CHAPTER IV
RESULTS AND DISCUSSION

There have been many researchers raising questions on how fully folded biological proteins can permeate through a lipid bilayer of living cell, even though they are the hydrophilic and charged proteins (Rothman and Kornberg, 1986). Bychkova et al (1988) found that the state of dihydrofolate reductase which translocated into mitochondria membrane was a non-native state. It was assumed to be a molten globule (MG) state. MG state or partially folded state is an intermediate state of protein folding. It is characterized as a loss in the tertiary structure, retaining the native-like secondary structure and an evidence of compactness. Moreover, MG state shows higher hydrophobicity than native state (Ohgushi and Wada, 1983; Bychkova et al, 1988; Oas and Kim, 1988; Creighton, 1990; Ptitsyn et al, 1990; Peng and Kim, 1994; Ptitsyn and Uversky, 1994; Privilov, 1996).

From MG characteristics, the hypothesis of the present study was raised, if MG state of lysozyme could penetrate into the epidermal layer of the model skin by its hydrophobic property. Thus, this study was intended to induce lysozyme to MG state by adding external factors such as organic solvent and acid. After that the penetration of modified lysozyme was determined using an in vitro animal skin model.

The present study was divided into two parts which were characterization of lysozyme folding mechanism and penetration parts.

Part I Characterization of lysozyme folding mechanism

This section was focused on the identification of specific environments for various stages of conformational changes of a model protein (lysozyme) using circular dichroism (CD) as major analytical equipment.

1. Identification of raw materials

1.1 Hen egg white lysozyme

Hen egg white lysozyme obtained from Sigma (L-6876) was identified by 2 techniques as follows:

1.1.1 Gel electrophoresis technique

Lysozyme, which was solubilized in water, generally was found to be mostly intact. It was analyzed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoretic band of lysozyme raw material was at 14.4 kDa molecular weight standard which corresponded with the reference lysozyme (Figure 25).

1.1.2 Circular dichroism technique

In general, the measurement of protein structure by CD technique is determined in the far-UV (<250 nm) and near-UV (250-300nm) regions for the secondary and tertiary structure, respectively. In the far-UV region, the CD intensities at 208 and 222 nm in negative side corresponds to α-helix secondary structure. Thus, the alteration of the CD intensities at 208 or 222 nm correlates to the changes in the α-helical contents. In the near-UV region, the alteration of the CD intensity represents the changes in tertiary structure (Kelly and Price, 1997; Sreerama and Woody, 2000).

This experiment was done to identify lysozyme structure in water which was believed to be in its native conformation. Researchers have found that the CD spectrum of native lysozyme shows a helical pattern of secondary structure where overshot of ellipticity is presented at 208 and 222 nm. The tertiary structure spectrum showed aromatic residues of tryptophan at 289 nm (Rothwoarf and Scheraga, 1996). It was correlated with the results of the present study which are illustrated in Figure 26 A and Figure 26 B, respectively.

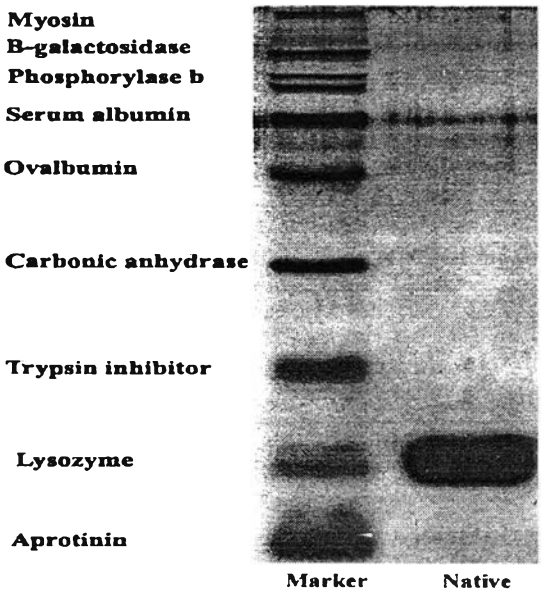
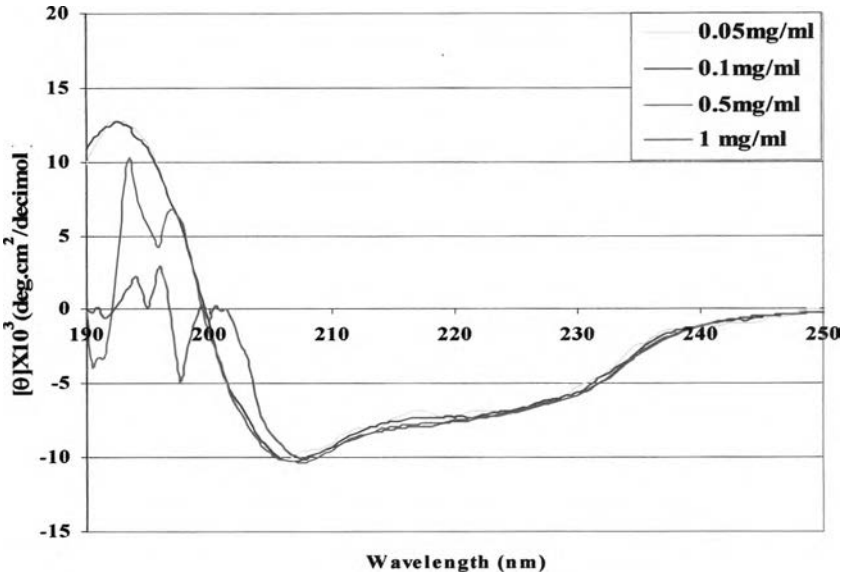


Figure 25 SDS-PAGE analysis of lysozyme dissolved in water

In the next techniques used to determine the structural transformation of lysozyme, suitable lysozyme concentrations for each technique was different. Therefore, it was necessary to confirm the native structure at various concentrations of lysozyme which would be used. Lysozyme was solubilized in water at 0.05, 0.1, 0.5 and 1 mg/ml. The CD spectral patterns confirmed that the secondary structure (Figure 26 A) and tertiary structure (Figure 26 B) of lysozyme at each lysozyme concentration were not different. Similarly, CD intensities of each lysozyme concentration were not different when determined at 222 and 289 nm (Figure 27).

Although the secondary spectrum of lysozyme concentration at 1 mg/ml shows some background noise at the wavelength lower than 200 nm (Figure 26 A), its CD intensity at 222 nm which is used to determine α-helix content is the same as the CD intensities at other concentrations (Figure 27). Thus, all lysozyme concentrations used in this study gave similar CD spectra.



A

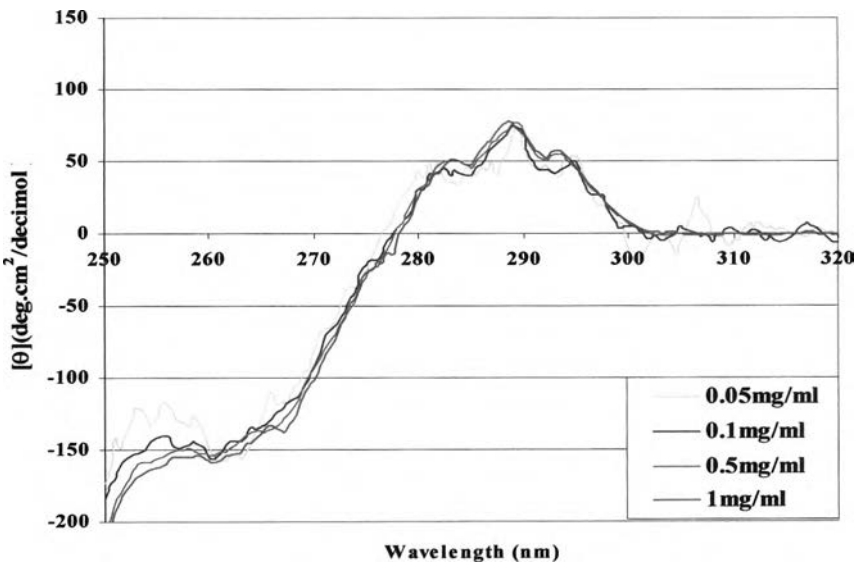


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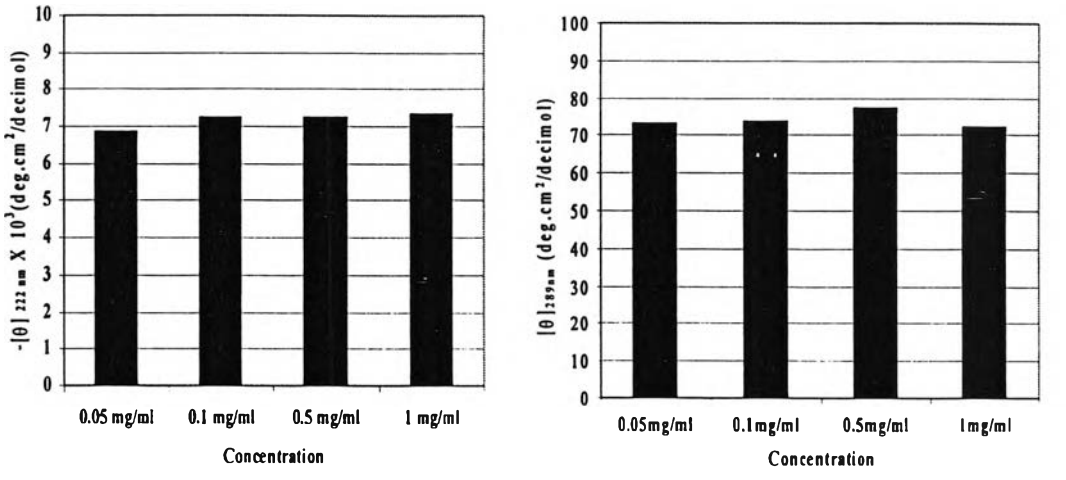
Figure 26 The CD spectra of native lysozyme dissolved in water at various concentrations in the far-UV (A) and near-UV (B) regions



A B

Figure 27 The CD intensities at 222 (A) and 289 (B) nm of native lysozyme dissolved in water at various concentrations.

Reduction of lysozyme concentration to 0.02 mg/ml showed some background noises at both the secondary and tertiary structure spectra (Figure 62 A and Figure 62 B in Appendix A, respectively). It may be because the instrument could not detect lysozyme contents beyond a certain range. Hence, for this study CD should be used to detect lysozyme structures in the range of lysozyme concentrations of 0.05-1 mg/ml. The proper lysozyme concentration was found to be at 0.1 mg/ml which did not present any background fluctuation. This concentration was used throughout the CD study.

1.2 Absolute ethanol

Absolute ethanol was characterized for the amount of water by Karl Fisher titrimetry. It was found that there was only 0.0434% v/v of water in ethanol. Thus this small amount of water in ethanol should not affect the total ratio between ethanol and water in the future experiments.

2. Conformational transformation of lysozyme

Globular proteins can be transformed into MG state by many external factors such as the use of organic solvents, acids, salts and thermal variations (Bychkova et al, 1988; Goto et al, 1990a, 1990b; Van der Goot et al, 1991, 1992; Privilov, 1996; Bhattacharjya et al, 1997; Bakuni, 1998; Dubey and Jagannadham, 2003). Ethanol and dilute hydrochloric acid were selected to be used in the present study. It was due to the fact that the environments were not too complicated to control. Furthermore, up to a certain concentration, ethanol is regarded as safe to be used on human skin (Inamori et al, 1994; Megrab et al, 1995). This experiment was done to determine the conformation of lysozyme under three environments.

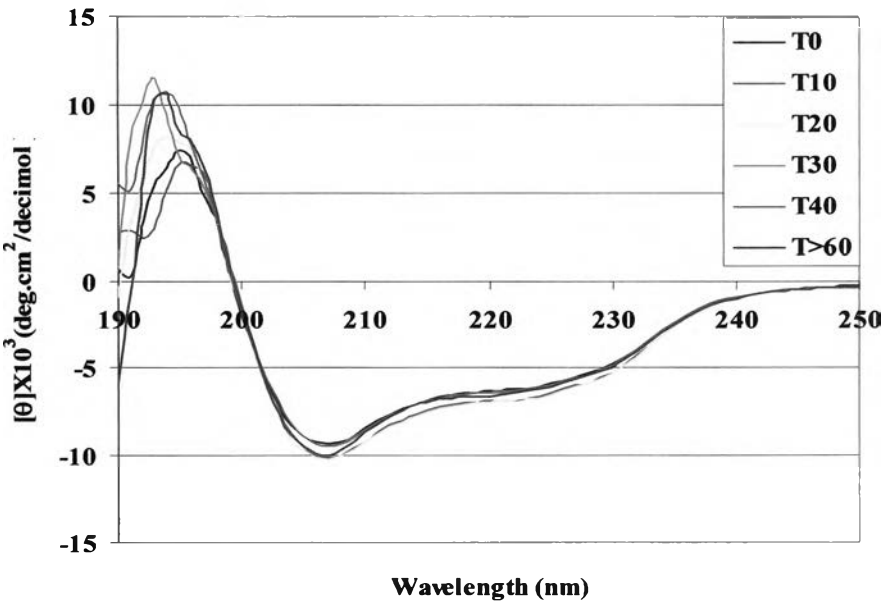
2.1 Aqueous-ethanolic environment

This section was carried out to evaluate the effects of mixing time, ethanolic concentration and lysozyme concentration on lysozyme conformation.

2.1.1 Effect of mixing time

Time is a necessary factor to determine the equilibrium protein conformation. Thus, this section was intended to consider if the mixing time affected lysozyme conformation. The ethanolic solution at 50% v/v was chosen as a model solution to avoid extreme domination of either water or ethanol. Conformation of lysozyme solubilized in 50% v/v ethanol was observed at 0, 10, 20, 30, 40 and 60 minutes using CD method, to study the effect of mixing time.

Lysozyme secondary spectra obtained for each time period were similar (Figure 28 A). The similarity was also observed for the tertiary pattern (Figure 28 B). CD intensities were not different at 222 and 289 nm (Figure 29). Researchers have found that the folding kinetics of the globular proteins to generate an equilibrium state is very fast in the range of milliseconds (Kato et al, 1982; Radford et al, 1992; Dobson et al, 1994). Thus, this finding was done to confirm that the mixing time used did not affect lysozyme conformation because it was already in its equilibrium conformation.



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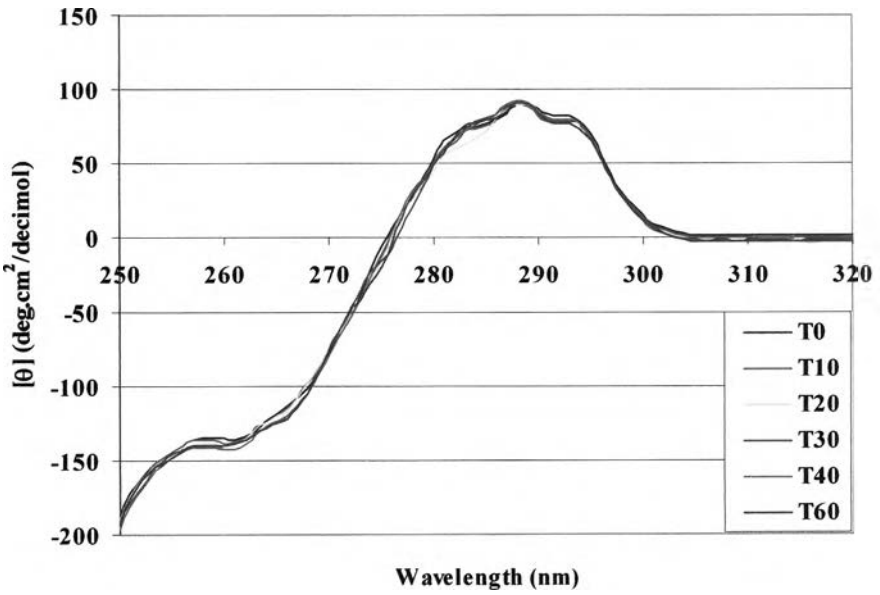
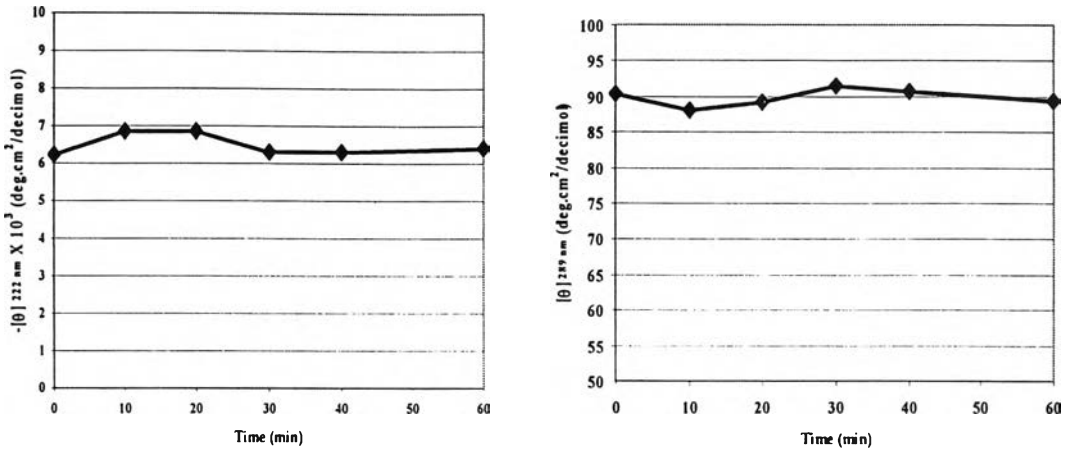


B

Figure 28 The CD spectra of lysozyme dissolved in 50% v/v ethanol at various solubilization times in the far-UV (A) and near-UV (B) regions.



A B

Figure 29 The CD intensities at 222 (A) and 289 (B) nm of lysozyme dissolved in 50% v/v ethanol at various solubilization times.

2.1.2 Effect of ethanol concentrations

Ethanol is one type of alcohol which is widely studied regarding its effect on protein folding, especially folded lysozyme (Lehmann et al., 1985; Calandrini et al, 2000; Goda et al, 2000 Tanaka et al, 2001). The difference in its reported characteristics was due to not only different techniques of determination but also the use of different sources of lysozyme and ethanol. These factors could affect structural alteration. Therefore, this section had to confirm the effects of ethanol concentration on lysozyme conformational modification.

The available techniques used to monitor the transformation of the model protein were carried out as follows:

2.1.2.1 Circular dichroism technique

Lysozyme was exposed to various concentrations of ethanol in water (0-99% v/v) to determine the shifts of conformation when the ethanol concentration was increased.

At 10-60% v/v ethanol concentrations, [L(10)(0)-L(60)(0)], the spectra shifted only slightly from its native state, [L(0)(0)], which were shown in the far and near-UV CD spectra. As ethanol concentration was increased to 70% v/v [L(70)(0)], some trace of tertiary structure was disrupted, as secondary structure was retained. The conformation was dramatically altered at 80% v/v ethanol concentration, [L(80)(0)], where tertiary structure was destroyed while secondary structure was especially high with helical contents. Similarly, at 90% and 99% v/v ethanol concentrations, [L(90)(0) and L(99)(0)], there was no defined tertiary structure, as helical structures were expanded as the concentration of ethanol was increased (Figures 30 and 31).

