CHAPTER II
LITERATURE REVIEW

Allergy is an immune reaction of a person who is specifically allergic to a specific allergen. Allergens are usually proteins which were a cause of allergic reaction. One type of allergy commonly occurs in infancy or childhood is food allergy (Kjellman and Björkstén, 1997; Sampson, 1998).

1. Food allergy or food hypersensitivity

Food allergies or food hypersensitivities are adverse reactions and usually occur after ingestion of food or food additives. Adverse reactions are divided into two types of reactions as follows:

1.1 Toxic reaction

This reaction occurs when anyone provides a sufficient dose of toxin such as *Salmonella, Shigella* and *Campylobacter* species etc.

1.2 Nontoxic reaction

This reaction is defined by immunoglobulin E (IgE) mediator. There are non immune reactions (intolerances) and immune reactions (allergic or hypersensitive reactions). The individual immunologic systems are differently responsive to the same allergens (Sampson, 1998).

After patients obtain allergens into body (initial sensitization), the immune systems recognize the allergen as foreign. B cells are stimulated by the allergens to produce specific IgE antibodies. The receptors of mast cells bind to these specific IgE antibodies. If the patients subsequently receive the allergens, they will react with the antibodies in order to lyse mast cells. Histamines and other substances in mast cells are released, leading to immediate allergic symptoms. This reaction is called IgE mediated (Type I) immune reaction illustrates in Figure 1 (Arshad, 2002).

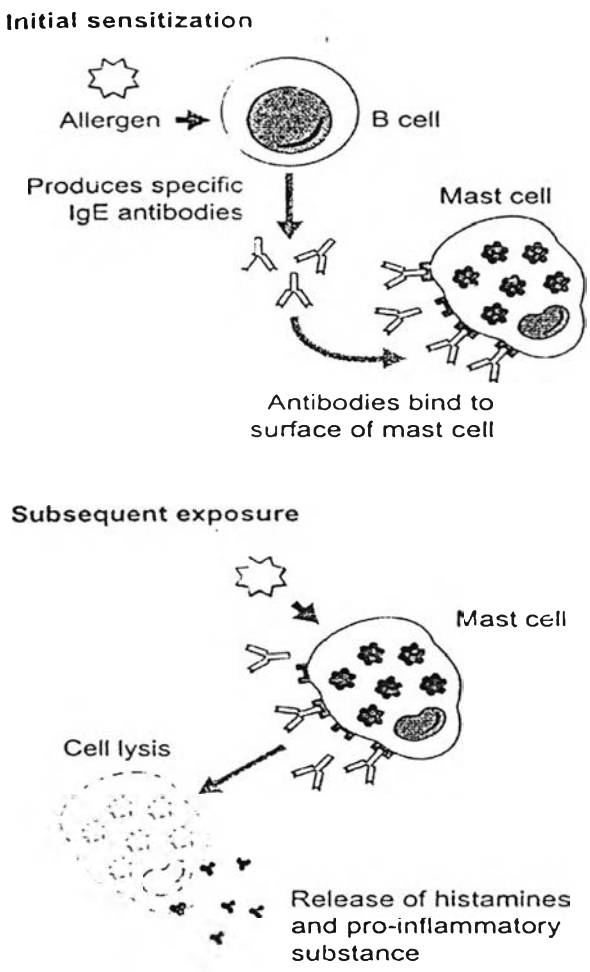


Figure 1 The IgE mediated (Type I) immune reaction (Obtained from Arshad, 2002)

The food hypersensitive symptoms from IgE-mediated reaction are generally atopic dermatitis or eczema, asthma, gastrointestinal anaphylactic reaction and anaphylaxis (www.allergyasthmatherapy.com/Conditions/Food-Allergies.htm).

Allergens in food products are commonly proteins such as eggs, cow's milk, wheat, nuts, fish, soy (www.allergyasthmatherapy.com/Conditions/Food-Allergies.htm). In general, cow's milk or chicken eggs or egg products are a cause of food allergy in infancy and childhood (Kjellman and Björkst'en1997; Leduc et al, 1999). This problem affects their growth later. The major allergens are found in egg whites rather than egg yolks. Proteins in egg white are consisted of ovomucoid, ovalbumin, ovotransferin and lysozyme (Atkins, 2000).

A model protein is used in the present study is lysozyme from hen egg white. In some countries, lysozyme is used as a preservative in food and maybe present in some cosmetics, shampoos and drugs (Leduc et al, 1999). Although lysozyme is approximately 3.4 % of total protein in egg white. There are a high percentage of people in Japan (67%), United States (61%) and France (35%) are specifically allergic to lysozyme (http://www.food-allegens.de/).

2. Diagnosis and treatment of food allergy

2.1 Diagnostic technique

Skin-prick-test is a primary diagnostic tool in allergy, including food allergy (Norgaard et al, 1992; Sporik et al, 2000; Arshad, 2002. The patients are injected with standard allergens which are purified allergen extracts to epidermal layer. The allergens react with IgE antibodies and then bind to mast cells. If the patients are allergic to any allergens, the wheal-and-flare will occur due to release of histamines. The reaction occurs after injection about 10-15 minutes. The diameter of the wheal-and-flare is measured and calculated, guiding subsequent treatments. Although, diagnosis of this treatment is very simple and fast, it requires physicians or allergists who experienced (Demoly et al, 1998; Arshad, 2002).

2.2 Treatment of food allergy

2.2.1 Avoid

The best treatment of food allergy is to avoid the specific food which is induced an allergic reaction (www.allergyasthmatherapy.com/Conditions/Food-Allergies.htm). Food allergy generally occurs in childhood. Therefore, if they avoid the essential food, they will get problems in their growth later.

2.2.2 Specific allergen immunotherapy

Specific allergen immunotherapy is one technique is widely used to desensitize the allergen in all types of allergy. The efficiency of this treatment is dependent on the response of individual immune system and other factors such as age, gender, race, disease and drug (Demoly et al, 1998; Arshad, 2002).

The mechanism of this treatment is to produce allergen-specific blocking IgG antibodies to block the binding of IgE antibodies and specific allergens (Figure 2). The degree of improvement do not relate to the level of blocking IgG antibodies. However, the increase of IgG may be influential to induce tolerance. In long term of treatment, mast cells and eosinophils are gradually reduced (Arshad, 2002).

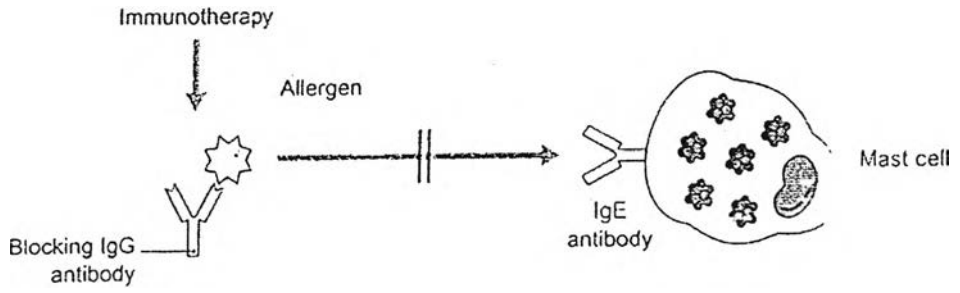


Figure 2 The mechanism of specific allergen immunotherapy (Obtained from Arshad, 2002)

The schedules of immunotherapy are divided into two types as follows:

A. Conventional immunotherapy schedule

The purified allergen extracts are diluted as series of diluted solutions. The allergen is injected into epidermal layer of the patients. The starting-dose is found an appropriate dose for each patient. After that the amount of allergens is continuously increased every week or twice a week to find the maximum dose which is tolerated by the patients. This dose is used as a maintenance dose. The regimen is dependent on individual immune response. In general, the duration of treatment is longer than 3 years (Demoly et al, 1998; Arshad, 2002).

B. Modified immunotherapy schedule (Cluster or rush immunotherapy)

This technique, the patients gain the rapidly increasing dose in a short time may be within a period of days or weeks to achieve the maintenance dose. Nevertheless, it highly risks occurring of adverse reactions (Demoly et al, 1998; Arshad, 2002).

Although the specific allergen immunotherapy is useful for some patients who respond to this treatment, some of them always suffer from the treatment, especially children. It is due to the fact that they are painful from injection and a long duration of the treatment. Therefore, the present study is focused on the possibility of the allergen or protein delivery into epidermal layer without needles.

Some researchers are interested in the state of biological proteins which is translocated into a lipid bilayer of living cells. They raised questions on how fully folded proteins (native state) could permeate through the membrane, in spite of the characters of native protein being hydrophilic and charged (Rothman and Kornberg, 1986).

