CHAPTER III

Materials and Methods

Materials

1. Raw materials and reagents for characterization of lysozyme folding mechanism:

1.1 Hen egg white lysozyme (L-6876, lot no. 09351455, Sigma, Canada)

1.2 Absolute ethanol (Lot no. K32983183409, Merck, Germany)

1.3 Sterile water for injection (Lot no. 2004/0049, Queen Saovabha Monorial Institution, Bangkok, Thailand)

1.4 Hydrochloric acid (Lot no. 7647-01-0, J.T.Banker, USA)

1.5 8-Anilino-1-naphthalenesulforic acid ammonium salt (ANS) (A3125, Lot no. 044K2502, Sigma, Switzerland)

1.6 Rhodamine Red (R6160, Lot no. 39188A, Sigma, The Netherlands)

1.7 SDS-PAGE molecular weight standards, broad range [Myosin (200.00 kDa), β-galactosidase (116.25 kDa), Phosphorylase b (97.40 kDa), Serum albumin (66.20 kDa), Ovalbumin (45.00 kDa), Carbonic anhydrase (31.00 kDa), Trypsin inhubutor (21.50 kDa), Lysozyme (14.40 kDa) and Aprotin (6.50 kDa)] (Lot No. 82442, Bio-Rad, USA.)

1.8 Acrylamide (Batch No. 0109539, Fisher Chemicals, UK)

1.9 N,N″-methylene bisacrylamide (Lot No. 89K0943, Gibco BRL Life Technologies, USA.)

1.10 TRIS, crystallized free base (Lot No. 034964, Fisher Chemical, UK)

1.11 Sodium dodecyl sulfate (Batch No. 0248390, Fisher Chemical, UK)

1.12 Ammonium peroxodisulfate (Lot No. 453008/1, Fluka Biochemika, Switzerland)

1.13 2-mercaptoethanol (Batch No. 004508, Fisher BioReagents)

1.14 TEMED (Batch No. 006702 , Fisher BioReagents)

1.15 Novex® Tris-Glycine SDS Sample buffer (2X) (Lot No. 1184833, Invitrogen™, USA.)

1.16 Novex® TrisGly SDS Running buffer pH 8.3 (10X) (Lot No. 1194870, Invitrogen™, USA.)

1.17 Simply Blue™ SafeStain (Lot No. 1194868, Invitrogen™ Life Technologies, USA.)

2. Materials for penetration study:

2.1 Pig's ear skin (Local market)

2.2 *Micrococcus lysodeikticus* (M 3770, ATCC no.4698, Lot no.052K8618, Sigma, USA)

2.3 Propranolol HCl (Batch no. 950915, China National Chemical IMP& EXP CORP, China)

2.4 Acetic acid (Lot no. K18049863, Merck, Germany)

2.5 Potassium Dihydrogen orthophosphate (Lot no. B/NO.F2H145, Asia Pacific Specialty Chemical Limited, Australia)

Equipment

1. Analytical balance (AG 204, Mettler Teledo, Switzerland)
2. Analytical balance (PB3002, Mettler Toledo, Switzerland)
3. Spectropolarimeter/Circular Dichroism (J-715, Jasco, Japan)
4. Spectrofluorometer (FP-777, Jasco, Japan)
5. UV/Vis spectrophotometer (V-530, Jasco, Japan)
6. Scanning electron microscope (JSM-5410 LV, JEOL, Japan)
7. pH meter (Thermo Orion model 210,USA)
8. Magnetic stirrer (Model M21/1, Framo®-Gerätetechnik, Franz MORAT KG (GmbH & Co.), Germany)

Methods

This experiment was studied in 2 sections as follows:

Part I. Characterization of lysozyme folding mechanism

Hen egg white lysozyme was used as the model protein for this study. It was dissolved in water as stock solution and the concentration was 10 mg/ml. This section was emphasized on the identification of specific environments for various stages of conformational changes of the model protein (lysozyme).