The conclusive tendency of the lysozyme intensity modifications in various ethanolic contents is displayed in Figure 32. The change in intensities at 222 (Figure 32 A) and 289 nm (Figure 32 B) were initiated when the amount of ethanol exceed 70% v/v. In addition, at higher concentrations of ethanol, the intensities were continuously increased in far-UV while there were no detectable intensities in the near-UV region.

Generally, globular proteins such as lysozyme solubilized in water are found to be in their native state as spherical or globular shape. Its nonpolar side chains are buried and compacted to form hydrophobic core, while distribution of hydrophilic residues is predominantly expressed over the molecular surface (Creighton, 1990; Thornton and Barlow, 1991; Brange, 2000). Thus, water molecules fully hydrate the protein surface. The protein-water interaction occurs and played a pivotal role in the folding pathway, protein functions and stability (Rupley et al, 1983; Brange, 2000). The protein structures are mainly stabilized with noncovalent forces such as electrostatic interactions, hydrogen bonding and hydrophobic effects (Timasheff, 1970 and Nölting, 1999).

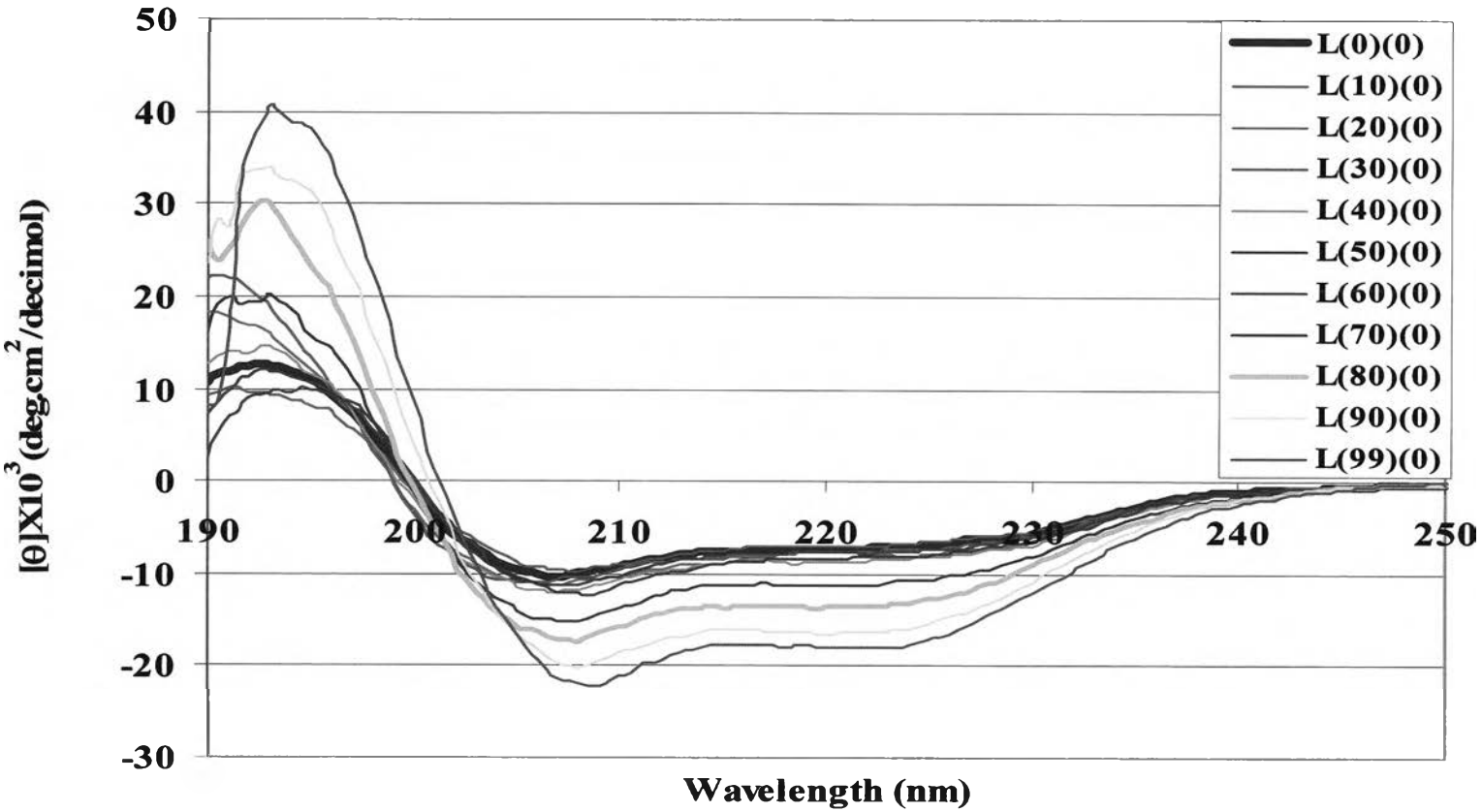


Figure 30 The CD spectra of lysozyme dissolved in various concentrations of aqueous-ethanolic solutions (0-99% v/v) in the far-UV region

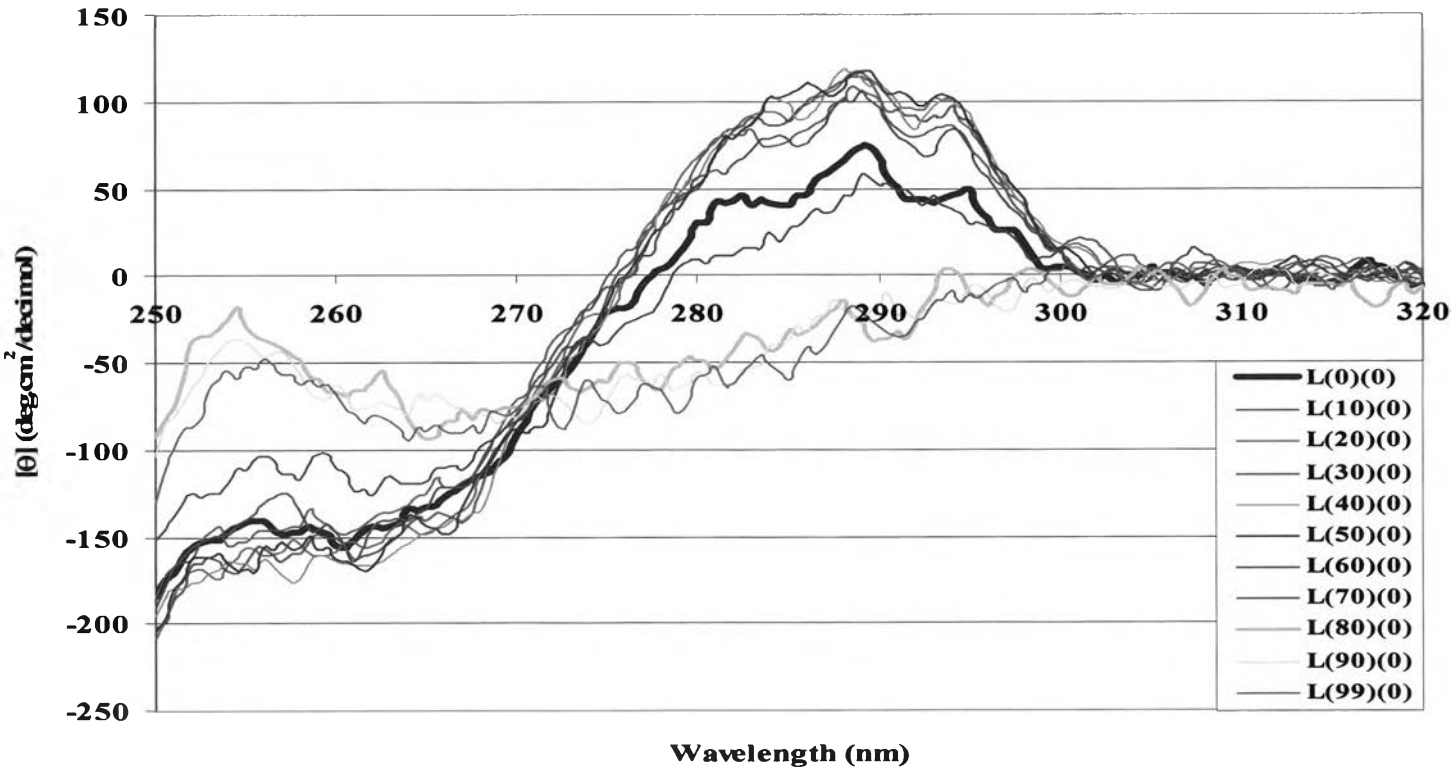
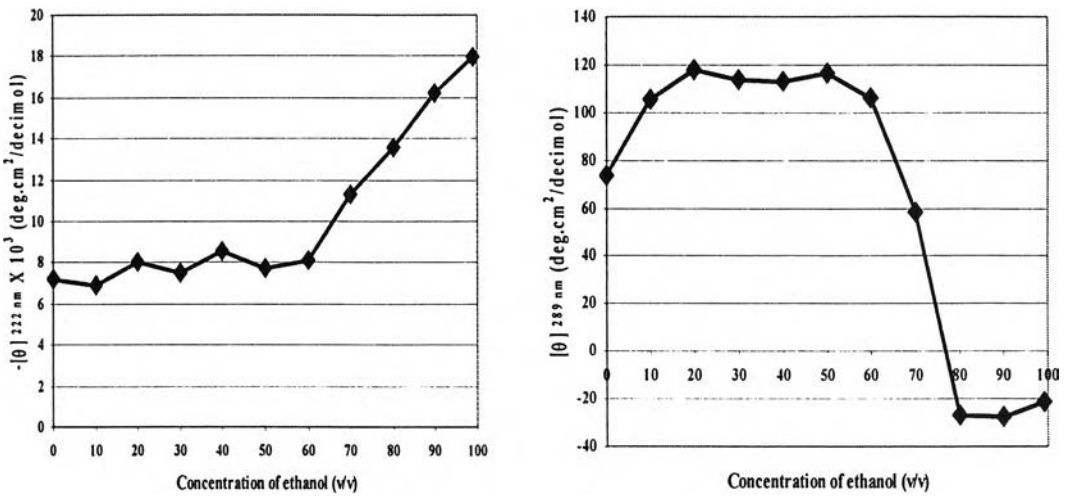


Figure 31 The CD spectra of lysozyme dissolved in various concentrations of aqueous-ethanolic solutions (0-99% v/v) in the near-UV region



A B

Figure 32 The CD intensities at 222 (A) and 289 (B) nm of lysozyme dissolved in various concentrations of aqueous-ethanolic solutions (0-99% v/v)

Addition of ethanol into native environment of the protein is to affect the folding mechanism. The interaction between alcohol and protein on its surface is divided into two parts; hydrophobic segment consisting of aliphatic and aromatic groups, and hydrophilic portion containing polar and charged groups (Lehmann et al., 1985; Mattos and Ringe, 2001).

Lehmann et al. (1985) reported that introduction of ethanol at low concentrations did not meaningfully affect the protein structures. However, at higher than 50% v/v ethanol concentrations, the expanded hydrophobic parts of solvent will decrease the polar interaction on the protein surface. Water molecules which surrounded the surface were replaced by hydrophobic molecules which eventually shown to be protein dehydration and initiation of protein conformational rearrangement (Timasheff, 1970; Rupley et al, 1983; Lehmann et al., 1985; Calandrini et al, 2000).

At the greater amount of ethanol, the tertiary structure of globular protein is disrupted, while retaining the secondary structures. It is due to the fact that dehydrated environment induces excellent interaction between hydrophobic side

chains and nonpolar solvent, leading to hydrophobic residue exposure (Timasheff, 1970; Mattos and Ringe, 2001). Furthermore, intramolecular interactions between peptide units are greatly enhanced resulting in the initiation of high helical contents (Lehmann et al., 1985). This is the reason why at higher concentrations of ethanol, destabilization of hydrophobic interaction of the interior residues occurred, leading to the extensive formation of α-helix structures.

2.1.2.2Fluorometric technique

The fluorometric technique was used to confirm the MG or partially folded state of the protein using 1-anilino-8-naphthalene sulfonate anion (ANS) which is an extrinsic hydrophobic probe (Semisotnov et al, 1991; Matulis and Loverien, 1998; Matulis et al, 1999). The anilinonaphthelene group of ANS is protected from water quenching by mixing this group into the nonpolar environment of the modified globular proteins, inducing fluorescent emission intensity (Matulis and Loverien, 1998; Matulis et al, 1999).

Figure 33 shows that there are little detectable fluorescent intensities in the aqueous-ethanolic range of 0-70% v/v. When ethanol concentration was increased to 80% v/v, the protein was tightly bound with ANS resulting in a sharp increase in the fluorescent emission intensity, which signified a molten globule (MG) or partially folded state. Nevertheless, at higher concentration, no observable fluorescent intensity was detected. This might be due to the fact that at higher than 80% v/v ethanol, lysozyme was induced to the expanded helical state and lost its globularity; hence, ANS binding did not occur (Bhattacharjya and Balaram, 1997; Kamatari et al, 1998).

Figure 34 shows fluorescent intensities (obtained from Figure 33) of ANS binding with lysozyme solubilized in various ethanolic concentrations. Lysozyme dissolved in 80% v/v ethanolic solution gave the highest intensities at 490 nm, implying occurrence of MG state. While lysozyme solubilized in other concentrations did not signify MG state.

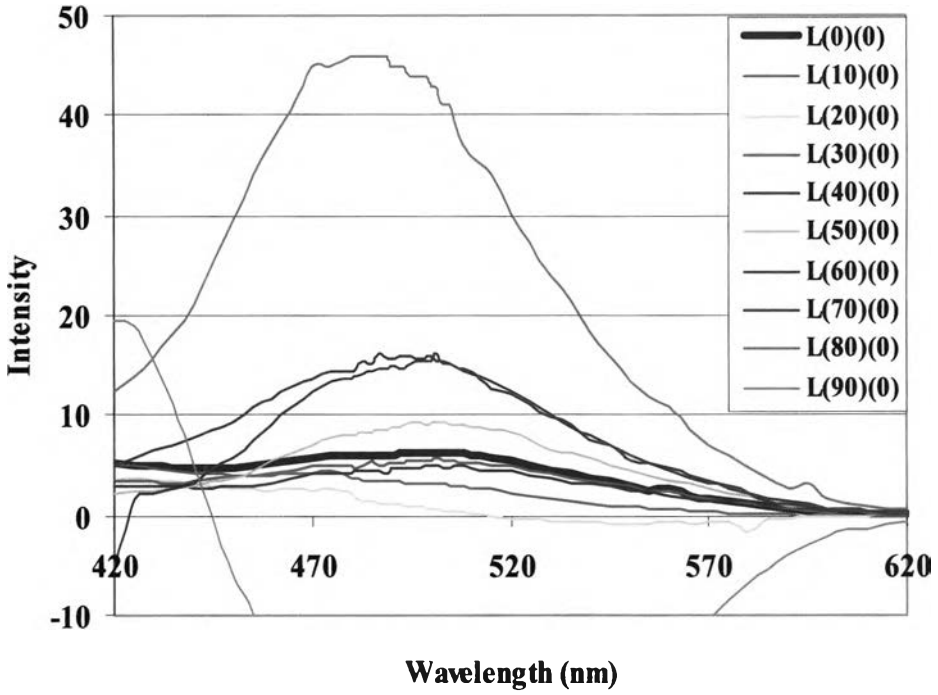


Figure 33 Fluorometric emission spectra of lysozyme bound to ANS after exposed to various concentrations of aqueous-ethanolic solutions (0-90% v/v)

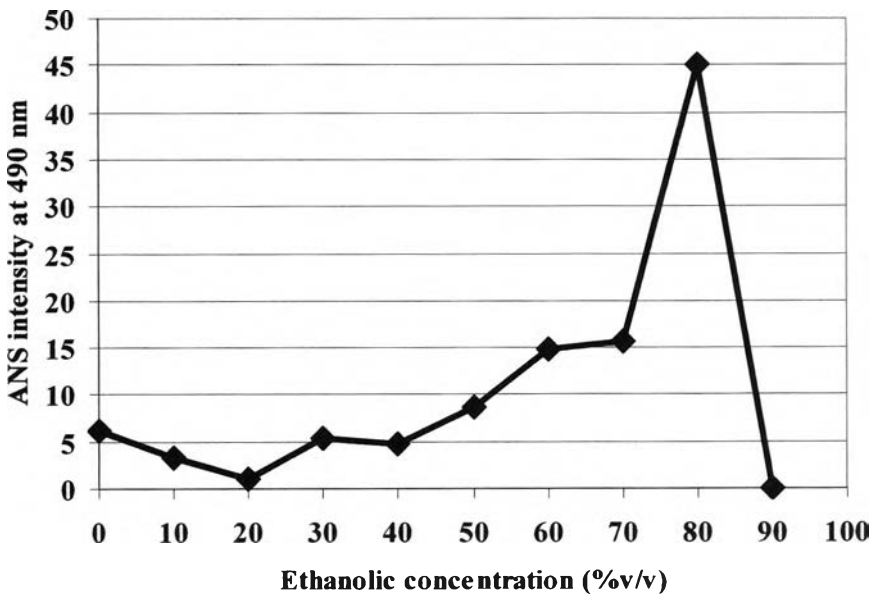


Figure 34 Fluorometric emission intensities at 490 nm of lysozyme bound to ANS after exposed to various concentrations of aqueous-ethanolic solutions (0-90% v/v)

The investigation of Tanaka et al (2001) revealed the possible diagram of lysozyme structural transformations in various percentages of ethanolic concentrations using dynamic light scattering technique as shown in Figure 19 in Chapter II. They reported that the native conformation was not significantly perturbed by the addition of ethanol at low contents (0-63% v/v). At higher ethanol concentrations (72-81% v/v), lysozyme conformation converted to have very high helical contents. At 90% v/v ethanol, unfolded polypeptide chains of the globular protein were increased, leading to protein aggregation. The results correlate well with the CD and fluorometry findings discussed above.

At 80% and 90% v/v ethanol, the CD data showed high helical pattern of the secondary structure while the tertiary structure could not be observed. Fluorometric results (Figure33) illustrate that lysozyme dissolved in 80% v/v of ethanol in the aqueous-ethanolic solution generated MG characteristics. The results also imply that the structure of lysozyme solubilized in 80% v/v aqueous-ethanolic solution was more compact than the structure of lysozyme dissolved in 90% v/v of ethanol. At 90% v/v, there was no detectable fluorescent emission intensity due to the fact that lysozyme had lost its globularity.

2.1.2.3 Gel electrophoresis

The molecular weight of lysozyme in each composition was detected using SDS-PAGE. It was found that lysozyme structure was still intact at every concentrations of ethanol used as shown in the complete electrophoretic bands in Figure 35.