3. Determination of the state for protein translocation

Dihydrofolate reductase (DHFR) is a protein that has been extensively studied about its translocation. Bychkova et al (1988) reported that urea was added in native environment in order to destabilize the rigid tertiary structure of DHFR, leading to increase translocation into mitochondrial membrane. They therefore proposed that this state was non-native transition which was assumed to be 'molten globule (MG) intermediate state'. Besides, some researchers revealed the characters of MG state are compact, flexible side chains, shielded charges and exposure of hydrophobic residues. (Ohgushi and Wada, 1983; Oas and Kim, 1988; Creighton, 1990; Van der Goot et al, 1991, 1992) The translocation of other proteins such as cytochrome C, the precursors of cytocrome c_1, b_2 and β-lactamase also increased when induced by other external factors such as temperature and pH (Bychkova et al, 1988; Endo et al 1988).

Van der Goot et al (1991, 1992) considered the structure of toxins such as colicin A (a pore-forming bacteriocin), diphtheria toxin, acid-tiggered toxins and non toxins, C9 complement protein when inserted into membrane. In vitro study, they found that the affinity of pore formation of colicin A significantly increased at low pH. In addition, the conformation of colicin A was observed by CD and intrinsic fluorescence presented as MG transition. Consequently, they summarized that pore formation of colicin A was predominately dependent on pH to alter the structure from native state to the MG state when translocation. Moreover, Van der Goot et al (1992) also proposed the schematic representation of the native and the MG state which illustrated in Figure3 and 4. Figure 3 shows the position of hydrophobic residues in either the native or the MG. Figure 4 displays the charge position of both states adapted from Bychkova et al (1988)'s hypothesis.

These evidences were correlated well with the interest of the present study. Otherwise, there has been no report about the use of the MG state of any protein in transdermal delivery. Therefore, the hypothesis of the present study was raised on the induction of the model protein to the MG state could increase the permeability.

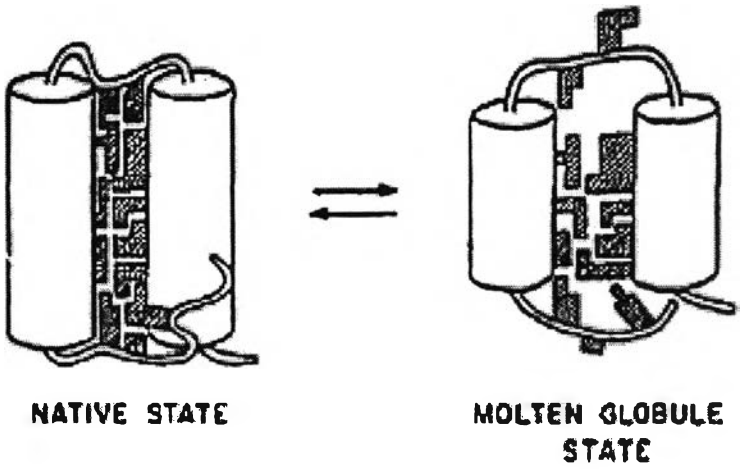


Figure 3 The schematic representation of the positions of non polar side chains when present in the native and the MG state (Obtained from Van der Goot et al, 1992)

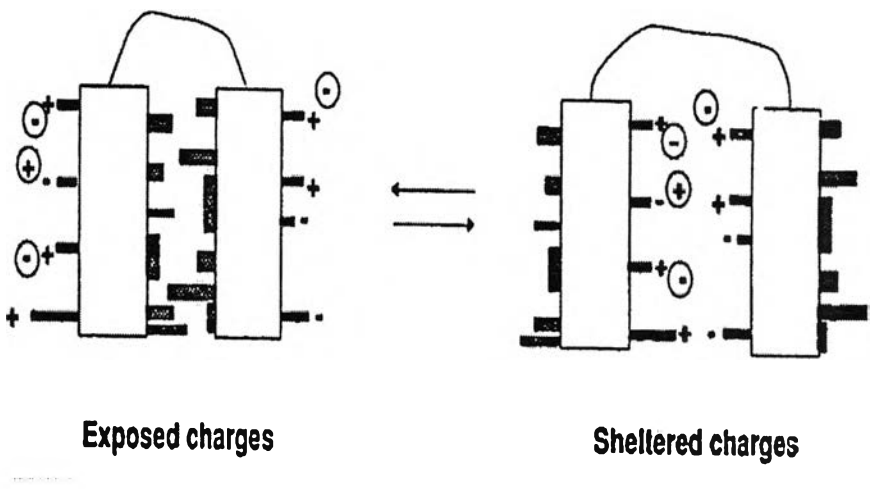


Figure 4 The schematic representation of the charges in the native and the MG state (Obtained from Van der Goot et al, 1992)

As a consequence, the study of the basic principles of protein is necessary for the study of protein conformational modification.

4. Basic principles of protein

The present study was focused on protein structure and protein folding.

4.1. Protein structures

Proteins are polymers of amino acids. All of the 20 amino acids are found in proteins have a common structure which is a carbon atom (the α-carbon) is linked to a carbonyl group, a primary amino acid group, a proton and a side chain (R) which is different in each amino acid (Turner et al, 1998). The general structure of amino acid residue is shown in Figure 5.

There are four levels of protein structure as follows:

4.1.1 Primary structure

Two amino acid residues are linked together by peptide bond as illustrated in Figure 6. Many amino acids linked by peptide bond from a polypeptide (Nölting, 1999). The repeating sequence of α-carbon atoms and peptide bond provides the backbone of the polypeptide. The different amino acid at one and end of a polypeptide has an unattached α-amino group while the one at the other end has a free α-carbonyl group. Hence, the direction of polypeptide is N-terminus and a C-terminus. The sequence of amino acid from the N to the C terminus is the primary structure of the polypeptide (Turner et al, 1998).

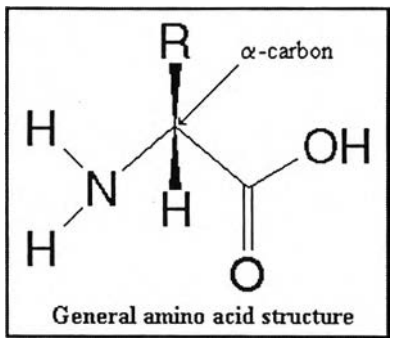


Figure 5 The general structure of amino acid residue (Obtained from http://www.fst.rdg.ac.uk/courses/fs916/index.htm)

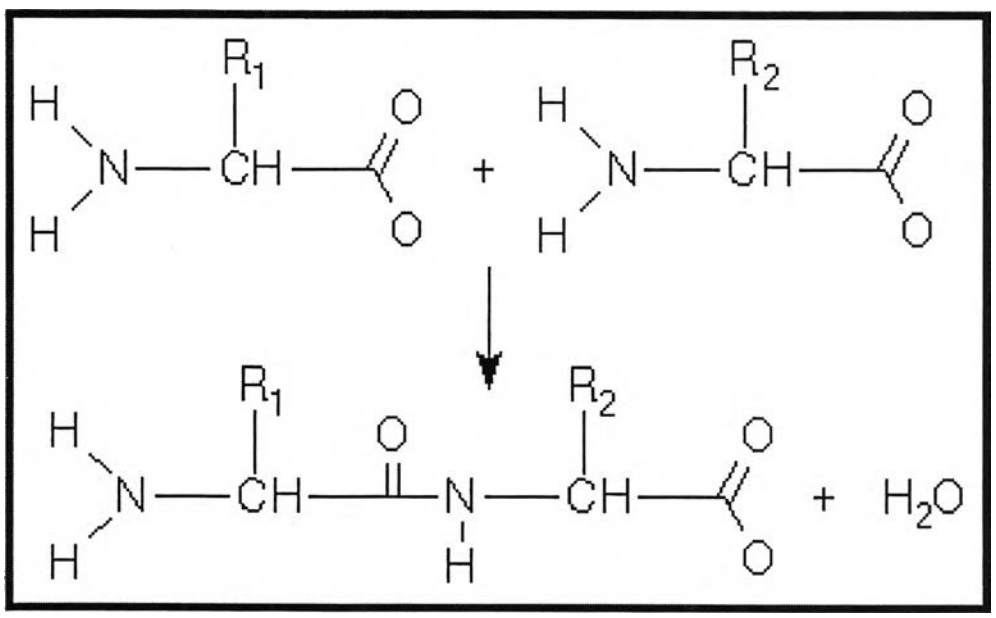


Figure 6 Peptide bond formations between two amino acid residues (Obtained from http://www.fst.rdg.ac.uk/courses/fs916/index.htm)

4.1.2 Secondary structure

The secondary structure represents some local conformations of polypeptide (Nelson and Cox, 2000). This structure is formed by hydrogen bond which is an efficient mechanism of paring polar groups of the polypeptide backbone (Nölting, 1999). This polarity also flavors hydrogen bond formation between appropriately spaced and oriented peptide bond units. Therefore, polypeptides can fold into a number of structures (Turner et al, 1998).