1. Identification of raw materials

1.1 Hen egg white lysozyme

Hen egg white lysozyme was identified by 2 techniques as follows:

1.1.1 Gel electrophoresis

The solution of lysozyme was prepared at 1 mg/ml. Gel electrophoresis technique was performed at 15% w/v of polyacrylamide gel (http://faculty.mansfield.edu/bganong/index.cfm) and used to identify lysozyme structure.

1.1.2 Circular dichroism (CD) technique

The solution of lysozyme was examined for its conformation using CD technique. Determination of protein conformations was done in far-UV (190-250 nm) and near-UV (250-320 nm) using the path lengths of cell at 1 and 10 mm, respectively. These path lengths of quart cell were used throughout the CD experiment.

Lysozyme displays its native structure when exposes to water (Rupley et al, 1983; Lehmann et al., 1985). The proper concentration for CD technique was determined by variation of lysozyme content in water as 0.02, 0.05, 0.1, 0.5 and 1 mg/ml. This suitable concentration of lysozyme was used throughout the CD study.

1.2 Absolute ethanol

Absolute ethanol was characterized for the amount of water by Karl Fisher titrimetry.

2. Conformational transformation of lysozyme

This section, three environments were examined, namely aqueous-ethanolic, aqueous-acidic and combination of the two environments. Lysozyme stock solution was added in each formula and its amount was the substitution of water in the solution while the other contents were fixed.

2.1 Aqueous-ethanolic environment

Lysozyme was exposed to various aqueous-ethanolic conditions. Several factors were studied which were expected to have effects on the protein's conformational structures.

2.1.1 Effect of mixing time

The effect of mixing time was obtained by solubilization of lysozyme in 50% v/v ethanol. The measurements were made at 0, 10, 20, 30, 40 and 60 minutes to determine the optimal time to analyze the protein using CD technique.

2.1.2 Effect of ethanol concentration

Various contents of absolute ethanol were introduced in water (0-99% v/v). This experiment was done to determine the effects of ethanol concentration on lysozyme conformations. For this section, the methods used to consider the transformation of the model protein were carried out as follows:

2.1.2.1 Circular dichroism technique

The preparation of lysozyme solution was done at 0.1 mg/ml in ethanolic solution with various concentrations (0-99% v/v). The conformational modifications of lysozyme were determined after mixing.

2.1.2.2 Fluorometric method

The solutions of lysozyme were prepared at 0.05 mg/ml at various ethanolic concentrations (0-90% v/v). Lysozyme was allowed to bind to an extrinsic probe, which was 1-anilino-8-naphthalene sulfonate anion (ANS), at the molar ratio of lysozyme to ANS of 1:1. The solutions were immediately characterized. The excitation wavelength was 380 nm and the emission wavelength was in the range of 420-620 nm.

2.1.2.3 Gel electrophoresis

The solutions of lysozyme dissolved in various concentrations of ethanol were prepared at 1 mg/ml. The structure of lysozyme was characterized using gel electrophoresis technique at 15% w/v of polyacrylamide gel (http://faculty.mansfield.edu/bganong/index.cfm).

2.1.3 Effect of lysozyme concentration

Lysozyme was dissolved at various concentrations of 0.05, 0.1, 0.5 and 1 mg/ml in 80% v/v of ethanol in the aqueous-ethanolic solution. This study was to investigate protein concentration effects on the conformational alterations in aqueous-ethanolic mixtures using CD technique.

2.2 Acidic environment

This section was to study the effects of acid concentrations on the model protein structure. Hydrochloric acid was diluted at the concentration of 0.1, 1, 2, 4 and 6 N. Lysozyme stock solution was dissolved in the prepared acidic solutions. CD measurements were made immediately after lysozyme and the acidic solution were mixed and after the mixtures were kept at room temperature overnight.