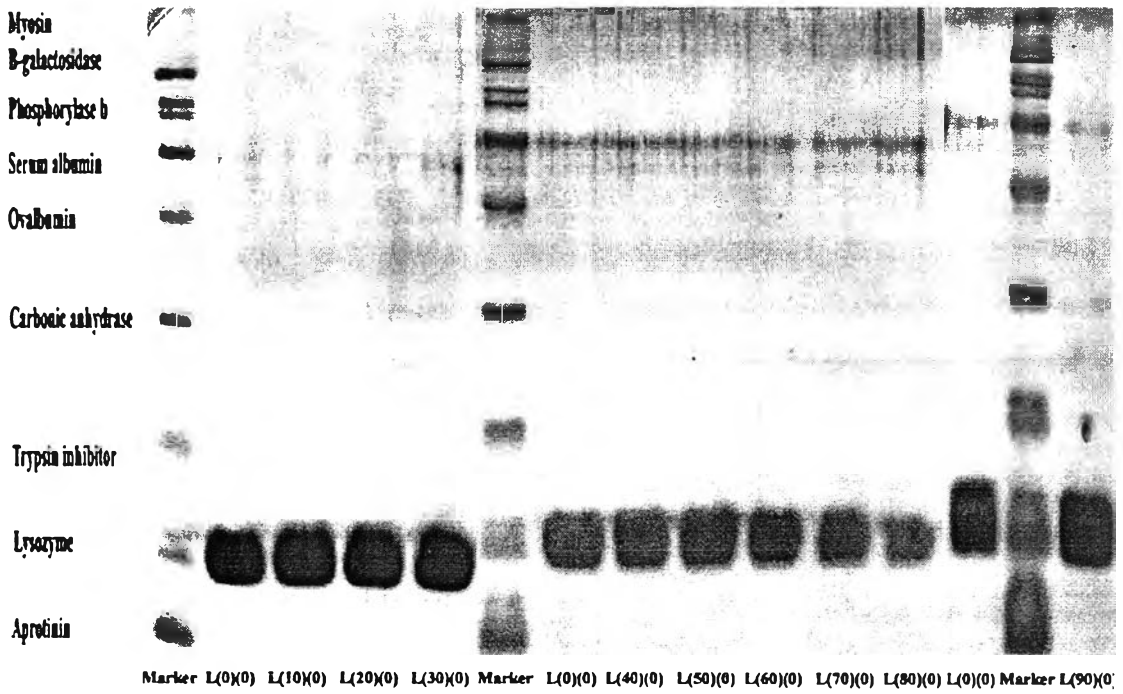


Figure 35 SDS-PAGE analysis of lysozyme dissolved in various concentrations of aqueous-ethanolic solutions (0-90% v/v)

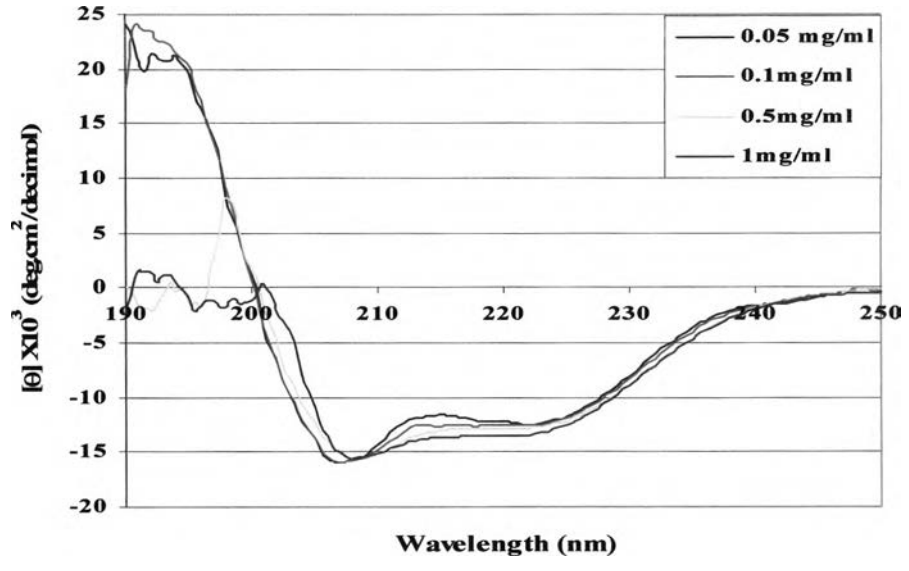
2.1.3 Effect of lysozyme concentration

Goda et al (2000) studied the conformational changes of lysozyme which was solubilized in various aqueous-ethanolic solutions (0, 50, 70, 80 and 85%v/v), at 1-5 mg/ml of protein contents using CD. Their results showed that the amount of ethanol added from 0 to 70% v/v, did not have pronounced effect on the secondary structures as they were the same as in the native structure. That is in accordance with the experiments previously discussed.

They also reported that lysozyme (5 mg/ml) dissolved in 80% v/v aqueous-ethanolic solution presented α-helix structure. However, the shape of their CD spectrum shown in Figure 18 in Chapter II was different from the finding in this present study. Furthermore, at 85% v/v ethanol, the conformations were altered to exhibit β-sheet instead of helical form in the far-UV region when lysozyme concentration was at higher than 1 mg/ml (Figure 20 in Chapter II). This is also different from this present study where lysozyme still exhibited the helical secondary structure even when exposed to ethanolic solution higher than 85% v/v.

Therefore, this section was aimed to confirm the lysozyme conformation which was exposed to 80% v/v of ethanolic solution at various lysozyme concentrations using CD technique. All lysozyme concentrations prepared in this study were 0.05, 0.1, 0.5 and 1 mg/ml at constant concentration of ethanol of 80% v/v. At 80% v/v of ethanol, the spectra of all lysozyme concentrations were similar and the secondary structures were retained as the loss in tertiary structures was observed (Figure 36 A and Figure 36 B).

From the results obtained in this section, lysozyme structural modifications in solution with various ethanol contents are summarized in Table 1. It was found that lysozyme needed high amount of ethanol, at least 70% v/v, to destabilize the tertiary structure. Although ethanolic concentration was increased to as high as 99% v/v, the secondary structure still retained. Lysozyme contains four disulfide bridges which are necessary for the stabilization of structural lysozyme. Disulfide bridge is considered a very strong intramolecular interaction, resulting in structural stabilization.



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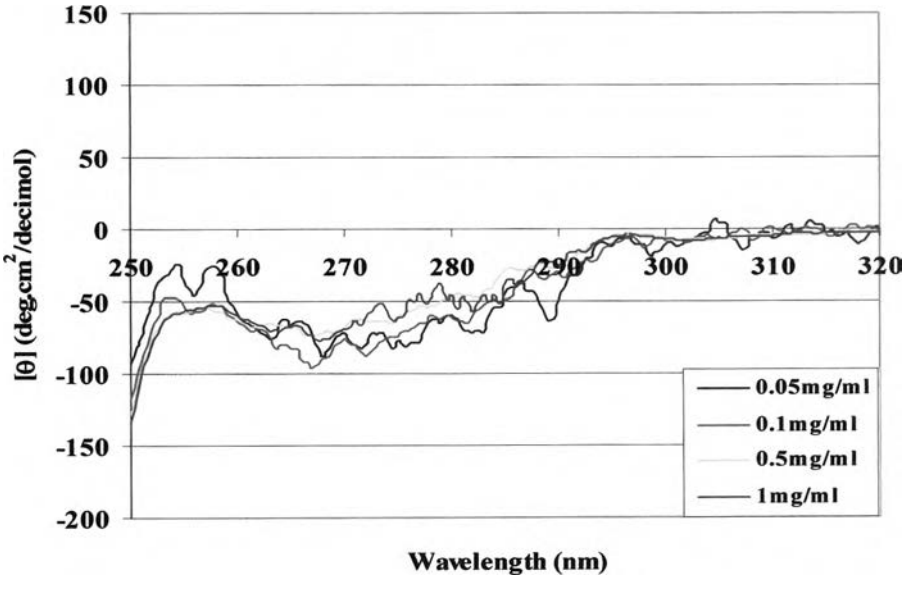


B

Figure 36 The CD spectra of lysozyme dissolved in 80% v/v ethanol at various concentrations in the far-UV (A) and near-UV (B) regions

The conformation of lysozyme was converted to partially unfolded or MG state when it was exposed to 80% v/v ethanolic solution while at other ethanolic concentrations this state was not generated when detected by fluorometric technique. The MG state is defined as a loss in tertiary structure but still retained the native-like secondary conformation and compactness (Peng and Kim, 1994; Privilov, 1996).

Table 1 The summary of lysozyme structure exposed to various ethanolic concentrations as characterized by CD and fluorometric techniques

Formula	CD		Fluorescence	Lysozyme structure
	Secondary structure	Tertiary structure	ANS binding	
L(0)(0)	+	+	-	Folded
L(10)(0)	+	+	-	Folded
L(20)(0)	+	+	-	Folded
L(30)(0)	+	+	-	Folded
L(40)(0)	+	+	-	Folded
L(50)(0)	+	+	-	Folded
L(60)(0)	+	+	-	Folded
L(70)(0)	+	-	-	Partially folded
L(80)(0)	+	-	+	MG
L(90)(0)	+	-	-	Unknown
L(99)(0)	+	-	-	Unknown

\+ = existence of specific response

\- = absence of specific response

From the findings, lysozyme was induced to MG state at very high ethanolic concentration. It will not be appropriate to used or apply such high concentration of ethanol to animal or human skin. Goto et al (1990a, 1990b) reported that introduction of HCl acid in native environment at low ionic strength affected folding mechanism of some proteins. The future experiment was aimed to reduce the ethanolic concentration in the environment using hydrochloric acid (HCl). It was expected that the tertiary structure of lysozyme would be destabilized by the addition of HCl acid to generate the temporary unfolded state. After that, a low ethanolic concentration would be added to the environment to induce refolding of unfolded lysozyme expected to result in MG state. Therefore, the following experiment was done to find out the effective concentration of HCl that would result in the conformational modification of lysozyme.

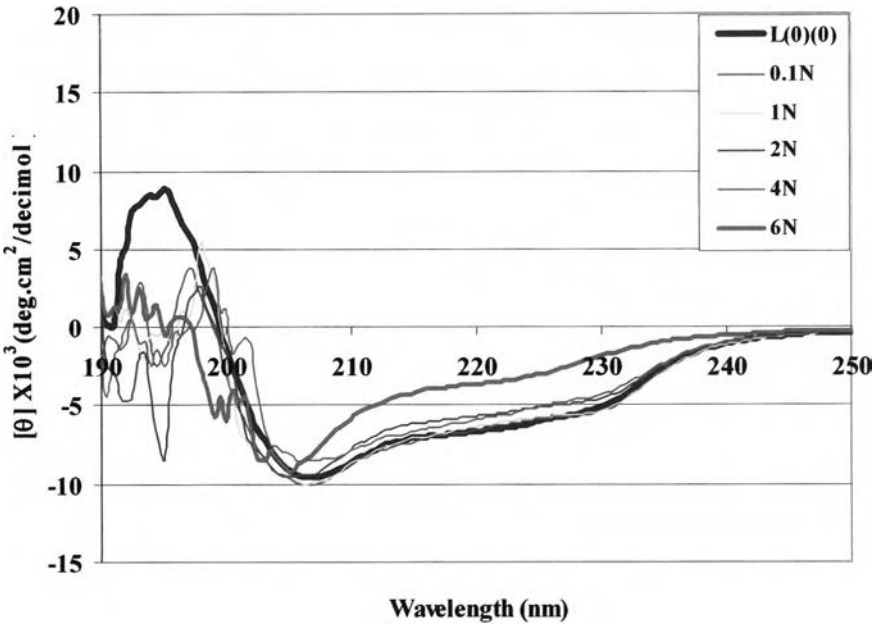
2.2. Acidic environment

This section was carried out to evaluate two factors, which were HCl acid concentration and the effect of time, on lysozyme conformation.

2.2.1 Effect of hydrochloric acid (HCl) concentration

The effect of acidic environment on the conformational transformation of lysozyme was determined at various concentrations of HCl. Previous discussion describes that the structural lysozyme was quite stable at various ethanolic concentrations. Hence, this experiment was aimed to determine the range of HCl acid concentrations of 0.1, 1, 2, 4 and 6 N on lysozyme conformation.

Lysozyme was solubilized at various HCl acid concentrations and its conformation was immediately detected by CD technique. The CD spectra of 0.1- 4 N HCl show similar secondary and tertiary patterns as in the native state. However, at 6 N HCl, the spectrum was changed dramatically. Secondary and tertiary structures were least pronounced (Figure 37 A and Figure 37 B). The fluctuation of the spectra at lower than 200 nm was found to be caused by Cl^- ion absorption at approximately 195 nm (Kelly and Price, 1997).



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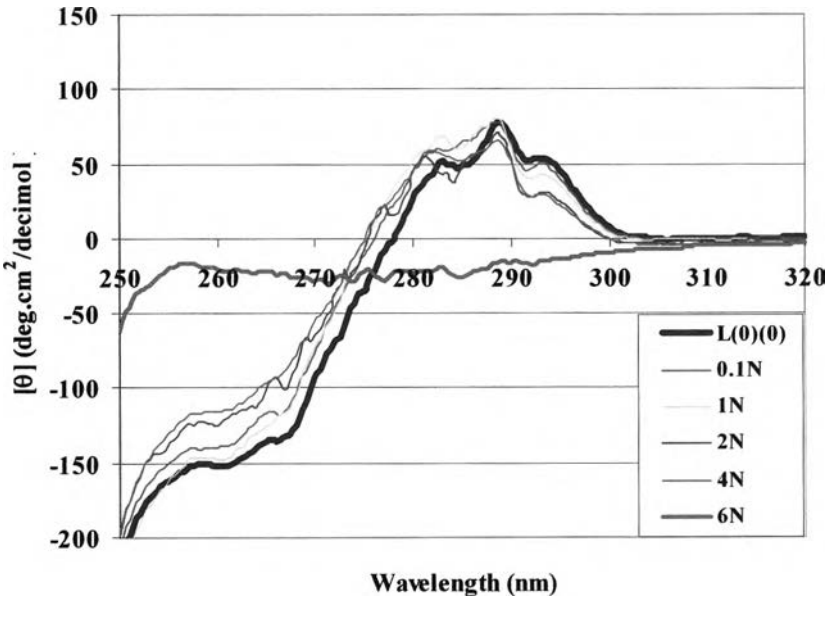


B

Figure 37 The CD spectra of lysozyme dissolved in various concentrations of HCl in the far-UV (A) near-UV (B) regions

2.2.2 Effect of time

Lysozyme was solubilized at various concentrations of HCl as in the previous study. It was then incubated at room temperature overnight to study the stability of lysozyme conformation in acidic environment.

The CD spectra of lysozyme which were exposed to 0.1- 2 N HCl overnight still showed the same pattern as the native. In contrast, at 4 N and 6 N HCl, CD spectra were not pronounced in far (Figure 38) and near-UV (Figure 39) regions. It was observed that lysozyme which dissolved in 4N HCl showed turbidity; however, at 6 N HCl the solution of lysozyme was clear.

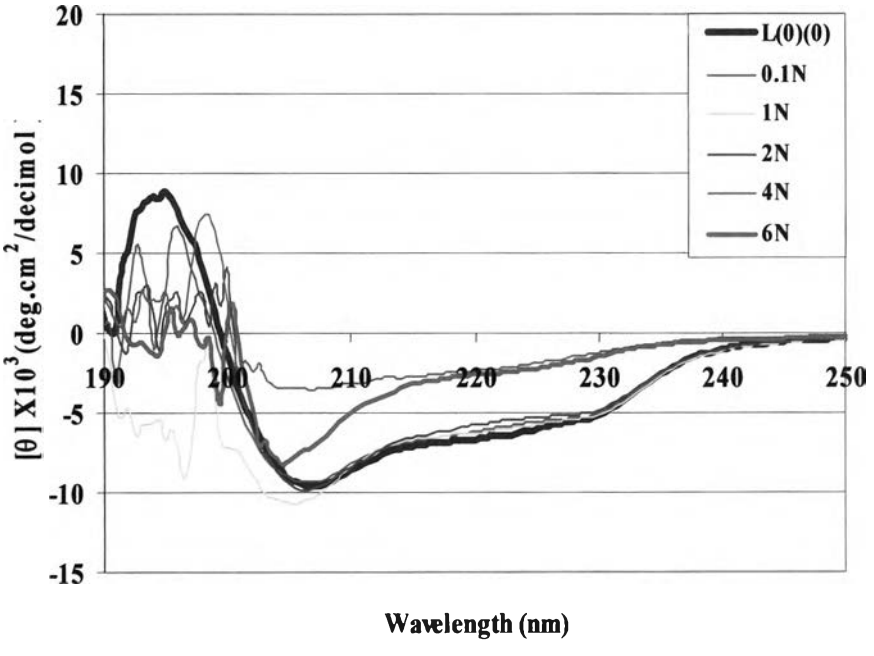


Figure 38 The CD spectra of lysozyme dissolved at various concentrations of HCl overnight at room temperature in the far-UV region

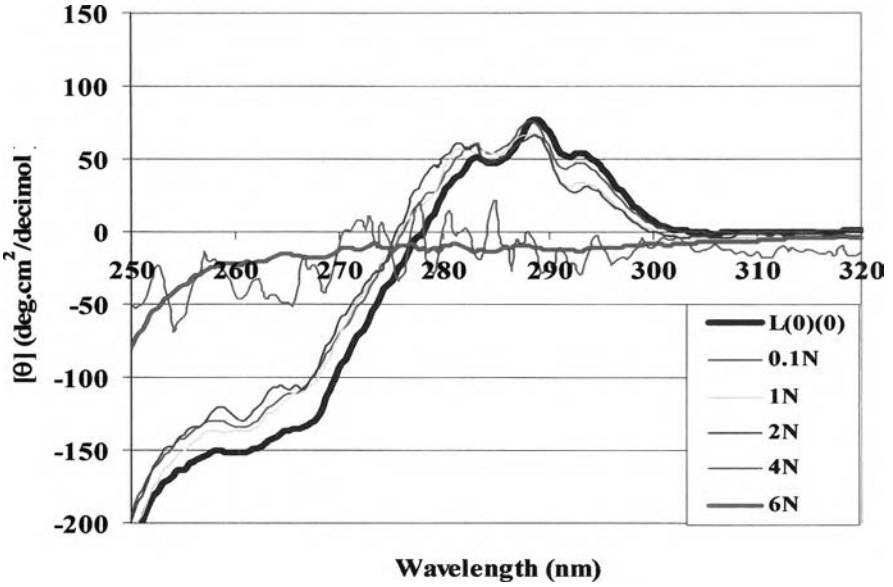
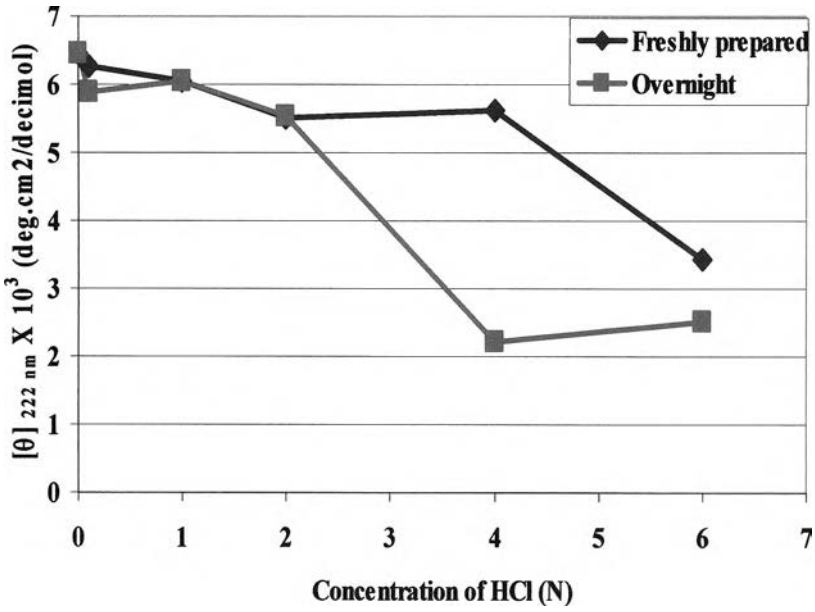


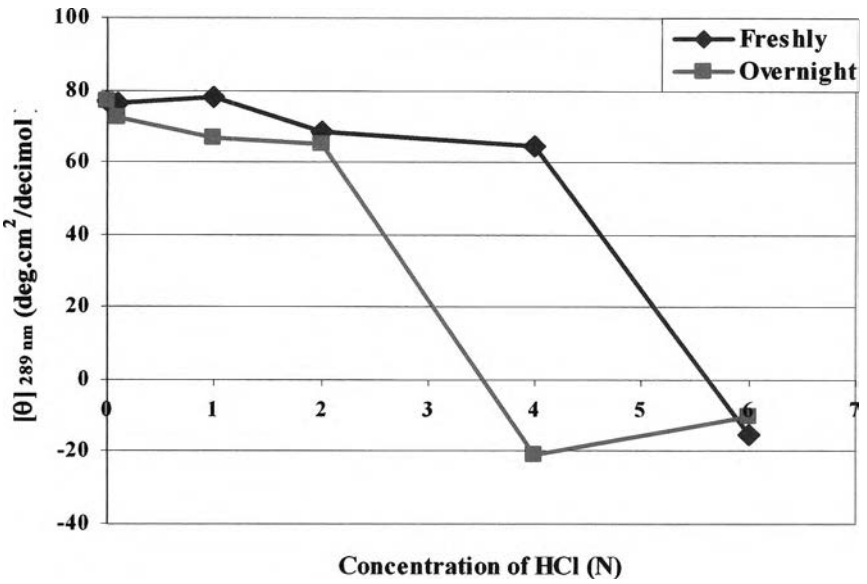
Figure 39 The CD spectra of lysozyme dissolved at various concentrations of HCl overnight at room temperature in the near-UV region

From the results the acidic condition was an environment which affected protein structural modification. Figure 40 shows CD peak intensities of lysozyme in solution at various HCl contents comparing samples that was freshly prepared and that was kept overnight. At 0.1-2 N HCl, time of preparation did not cause conformational shift of lysozyme.