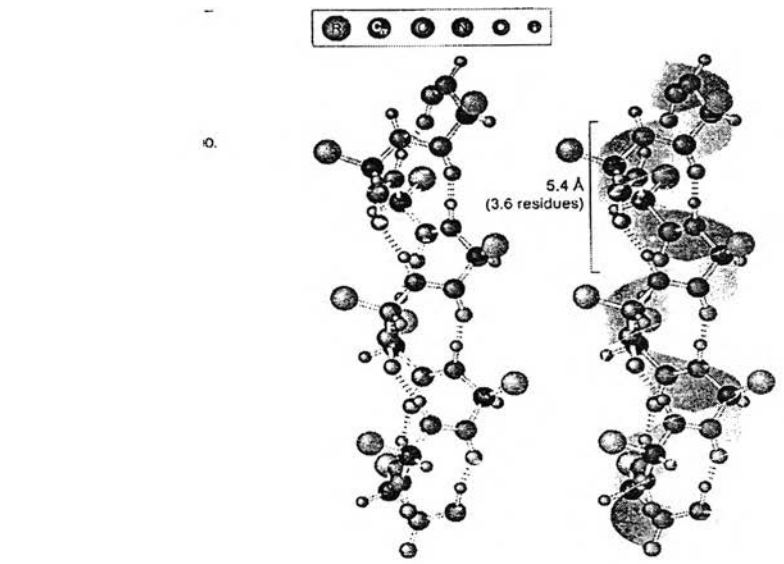
The secondary structures are divided as follows:

A. α- helix: The polypeptide backbone forms a right-handed helix with 3.6 amino acid residues per turn. It is stabilized by hydrogen bond between C=O group of amino acid residue at position *n* in the polypeptide chain with N-H group of residue *n* + 4 (Turner et al, 1998; Nölting, 1999).

B. β-sheet (The β-pleated sheet): The polypeptide is form by hydrogen bonding N-H and C=O groups to the complementary group of another section of the polypeptide chain. These sections run in the same direction (e.g. N-terminus to C terminus), the sheet is parallel. Several sections of polypeptide chain may be involved side-by-side, giving a sheet structure with the side chains (R) projecting alternately above and below the sheet (Turner et al, 1998).

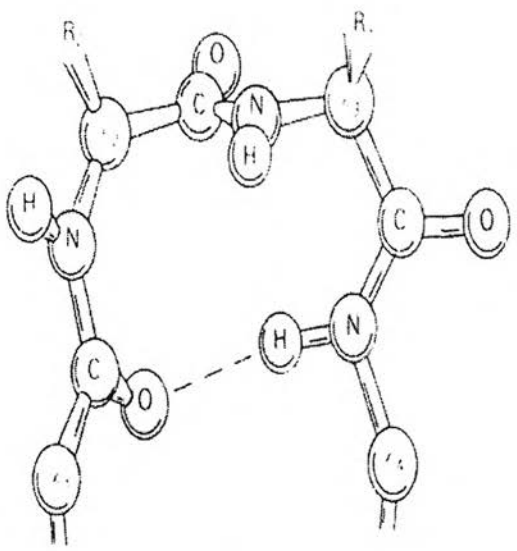
C. β-Turn: A direction of the polypeptide chains is a 180° change and are stabilized by a hydrogen bond between C=O group of the residue at the position *n* with NH group, of the residue *n* + 3 (Turner et al, 1998).

All types of the secondary structures are displayed in Figure 7 (Watson et al, 2004).



(A)

(B)



(C)

Figure 7 The schematic representation of the secondary structures; (A) α-helix (B) β-sheet (C) β-turn (Obtained from Watson et al, 2004)

4.1.3 Tertiary structure

Tertiary structure is composed of the different sections of the α-helix, β-sheet and other minor secondary structures (Turner et al, 1998). A compact tertiary conformation is dominantly stabilized by hydrophobic effect and van der Waal's interaction, leading to be a rigid structure (Brange, 2000). Some proteins contain disulfide bridges or can incorporate cofactor. The unique tertiary structure of each protein is determined by amino acid sequence (Nölting, 1999).

4.1.4 Quaternary structure

Many proteins are composed of two or more polypeptide chain (subunits). They may be identical or different. The same forces stabilize tertiary structure hold these sub units together, including disulfide bonds between cysteines on separate polypeptides (Turner et al, 1998).

The relationship of four levels of protein structures is shown in Figure 8 (Watson et al, 2004).

4.2 Protein folding

The protein folding process spontaneously generates in the range of milliseconds (Nölting, 1999). Although the protein is purified or synthesized, the in vitro unfolded protein can spontaneously refold (Creighton, 1990). This pathway is supposed to start with fully unfolded state and process to reach fully folded state (native state) which is a stable state. This pathway is illustrated in Figure 9 (Tornton and Barlow, 1991).

The states of protein folding are divided into three major states as follows:

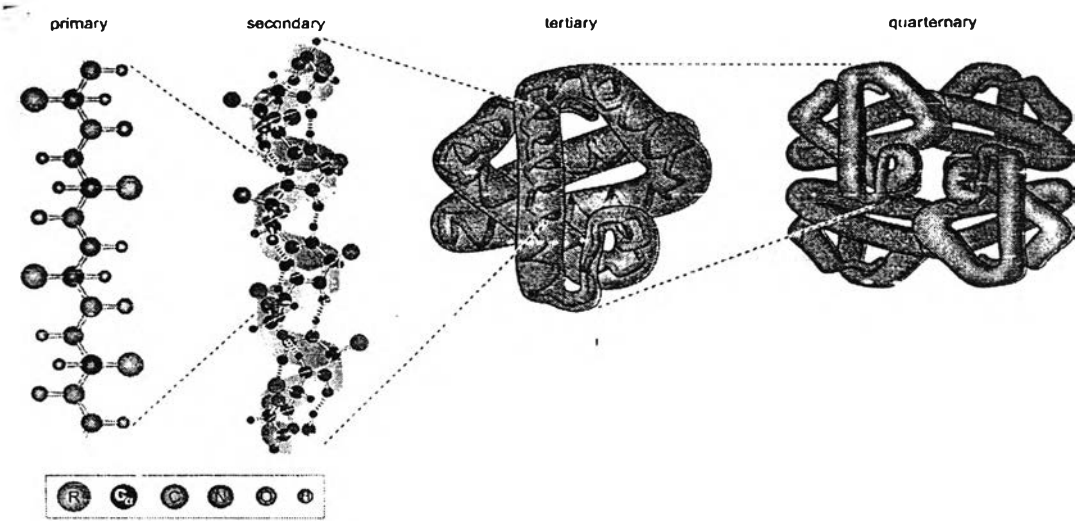


Figure 8 The schematic representation of four levels of protein structures (Obtained from Watson et al, 2004)

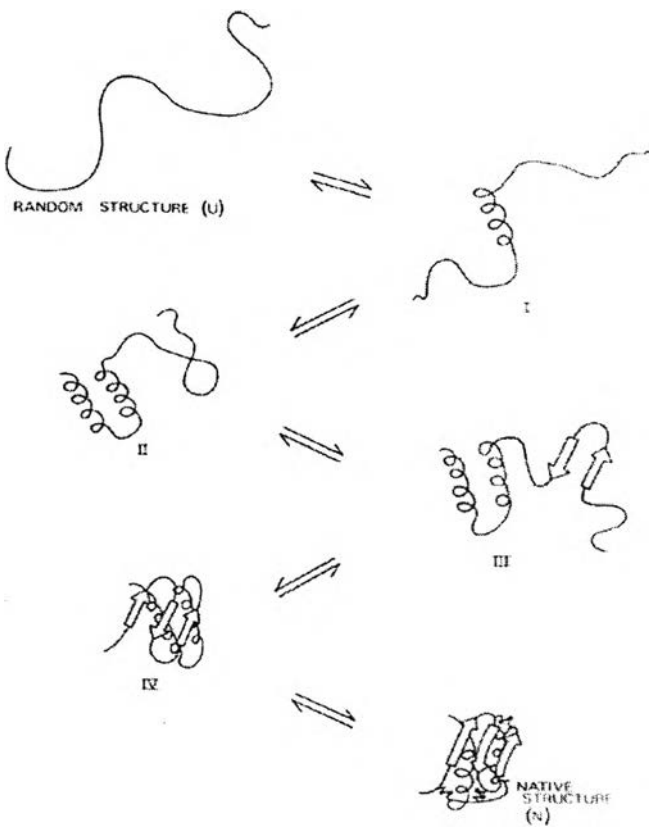


Figure 9 The schematic representation of process of protein folding; the native state (N), the intermediated state (I-IV) and fully unfolded state (U) (Obtained from Tornton and Barlow, 1991)

4.2.1 The native, fully folded state (N)

Amino acid side chains which are located in back bone are hydrophilic and hydrophobic residues. In the native environment, the globular proteins commonly stabilize their tertiary structures by dominant forces such as hydrophobic interaction, hydrogen bond formation and electrostatic interaction (Timasheff, 1970). The protein surfaces are fully hydrated with water. Hence, the hydrophilic side chains are exposed to water molecules, as the non-polar residues are buried in the structure in order to form hydrophobic core (Rupley et al, 1983; Nölting, 1999; Brange, 2000). These native tertiary structures are virtually considered conformation by X-ray crystallography which is the great instrument and NMR also (Creighton, 1990).

