CHAPTER 3 MATERIALS AND METHODS

3.1 Instruments and Chemicals

3.1.1 Instruments

- 96 well plate: NUNC, Thermo fisher scientific, Denmark
- Autopipette: Biohit (proline 50-250), Sartorius, Finland
- Autoclave: Hirayama, Japan
- Centrifuge: Hehich zentrifuen, Germany
- Larminar air flow: Esco Class II Biohazard safety cabinet
- Microscopic slide: HAD, Yancheng huida medical instruments co.,Ltd. China
- Plate reader: GE healthcare, United Kingdom
- Spectrophotometer: Spectronic 21, Milton Reg Ltd., USA
- Vortex mixer: Vortex-2GeneG-560E, Scientific Industries Inc., USA
- Water bath: EYELA, SB-9, Japan

3.1.2 Chemicals

- Anti-chicken IgY FITC (fluorescein isothiocyanate) conjugate, Disodium hydrogen phosphate dodecahydrate (Na₂HPO₄.12H₂O), Sodium taurodeoxycholate hydrate, Trypsin: Sigma, Germany
- Bovine serum albumin (BSA): PAA Laboratories GmbH, Austria
- Coccivac®-D: Schering-Plough company, USA
- Disodium hydrogen phosphate (Na₂HPO₄), Glycerol, Potassium dichromate (K₂Cr₂O₇), Potassium dihydrogen phosphate (KH2PO4), Sodium hydrogen carbonate (NaHCO3), Sucrose (C₁₂H₂₂O₁₁), Tween 20, Tween 80: Ajax Finechem Pty Ltd., New Zealand
- Formadehyde Solution 37%, Phenol, Potassium chloride (KCI): MERCK, Germany
- Hydrogen Chloride (HCl): J.T. Baker, Thailand
- Magnesium Chloride (MgCl), Phenylmethanesulfonylfluoride (PMSF), Polyethylene glycol 6000 (PEG 6000), Sodium carbonate (Na₂CO₃): Fluka, Switzerland

- Protein assay (Dye reagent concentrate): Bio-rad, USA
- Rabbit Anti-IgY HRP (Horseradish peroxidase): Pierce, Thermo scientific, USA
- Sodium Chloride (NaCl): Carlo Erba reagent, Italy
- t-Octylphenoxy polythoxylethanol (Triton X-100): Research organics, USA
- TMB substrate kit [Peroxide solution (H₂O₂), Peroxidase substrate (TMB)]: Thermo scientific, USA

3.2 Production of anti-chicken coccidia IgY at large scale

3.2.1 Antigen preparation

Coccivac®-D, a commercial coccidiosis vaccine from Schering-Plough company (USA), was used as antigen for immunization of hens. This vaccine consists of 8 different species of Eimeria unsporulated oocyst as follows: E.acerulina, E.mivati, E.maxima, E.tenella, E.necatrix, E.praecox, E.brunetti and E.hagani. Sporulation of the unsporulated oocysts was activated using 2.5% potassium dichromate and shaking at 30°C for 5 days. Sporulation of oocyst was observed under microscope. The sporulated oocysts were centrifuged at 5,000 rpm for 5 min and supernatant was discarded. The sporulated oocysts were separated from contaminant materials by floatation technique using Modified Sheather solution (500 grams of sucrose, 5 ml of phenol and 350 ml of tap water). The sporulated oocyst pellet was resuspended in Modified Sheather solution, inversely mixed for several times then centrifuged at 5,000 rpm for 5 min. Floating phase containing sporulated oocysts was collected whereas lower phase supernatant was refloated until the floating phase was clear. All of harvested floating phase were pooled and centrifuged at 14,000 rpm for 20 min, then the pellets were washed several times in Phosphate Buffer Saline (PBS). Impurity check was performed under microscope. Subsequently, the pure sporulated oocysts were extracted in PBS supplemented with 1% triton X-100 and 4 mM Phenyl Methyl solfonyl Fluoride (PMSF) using sonicator. Protein concentration of extracted coccidia was determined using Bradford protein assay providing that bovine serum albumin (BSA) was used as the standard protein sample for the standard curve construction. The extracted coccidia was kept at -20°C until used.