Fink (1994) and Brange (2000) reported that some protein exhibited their partially folded state under salt-free and strong acidic environment (pH~ 1-2) by electrostatic interaction. On the other hand, some proteins still retained their native state even when decreasing the pH to 0.5. This was due to the fact that protein structures composed of disulfide cross-links which had very high intramolecular interactions and were predominant than electrostatic repulsion (Goto et al, 1990; Fink et al, 1994). Lysozyme is a globular protein which contains 4 disulfide bridges in its structure (Caffotte et al, 1992; Radford et al, 1992; Morgan et al, 1998). Thus, the native state of this protein should be rather stable in acidic condition.



A



B

Figure 40 Comparison of CD intensities at 222 (A) and 289 (B) nm of freshly prepared lysozyme solution and lysozyme solution that was kept overnight at various HCl concentrations

Addition of HCl at higher concentrations promotes proton and anion generation in the medium. This will induce an increase in interior repulsion between positive charges and the disturbance of anions on the water molecules surrounding protein surface. This could lead to the destabilization of hydrophobic interaction of globular protein and exposed non-polar side chains to the environment, resulting in an unfolded structure (Goto et al, 1990a, b; Fink et al, 1994).

The CD intensity of lysozyme in 4 N HCl maintained at room temperature overnight showed that the secondary and tertiary structures obviously decreased from the freshly prepared samples as illustrated in Figure 40. Unfold proteins generate intermolecular interactions, leading to protein aggregation. In such case is an irreversible form, called precipitation (Goto et al, 1990a; Bange, 2000). This was in accordance with the physical observation that the solution was turbid when lysozyme had been dissolved in 4 N HCl overnight.

Nevertheless, exposure of lysozyme to 6 N HCl overnight significantly diminished the CD intensities (Figure 40) of the native state. This result was similar to that of the sample which were prepared and measured instantly. Furthermore, both mediums showed no turbidity although it was kept overnight. When protein was exposed to the extreme acidic condition for a while, the main chain was cleaved into fractions (Bammer and Keppenol, 2000). Consequently, each segment could still be dissolved in the solution giving it a clear appearance.

The results in the acidic environment are summarized in Table 2. There was no detectable MG characteristic at any concentration of HCl. Each HCl concentration did not induced the characters of MG state with disruption of tertiary structure and still retaining the secondary structure (Peng and Kim, 1994; Privilov, 1996). It could be summarized that lysozyme could retain its native conformation to a certain concentration of acid in solution overnight, in this case at a concentration of less than 4 N HCl. However, in an extremely low pH or extreme acidic conditions the structures of both secondary and tertiary conformations were completely destroyed.

Table2 The summary of lysozyme structures which was freshly prepared and kept overnight at various concentrations of HCl as characterized by CD

HCl content (N)	Freshly prepared samples		Samples kept overnight	
	Secondary structure	**Tertiary structure**	**Secondary structure**	**Tertiary structure**
0.1	+	+	+	+
1	+	+	+	+
2	+	+	+	+
4	+	+	-	-
6	-	-	-	-

+ = evidence of specific response

- = absence of specific response

2.3 The combined aqueous-ethanolic and acidic environment

The conformational transformation of lysozyme occurred when it was solubilized in either hydroethanolic or acidic condition which had previously been evaluated in sections 2.1 and 2.2, respectively. Therefore this section was aimed to minimize the concentration of ethanol using HCl acid in the solution and still to induce lysozyme structure to MG or partially folded state. The combination was carried out by variation of one factor concentration while keeping another constant. The observation was separated into two parts as follows:

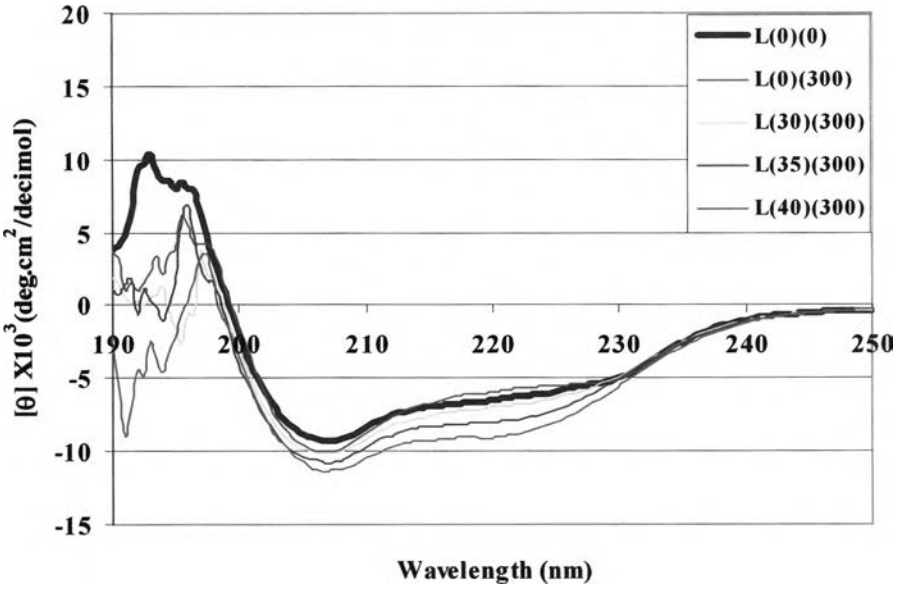
2.3.1 Preliminary determination for optimal ethanolic concentration

This study was done to determine the lowest possible range of ethanol to induce MG state of lysozyme in the presence of constant HCl acid. The preliminary study of the combined condition was roughly shown to be able to reduce the amount of ethanol used to induce MG state (data not shown). From the result, it suggested focusing on the narrower range of ethanol contents of 30-40% v/v in the constant low HCl acid concentration that meaningfully affected the lysozyme structure.

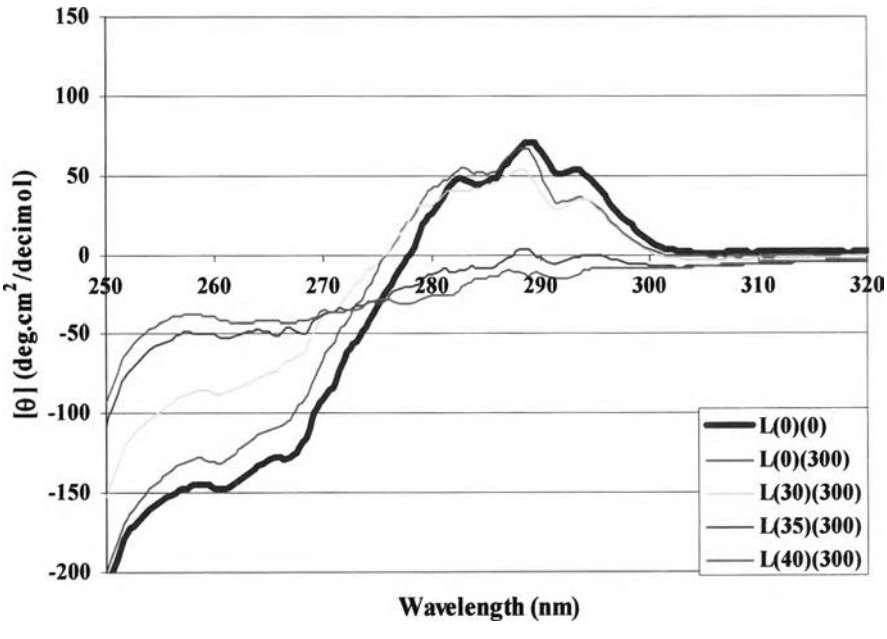
Figures 41 shows the CD spectra of lysozyme at constant concentration of HCl (300 mM) and variation of ethanolic concentrations in the far-UV (Figure 41 A) and near-UV (Figure 41 B) regions. At 30% v/v ethanol [L(30)(300)], the spectrum was not different from the spectrum of native condition and the spectrum with only HCl acid. However, with increasing ethanolic concentration to 35% and 40% v/v [L(35)(300) and L(40)(300), respectively], the tertiary structures were disrupted while the secondary structure was retained with higher helical contents.

2.3.2 Effect of HCl acid concentration on lysozyme structure at constant ethanolic concentrations

From the previous results it was suggested that at least 35% v/v ethanol at a low constant HCl acid concentration could initiate lysozyme conformational change. Hence, in this study, the aim was to find the lowest concentration of HCl acid in constant hydroethanolic solution. The concentrations of ethanol at 35% and 40% v/v were used in this examination. Lysozyme structure was investigated by two techniques.



A



B

Figure 41 The CD spectra of lysozyme at various concentrations of ethanol and constant HCl content of 300 mM in the far-UV (A) and near-UV (B) regions

2.3.2.1 Circular dichroism method

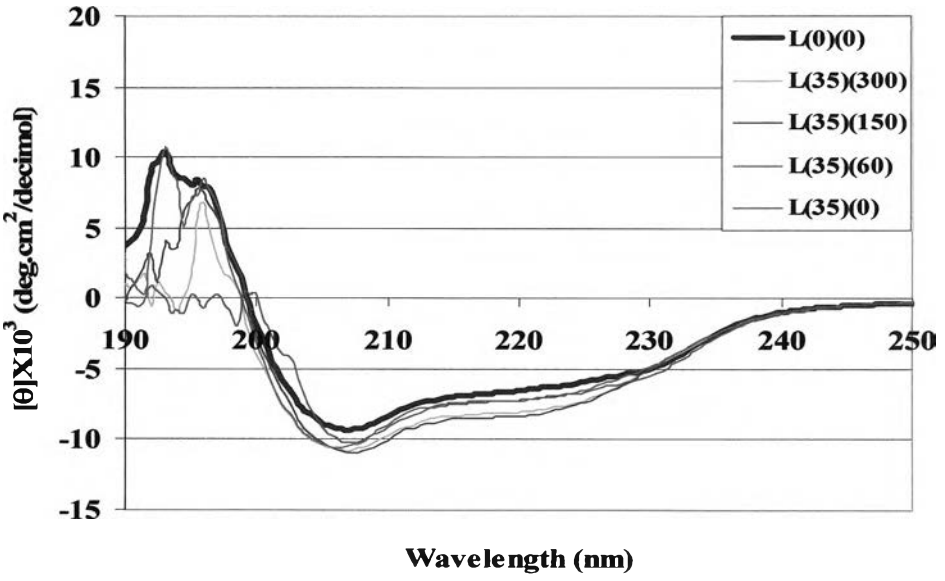
A. At 35% v/v ethanol

Lysozyme was dissolved in the medium of constant ethanol concentration at 35%v/v with variation in HCl acid contents. The amounts of HCl acid was decreased from 300 to 150 and 60 mM which were represented by L(35)(300), L(35)(150) and L(35)(60), respectively.

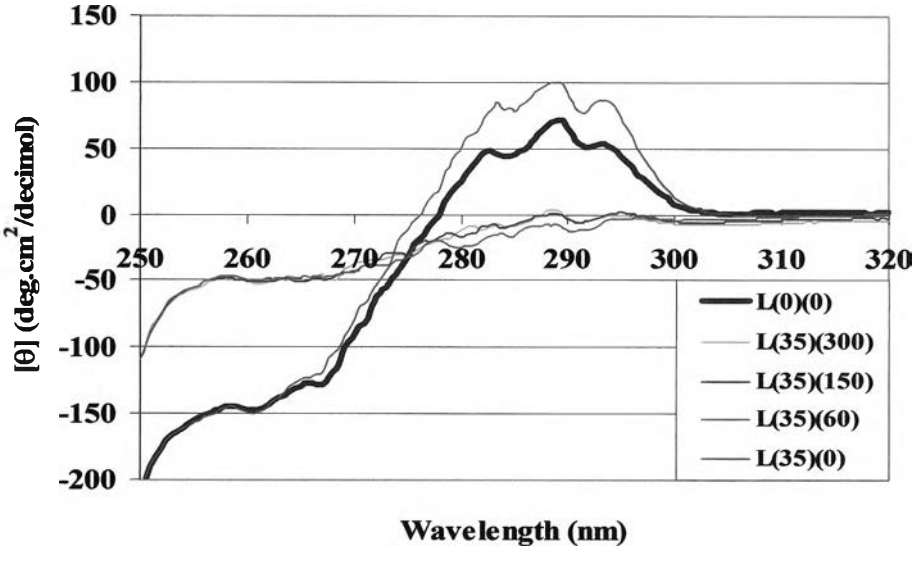
Secondary and tertiary patterns of lysozyme in 35% v/v ethanol without acid [L(35)(0)] were only slightly shifted from the native. In contrast, the tertiary structure was greatly interrupted when 60 mM HCl acid was added to the hydroethanolic solution. However, their secondary structures were stabilized with higher helical contents (Figure 42).

Further, reduction of HCl acid concentration was done to find the lowest possible amount of HCl acid in 35% v/v ethanol which generated MG characteristics. HCl acid concentrations were decreased to 30, 22.5, 20, 15 and 10 mM in constant 35% v/v ethanol contents. The protein's secondary and tertiary conformation results are displayed in Figure 43. In Figure 43 A, the CD spectra presented similar helical conformations to the native pattern when lysozyme was solubilized in 35% v/v ethanol with 30 mM HCl [L(35)(30)]. There was also the loss in the tertiary structures when observed in the near-UV region (Figure 43 B).

Reduction of HCl acid concentration to 22.5-15 mM in 35% v/v of ethanol in the aqueous-ethanolic mixture showed that some traces of tertiary structures still retained. The tertiary structure was increasingly detectable when the HCl acid content was reduced. Secondary conformations, however, were detectable with similar helical structure to the native state. Besides, when HCl concentration decreased to 10 mM in hydroethanolic solution, the spectra became similar to the secondary and tertiary conformation of the native lysozyme (Figure 43).