4.2.2 The unfolded state (U)

The ideal unfolded protein is random coil, in which the rotation angle about each bond of the backbone and side chains is independent on bonds distance in the sequence, and where all conformations have comparable free energies.

For in vitro study of protein folding, a strong denaturant such as 6M Guanidium HCl (GdmCl) or 8M urea is used to generate unfolded protein. Each molecule in a typical sample of a fully unfolded protein will probably have a unique conformation at any instant of time. Consequently, the initial states of folding must be nearly random (Creighton, 1990).

4.2.3 The intermediate states

The intermediate states are between fully folded and fully unfolded states of protein folding (Privilov, 1996). The stable conformation of these states is called molten globule (MG) state which is a compact intermediate. This state was named by Drs. Ptitsyn's and Crane-Robinson's discussion at the 'International Symposium on Peptides, Polypeptides and Proteins' in Italy, 1982 (Ohgushi and Wada, 1983). The general characteristics of the MG state are

A. The rigid tertiary structure is disrupted and the native-like secondary structure retains, in order to present a flexible conformation.

B. Hydrophobic side chains of the MG state are exposed to the environment. Therefore, the MG state is more hydrophobic than native state.

C. The radius gyration of the MG state is close to native state. Its topology is more compact than fully unfolds.

D. The MG state shield charged group, leading to less reveal polar surface.

E. Enthalpy of this state is near fully unfolded form.

(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)

5. The in vitro study of the MG state

Many scientists are interested in the MG or partially folded states of some proteins such as α-lactalbumin, apomyoglobin, cytochrome C, staphylococcal nuclease, procerain and lysozyme (Goto et al, 1990a, 1990b; Radford et al 1992; Itzhaki et al, 1994; Bychkova et al, 1996; Privilov, 1996; Morgan et al, 1998; Dubey and Jagannadham, 2003). They therefore intend to generate the MG state of the globular proteins in various conditions. Induction of the globular proteins to be MG state is simply prepared by adding of external factors which are mild denatured conditions. Researchers reported that the conditions such as the use of organic solvents, pH alteration, salts, suitable concentrations of denaturing agent and thermal variation could generate the MG state (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).

The external factors used in the in vitro study of the MG state are determined as follows:

5.1 Types of external factors

The effects of types of external factors on protein conformational modifications are shown as follows:

5.1.1 Organic solvents

Many organic solvents are used to induce globular protein to the MG state such as:

A. Methanol

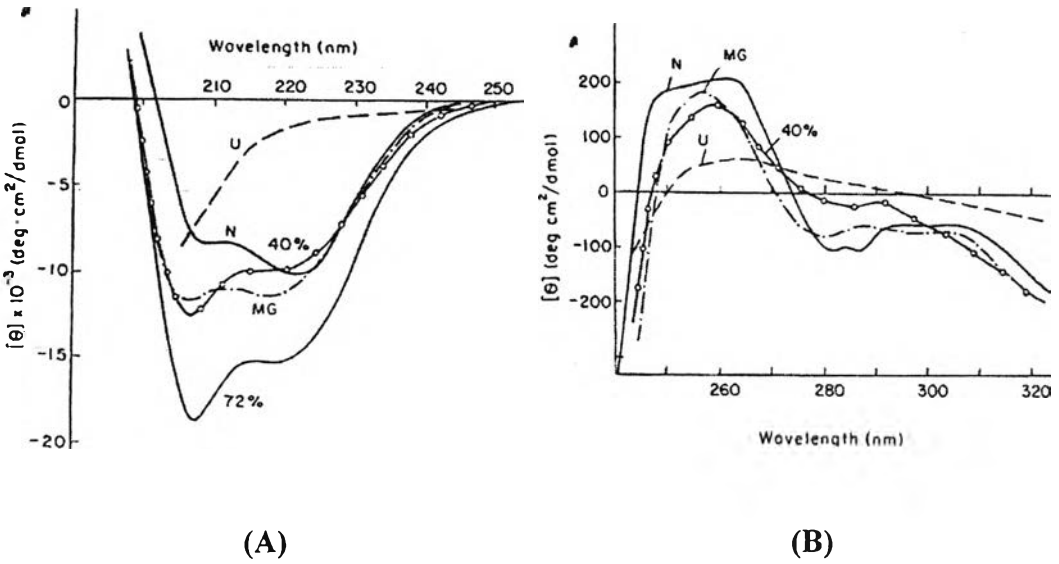
Cytochrome C dissolved at various concentrations of methanol (Bychkova et al, 1996). At 40% v/v of methanol, cytrochrome C presented a native-like secondary structure, but some traces of the tertiary structure were disrupted (Figure 10). These were the characteristics of the MG state.

B. Trifluoroethanol (TFE)

α-Lactalbumin dissolved in aqueous-TFE solution at various concentrations (Alexandrescu et al, 1994). At 50% v/v of TFE showed a pattern of nuclear magnetic resonance (NMR) correlated to the MG state of α-Lactalbumin at pH 2 (Figure 11).

C. Hexafluoroacetone (HFA)

Bhattacharjya and Balaram (1997) revealed that at 25% v/v of hexafluoroacetone (HFA), lysozyme exhibited the MG state determined by CD, fluorometry and nuclear magnetic resonance (NMR). At higher concentration (50% v/v of HFA), the conformation of lysozyme lost it globularity (Figure12).



(A) **(B)**

Figure 10 The CD spectra of the methanol-induce intermediate state and pH-induced MG state of cytochrome C in the far-UV and near-UV regions (Obtained from Bychkova et al, 1996)

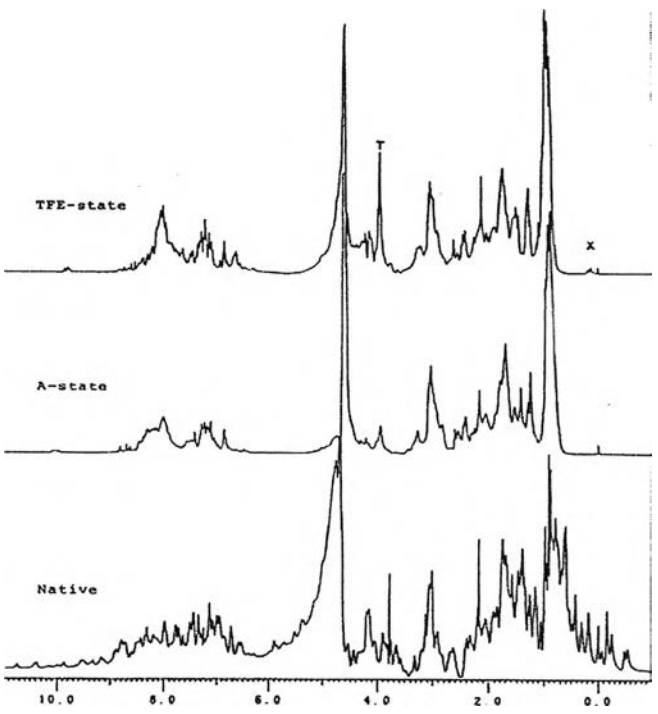


Figure 11 The NMR spectra of MG characteristics of α-Lactalbumin (Obtained from Alexandrescu et al, 1994)