3.2.2 Immunization of chickens

To produce anti-chicken coccidia IgY in large scale, 855 six month old Hisex hens from Ayudya province were immunized intramuscularly 3 times. For the first immunization, the hens were injected at two different sites of breast muscle with extracted coccidia antigen (as described in 3.2.1) emulsified with equal volume of Freund's complete adjuvant (30μg protein antigen/site). The second and third immunization, antigens in combination with Freund's incomplete adjuvant were administrated at 2 and 6 weeks after first immunization, respectively. Eggs were collected at 6 weeks after first immunization and IgY titer produced in the yolk was determined using indirect Enzyme Linked Immunosorbent Assay (ELISA).

3.2.3 Sample preparation for IgY detection

To detect anti-chicken coccidia IgY produced in immunized chicken eggs, 200 IgY samples were prepared from fresh yolk. 200 eggs were collected randomly from immunized chickens from week 13 and 14 after first immunization. The eggs then were washed with tap water. Subsequently, fresh egg yolks were separated individually from egg white. Each fresh yolk sample was kept individually in microcentrifuge tube at -20°C until tested. 0.33 grams of each yolk sample was dispensed into new microcentrifuge tubes (each sample was prepared in triplicate) and dissolved in 1 mL of PBS pH 7.4. The yolk suspensions then were mixed well using vortex followed by centrifugation at 10,000 rpm for 10 min. Then, supernatants were transferred into new tube and diluted to 1:640 in PBS pH 7.4 containing 1.5% BSA (blocking buffer). These supernatants were further used as primary antibody for indirect ELISA. The negative control was prepared similarly using yolk from chickens from the same farm that were immunized with other non related antigen, adipocyte membrane proteins.

3.2.4 Indirect Enzyme Linked Immunosorbent Assay (ELISA) for detection of anti-chicken coccidia IgY

Coccidia antigen prepared as described in 3.2.1 was suspended in coating buffer (Carbonate/Bicarbonate pH 9.6) at concentration of 1 μ g/ μ l (w/v), then 100 μ l of coccidia suspension was added into wells of 96 well-plate and placed at 4°C for overnight followed

by washing 3 times with 250 μ l/well of washing buffer (PBS-Tween, pH 7.4). To block any remained uncoated area by the test antigen, 200 μ l of blocking buffer (PBS-Tween, pH 7.4 containing 1.5% BSA) was added into each well and incubated at 37°C for 2 hours followed by washing 3 times with washing buffer (250 μ l/well). Then 100 μ l of each diluted sample including negative control was added into each well and incubated at 37°C for 1 hour. After incubation, the plate was washed 3 times with washing buffer (250 μ l/well) to remove unbound antibodies. Then 100 μ l of anti-IgY HRP solution at dilution 1:10,000 was added into each well and incubated at 37°C for 1 hour followed by washing 3 times with washing buffer (250 μ l/well). Then, 100 μ l of peroxidase substrate solution was added into each well and incubated in dark place at 25°C for 30 min. Finally, 100 μ l of 1% HCL solution was added into each well to stop the reaction. Then OD (at wavelength 450 nm) of all samples then was measured immediately using spectrophotometer.

3.2.5 Determination of anti-chicken coccidia IgY immunization efficiency

To study immunization efficiency in immunized hens, anti-chicken coccidia IgY from each egg yolk was determined. 200 IgY samples were prepared as described in 3.2.3. All samples were detected for anti-chicken coccidia IgY using indirect ELISA technique. The ELISA steps were performed as described in 3.2.4. Samples whose OD was higher than OD of negative control were classified as positive samples (contain the IgY). The percentage of anti-chicken cocidia IgY positive egg determined the efficiency of immunization.

