CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. Organisms

Pasteurella multocida serotype 2T35 mutant strain was obtained from Animal Vaccine Research Unit, Chulalongkorn University.

2. Media

Brain heart infusion broth (Difco, USA)

Tryptose blood agar base (Difco, USA)

3. Chemicals

Bovine serum albumin Batch no. 48177A, BIORAD, USA

Carboxymethylcellulose, Medium viscosity Batch no. 64H1176, SIGMA, USA

Carboxymethylchitosan, UNICORD, Thailand

Cholesterol Batch no. 111H8485, SIGMA, USA

Coomassie brilliant blue G-250, BIORAD, USA

Dichloromethane, Batch no. 921-K12277250, E.merck, Germany

Disodium hydrogen phosphate, Batch no. 120A578486, E.merck, Germany

Egg yolk lecithin, SIGMA, USA

Potassium dihydrogen phosphate Batch no. A776073, E.merck, Germany

Potassium thiocyanate Batch no. 328647/2 , Fluka , Japan

Sodium chloride Batch no. 049K14521804, E.Merck, Germany

TRIS-hydrochloride , Batch no. 41H5619, SIGMA, USA

Triton X-100 Batch no. 305142/1692, Fluka, Japan

The Bio-Rad DC Protein Assay Reagents, BIORAD, USA

Reagent A 1-5 % sodium hydroxide solution (an alkaline copper tartrate solution)

Reagent B (a dilute Folin reagent)

Reagent S (1-5 % sodium dodecyl sulfate solution)

4. Instruments

Analytical balance, Sartorius 1615MP., Germany

Autoclave, Hirayama, Japan

Freeze dryer, FTS, USA

Freezer -20 ^{o}C, Tropical Ariston, Italy

Incubator, Memmert, Germany

Layser Particle Sizer

Magnetic stirrer, Thermolyne, USA

Microplate Reader (BIORAD, USA)

Microscope, Olympus BH-2, Japan

Mixer, Franz MORAT Type R25, USA

Milipore filter set, Gelman Science, Finland

pH meter, Model 420A, Orion, USA

Scanning Electron Microscope, JSM-T220A, Joel Co. Ltd., Japan

Refrigerated centrifuge SIGMA, Japan

Refrigerator, Hitachi, Japan

Thermostatted water bath, HETO FRIG CB60, Heto, USA

Transmission Electron Microscope

Ultracentrifuge Beckman, USA

Viscometer, Brookfield, USA

Vortex mixer, Scientific Industries, Inc.

5. Glassware (Pyrex , USA)

Beaker

Centrifuge tube

Erlenmeyer flasks

Glass slide

Glass plate

Measuring cylinders

Measuring pipettes

Pasteur pipette

Petri dishes

Sterring rods

Test tubes

6. Others

Dialysis tubing and clips, Medicap, England

Disposable gloves

Disposable syringes

Filter membrane Q.22 µ, Millipore, USA

Microtiter plates

Micropipette tip

Microtube plastic , Treff Lab , Switzerland

Methods

1. Calibration curve of standard protein (Assay by Bradford Method) (90)

This assay, based on the method of Bradford, is a simple and accurate procedure for determining concentration of solubilized protein. It involves the addition of an acidic dye (Coomassie Brilliant Blue G-250) to protein solution. The different color change of a dye occurs in response to various concentrations of protein, and subsequent measurement at 595 nm with a spectrophotometer or microplate-reader. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. The extinction coefficient of a dye - albumin complex solution was constant over a 10 fold concentration range. Thus, Beer's law may be applied for accurate quantitation of protein by selecting an appropriate ratio of dye volume to sample concentration.