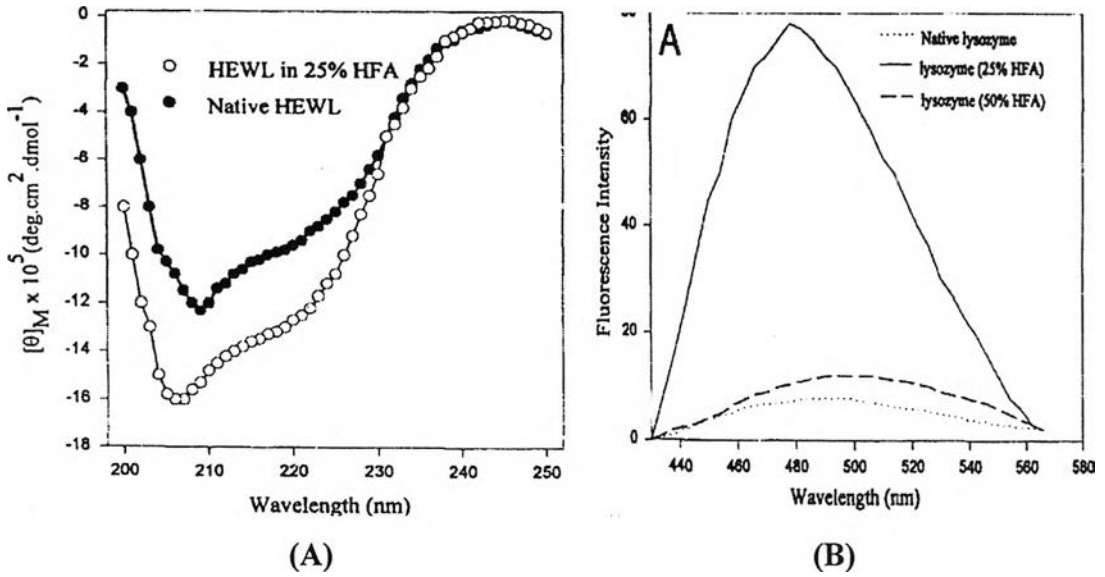


Figure 12 The CD spectra in the far-UV region (A) and fluorometric emission spectra (B) of lysozyme dissolved in aqueous-HFA solution (Obtained from Bhattacharjya and Balaram, 1997)

5.1.2 pH alteration

Cytochrome C in solution containing 0.5M NaCl at pH 2 presented the MG state when detected by CD technique. The CD spectra displays in Figure 10 (Bychkova et al, 1996).

Alexandrescu et al (1994) reported that α-Lactalbumin presented partially folded or the MG state at pH 2. NMR result illustrates in Figure 11.

5.1.3 Temperature

Koshiba et al (2000) reported apo-canine milk lysozyme in solution pH2 presented the MG state at 63°C. The differential scanning calorimetry (DSC), CD and fluorometric results were well defined as the characters of the MG state. Figure13 shows the fluorometric results of this experiment, apo-canine milk lysozyme at 63°C presents the highest fluorometric intensity which signified the MG state.

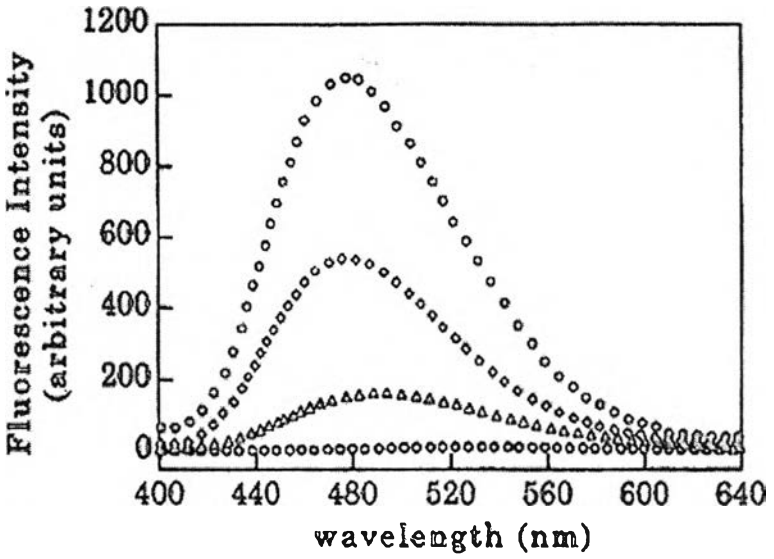


Figure 13 The fluorometric emission spectra of the lysozyme-ANS binding at 9° (♦), 63° (●), 98°C (▲) and at 25°C ANS without lysozyme (O) (Obtained from Koshiba et al, 2000)

5.2 The effect of external factor on the structural transformation

Each type of external factors affects the structural proteins in different mechanisms. Moreover, the effect of one factor on the conformation of one protein is individual. (Timasheff, 1970; Rupley et al, 1983; Lehmann et al., 1985; Goto et al, 1990; Fink et al, 1994). The present study was focused on the effects of organic solvent and acid on the structural modification of the model protein.

5.2.1 The effect of organic solvent

Organic solvents such as acetonitrile, methanol, ethanol, trifluoroethanol (TFE) and hexafluoroacetone are widely used to study protein folding. Introduction of organic solvent in the native environment is in order to destabilize the native tertiary structures (Bychkova et al, 1996; Bhattacharjya et al, 1997; Bakuni, 1998; Katamari et al, 1998; Calandrini et al, 2000, Chittchang et al, 2002).

It is due to the fact that the polar interactions between hydrophilic residues and water molecules on protein surface are reduced. Consequently, the removal of water molecules occurs. Increasing of organic solvent in the environment, hydrophobic molecules are continuously placed instead of water, leading to be dehydrated proteins (Rupley et al, 1983; Lehmann et al., 1985; Brange, 2000; Mattos and Ringe, 2001). At higher concentration of organic solvent, the hydrophobic residues excellently bind to hydrophobic group of organic solvent, in order to expose the hydrophobic residues to the environment (Timasheff, 1970; Brange, 2000). Besides, hydrogen bonding is formed among peptide units of globular protein, leading to originate α-helix structures (Lehmann et al., 1985)

Moreover, in 1998 Bhakuni studied protein folding under various monohydric alcohols as methanol, ethanol, propanol and TFE. He found that disruption of native conformations depended on alkyl chain length, amounts of alcohol and stearic effects. Besides, his results revealed that stearic side chain was a dominant factor which affected the tertiary conformations. Therefore, TFE was more efficient than propanol.

5.2.2 The effect of acid

Goto et al (1990a, 1990b) studied adding of HCl acid in native environment at low ionic strength affected folding mechanism of some proteins. Fink (1994) and Brange (2000) reported that under strong acidic environment (pH~ 1-2 with salt-free), some proteins were induced to partially unfolded state by electrostatic interaction. In contrast, although decreasing pH to 0.5, some proteins still stabilize its native state. It is due to the fact that these structures contain disulfide cross-links. These interactions are very strong intramolecular interactions and more predominant than electrostatic repulsion (Goto et al, 1990; Fink et al, 1994).

At higher concentrations of HCl acid, protons and anions are promoted in the solution. This condition will induce to increase interior repulsion between positive charges and the disturbance of anions on the water molecules surrounding protein surface. Thus, the removal of water molecules occurs easily because of weak interaction between water and hydrophilic molecules in protein. The native tertiary structure of protein is destabilized and exposed hydrophobic side chains to the environment (Goto et al, 1990a, b; Fink et al, 1994).

6. Hen egg white lysozyme

Hen egg white lysozyme is used as the model protein for this experiment. Lysozyme containing 129 amino acid residues is a small monomeric protein. It consists of three phenylalanines, three tyrosins and six tryptophans (Timasheff, 1970). There are two domains which are α-domain and β-domain. The α-domain consists of four α-helices (A to D) and 3^{10}helix, β-domain includes triple stranded β-sheet, a short double stranded β-sheet, 3^{10}helix and a long loop (Radford et al, 1992; Itzhaki et al, 1994; Chen et al, 1996). In addition, Lysozyme has four disulfide bridges. Lysozyme structure is illustrated in Figure 14.

Figure 14 The native tertiary structure of lysozyme (Obtained from http://www.rcsb.org/pdb/)

Many scientists studied about lysozyme folding. They also proposed that an early intermediate state of folding exhibited the properties like MG or partially folded state. This state could be determined by circular dichroism CD (Radford et al, 1992), fluorometry (Itzhaki et al, 1994; Morgan et al, 1998), heteronuclear nuclear magnetic resonance spectroscopy (Buck et al, 1995), hydrogen exchange labeling (Gladwin and Evan, 1996), solution X-ray scattering (Chen et al, 1996) and differential scanning calorimetry (Bhakuni, 1998).

6.1 The effect of type and the concentration of organic solvent on structural lysozyme

Lysozyme has been widely used as a model protein to determine its conformational modification under various types of organic solvents. Each organic solvent affects structural alteration of lysozyme at different concentrations.

For in stance, in 1970, Timasheff studied the protein-solvent interaction between lysozyme and 2-chloroethanol. The conformational modification was detected by circular dichroism (CD) technique. He found that the conformation of lysozyme dramatically changed when lysozyme solubilized in 2-chloroethanol between 12.5% v/v–17.5% v/v. The tertiary structure is disrupted, as the secondary structure retained.