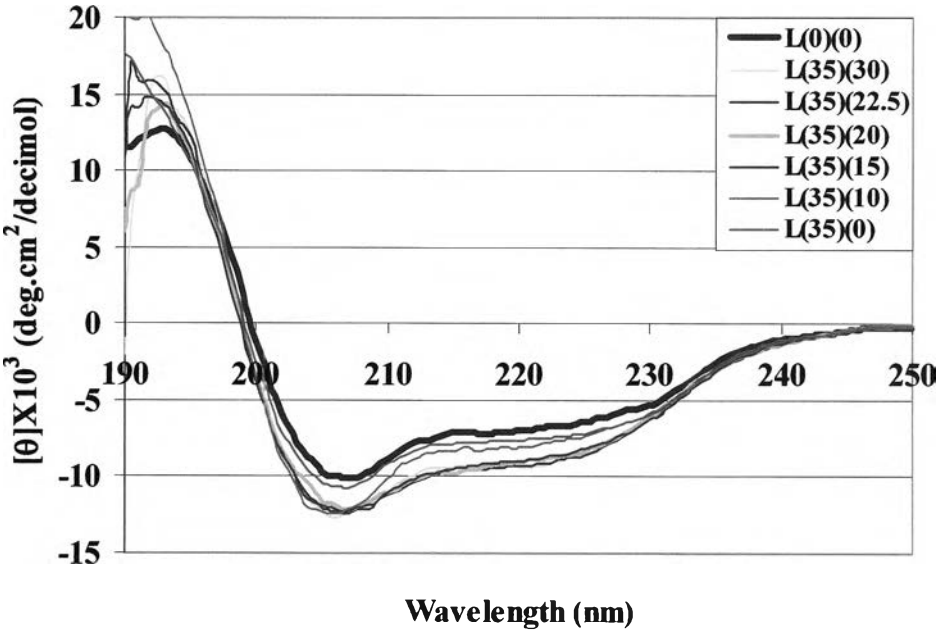


A

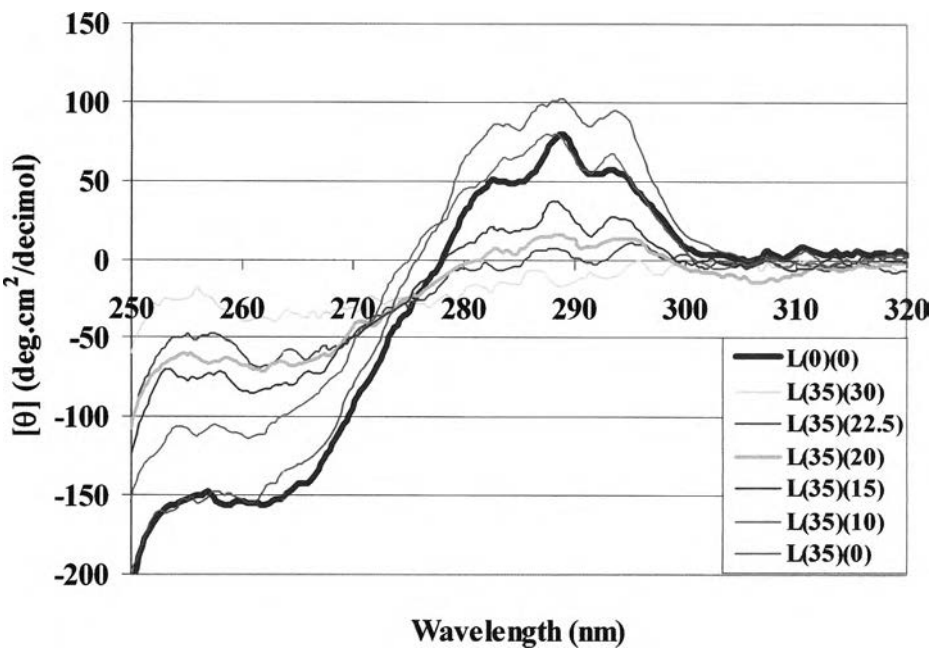


B

Figure 42 The CD spectra of lysozyme at various concentrations of HCl (60-300 mM) and constant 35% v/v ethanol in the far-UV (A) and near-UV (B) regions



A



B

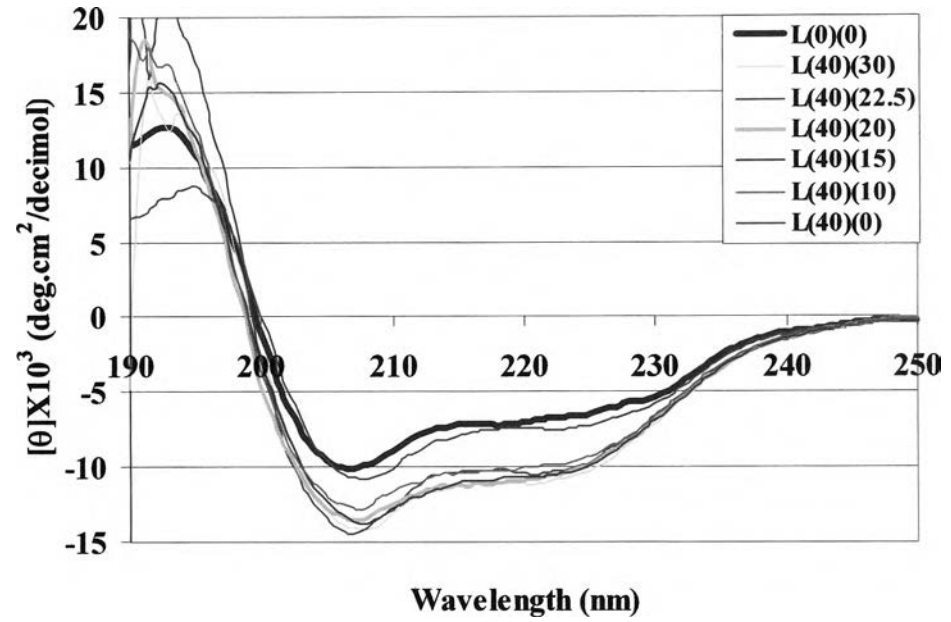
Figure 43 The CD spectra of lysozyme at various concentrations (10-30 mM) of HCl and constant 35% v/v ethanol in the far (A) and near-UV (B) regions

It can be concluded that, at constant 35% v/v ethanol, the initiation of lysozyme structural modification to MG state occurred when HCl acid content in the medium was at least 15 mM. At higher HCl acid concentration in 35% v/v ethanolic solution, the tertiary structure was further disrupted, but the secondary structure was stabilized with high α-helix content. At HCl acid of lower than 15 mM, no significant changes in tertiary and secondary conformation were found.

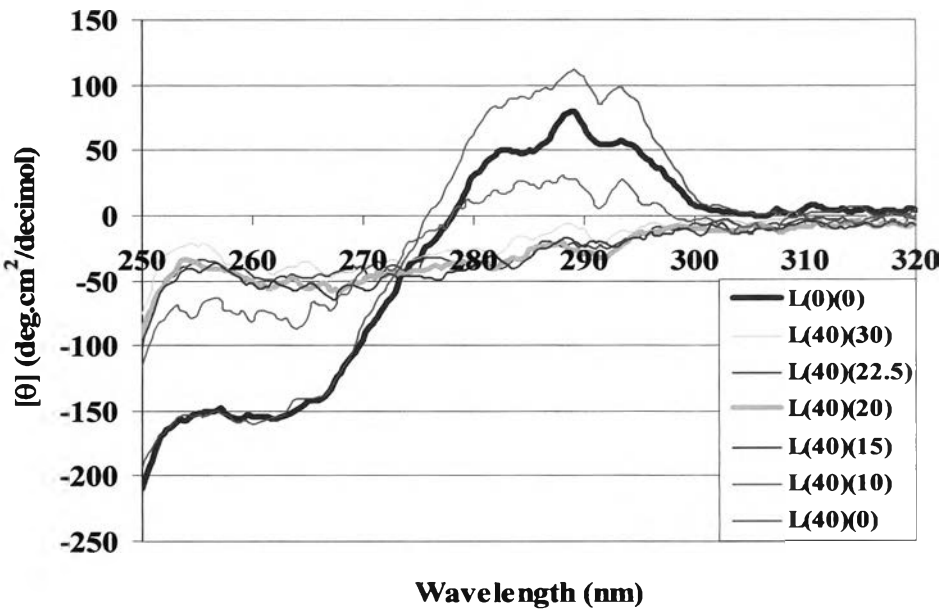
B. At 40% v/v ethanol

The comparison of ethanolic content of 40% v/v with varied acidic condition was done. Lysozyme was exposed to content 40% v/v of ethanol in the aqueous-ethanolic solution and variation of HCl acid concentrations of 30, 22.5, 20, 15 and 10 mM. Solubilization of lysozyme in 40% v/v ethanol without acid showed that the conformation was not different from the native spectrum as illustrated in Figure 44. A significant reduction in lysozyme tertiary structures was seen when the amount of ethanol was kept constant at 40% v/v and HCl acid concentration was in the range of 30 to 15 mM. The secondary spectra still revealed high α-helix contents. Reduction of HCl acid concentration to 10 mM at constant 40% v/v ethanol started to disrupt the tertiary structure. The secondary structures were still retained in the expanded helical conformation.

It could be concluded that at constant ethanol contents of 40% v/v, the amounts of HCl acid of at least 10 mM initiated lysozyme conformational change. At higher HCl acid concentrations in 40% v/v ethanolic solution, the tertiary structure of lysozyme was completely destroyed. The secondary structure retained with high helical content. Hence, the combined environment which contained 40% v/v ethanol was more efficient in inducing lysozyme structural modification than that contained 35% v/v ethanol using the same acidic condition.



A



B

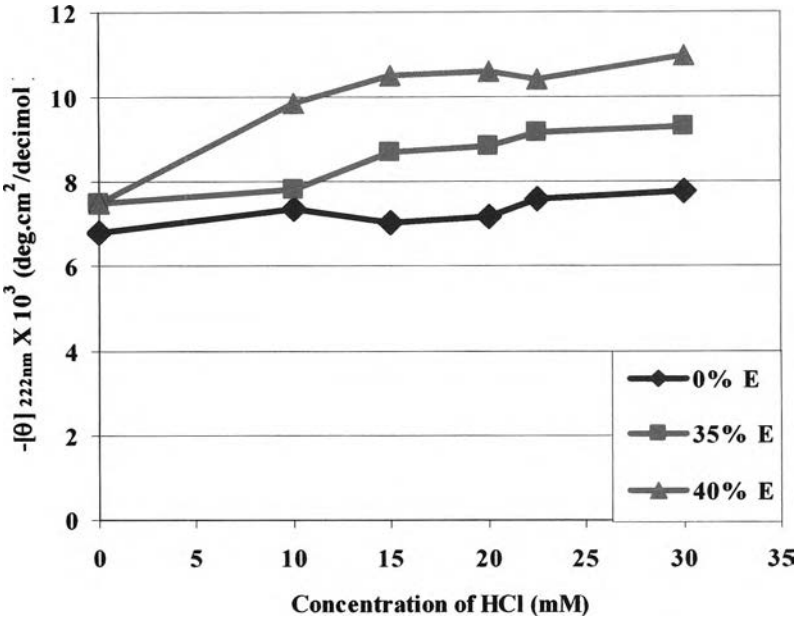
Figure 44 The CD spectra of lysozyme at various concentrations (10-30 mM) of HCl and constant 40% v/v ethanol in the far-UV (A) and near-UV (B) regions

Figure 45 shows the secondary (Figure 45 A) and tertiary (Figure 45 B) CD intensities of lysozyme in combined solution which consisted of hydroethanolic solution and low HCl content. The secondary and tertiary intensities of lysozyme were close to native intensities when it was solubilized in either 35% or 40% v/v ethanol or acidic environment (10-30 mM). The secondary and tertiary intensities of lysozyme in combined environments were significantly different from conformation of lysozyme that was dissolved in each environment. At far-UV region, the intensities of lysozyme dissolved in constant 35% or 40% v/v ethanol with various HCl concentrations were shown to have helical intensities greater than lysozyme in the same acidic condition without ethanol. However, the decrease in tertiary intensities was initiated when the amounts of HCl acid was at least 15 mM in 35% v/v ethanol and at least 10 mM in 40% v/v of ethanol.

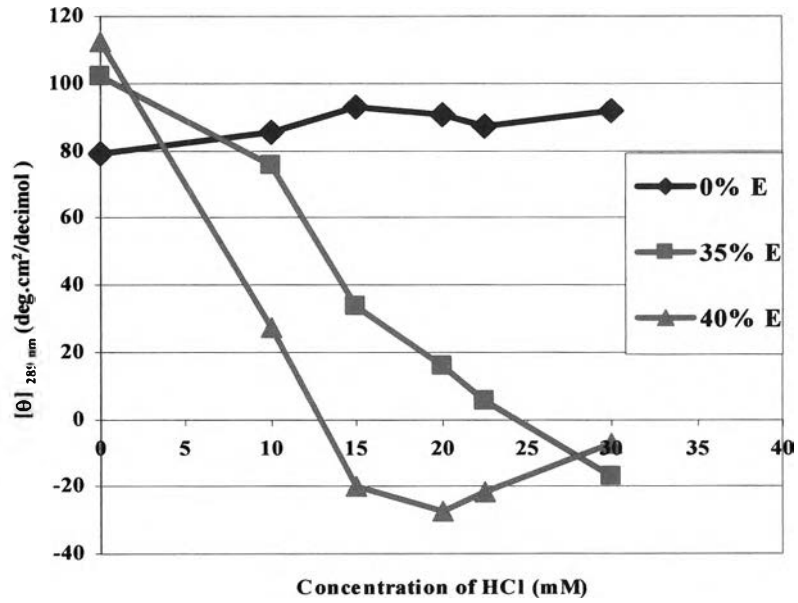
2.3.2.2 Fluorometric technique.

Fluorometric technique was used to detect the conformational alteration of lysozyme in the combined solutions. This was to confirm with the prior CD results. The formulas which contained 35% and 40% v/v ethanol with low contents of HCl (10-30 mM) were selected for this study.

Figure 46 A and 46 B shows that at 35% v/v and 40% v/v ethanol without acid, respectively, fluorescent intensities were very low. Introduction of HCl acid in both 35% v/v and 40% v/v ethanol solution from 10 to 20 mM caused the intensities to continuously increase. Both ethanolic concentrations that contained 20 mM HCl acid gave the highest fluorescent emission intensities due to strong binding with ANS, indicating partially unfolded or MG state. On the contrary, at higher contents of HCl acid, the fluorescent intensities were decreased. It might be because the globularity of lysozyme was disrupted by the acid, resulting in the reduction in ANS binding (Bhattacharjya and Balaram, 1997; Kamatari et al, 1998).

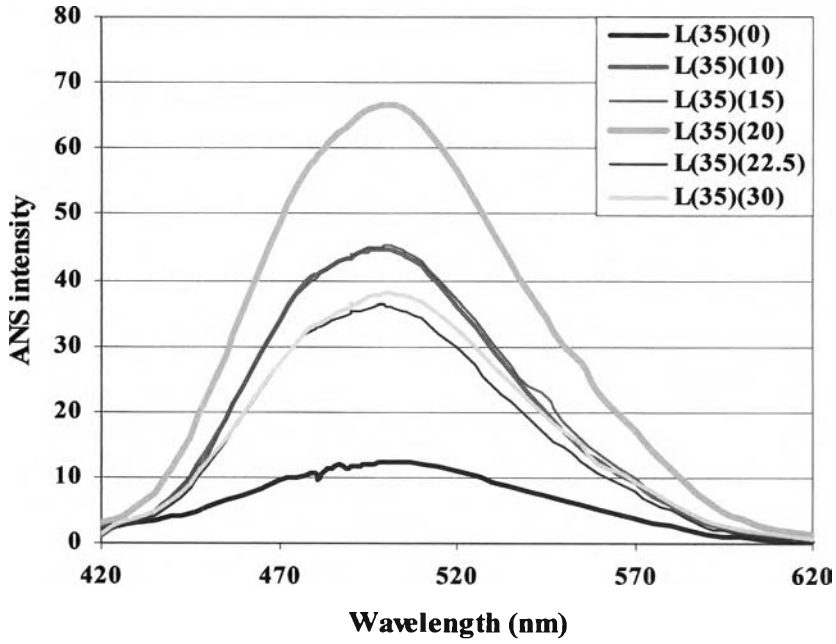


A

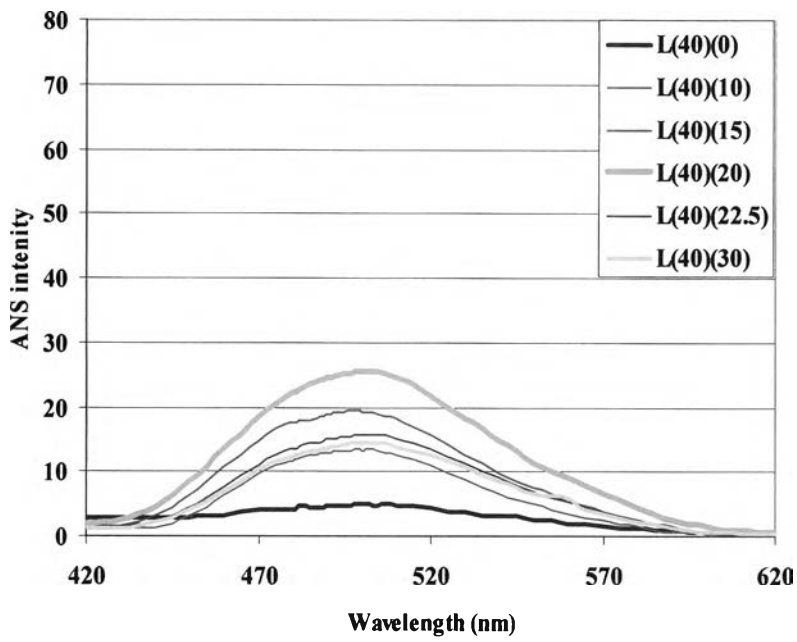


B

Figure 45 Comparison of CD intensities at 222 (A) and 289 (B) nm of lysozyme in 0% v/v, 35% v/v and 40% v/v ethanol with varying HCl acid concentrations (10-30 mM)



A



B

Figure 46 Fluorometric emission spectra of lysozyme bound to ANS after being exposed to 35% v/v (A) and 40% v/v ethanol (B) with low concentrations (10-30 mM) of HCl acid

Figure 47 illustrates a tendency of ANS-binding with lysozyme solubilized in 35%v/v and 40%v/v ethanol with various HCl concentrations. From Figure 47, the optimum concentration of HCl in 35%v/v and 40% ethanol to generate MG state is 20mM because lysozyme solubilized in 20 mM HCl acid solution with 35% v/v and 40% v/v ethanol gave the highest intensity compared with other HCl acid concentrations.

The combined effect of HCl acid and ethanol was due to the electrostatic effect and the removal of water on the protein surface. At low pH, the anions from HCl acid would decrease the electrostatic repulsion which was an important force on the positive charge of the protein structure and they also affected water molecules. Therefore, with introduction of ethanol into this condition, the protein could be easily dehydrated and MG state was more pronounced even at low ethanol concentrations (Goto et al, 1990; Bychkova et al, 1996; Konermann and Douglas, 1997).

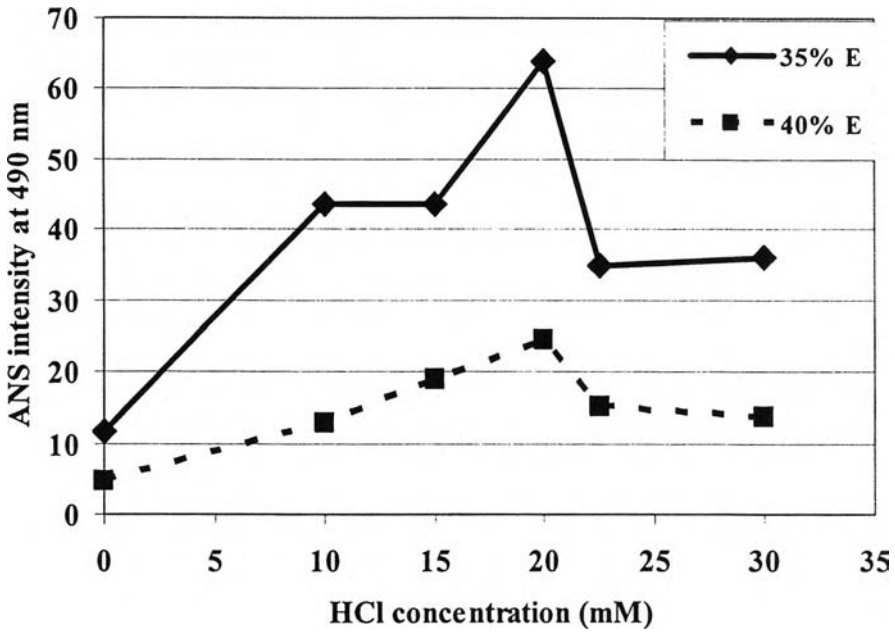


Figure 47 Fluorometric emission intensities at 490 nm of lysozyme bound to ANS after being exposed to 35% v/v and 40% v/v ethanol with low concentration (10-30 mM) of HCl acid

Table 3 and 4 briefly describe the secondary and tertiary structures of lysozyme in 35% and 40% v/v ethanol with low HCl concentrations,

respectively. The addition of HCl acid (at least 20mM) in both 35% and 40% v/v ethanol could initiate MG state.

