, in this part, 2-ethyl-1-hexanol and 1-hexanol were used as a cosurfactant in comparison with TBP previously studied.

4.3.4.1 Effect of type of cosurfactants on forward extraction

Table 4.3 shows the effects of type of cosurfactants used in NaDEHP reverse micellar systems on the extraction of α-chymotrypsin. The initial protein concentration of 0.5 mg/ml was used in all of experiments. From Table 4.3, it can be seen that when system had TBP as a cosurfactant, the water content in reverse micelles and hydrodynamic radius of reverse micelles before forward extraction were higher than those of the systems with 1-heptanol and 2-ethyl-1-hexanol. This is partly due to the higher salt concentration (1 M) required to form Winsor type II when 1-heptanol and 2-ethyl-1-hexanol were used as cosurfactant. Another reason can be attributed to the structure of two alcohols which are quite

straight chain molecules and less bulky than TBP, resulting in smaller size of reverse micelles and thus less water content in the micelles than the micelles of the system with TBP as a cosurfactant as clearly shown in Table 4.3. According to the limitations of the water-pool and size of the reverse micelles, the systems with 1-heptanol and 2-ethyl-1-hexanol were less effective in extracting the protein as indicated by low % extraction. The highest percentage of protein transferred into reverse micelle (~93%) was observed when cosurfactant was TBP as seen in Table 4.3.

Table 4.3 Effect of type of cosurfactant on forward extraction, water content and size of reverse micelles.

Type of cosurfactant	Forward Extraction (%E)	Water content (ωo) in reverse micelles before forward extraction	Hydrodynamic radius (nm) of reverse micelles before forward extraction	Hydrodynamic radius (nm) of reverse micelles after forward extraction
TBP	93.71±0.75	22.4±0.50	17.53±0.54	44.3±0.43
1-heptanol	72.55±3.5	7.09±0.13	6.03±0.26	10.23±0.75
2-ethyl-1-hexanol	77.281±8.0	10.38±0.34	5.5±0.16	10.17±0.87

4.3.4.2 Effect of type of cosurfactants on backward extraction

Figure 4.17 presents the effect of type of cosurfactants on backward extraction. The results show very little effect of type of cosurfactants on the percentage of backward extraction. In all cases, the backward extraction percentages were found to be approximately 70% with TBP system giving slightly higher percentage in this recovery step. This can be explained that the cosurfactant formed in the reverse micelle neither caused any effect on nor involved in the mechanism of backward extraction.

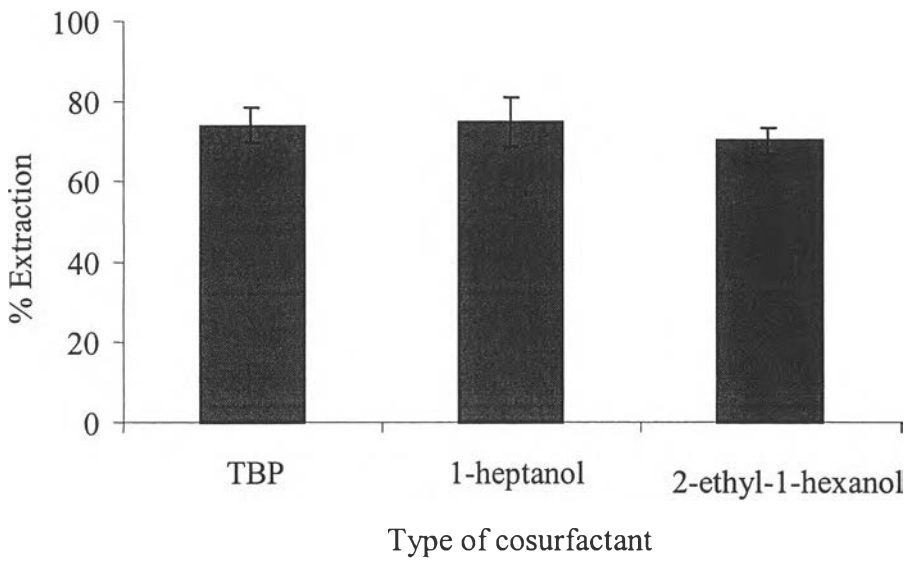

Figure 4.18 Effect of type of cosurfactant on backward extraction.

4.3.4.3 Effect of type of cosurfactants on activity test

Using the same procedure described in the previous section, after backward extraction the activity test was performed on the systems with various cosurfactants. Figure 4.19 shows the percentage of the enzyme activity of α-chymotrypsin obtained from the systems using TBP, 1-heptanol and 2-ethyl-1-hexanol, respectively. From this figure, type of cosurfactants used to form the reverse micelles had a major impact on the activity of the recovered protein. When the NaDEHP micellar system had TBP as a cosurfactant, the highest enzymatic activity (about 50%) was observed whereas only 20% of its original activity was retained in the system using 1-heptanol. In the system using 2-ethyl-1-hexanol as a cosurfactant, the recovered protein nearly lost its enzymatic activity completely. This is partly due to the concentration of Na^+ ions in water-pool as described previously. Another part is attributed to the structure of 1-heptanol and 2-ethyl-1-hexanol molecules which are quite straight in nature and thus they could penetrate closer into the reverse micelle and might form hydrogen bond with protein in the reverse micelles, leading to denaturation of the protein. It is difficult to make any conclusion in this study and further investigation is being suggested.

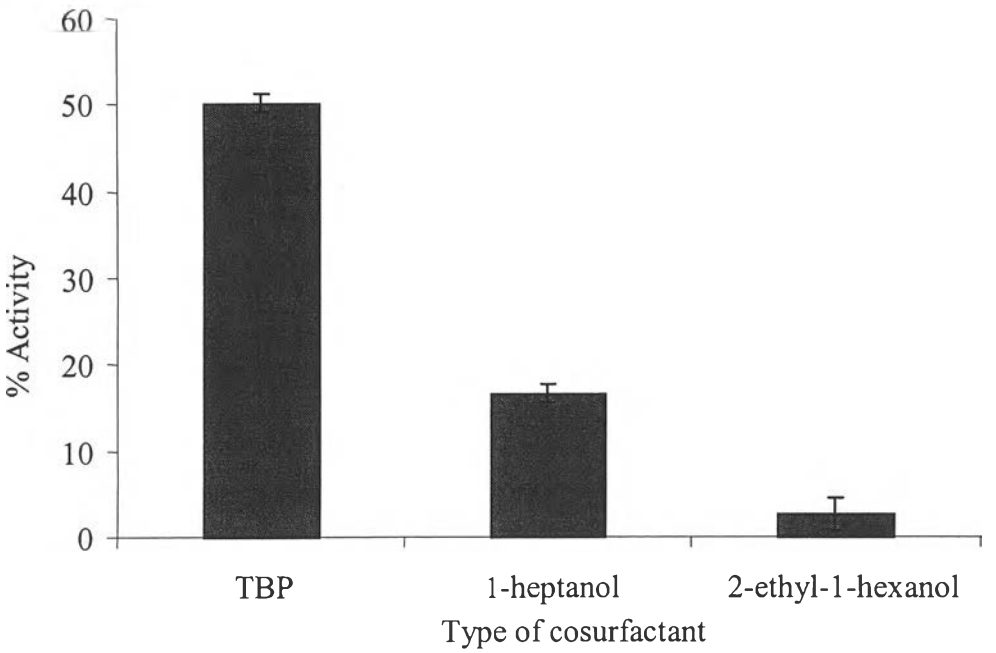

Figure 4.19 Effect of type of cosurfactant on activity test.