Buck et al (1993) reported that 50% v/v of TEF was introduced in the lysozyme solution at pH 2. Lysozyme conformation slightly changed. At higher concentration, lysozyme presented as partially folded state characteristics which were loss in the tertiary structure and retaining the secondary structure (Figure15).

Goda et al (2000) and Tanaka et al (2001) studied the conformational changes of lysozyme solubilized in various ethanolic concentrations using CD (Figure 16) and dynamic light scattering (Figure 17), respectively. At higher 70% v/v of ethanolic solution, the tertiary structure of lysozyme was disrupted. The secondary structure presented a high content of α-helix conformation. At 90% v/v of ethanol the aggregation of lysozyme occurred due to intermolecular β-sheet formation.

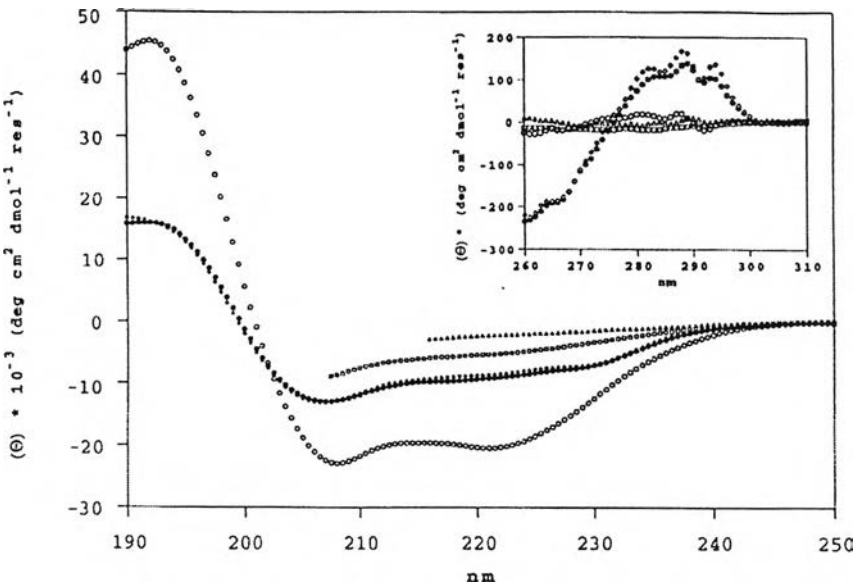


Figure 15 The CD spectra of lysozyme dissolved in various TFE concentrations in the far-UV (inset) and near-UV regions, the native state (●), 12.5% v/v TFE (◊), 50% v/v TFE (○) and the thermally (65°C) (□) and urea denatured state (8M) (△) (Obtained from Buck et al, 1993)

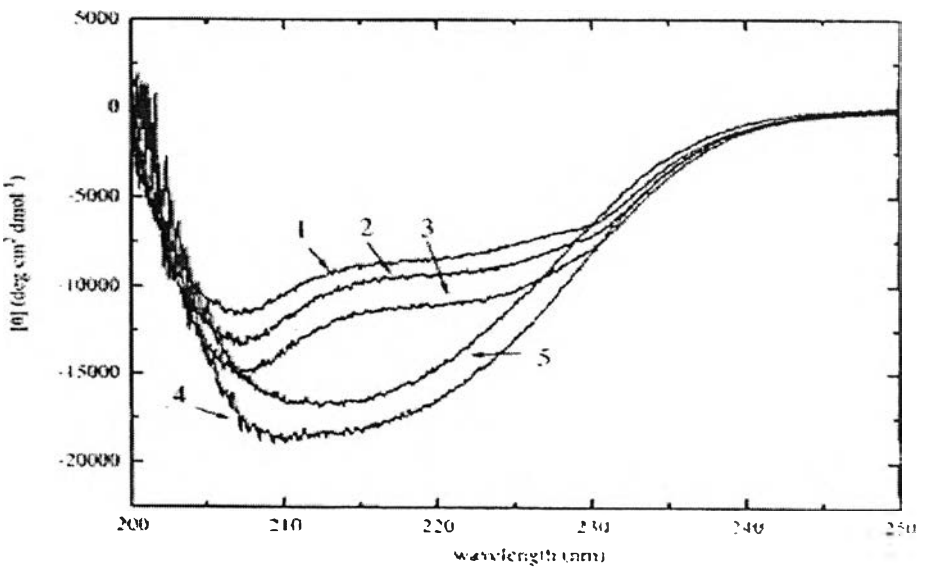


Figure 16 The CD spectra of hen egg white lysozyme (5 mg/ml) in various concentration of ethanol incubated for 24 h at 25°C. 1) 0% v/v; 2) 50% v/v; 3) 70% v/v; 4) 80% v/v; 5) 85% v/v ethanolic solution in the far-UV region (Obtained from Goda et al, 2000)

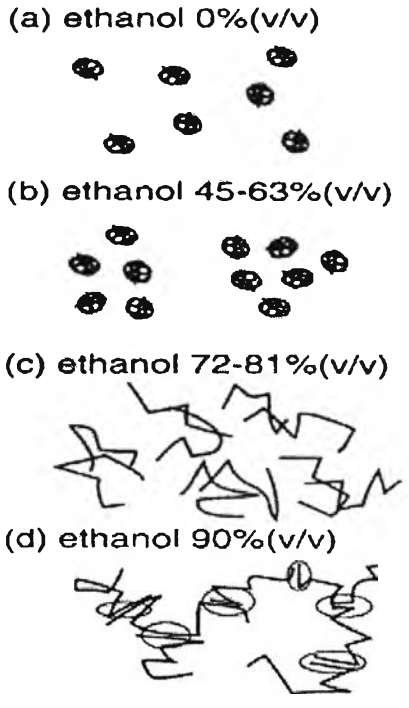


Figure 17 The possible structure of lysozyme dissolved in various contents of ethanol using dynamic light scattering technique. (Obtained from Tanaka et al, 2001)

From the results of many researchers indicate that induction of the MG state is dependent on type and the optimal concentration of organic solvent. If the concentration is higher than the optimum, the structure would lose its globularity.

Nevertheless, in some cases, lysozyme dissolved in 60% v/v methanol at pH 3 presented like MG characteristics when detected by CD technique (Figure18). However, the fluorometry (Figure19) and small-angle X-ray scattering results did not show the character's MG (Katamari et al, 1998). Consequently, the detection of protein conformations requires more than one technique to confirm the results.

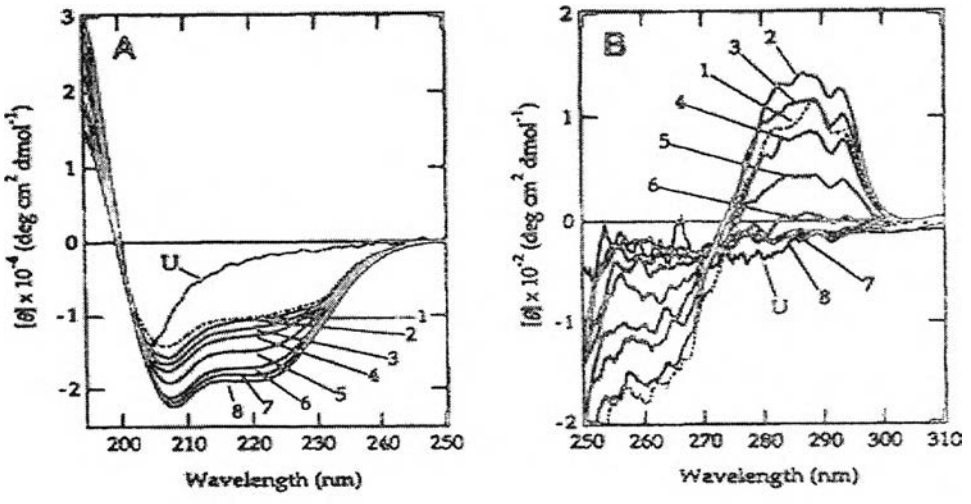


Figure 18 The CD spectra of lysozyme dissolved in various concentrations of methanol at pH 3 in the far-UV (A) and near-UV (B) regions, 1) 0% v/v, 2) 50% v/v, 3) 60% v/v, 4) 62% v/v, 5) 64% v/v, 6) 66% v/v, 7) 68% v/v, 8) 70% and U) urea- denatured state at pH 1.9 (Obtained from Katamari et al, 1998)