2.3 The combined aqueous-ethanolic and acidic environment

This condition consisted of both aqueous-ethanolic and acidic condition. Six normal HCl stock solution was gradually added in different quantities to reduce the amount of ethanol required in the aqueous-ethanolic solution to induce the specific conformation detected by various techniques as follows:

2.3.1 Circular dichroism technique

The preparation of lysozyme solution was done at 0.1 mg/ml in various combinations. The conformational modifications of lysozyme were determined after mixing.

2.3.2 Fluorometric method

The preparations of the combined solutions of lysozyme were carried out at 0.05 mg/ml. Lysozyme was allowed to bind to ANS at the molar ratio of lysozyme to ANS of 1:1. The solutions were immediately characterized. The

excitation wavelength was 380 nm and the emission wavelength was in the range of 420-620 nm.

2.3.3 Gel electrophoresis

The solutions of lysozyme dissolved in the solvent of the combined environment were prepared at 1 mg/ml. The determination of lysozyme structure was done using gel electrophoresis technique at 15% w/v of polyacrylamide gel.

3. Determination of reversibility of the model protein

This part was to determine the reversibility of lysozyme conformation in the condition which significantly affected the structures. The initial concentration of lysozyme was 1 mg/ml in specified hydroethanolic and acidic conditions. After that, it was diluted 20 times the original volume with water and the protein conformation was analyzed by using CD. Lysozyme conformation was investigated at 0, 5, 10, 15, 30, 60 and 120 minutes after being diluted with water at 1 mg/ml lysozyme in 80% v/v aqueous-ethanolic solution. This experiment was done to observe the kinetics of lysozyme conformational changes over time.

3.1 Reversibility of lysozyme

The predetermined conditions which caused lysozyme conformational modification were used to evaluate the reversibility of the protein. Each formula was divided into three groups. In the first group, lysozyme was dissolved in the medium of interest and immediately diluted with water. In the second group, water was added after the protein had been exposed to the specified solution overnight. In the third group, lysozyme was directly incorporated in the solution already diluted to the final concentration. The third group was used as a control group.

All formulas in this study were represented by L(a)(b) where a and b were the concentration of ethanol (% v/v) and the concentration of acid (mM), respectively.

Part II. Penetration

This part was intended to study penetration of modified lysozyme which was presented as MG or partially folded form. Pig's ear skin was selected as the in vitro model skin in modified Franz diffusion technique. The receiver compartment of this experiment was water controlled at 37 °C.

An unshaved pig's ear skin was prepared by heat separation technique. The pig's ear was boiled in deionized water at 60°C for 45 seconds. After that removal of whole dorsal skin membrane on the cartilage was done. The prepared skin used within 24 hours or kept wrapped in aluminium foil at -20°C for up to 7 days (Dick and Scott, 1992). This technique was used throughout the penetration part.

There were two sections of permeation study as follows:

1. Pig's ear skin integrity test

Pig's ear skin was evaluated by two techniques as follows:

1.1 Franz diffusion technique

This experiment was used to test the leakage of the model skin. A hydrophilic drug model, propranolol HCl, was added in four solvents of interest at 10 mg/ml. In the first group, propranolol HCl was dissolved in water [P(0)(0)] which was a representative of the native environment. In the second group, two solutions which generated MG state of lysozyme were used where propranolol HCl was solubilized in 80% v/v of ethanol in the aqueous-ethanolic solution [P(80)(0)] and in 35% v/v of ethanol in the aqueous-ethanolic solution in 20 mM HCl acid [P(35)(20)]. In the third group, propranolol HCl was dissolved at low concentration of ethanol at 35% v/v [P(35)(0)]. The amount of propranolol HCl in the receiver side was determined at 0, 1, 2, 3, 4, 5 and 6 hours using spectrophotometry at 289 nm.