Table 3 The summary of lysozyme structures exposed to 35% v/v ethanol with 10-30 mM HCl contents as characterized by CD and fluorometric techniques

Formula	CD		Fluorescence	Lysozyme structure
	Secondary structure	Tertiary Structure	ANS binding	
L(35)(0)	+	+	-	Folded
L(35)(10)	+	+	-	Partially folded
L(35)(15)	+	+	-	Partially folded
L(35)(20)	+	-	+	MG state
L(35)(22.5)	+	-	-	Unknown
L(35)(30)	+	-	-	Unknown

Table 4 The summary of lysozyme structures exposed to 40% v/v ethanol with 10-30 mM HCl contents as characterized by CD and fluorometric techniques

Formula	CD		Fluorescence	Lysozyme structure
	Secondary structure	Tertiary Structure	ANS binding	
L(40)(0)	+	+	-	Folded
L(40)(10)	+	+	-	Partially folded
L(40)(15)	+	-	-	Partially folded
L(40)(20)	+	-	+	MG state
L(40)(22.5)	+	-	-	Unknown
L(40)(30)	+	-	-	Unknown

\+ = existence of specific response

\- = absence of specific response

The environment that contained 35% v/v ethanol in 20 mM of HCl concentration 'L(35)(20)' was selected for future examination. Both L(35)(20) and L(40)(20) initiated MG state; however, lowest concentrations of the two factors were chosen and believed to be most suitable for transdermal application.

This combination of solvent [L(35)(20)] was also determined using various lysozyme contents to confirm that each concentration of lysozyme would result in the same conformation. The spectra of various lysozyme concentrations (0.05, 0.1, 0.5 and 1 mg/ml) were examined both in the far-UV and near-UV regions as shown in Figure 48 and Figure 49, respectively.

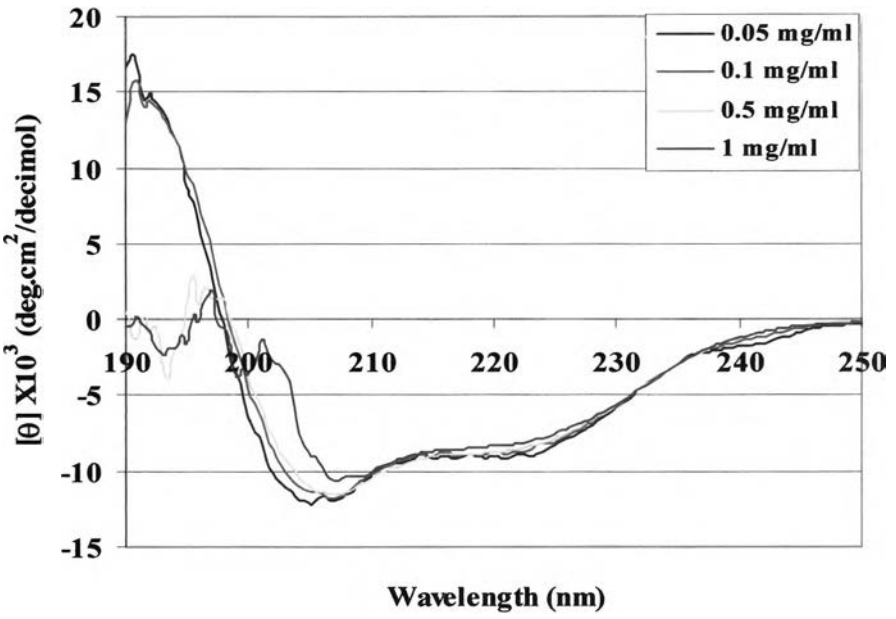


Figure 48 The CD spectra of lysozyme dissolved in 35% v/v ethanol with 20 mM HCl at various lysozyme concentrations in the far-UV region

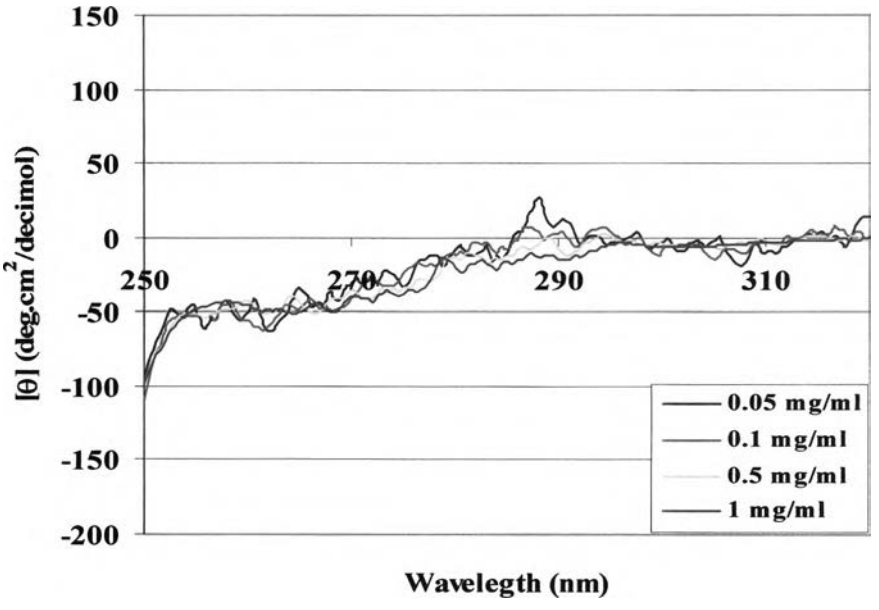


Figure 49 The CD spectra of lysozyme dissolved in 35% v/v ethanol with 20 mM HCl at various lysozyme concentrations in the near-UV region

2.3.2.3 Gel electrophoresis

The integrity of lysozyme was directly determined by SDS-PAGE technique. The formulas used were the formulas that contained 6 N HCl, 35% v/v ethanol with 20 mM HCl [L(35)(20)] and 20 mM HCl without ethanol [L(0)(20)], which was used as control for formula L(35)(20). Figure 50 displays the intact electrophretic bands of L(35)(20) and L(0)(20). In contrast, there was no defined lysozyme band present when it was exposed to 6 N HCl. This was due to the strong acidic condition of 6 N HCl which destroyed the structure of lysozyme into smaller molecular weight fractions.

This study showed that lower concentrations of HCl acid alone did not disrupt the polypeptide chains of lysozyme even when 35% v/v ethanol was added in the solution. However, higher concentration of acid (6 N HCl) broke lysozyme into small fragments which were not detectable under the gel electrophoretic conditions used.

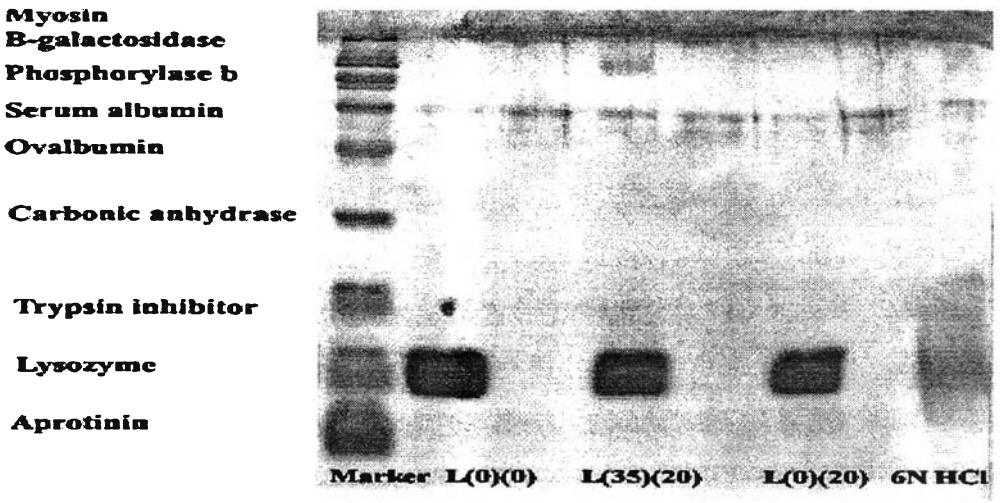
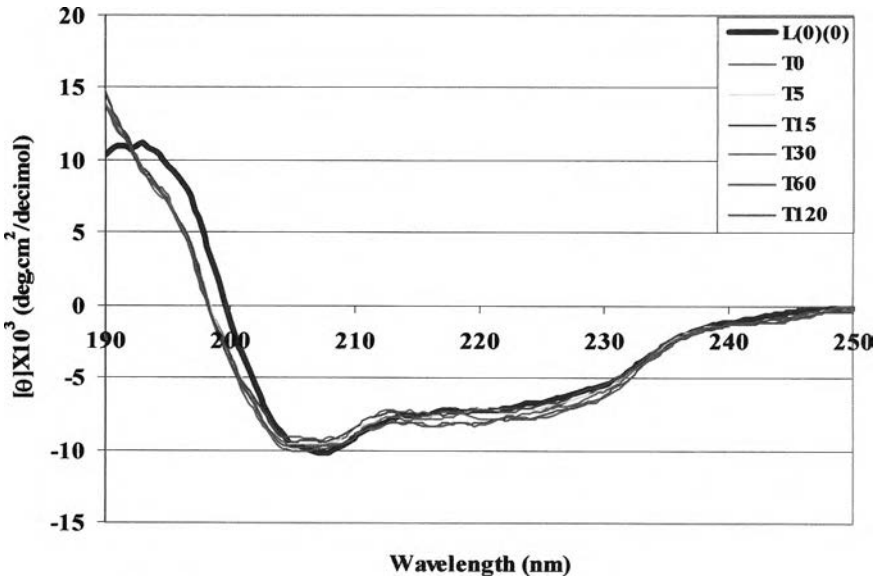


Figure 50 SDS-PAGE analysis of lysozyme under various conditions

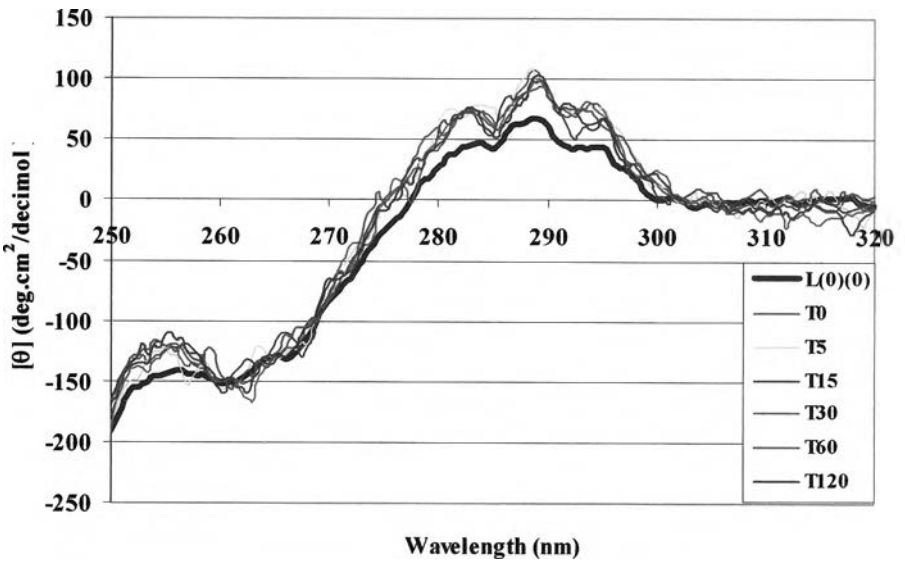
3. Reversibility study

The goal of this experiment was to study the conformational reversibility of lysozyme which was converted to MG or partially folded states after exposure to ethanolic and acidic environments. This was to investigate whether MG lysozyme will reverse to the native structure after penetration through the stratum corneum into a more diluted condition within the epidermis. Therefore this section was intended to determine the state of modified lysozyme in the diluted environment.

The conditions which generated dramatic transformation of lysozyme structure to MG state were obtained from sections 2.1 and 2.3. The protein was solubilized in each condition at the concentration of 1 mg/ml and water was then added twenty times the original volume. The conformational observation was carried out using CD technique. The formula contained 80% v/v ethanol [L(80)(0)] which initiated MG state of lysozyme was used as model condition in the preliminary of reversibility study. It was done to investigate effect of time on the conformational alteration. The solution was diluted and the conformation was determined at 0, 5, 15, 30, 60 and 120 minutes. The spectra at each time period were similar for both the secondary and the tertiary structures as shown in Figure 51. This confirmation study was done to reveal that the time of detection did not affect the change in conformation.



A



B

Figure 51 The CD spectra of lysozyme dissolved in L(80)(0) solution after being diluted 20 times with water at various times in the far-UV (A) and near-UV (B) regions

3.1 Reversibility of lysozyme

This determination was emphasized at the conditions which caused the conformational transformation. Each condition was divided into three groups. The first group [reversed (F)], lysozyme was dissolved in the medium of interest and immediately diluted with water. The second group [reversed (O)], the water was added after the protein had been exposed to the specific solution overnight. The third group [reversed (C)], lysozyme was directly incorporated in the diluted solution at a final concentration. This group was used as a control group.

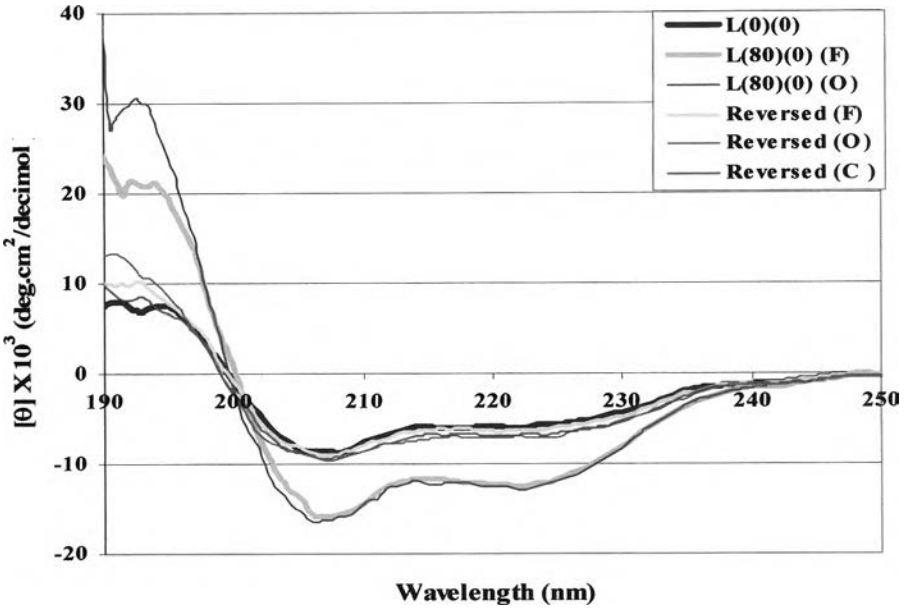
3.1.1 Partially folded or MG states

From the results, L(80)(0) and L(35)(20) were the formulas which generated partially unfolded or MG state of lysozyme.

3.1.1.1 L(80)(0)

Lysozyme solubilized in 80% v/v ethanol for a few seconds [L(80)(0)(F)] and overnight [L(80)(0)(O)] displayed similar MG spectra for both secondary and tertiary patterns. There was no observable tertiary structure while there was a pronounced high contents of α-helix as illustrated in Figure 52.

Upon introduction of water into both solutions, the disrupted tertiary conformations were reformed to the native state and the helical secondary contents were reduced back to the native lysozyme (Figure 52).



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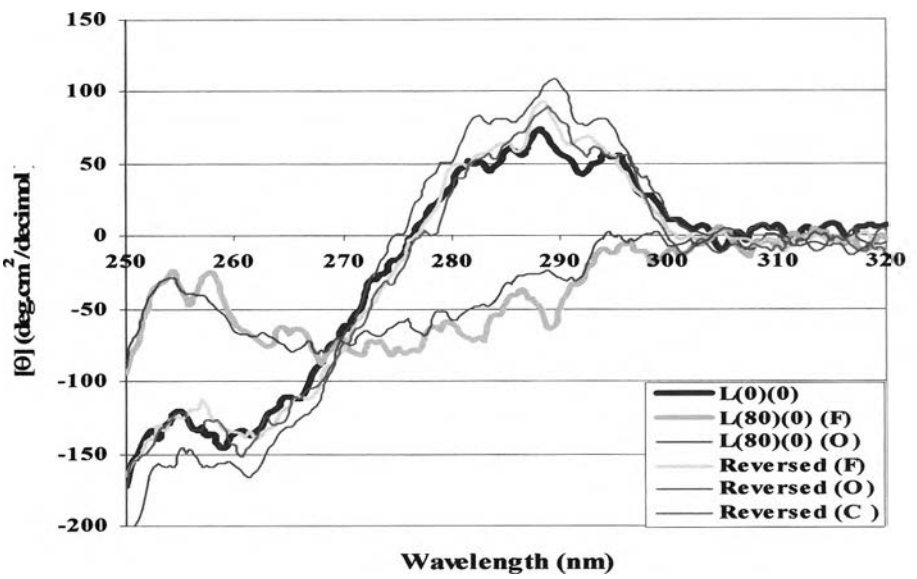


B

Figure 52 The CD spectra of lysozyme dissolved in L(80)(0) solution which was freshly prepared and kept overnight, after the solutions were diluted 20 times with water, and a control solution in the far-UV (A) and near-UV (B) regions

3.1.1.2 L(35)(20)

The spectra of the conditions under which lysozyme was dissolved in 35% v/v ethanol with 20 mM HCl [L(35)(20)(F)] and incubated overnight [L(35)(20)(O)] presented similar secondary and tertiary patterns. Upon adding water into both solutions, the conformations reversed to lysozyme in the native form as seen in the control spectra (Figure 53).

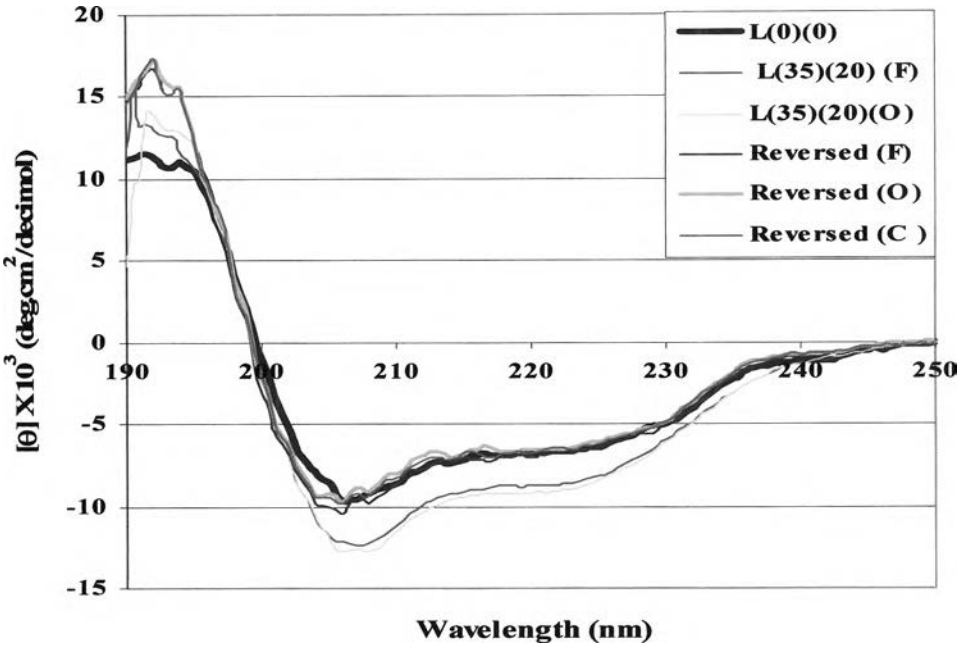
Mottos and Ringe (2001) reported that some dehydrated proteins could be reversed to the native state with increased water contents in the environment. Consequently, it was pointed out that the conditions which generated MG state induced only temporary conformational rearrangement but it did not irreversibly disrupt the polypeptide chains of lysozyme.