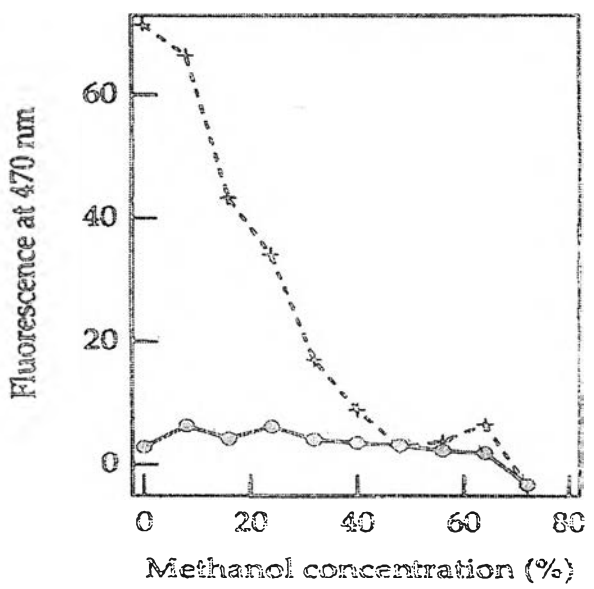


Figure 19 The fluorometic emission intensities at 470nm of lysozyme which dissolved in various concentrations of methanol at pH 3 (Obtained from Katamari et al, 1998)

6.2 The effect of lysozyme concentration on structural lysozyme

Generally, the study of protein conformation requires various techniques to confirm the conformational changes. A suitable concentration of protein used in each technique is different. Goda et al (2000) reported the effect of protein concentration on the structural alteration. At 85% v/v of ethanol, 1 mg/ml of lysozyme highly presented α-helix conformation. However, at higher 1 mg/ml of lysozyme concentration, lysozyme exhibited β-sheet conformation (Figure 20). Consequently, the determination of protein structure has to concern about the concentration of protein when detects different techniques.

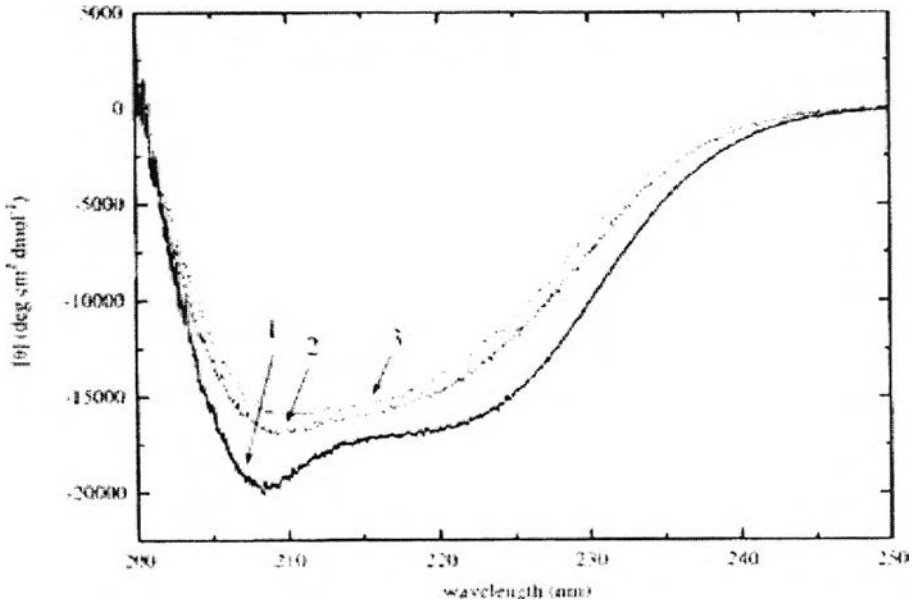


Figure 20 The CD spectra of dependence of protein concentration on the protein structure in the far-UV region. All solutions were incubated at 25°C for 24 h. in 85% ethanolic solution. 1) 1 mg/ml; 2)2 mg/ml/; 3) 3 mg/ml hen egg white lysozyme (Obtained from Goda et al, 2000)

7. The characterization of protein conformational modification

The conformational modification of globular proteins in solution has been determined using several techniques such as circular dichroism (CD), fluorometry, differential scanning calorimetry (DSC), fourier transform infrared (FT-IR) and nuclear magnetic resonance spectroscopy (NMR), etc (Privilov, 1996; Bhattacharjya et al, 1997; Katamari et al, 1998).

The available methods for this experiment are circular dichroism, fluorometry and gel electrophoresis.

7.1 Circular dichroism (CD)

The secondary and tertiary structures of the proteins have been extensively considered by circular dichroism (CD) because the CD is really sensitive to the conformational modifications and it is not complicated to analyze the structures (Kelly and Price, 1997; Sreerama and Woody, 2000).

7.1.1 The principles of CD

The protein structures which consist of chiral chromophores, covalent linkages to chiral center or being located in the asymmetric environment can be characterized by CD. This is based on the differences of absorption (ΔA) or ellipticity (θ) between left (A_L) and right (A_R) circularly polarized light of plane polarized light (Kelly and Price, 1997). For example, Figure 21 the molecule of glucose is determined by CD and illustrates the direction of differential circularly polarized absorption.

7.1.2 The composition and operation of CD

CD apparatus is consisted of light source, photoelectric modulator, photomultiplier tube, amplifier and detector as displays in Figure22. The light source is most important thing for this equipment. It has been commonly used

xenon arc which pronounces delight signals in the range of 178-1000 nm. In addition, it is necessary to protect lamp compartment and sample chamber from O_2 by flushing with N_2 gas because generation of ozone affects on the optical system. The circular dichroism should be calibrated with a chiral standard; 1S-(+)-10-champhorsulfonic acid (Kelly and Price, 1997).

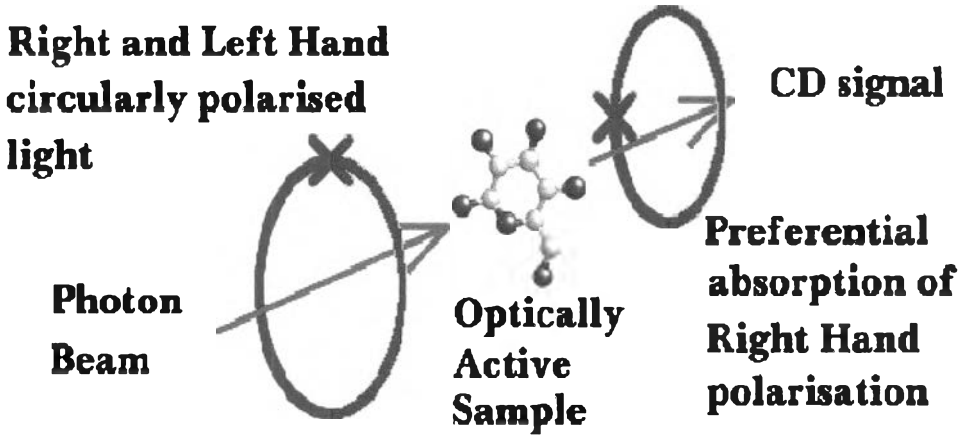


Figure 21 The molecule of glucose is observed by circular dichroism (Obtained from http://www.isa.au.dk)

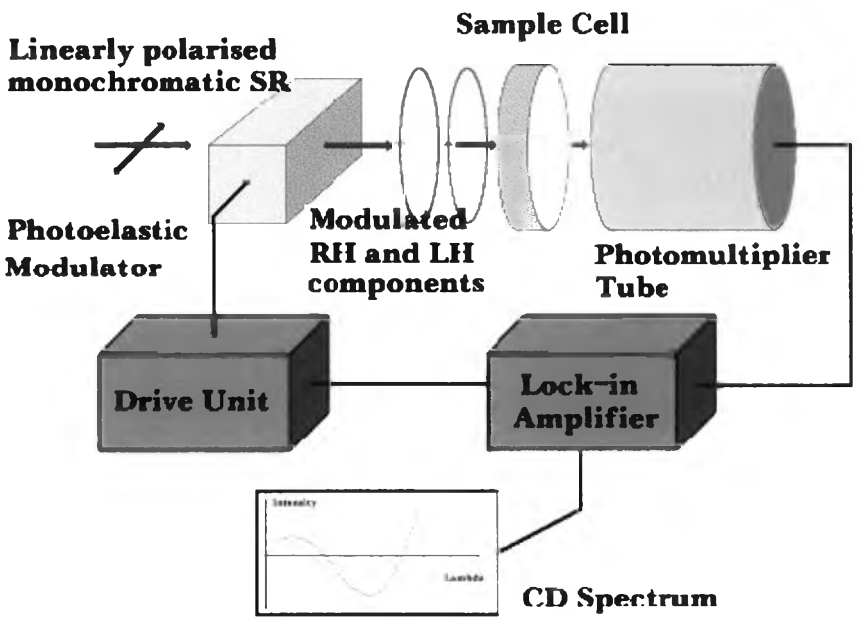


Figure 22 The composition of circular dichroism (Obtained from http://www.isa.au.dk)

7.1.3 Determination of structural peptides/ proteins

Measurement of protein conformations by using CD is divided into three regions which perform the CD signal; far-ultraviolet (UV), near-UV and near UV visible (Kelly and Price, 1997; Sreerama and Woody, 2000).