The penetration profiles of propranolol HCl dissolved in various environments were evaluated by the similarity factor (f_2) using equation [3] (Yuksel et al, 2000).

$$(f_2) = 50 \log \{[1+ (\frac{1}{n}) \sum_{t=1}^{n} (Rt-Tt)^2]^{-0.5}) X 100 \} \qquad [3]$$

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

Physical observation of the pig's ear skin was done after exposure to the solutions of interest without lysozyme for six hours. The solutions were water [V(0)(0)], 35% v/v ethanol of the aqueous-ethanolic solution [V(35)(0)], 80% v/v ethanol of the aqueous-ethanolic solution [V(80)(0)], 20 mM HCl acid [V(0)(20)] and 35% v/v ethanol in 20 mM HCl acid [V(35)(20)]. Each treated pig's ear skin was examined for its skin's surface by Cryo-Scanning electron microscopy (Cryo-SEM).

2. Penetration study

2.1 Detection of the penetrated amount of lysozyme by enzymatic activity

The lysozyme enzymatic activity technique was used to evaluate the amount of lysozyme which permeated into the receiver compartment. *Micrococcus lysodeikticus* was used as a substrate. This experiment followed the Sigma guidelines.

Determination of the penetration of lysozyme was divided into three groups and the concentration of lysozyme was 1 mg/ml. In the first, lysozyme was dissolved in water and represented the native conformation [L(0)(0)]. In the second, lysozyme was solubilized in 35% v/v ethanol with 20 mM of HCl acid [L(35)(20)] which represented the MG state group. The third group was a control group containing water without lysozyme (H_2O).

The basic principle of this technique is *Micrococcus lysodeikticus* was hydrolyzed with lysozyme. The condition was at pH 6.24 and temperature at 25 °C

and the reaction was evaluated by spectrophotometer at 450 nm. The reagents were the following.

A. 66 mM potassium phosphate buffer, pH 6.24 (adjusted to pH 6.24 with 1 M KOH)

B. 0.015% (w/v) *Micrococcus lysodeikticus* cell suspension (substrate). The A_{450nm} of this suspension should be between 0.6 and 0.7.

C. Lysozyme enzyme solution (Prepare solution containing 200–400 units/ ml of lysozyme in cold reagent A)

The procedure was to pipet (in milliliters) the reagents into cuvattes as follows:

	Test	Blank
Reagent B (Substrate)	2.50	2.50
Reagent C (Enzyme solution)	0.10	-
Reagent A (Buffer)	-	0.10

This solution was immediately mixed and recorded the decrease in A_{450nm} for approximately 5 minutes to obtain the A_{450nm}/ min using the maximum linear rate for both test and blank. It was calculated as units/ml enzyme using the equation [3].

$$\text{Units/ml enzyme} = \frac{(A_{450nm}/\text{min Test} - A_{450nm}/\text{min Blank})(df)}{(0.001)(0.1)} \qquad [3]$$

df = Dilution factor

0.001 = Change in absorbance at A_{450nm} per the unit definition

0.1 = Volume (in milliliter) of enzyme used.

It was then calculated as units/mg protein using the equation [4].

$$\text{Units/mg protein} = \frac{\text{Units/ml enzyme}}{\text{mg protein/ml protein}} \quad [4]$$

2.2 Detection of the amount of lysozyme penetrating the skin by CD technique.

This experiment used modified Franz diffusion cell technique and analyzed the amount of lysozyme remaining in the donor compartment by CD method. Lysozyme was solubilized at a concentration of 1 mg/ml in various conditions.

The interested formulas were lysozyme dissolved in water [L(0)(0)], 35%v/v ethanol in the aqueous-ethanolic solution [L(35)(0)], 80% v/v ethanol in the aqueous-ethanolic solution [L(80)(0)] and 35%v/v ethanol in 20mM HCl acid [L(35)(20)]. The CD spectra of each formula (Figures 65, 67, 69 and 71) were displayed in ellipticity (mdeg) unit. It was not normalized to molar ellipticity unit (deg.cm^2/decimol) as in other previous studies. All CD intensities were evaluated at 289 nm. It was calculated from the calibration curve of each formula. The intensity of lysozyme was determined at 0, 1, 2, 4 and 6 hours.

The profiles of remaining amount of lysozyme in various environments were evaluated by the similarity factor (f_2) using equation [3].