The procedure of assay explained as the following:

Standard solution containing an accurate amount of bovine serum albumin (BSA) 1.35 mg/ml were diluted to five dilutions (10, 20, 40, 60, 80

μg/ml) with phosphate buffer solution (PBS) pH 7.4. Triplicate, the 160 μl of each standard solution were pipetted into separate microtiter plate wells. The 40 μl of concentrate dye reagent (Coomassie Brilliant Blue G-250) were added to each well. The standard solution and dye reagent, were mixed thoroughly using a microplate mixer, incubated at room temperature for 15 minutes. Absorbances were measured at wavelength of 595 nm. The plot of average absorbance versus known concentrations was made. The linearity of the relation between absorbance and concentration was determined by linear regression .

2. Calibration curve of standard protein

(Assay by Lowry method) (91,92)

This assay is a colorimetric assay for protein concentration following detergent solubilization. The reaction is similar to the Lowry assay, but with some improvements.

The assay is based on the reaction of protein with an alkaline copper tartrate solution (Reagent A) and Folin reagent (Reagent B). There are two steps which lead to color development. The reaction between protein and copper in alkaline medium, and the subsequent reduction of the Folin reagent by copper treated protein. Protein affects a reduction of the Folin reagent by loss of 1, 2 or 3 oxygen atoms, thereby producing one or more of several possible reduced species which have a characteristic blue color with maximum absorbance at 750 nm and minimum absorbance at 605 nm.

The procedure of assay was explained as the following: The working reagent $A^{/}$ was prepared by adding 20 μl of reagents to each ml of reagent A. (This

working reagent A/ is stable for 1 week even though a precipitate will form after 1 day. If precipitate forms, warm the solution and vortex)

Standard solution containing an accuratae amount of bovine serum albumin (BSA) 1.35 mg/ml was diluted to 5 dilutions. 50, 100, 200, 400, 800 µg/ml. Triplicate, 5 µl of each standards was pipetted into separate microtiter plate wells. The 25 µl of reagent A was added into each well, subsequently 200 µl of reagent B was added into each well. Then, let the plate mix for 5 sec. by microplate mixer. After 15 min, absorbances can be read at 655 nm. The plot of average absorbances versus known concentrations was made. The linearity of the relation between absorbances and concentrations was determined by linear regression.

3. Preparation of Protein Extract from *Pasteurella multocida*

3.1 Culture Organisms

The culture used for protien extraction was *Pasteurella multocida*, serotype 8:A(2T35). They were thawed from stock cultures (kept at - 80 °C) and streaked for isolation on tryptose blood agar (TBA) plates. Isolated colony was streaked on surface of TBA plates and incubated at 37°C for 24 hr. Cells were harvested by washing cultures on agar surface with normal saline solution (NSS). The suspension was adjusted to contain $1x10^9$ cells/ml. The 2 ml suspension of *P. multocida* was inoculated in a flask of 400 ml of brain heart infusion broth (BHI), 2 flasks were used, then incubated in shaker incubator at 37 °C for 24 hr. Cells were harvested from broth and washed three times with NSS by centrifugation at 2,000 g for 30 min at 4°C. The cells were collected.

3.2 Preparation of Potassium Thiocyanate (KSCN) Antigen Extracts

The collected cells were suspended in 10 ml of 0.5 M potassium thiocyanate (KSCN) in 0.08 M sodium chloride, pH 6.3 and slowly stirred in shaker water bath at 37 ^{o}C for 5 hr. This suspension was centrifuged at 19,000 g for 30 min at 4°C to pellet the cells. The supernatant was dialyzed four times with 0.1 M. Tris - hydrochloride, 0.32 M sodium chloride buffer, pH 8.0 for 48 hr at 4 ^{o}C to remove any possible dialyzable fragments. The dialysate was protein antigen collected and concentrated by freeze-drying using lyophilizer (FTS System). They were stored at - 20°C until used.

4. Protein Determinations of *P. multocida* Antigen Extracts (83)

The protein contents of antigen extracts were determined by dye-binding method described by Bradford (90). Bovine serum albumin (BSA) was prepared for standard protein at the concentration of 10, 20, 40, 60 and 80 μg/ml. Standard BSA and antigen extracts in a volume of 160 μl were added in each well of microtiter plates. For dye-binding reaction, 40 μl of dye reagent (Coomassie brilliant blue G-250, BIORAD) was added to each well. The mixture was mixed immediately and incubated at room temperature for 15 min. The absorbance was measured at wavelength of 595 nm by using Microplate Reader model 450 (BIORAD). The protein contents were estimated from standard curve.