7.1.3.1 Far-UV (below 250 nm)

In this region, CD is sensitive to secondary conformations of the globular proteins. Amide chromophore from the polypeptide backbone plays an important role to reveal the CD signal (Pelton and McLean, 2000; Sreerama and Woody, 2000). The secondary structures of the proteins are consisted of α-helix, β-sheets and β-turns or random coils where pronounce CD spectra in different patterns as illustrates in Figure 23.

A. α-helical conformation: The pattern of this conformation presents negative band at 208 and 222 nm, while positive one at 192 nm.

B. β-sheet conformation: The CD signal is displayed at 175 and 216 nm for negative band and between 195 and 200 nm for positive band.

C. β-turn or random coil conformation: This conformation reveals strong positive band near 205 nm and negative band near 190 nm (Sreerama and Woody, 2000).

7.1.3.2 Near-UV (250-300 nm)

Tertiary structures are represented with CD spectra where aromatic residues and disulfide group strongly pronounce in this region. Each type of aromatic side chains which are phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Typ) is overshot CD signal in different wavelengths: a peak between 255-270 nm,

275-282 nm and near 290 nm for Phe, Tyr and Typ, respectively (Kelly and Price, 1997, Sreerama and Woody, 2000).

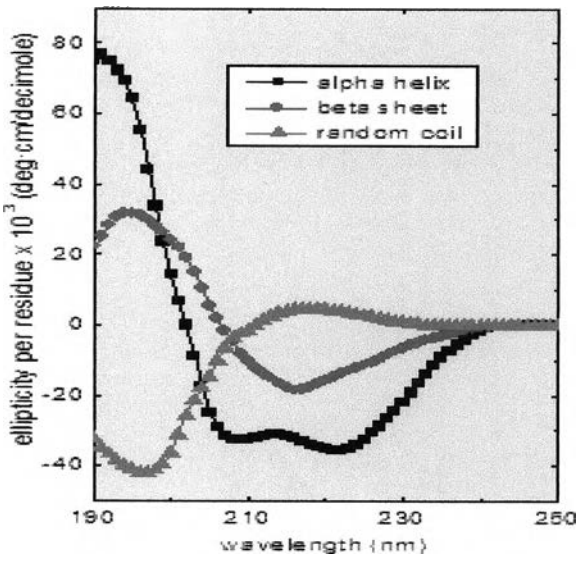


Figure 23 The CD spectra of all secondary structure patterns in the far-UV region (Obtained from http://www.ap-lab.com/circular_dichroism.htm)

7.1.3.3 Near-UV/ visible CD of protein (cofactor and ligand)

This region is suitable for proteins which are observed binding of proteins and cofactors or ligands (Kelly and Price, 1997; Sreerama and Woody, 2000).

7.1.4 Preparation of sample and calculation

The sample is simply prepared by dissolving of protein in the environment of interest. The sample should be clear. If the solution is not homologue, scattering of detection would occur. Kelly and Price (1997) proposed the suitable parameter for general protein as follows:

Resolution	:	0.5 nm
Bandwidth	:	2.0 nm
Sensitivity	:	10 mdeg
Response	:	2 sec
Accumulation	:	4
Speed	:	50 nm / min

The CD absorbance is shown in term of ellipticity (mdeg). It is calculated as molar ellipticity [θ] using the equation [1]

$$[\theta] = \theta / (10\ C.l) \qquad [1]$$

[θ] = Molar ellipticity; (deg.cm^2/decimol)

θ = Ellipticity (mdeg)

C = Molar concentration (mol/l)

l = Path length of cell (cm)

For macromolecules, mean residue molecular concentration (Cr) is obtained instead of molar concentration. The calculation is performed as in equation [2].

$$Cr = n.Cp = 1000\ n\ C' / Mp \qquad [2]$$

Cr = Mean residue molecular concentration

n = Number of constructed residues of macromolecule

Cp = Molar concentration of macromolecule (mol/l)

C ' = Weighing weight (g/ml)

Mp = Molecular weight of macromolecules

7.2 Fluorometry

In native environment, the hydrophobic residues are buried in the protein structures. Addition of organic solvents, salts, temperature or acidic/basic solutions in the native environment induces exposure of non-polar side chains. Observation of the conformational changes by fluorometric technique commonly uses two types of probes are intrinsic and extrinsic fluorescent probes.

7.2.1 Intrinsic fluorescent probe

The tryptophan residue is the hydrophobic residue in protein structure. It performs as the intrinsic fluorescent probes to monitor the transform of protein conformations during refolding process. The tryptophan residue is quenched by iodide titration, called the quenching of tryptophan fluorescence (Itzahaki et al, 1994; Dubey and Jagannadham, 2003).

7.2.2 Extrinsic fluorescent probe

1-anilino-8-naphthalene sulfonate anion (ANS) is the hydrophobic probe is widely used as extrinsic fluorescent probe in fluorometric technique. It is excellently bound to molten globule (MG) or partially folded state and give the highest fluorometric emmission intensity when compares with the native and fully unfolded state. It is due to the fact that the MG state of the globular proteins present more hydrophobic than naïve state and more compact than unfolded state (Semisotnov et al, 1991; Matulis and Loverien, 1998, Dubey and Jagannadham, 2003). In addition, the mechanism depends on pH of the solutions and the protein cationic charges which bind to sulfonate group of ANS. The mechanism is protected the anilinonaphthelene group from water quenching. If this group immerses into nonpolar environment of globular protein, the fluorescence of protein-ANS binding will express as illustrates in Figure 24 (Matulis et al, 1999).

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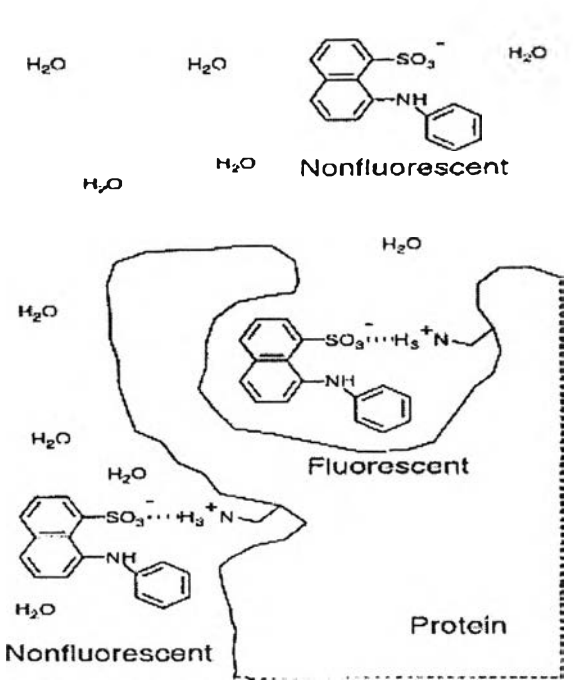

Figure 24 The mechanism of protein-ANS binding (Obtained from Matulis et al, 1999)

7.3 Gel electrophoresis

Gel electrophoresis technique is a well-known analytical technique is high resolution to separate sizes of proteins by their molecular weight. In addition, this technique can characterize the impurity and stability of proteins. However, only proteins in a limited of range can be separated.

In general, the characterization of protein using gel electrophoresis, the concentration of polyacrylamide gel was at 12.5% w/v. However, lysozyme gave a low resolution due to lysozyme being a small molecule. Hence, the concentration of polyacrylamide should increase to 15% w/v gel (http://faculty.mansfield.edu/bganong/index.cfm).

In penetration study, pig's ear skin was selected to use as in vitro animal skin.

8. The in vitro animal skin

Actually, an ideal in vitro skin for penetration study is human skin obtained from plastic surgery. However, it is limited and is not available in some cases. Therefore, researchers intend to propose the animal skins such as rat skin, mice skin and pig skin (Moser et al, 2001; Schmook et al, 2001). Pig skin is one type of the in vitro animal skins which widely uses in penetration study, especially, pig's ear skin as follows:

Dick and Scott, 1992 reported that pig's ear skin was as good model for human skin permeability. Its permeability character is closer to human skin than rat skin, particularly when lipophilic molecules are used.

Schmook et al, 2001 showed the comparison of human skin and other animal skins was evaluated using topical dermatological drugs. The result indicated that pig's skin was the most suitable model for human skin.

Hadgraft, 2004 reported animal skin was extensively used and the most reliable tissue appeared to be form pig's ear. From these evidences revealed that pig skin presented a similarity of histological and physical property to human skin. In addition, the permeability of drugs through pig skin has been shown to be similar to that through human skin (Moser et al, 2001). Therefore, pig's ear skin was selected to use in penetration section of the present study.