3.2.6 Determination of IgY production period

The titer of anti-chicken coccidia IgY in eggs collected after immunization was monitored in order to determine the IgY production period of immunized chickens. Approximately 2,000 eggs were collected weekly from immunized hens from week 6 to 21 after first immunization. All collected eggs from each week were washed with tap water and egg yolk was separated from egg white. All separated egg yolks then were pooled and homogenized. Pooled fresh yolk samples from each week were subjected to measurement for anti-chicken coccidia IgY activity at various dilutions using indirect ELISA technique (as described in 3.2.4). IgY samples were prepared from pooled fresh yolk samples from each week using the same protocol as described in 3.2.3. Each IgY sample was serially diluted with blocking

buffer as follows: 1:160, 1:320, 1:640, 1:1280 and 1:2560 before subjected to indirect ELISA. Negative control adopted was yolk from chickens from the same farm which were immunized with other antigen (adipocyte membrane protein), which was similarly prepared. IgY titer of sample is defined as reciprocal of the highest dilution of sample whose OD is higher than that of the negative control at same dilution. IgY production period which is the time period when level of IgY titer remained higher than that of the negative control was determined.

3.2.7 Extraction of anti-chicken coccidia IgY

Ten eggs of immunized hens were collected from week 13 after first immunization. Then, egg yolk of ten eggs was separated from egg white and pooled. Next, anti-chicken coccidia IgY was extracted from the pooled fresh yolk sample using chloroform-polyethylene glycol procedure.

4 grams of pooled fresh yolk sample was added into centrifuge tube containing 5 mL of 100 mM sodium phosphate buffer, pH 7.6 and mixed vigorously. Subsequently 4 mL of chloroform was introduced and the mixture was shaken until the semisolid phase was observed. The suspension then was centrifuged at 1,200 g for 30 min followed by the supernatant was transferred into new tube. Then the tube was added with solid polyethylene glycol (PEG) 6000 to a final concentration of 12% (w/v), mixed and centrifuged at 15,700 g for 10 min. After centrifugation, the supernatant was discarded whereas the pellet was resuspended in 5 mL sodium phosphate buffer. Then the protein concentration of suspension containing IgY was determined using Bradford protein assay and stored at -20°C for determination of IgY activity.

3.2.8 Excystation of sporulated oocyst

Sporulated oocysts (as prepared in 3.2.1) were broken by mechanical grinding with glass beads to release sporocysts after which sporozoites were released from the sporocysts by the action of pancreatic enzymes. The oocyst wall was cracked using 0.5 mm glass beads to release sporocysts. An equal volume of glass bead was added into the tube containing oocyst suspension. Then the mixture was subjected vortexing at maximum speed to

rigorously mix the contents until most of the oocysts were broken leading to release of sporocysts. It is important to check the process by examining small sample at regular interval, e.g., every 4-5 bursts. If too many burst are used, sporocysts may also be damaged, when free sporozites are observed in the mixture the cracking procedure should be terminated. To recover the sporocysts from the glass balls, PBS was added and the supernatant containing sporocysts was transferred to new tube. The process was repeated until most sporocysts were collected. To release sporozoites, the collected sporocysts were incubated in water bath at 41°C for 60-90 min in the presence of 0.25% trypsin in saline solution containing 0.5% taurodeoxycholic acid and 5 mM magnesium chloride. After incubation, the released sporozoites were washed in PBS several times by centrifuging at 14,000 rpm and kept at 4°C until used.

3.2.9 Specificity of anti-chicken coccidia IgY

Specific binding of anti-chicken coccidia IgY to coccidia cells was investigated by using immunofluorescence technique. To prepare coccidia antigen, two different development stages of coccidia, unsporulated oocyst and sporulated oocyst, were prepared from Coccivac®-D vaccine antigen. Sporulated oocysts were extracted for sporocysts and sporozoites using the same extraction protocol as described in 3.2.8. Unsporulated oocysts, sporulated oocysts and extracted sporulated oocysts were spotted onto positive charged slides [HDA] and air dried at room temperature for 15-20 min. After drying, 200 µl of 4% para-formaldehyde was dropped onto all slides to fix the coccidia antigen and incubated at room temperature for 15-20 min. Subsequently, the sides were washed 3 times by soaking in washing buffer (PBS-Tween, pH 7.4) for 5 min. Then any free space on the slides were blocked by dropping 200 µl of blocking buffer (PBS-Tween, pH 7.4 containing 1.5%BSA) onto all slides and incubated at room temperature for 1 hour. Then, the slides were washed 3 times by soaking in washing buffer for 5 min.