5. Preparation of Liposomes Containing Protein Extract from *P. multocida* by Double Emulsion Technique.

A prime objective of this study is to prepare the stable liposomes from appropriate membranes by using double emulsion technique. To select the appropriate membranes, the experiment was investigated in two main studies which was the roles of cholesterol content and the roles of polymer coating on physicochemical properties of liposomes.

5.1 Study of the Roles of Cholesterol Content

5.1.1 Preparation of liposomes with various ratio of egg yolk lecithin to cholesterol

Liposomes containing protein extract from *P. multocida* were prepared by double emulsion technique (modified from method of Kato A., Arakawa M., and Kondo T. (24) as following :

Total lipid mixture in various molar ratios of 1:0 of egg yolk lecithin to cholesterol (non cholesterol liposomes), 7:2 of egg yolk lecithin to cholesterol (cholesterol poor liposomes), and 1:1 of egg yolk lecithin to cholesterol (cholesterol rich liposomes) was dissolved in 10 ml of dichloromethane at 50 mg/ml. Protein extracted from *P. multocida* was dissolved in a phosphate buffer solution (PBS), pH 7.4 to obtain protein concentration at 1000 μg/ml. To 10 ml of protein solution was added an equal volume of the lipid solution, the mixture was vigorously agitated by vortex mixer for 30 second to give a w/o emulsion. The emulsion obtained was quickly added with stirring to 100 ml of pH 7.4 PBS to yield a w/o/w emulsion. After 10 minutes stirring, another 100 ml of

PBS pH 7.4 was added to complex emulsion under stirring and the stirring was further continued for 18 hours until the dichloromethane was completely evaporated out. In this way, liposomes containing protein extract from *P. multocida* were obtained.

The aqueous dispersion of liposome was centrifuged at 15,000 g for 30 min. The precipitates were washed three times with the PBS pH7.4 on the centrifuge and redispersed in 10 ml of the same medium for further analysis.

5.1.2 Studies of physicochemical properties of liposomes.

Liposomes, prepared with various ratios of egg yolk lecithin to cholesterol were investigated as following :

a) Entrapping efficiency

The volume of 1 ml liposome suspension was pipetted into microcentrifuge tube. The encapsulated protein was separated from free protein by centrifugation at 15,000 g for 30 min. The pellet containing entrapped protein was ruptured by adding 1 ml of 2% w/v Triton X-100. After incubated at room temperature for 30 min, the clear solution was assayed by modified Lowry method (Bio-Rad DC Protein Assay). The absorbances were read at 655 nm and compared with the corresponding standard curve (all analysis were done in triplicate). The entrapping efficiency was calculated by following equation :

$$\text{Entrapping efficiency} = \frac{\text{Total protein content in liposome vesicles x 100}}{\text{Initial protein content}}$$

b) Microscopic appearances

The generally appearances in shape, size, size distribution, aggregation,and type of liposomes containing protein extract from *P. multocida* were investigated and photographed under the microscope (magnification 1000x).

c) Transmission electron microscopy (TEM)

In order to examine the phospholipid bilayers of prepared liposomes, transmission electron microscopy was perfomed. Samples were stained with 1% phosphotungstic acid pH 7.0 on 300 mesh formyar film coated copper grids and air dried. The grids were photographed and magnified at 75,000 and 150,000 times.

d) Scanning electron microcopy (SEM)