3.1.2 Unfolded state

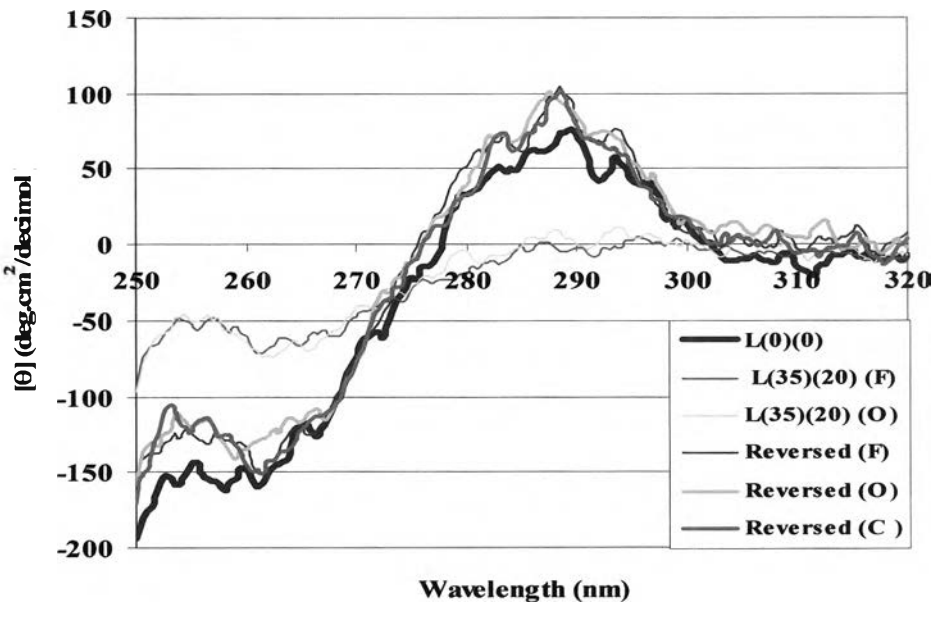
3.1.2.1 At 4 N HCl

The CD spectra of lysozyme which was freshly prepared in 4 N HCl, were similar to native spectra for both the secondary structure and tertiary structure spectra. Lysozyme solubilized in 4 N HCl overnight resulted in aggregation and did not show any structural data in CD spectra. Thus, this experiment was focused on this aggregated form if it was reversible or irreversible form. In the diluted environment, the spectra of secondary and tertiary conformations did not convert back to the native form as the control group (Figure 54).

It was a clear indication that the aggregation caused by incubating lysozyme in 4 N HCl overnight was irreversible. It did not reverse to the native lysozyme conformation in the diluted environment. It was also found that for irreversible aggregation to occur, sufficient time must be allowed.



A



B

Figure 53 The CD spectra of lysozyme dissolved in L(35)(20) solution which was freshly prepared and kept overnight, after the solutions were diluted 20 times with water, and a control solution in the far-UV (A) and near-UV (B) regions

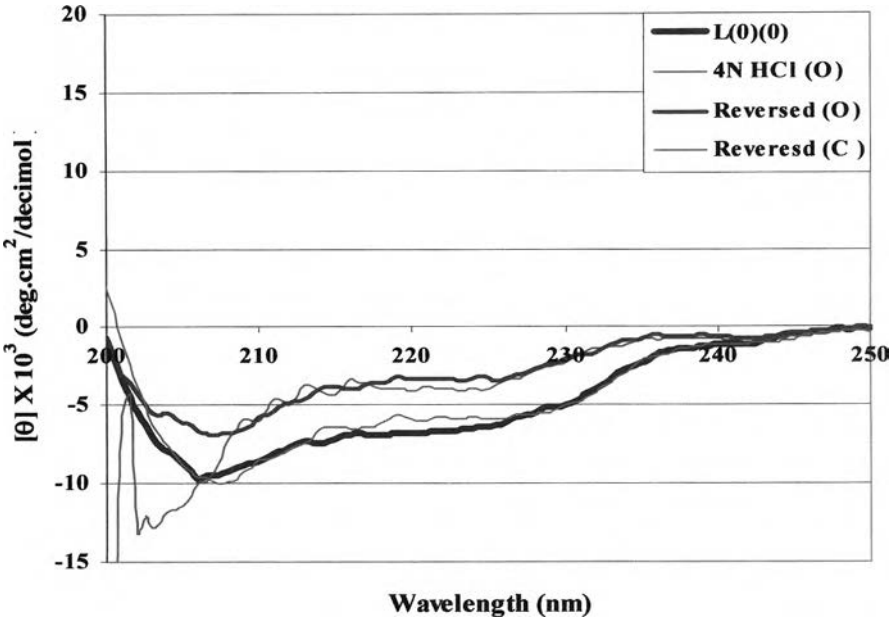
3.2.2.1 At 6 N HCl

The CD spectra of lysozyme that was immediately solubilized in 6 N HCl was least pronounced in both the secondary and tertiary structures. This result was the same as that of incubation of the solution overnight which obtained from section 2.2. They displayed the same spectra (6 N HCl in Figure 55). Introduction of water to the modified lysozyme reversed the protein to the native state corresponding to the control group (Figure 54). However, incubation of lysozyme in 6N HCl overnight and adding water afterward showed no observable spectra both in the far-UV and near-UV regions (Figure 55).

A brief exposure of lysozyme to the strong acidic environments did not irreversibly destroy the polypeptide chains of the protein. It might be because disulfide cross-links were still intact. Therefore random coil conformation could be reversed rapidly back to a globular protein under diluted condition. In contrast, solubilization of lysozyme in 4 N and 6 N HCl overnight caused irreversibility of lysozyme conformational change due to the fact that the disulfide cross-links were completely disrupted (Chaffotte et al, 1992; Rothwoarf and Scheraga, 1996) and aggregation was induced permanently.

The results of reversibility study of lysozyme are summarized in Table 5. All lysozyme conformations in various solutions if immediately prepared could reverse back to the native state. On the contrary, lysozyme in 4 N and 6 N HCl that was kept overnight was not able to convert back to the native form.

In summary HCl and ethanol were the two factors, with the appropriate ratios, could be used to introduce the modification of lysozyme structure. The considered states were the partially unfolded or MG states where helical secondary structures of lysozyme were stabilized with no defined tertiary conformation and the structure was still compacted (Peng and Kim, 1994; Privilov, 1996).



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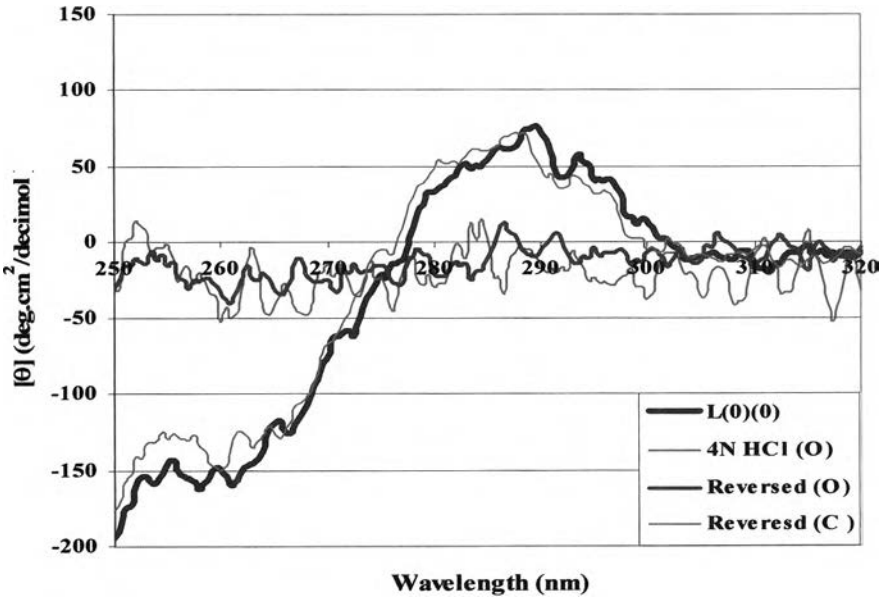
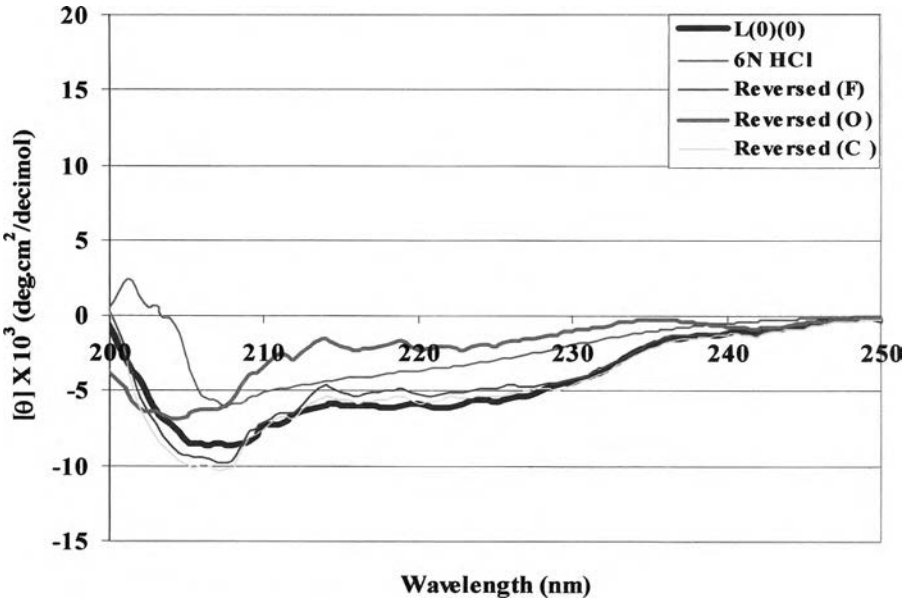


B

Figure 54 The CD spectra of lysozyme dissolved in 4 N HCl which was kept overnight, after the solutions were diluted 20 times with water, and a control solution in the far-UV (A) and near-UV (B) regions



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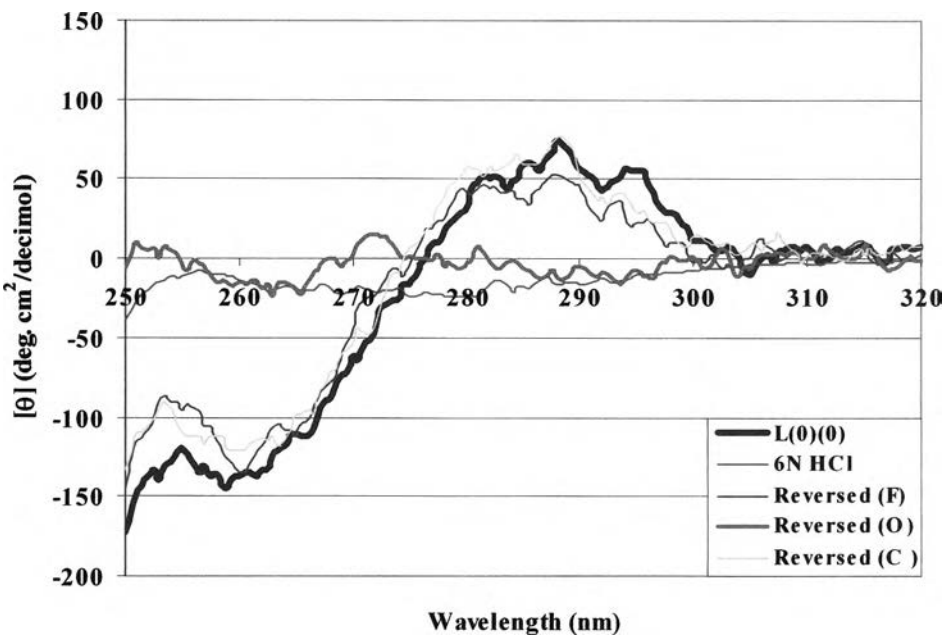


B

Figure 55 The CD spectra of lysozyme dissolved in 6 N HCl which was freshly prepared and kept overnight, after the solutions were diluted 20 times with water, and a control solution in the far-UV (A) and near-UV (B) regions

Table 5 The summary of lysozyme conformations in various conditions and lysozyme conformations after the solutions was diluted 20 times with water as characterized by CD technique

Formula	Freshly prepare Sample (F)		Sample kept Overnight (O)		Reversibility		
	Secondary Structure	Tertiary Structure	Secondary Structure	Tertiary Structure	F	O	C
L(80)(0)	+	-	+	-	R	R	R
L(35)(20)	+	-	+	-	R	R	R
L(4N HCl)	+	+	-	-	R	NR	R
L(6N HCl)	-	-	-	-	R	NR	R

F = freshly prepare sample
O = sample kept overnight
C = a control group
+ = existence of specific response
- = absence of specific response
R = reversed to native conformation
NR = not reversed to native conformation

The environments that contained only HCl could not convert lysozyme conformation to partially folded state or MG state even when highly acidic conditions were used. On the other hand, the MG state of lysozyme was generated at 80% v/v ethanol without acid. Interestingly, this MG state was also induced when the combined environment which consisted of only 35% v/v ethanol and 20 mM HCl acid were used. Therefore, the formulas which would be used in the future experiments were L(80)(0) and L(35)(20).

Part II Penetration

This part was aimed to study the possibility of modified lysozyme, which was presented as MG or partially folded characteristics, to permeate a model skin. The present study was expected that its molecular hydrophobicity could be a dominant character in the penetration mechanism. Pig's ear skin was selected as an in vitro model skin using modified Franz-diffusion technique.

The following sections represented the penetration results of lysozyme. Two methods of detection were used to determine the amount penetrating through the model skin.

1. Pig's ear skin integrity test

From the previous sections, formulas which induced lysozyme to MG state were L(80)(0) and L(35)(20). The composition of these solutions was quite harmful to both human and the model skin. From this reason, a question was raised whether the modified lysozyme permeated into the receiver by its molecular hydrophobicity or through leakage in the model skin. Consequently, 'Pig's ear skin integrity test' was proposed to evaluate the integrity of the model skin before it was used in the future experiment.

The aim of this section was to determine the effects of vehicles on the integrity of model skin. This section was separated into two parts as followed;

1.1 Modified Franz diffusion technique

The effect of vehicles on the conformation of the model skin was evaluated. If the integrity of the model skin was not altered when exposed to various conditions of interest, the hydrophilic molecules from the donor compartment should not pass through and be presented in the receiver compartment.

Propranolol HCl is known to be hydrophilic. It was used as a hydrophilic drug marker for this study. It is due to the fact that propranolol HCl does not degrade in acidic environment (Figure 63 in Appendix B) as opposed to other hydrophilic fluorescent probes.

In this experiment, propranolol HCl was added in four solutions of interest at 10 mg/ml concentration instead of lysozyme. The first group, propranolol HCl was dissolved in water [P(0)(0)] which was a representative of the native environment. The second group, two solutions which was shown to generate MG state of lysozyme were used and propranolol HCl was solubilized in 80% v/v aqueous-ethanolic solution [P(80)(0)] and in 35% v/v aqueous-ethanolic solution in 20 mM HCl acid [P(35)(20)]. The third group, propranolol HCl was dissolved in low concentration of ethanol at 35% v/v [P(35)(0)].

Figure 56 illustrates the percentages of cumulative amount of propranolol HCl in various conditions which penetrated through pig's ear skin and Table 6 presents cumulative percentages of propranolol HCl penetrated and the standard deviations (SD). Solution P(0)(0) gave the highest cumulative amount of propranolol HCl in the receiver compartment after six hours. The amounts penetrated of propranolol HCl in formula P(35)(0), P(35)(20) and P(80)(0) were lower than P(0)(0).

In addition, the penetration profiles of propranolol HCl (Figure 56) solubilized in various conditions were statistically compared using similarity factor (f_2). Generally, f_2 value of higher than 50 shows similarity of the profiles compared (Yuksel et al, 2000). Propranolol HCl dissolved in water (P(0)(0)) was used as reference. It was due to the fact that this condition was a representative of native environment which was found not to affect the model skin and the protein.

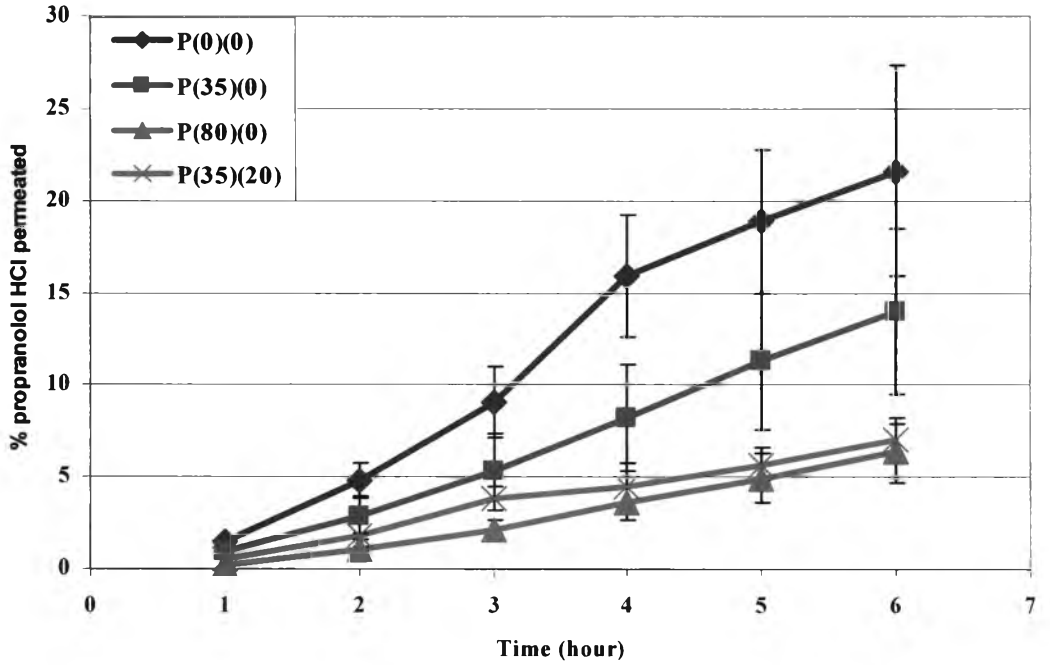


Figure 56 Graphical representation of cumulative percentages of propranolol HCl permeated through pig's ear skin over time.

Table 6 Cumulative percentages of propranolol HCl permeated through pig's ear skin over time.