For anti-chicken coccidia IgY (specific IgY) preparation, the previously extracted IgY from fresh yolk (as described in 3.2.7) was diluted to 1:320 with blocking buffer as diluent. The negative control IgY employed, yolk from chickens from the same farm that were immunized with adipocyte membrane proteins, was also prepared similarly providing that

PBS was used for autofluorescent test of IgY. The slides of each stage of coccidia were then valid with 200 µl of specific IgY sample, negative control IgY and PBS, and incubated at room temperature for 1 hour. In subsequent to incubation, all slides were washed 3 times in washing buffer. Fluorescein isothiocyanate (FITC)-conjugated rabbit-anti-IgY was diluted 1:500 in blocking buffer, then 200 µl of the FITC-conjugated rabbit-anti-IgY solution was introduced onto all slides and incubated in the dark at room temperature for 30 min. The slides were washed 3 times in washing buffer, mounted with 70% glycerol and covered with cover-glasses. Binding of anti-coccidia IgY to coccidia cells in each stage was observed immediately under fluorescence microscope.

3.3 Spray drying process of anti-chicken coccidia IgY

Dried anti-chicken coccidia IgY powder product was attempted using spray drying. Large amount of eggs were collected weekly from the immunized hens beginning at week 12 after the first immunization until level of the IgY in yolk of those eggs dropped. weekly, all collected eggs were washed and egg yolk was separated from egg white. All separated egg yolks then were pooled, homogenized and dried with spray dryer using an air inlet temperature of 140°C and an air outlet temperature of 72°C according to the condition reported by Kakohki and Kanagawa (1991). Finally, dried yolk powder was filled in aluminum foil bags in vacuum condition and stored at 4°C until its properties were analysed.

3.4 Characterization of anti-coccidia IgY produced at large scale

3.4.1 Effect of spray drying process on IgY activity

0.3 grams of pooled fresh yolk samples and 0.15 grams of dried yolk powder samples from week 15 to 17 after first immunization were dissolved in 1 ml of PBS. Each sample was centrifuged at 10,000 rpm for 10 min and the supernatant were transferred into new tubes. All samples were measured for anti-chicken coccidia IgY activities at various dilutions (namely, 1:160, 1:320, 1:640, 1:1280 and 1:2560) using indirect ELISA technique performed as described in 3.2.4. The IgY titers of both fresh and dried yolk samples were compared.

3.4.2 Determination of particle size distribution of dried IgY product

Particle size of dried IgY product was analyzed by Science and technology research center of King mongkut's university of technology thonburi using particle size analyzer (Model Mastersizer 2000).

3.4.3 Sedimentation of IgY yolk powder

The dilution of IgY yolk powder suspended in water to remain in suspension was investigated before sedimentation. I gram of IgY yolk powder was suspended in 100 mL tap water containing 0.1% and 0.3% tween 80. The negative control employed was 1 gram of IgY yolk powder suspended in tap water. Sedimentation was observed every 30 min with naked eyes until the suspension was clear.

3.4.4 Detection of bacterial contamination in fresh egg yolk and dried IgY product

Specification required for commercial feed product are as follows: Coliform germs should not be more than 10 cfu/gram, *E.coli*, *S. aureus* and *Salmonella spp.* should be absent in 25 grams sample (AOAC). In this study, Coliform germs, *E.coli* and *S. aureus* were determined by using 3M petrifilmTM (a commercial prepared plate) while *Salmonella spp.* was detected according to AOAC official method.

3.4.5 Shelf life determination of dried anti-chicken coccidia IgY product

Three samples of dried IgY products from week 15, 16, 18 after first immunization were tested. Each sample was filled into 30 aluminum foil bags (approximately 30 grams/bag) in vaccum condition. 30 bags of each sample were stored at 4°C and 30°C, The IgY titer of samples kept in both temperatures was monitored monthly until IgY titer of the stored product dropped. 50 mg of stored products containing anti-coccidia IgY was dissolved in 1ml PBS and diluted to 1:2560 dilution before subjected to the indirect ELISA (the ELISA steps as described in 3.2.4).