Surface characteristics of liposomes (JSM - T 220 A, Jeol) were studied by scanning electron microscope. The specimens were obtained by a specific fixation technique, which liposomes were adsorbed on the glass slide and were fixed with 2.5 % glutaraldehyde in 0.1 M PBS pH 7.2 for 1 hr. and subsequently fixed with 1 % osmium tetraoxide in 0.1M PBS pH 7.2,

washed three times with 0.1M PBS pH 7.2 for 5 min, dehydrated with a serial concentrations of 30 %, 50 %, 70 %, 90 % and 100 % v/v acetone solution for 5 min in each step, dried with criticle point drying method by Samtri 780 dryer, mounted, and coated with gold. The scanning electron micrographs were examined and photographed.

e) Particle size analysis

Laser particle sizer (Mastersizers long bed Ver 2.11 with 300 mm range lens, 240 mm beam length) was used to determine particle size of liposomes. Particle size distribution was analysed by the curve plotted between particle diameter versus percent volume of particles. Cumulative frequency curve was plotted between cumulative percent under size versus particle diameter, and the median diameters were determined.

f) Assessment of protein leakage rate from liposomes.

The initial preparations were diluted 1 to 10 dilution with PBS pH 7.4. The 200 μl/ml of diluted preparation were subdivided into microcentrifuge tubes, capped and shaken in a water bath at 37°C. Efflux of protein was assessed by periodic centrifugation of sample at 15,000 g for 60 min. The supernatant was assayed for protein by Bradford method (Biorad protein assay). The precipitates were also assayed for the remaining protein. They were dissolved in 100 μl of 2% Triton X-100 then assayed by Lowry method (Biorad DC protein assay). Percent of protein that released from liposomes was calculated as following equation :

$$\text{Percent releasing of protein} = \frac{[\text{Protein}]_s}{[\text{Protein}]_{ppt}+[\text{Protein}]_s} \times 100$$

$[\text{Protein}]_S$, Protein conent in supernatant

$[\text{Protein}]_{ppt}$, Protein content in liposome vesicle

5.2 Study of the Role of Polymer Coated Liposomes

From the studies of the roles of the ratio of egg yolk lecithin to cholesterol on the lipid content of liposomes, the preparation which gave a good properties such as entrapping efficiency, microscopic appearance, assessment of protein leakage rate from liposomes was selected and used for preparation of polymer coated liposomes. In this study, the two biopolymers were investigated with various concentrations which were studied as follows.

0.02, 0.2, 0.5% w/v Carboxymethylchitosan

0.02, 0.2, 0.5% w/v Carboxymethylcellulose

5.2.1 Preparation of polymer coated liposomes

The polymer coated liposomes containing protein extract from *P. muctocida* were prepared by double emulsion technique (modified technique that described by Kato A. and Kondo T, 1987).

The mixture of egg yolk lecithin and cholesterol in 1:1 molar ratio was dissolved in 10 ml of dichloromethane at 50 mg/ml. To 10 ml of 1000 µg/ml protein extract solution was added an equal volume of the lipid solution, and the mixture was vigorously agitated by vortex mixer for 30 second to

give a w/o emulsion (first emulsification). The emulsion obtained was quickly added with stirring to 100 ml of the polymer solution to yield a w/o/w complex emulsion (second emulsification). After 10 min stirring, another 100 ml of the polymer solution was added to the complex emulsion under stirring and the stirring was further continued until dichloromethane was completely evaporated out. In this way lecithin - cholesterol walled liposome coated with polymer, containing protein extract from *P. multocida* were obtained.

The aqueous dispersion of liposome was centrifuged at 15,000 g for 60 min. The precipitate were washed three times with PBS pH 7.4 on the centrifuge and redispersed in the same medium for further analysis.

5.2.2 Studies of physicochemical properties of liposome

The polymer coated liposomes, prepared with various concentrations and types of polymers were investigated by the same method as described in 5.1.2 (a), (b), (c), (d), (e), (f).

5.3 Stability study

The stability of all preparations were studied. They were stored in the closed seal container and kept at 4-8°C for 3 months. Percent of remained protein in liposome vesicles were determined every week. After 3 months storage, the physicochemical properties were investigated in term of particle size, particle size distribution, and microscopic appearance.