Time (Hour)	% cumulative amount of propranolol HCl (± SD)			
	P(0)(0)	P(35)(0)	P(80)(0)	P(35)(20)
1	1.50 ± 0.92	0.97 ± 1.01	0.21 ± 0.01	0.55 ± 0.17
2	4.81 ± 1.93	2.93 ± 2.08	1.06 ± 0.52	1.78 ± 0.63
3	9.03 ± 3.31	5.29 ± 2.87	2.14 ± 0.94	3.87 ± 1.31
4	15.94 ± 3.83	8.21 ± 3.71	3.58 ± 1.30	4.46 ± 0.94
5	18.93 ± 5.71	11.29 ± 4.51	4.96 ± 1.78	5.70 ± 0.90
6	21.59 ± 5.96	13.98 ± 4.92	6.43 ± 1.96	7.03 ± 0.88

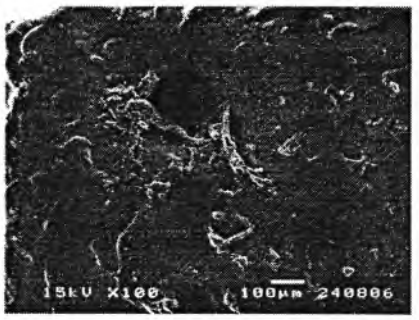
The f_2 values of P(35)(0), P(80)(0) and P(35)(20) when compared with the reference [P(0)(0)] were 61.94, 49.18 and 50.72, respectively. From the f_2 values, it was concluded that the penetration profiles of propranolol HCl of P(35)(0) and P(35)(20) formulas were similar to the reference profile (P(0)(0)). The f_2 value of formula P(80)(0) was close to the borderline of the classification. Statically, it was different than the profile of P(0)(0). However, the profile of P(80)(0) was not much different from the reference if observed visually from the graph (Figure 56). Thus, it was not appropriate to conclude that the penetration profile of formula P(80)(0) was similar or dissimilar to the profile of the reference [P(0)(0)].

The integrity test of the model skin might be concluded that the leakage in the model skin did not occur after exposure to various conditions of interest for six hours. If leakage of the skin occurred, the permeated amount of formulas P(35)(0), P(35)(20) and P(80)(0) would be higher than the permeated propranolol of formula P(0)(0) which represented the native environment. Although these solutions did not cause leakage of the model skin, they trend to decrease the penetration of propranolol HCl.

After six hours of this experiment with propranolol, the visual observation of pig's ear skin was done. It was found that pig's ear skin exposed to the solution which contained acid was swollen. However, other environments did not affect the model skin. From this evidence, the effects of ethanolic environment, acidic environment and the combined environment on pig's ear skin surface were further determined by Scanning Electron Microscopy.

1.2 Cryo-Scanning electron microscopy (Cryo-SEM) technique.

The treated pig's ear skin was evaluated using a specific method of Cryo-scanning electron microscopy (Cryo-SEM) technique. Pig's ear skin exposed to the conditions of interest without lysozyme for six hours to observe the effects of various environments on the model skin. The conditions were water [V(0)(0)], 35% v/v of ethanolic solution [V(35)(0)], 80% v/v ethanolic solution [V(80)(0)], 20 mM HCl acid [V(0)(20)] and 35% v/v of ethanol in 20 mM HCl acid [V(35)(20)]. The treated pig's ear skins detected by Cryo-SEM are illustrated in Figures 57.



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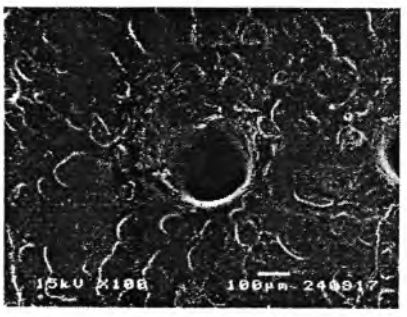

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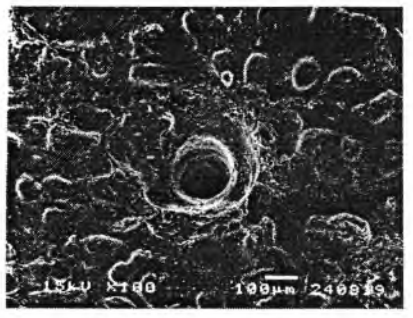

C

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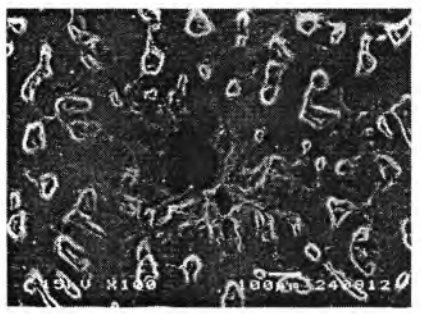

E

Figure 57 Treated pig's ear skin after exposure to various conditions for six hours using Cryo-SEM technique. A. V(0)(0), B. V(35)(0), C. V(80)(0), D. V(0)(20) and E. V(35)(20)

The micrographs of the skin in ethanolic solution, Figures 57 B and 57 C, show that the increasing ethanolic concentration induced dehydrated skin and well-defined pores of skin when compared with the skin surface which exposed to low ethanolic concentration [L(35)(0)] and without ethanol [L(0)(0)].

In the group which contacted with acid, the micrographs show that the skin was perturbed by both the combined environment (Figure 57 E) and when exposed to acid alone (Figure 57 D). The roughness on the surface of pig's ear skin was different from the skin that was exposed to ethanolic solution where it was dehydrated. Acidic solution affected the skin surface by inducing swelling and loss of the skin structure (Allenby, 1969). It was concluded that the solution that consisted of only acid or acid in ethanol had physical effect on the pig's ear skin more than the solution which contained only ethanol in water even though at very high concentration.

At low concentrations of ethanol (20-70%), increased fluidity of lipids in skin occurs, leading to enhance permeation of lipophilic drugs where hydrophilic drugs are less permeated (Pillai et al, 2004). Similarly, our study showed the decrease in permeability of hydrophilic propranolol HCl with increasing ethanolic concentrations. The effect of ethanolic system on skin was proven to be highly dependent on the concentration of ethanol.

At high concentrations (> 70%v/v of ethanol), lipids from the skin are extracted, as a consequence, pore formation exists. Moreover, in the stratum corneum layer, the keratin fibrils are swollen and extraction of protein occurs (Suhonen et al, 1999; Pillai et al, 2004). As the result, permeability of hydrophilic and hydrophobic drugs is both shown to enhance. Furthermore, Manabe et al (1996) reported the estimation of pore radius of hairless rat skin which exposed to ethanolic solution (0-100% of ethanol). The pore radius of rat skin was 0.98 ± 0.60 nm after exposure to water without ethanol. However, at 100% of ethanol gave the maximum pore radius of 1.89 ± 1.24 nm. When the binary mixtures of water and ethanol were used (20%-80% of ethanol), the pore radius was determined to have the mean values of 0.42–0.50 nm.

Otherwise, the permeation enhancement is also dependent on pH system. It is due to the fact that the alteration of pH in solution affects the ionization of drug and the pathway of permeation as well (Katayama et al, 2001). In addition, Allenby (1969) reported if the skin was exposed to solution at pH lower than 3, the structural changes of stratum corneum was irreversible. Therefore, the effect of the combined environment was quite harmful on the model skin and might be responsible to the decreased propranolol HCl permeation.

The results of two experiments were considered. It was concluded that even though the leakage in the model skin which exposed to the solutions for six hours did not occur, they affected the permeation rate and the surface structure of the model skin.

2. Penetration Study

2.1 Detection of the penetrated amount of lysozyme by enzymatic activity

In this preliminary study, lysozyme enzymatic activity method using *Micrococcus lysodeikticus* as a substrate was proposed. This experiment was used to evaluate the amount of lysozyme which permeated in to the receiver compartment of modified Franz diffusion cells.

Determination of the penetration of lysozyme was divided into three groups and the concentration of lysozyme used was 1 mg/ml. First, lysozyme was dissolved in water and presented as native conformation L(0)(0). Second, lysozyme was solubilized in 35% aqueous-ethanolic solution with 20 mM of HCl acid L(35)(20) which represented the MG state group. Third, a negative control group contained water without lysozyme (H_2O).

The lysozyme enzymatic activity results are illustrated in Figure 58 Every formula showed the amount of lysozyme during each time interval even the negative control group. It may be due to the fact that some endogenous substances in the pig's ear were extracted and interfered with the detection of lysozyme in the

receiver compartment. Thus, this technique could not detect the actual permeated amount of hen egg white lysozyme in the solution of interest.

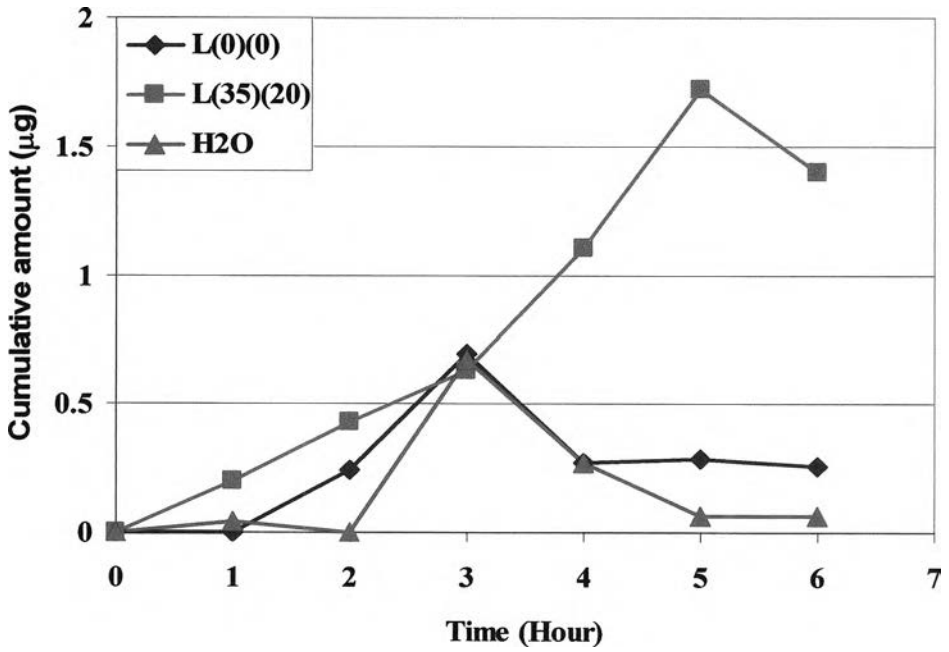

Figure 58 Graphical representation for the cumulative amount of lysozyme (n=2) which permeated through the pig's ear skin into the receiver compartment using lysozyme enzymatic activity detection technique.

Even through lysozyme enzymatic activity experiment was not suitable for the study, the result showed lysozyme potential to permeate the skin. Figure 58 shows that MG state group gave the highest cumulative amount of lysozyme in the receiver when compare with native and negative control groups after six hours. One possibility could be that the solvent containing ethanol and HCl acid might extract part of endogenous lysozyme from the model skin. However, it was proven in later experiments (Part 2.2 and Figure 59) that the same vehicle (35% v/v ethanol in 20 mM HCl acid) did not increase lysozyme amount in the donor compartment showing that the endogenous lysozyme was less likely to be extracted from the skin. Another possible explanation for the increased in lysozyme amount in the receiver side was that lysozyme when induced to MG state might be able to penetrate across the skin better than the native lysozyme. This result needed further confirmation which would be shown by other methods in the future experiments such as fluorescent labeling and CD techniques.

The next experiment was the labeling of lysozyme using fluorescent probe (Rhodamine Red, Sigma). It would be done to evaluate the penetration of the labeled lysozyme into pig's ear skin with a possibility to detect the penetrated labeled lysozyme with confocal microscopy. However, detection of the labeled lysozyme dissolved in water did not yield the native state. The tertiary structure was retained, while the secondary structure was disrupted (Figure 65 in Appendix B). These conformational patterns were different from the conformation of unlabeled lysozyme.

From this finding, the labeled lysozyme could not express its conformation in the native environment. Consequently, if the labeled lysozyme were to be solubilized in other environments use in previous studies, its conformation will be different from the conformation of unlabeled lysozyme dissolved in the same environment. Thus, the labeling of lysozyme using Rhodamine Red was not appropriate for this study.

Since the previous penetration studies could not evaluate the actual amount of hen egg white lysozyme in the receiver side because of some problems such as interference of endogenous substances. Therefore, the following study was to determine the remaining amount of lysozyme in the donor compartment instead of the receiver compartment.

2.2 Detection of the penetrated amount of lysozyme by CD technique

This experiment used modified Franz diffusion cell technique and analyzed the amount of lysozyme left in the donor compartment by CD method. Lysozyme was solubilized at concentration of 1 mg/ml in various solvent conditions selected from previous studies. The condition of interest were lysozyme dissolved in water [L(0)(0)], 35% v/v of ethanolic solution [L(35)(0)], 80% v/v ethanolic solution [L(80)(0)] and 35% v/v of ethanol in 20 mM HCl acid [L(35)(20)]. The amount of lysozyme remaining in the donor compartment was evaluated after 0, 1, 2, 4 and 6 hours. The CD spectra of each formula are presented in Figure 67, 69, 71 and 73 in Appendix B for L(0)(0), L(35)(0), L(80)(0) and L(35)(20), respectively.

These CD spectra were expressed in ellipticity (mdeg). It was not normalized to molar ellipticity (deg.cm^2/decimol) as in other previous studies. Therefore, the difference in spectra as a function of concentration could be determined. The decrease in the CD intensity was linearly related to the reduction in lysozyme concentrations. All CD intensities were evaluated at 289 nm.

The CD intensity of lysozyme was analyzed as the amount remaining using calibration curve for each condition (Figure 66, 68, 70 and 72 for L(0)(0), L(35)(0), L(80)(0) and L(35)(20), respectively in Appendix B). Figure 59 graphically presents the decreasing amounts of lysozyme in the donor compartment which were solubilized at various conditions as a function of time. Determination of the amount of lysozyme remaining in donor side by the end of the experiment showed that the amount of lysozyme in L(80)(0) and L(35)(20) were greatly decreased. The amounts of lysozyme remaining in L(0)(0) and L(35)(0) were slightly reduced in slow and constant manner. The patterns of reduction for both conditions above were similar. As the rate of reduction of lysozyme in L(80)(0) and L(35)(20) were initially high after first hour and then finally reaching plateau at the end of the study.

The similarity factor (f_2) (Yuksel et al, 2000) was used to compare the profiles of lysozyme which solubilized in various conditions. Lysozyme dissolved in water (L0)(0)) was designated as reference. Due to the fact that this condition represented native environment, it did not affect the lysozyme conformation and the model skin. The f_2 values of L(35)(0), L(80)(0) and L(35)(20) when compared with the reference [L(0)(0)] were 57.60, 36.23 and 34.27, respectively. The f_2 value implied that the profile of L(35)(0) was not different when compared with L(0)(0). However, the profiles of lysozyme solubilized in L(80)(0) and L(35)(20) were different from the reference profile [L(0)(0)].

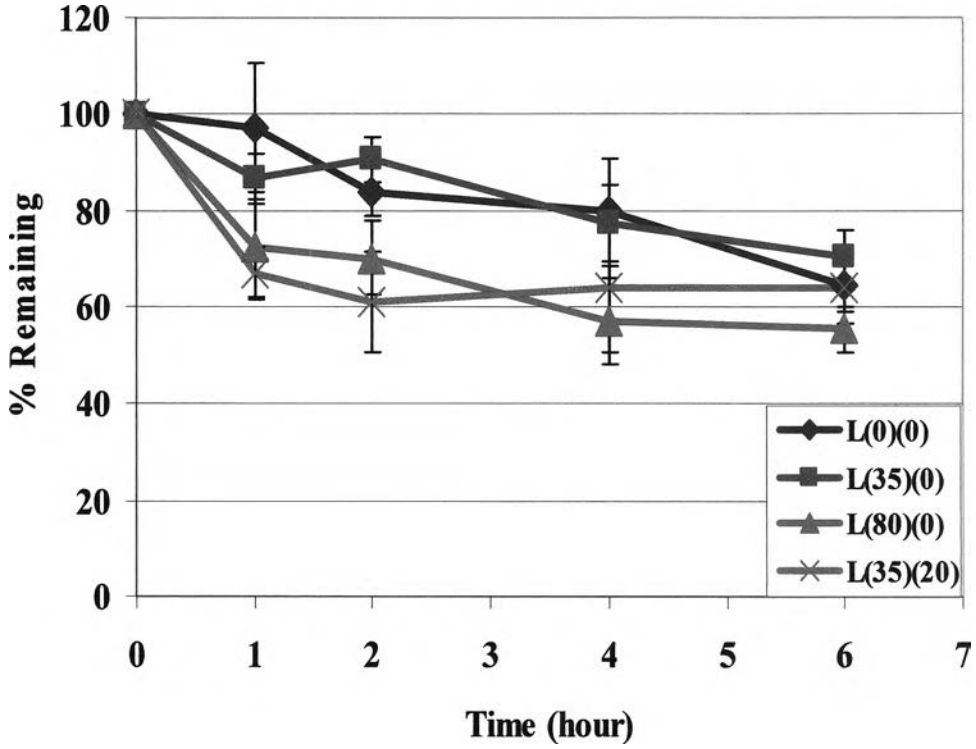

Figure 59 The graphical representation of the percentages for the remaining amount of lysozyme in donor compartment at various time intervals.

Table 7 The percentages of the remaining amount of lysozyme in donor compartment (± SD) at various time intervals.

Time	% Remaining amount of lysozyme in donor compartment (± SD)			
(Hour)	L(0)(0)	L(35)(0)	L(80)(0)	L(35)(20)
0	100	100	100	100
1	97.23 ± 13.50	86.63 ± 5.16	72.22 ± 10.01	66.88 ± 5.34
2	84.04 ± 5.06	90.54 ± 4.86	70.13 ± 7.56	61.14 ± 10.51
4	79.67 ± 11.19	77.36 ± 8.16	57.01 ± 8.96	63.75 ± 13.30
6	64.62 ± 7.86	70.50 ± 5.60	55.35 ± 4.72	64.08 ± 5.00

The reduction of lysozyme in donor compartment could be due to one or more of the following reasons.

1. Some lysozyme might be induced to exhibit aggregation form due to the temperature in modified Franz diffusion technique. Although the preliminary study (data not shown) revealed that lysozyme which dissolved in boiling water for 30 minutes still retained the native secondary structure. Confirmation of lysozyme which dissolved in conditions other than water after high temperature exposure was not determined. The solvent used might be the cause of temperature-induced aggregation of lysozyme and a reduction in CD intensities.

Or 2. Some lysozyme might adsorb on the glass walls of the donor compartment. This experiment did not test for protein adsorption on to glass. Thus, addition of bovine serum albumin (BSA) should be used in the future studies to reduce the effect of protein adsorption.

Or 3. The modified lysozyme which was transformed to MG state might be able to permeate into the epidermal layer by its hydrophobic characteristics.

Therefore, the results of this part could give only the likelihood of lysozyme penetration through the model skin when it was presented as MG state. Thus, MG state of lysozyme might be able to penetrate better than its native state.

Further experiment should be aimed to characterize the amount of lysozyme using monoclonal antibody to hen egg white lysozyme via ELISA technique. However, this technique was not used in the present study. It was due to the fact that the commercial hen egg white lysozyme monoclonal antibody was not available. Therefore, this monoclonal antibody should be produced in the future studies. Moreover, if this characterization goes well, the dosage form selection and development should be done to evaluate the suitability and possibility for this approach to be used on human skin in the future.