CHAPTER II

MATERIALS AND METHODS

Materials

1. Chemicals

Tryptic soy agar, Mueller Hinton broth, Mueller Hinton agar, thiosulfate citrate bile salt sucrose agar, Titriplex®III (ethylenediaminetetraacetic acid), 95% ethyl alcohol are analytical grade, glacial acetic acid, formaldehyde and glucose anhydrous from MERCK, Germany. Magnesium chloride hexahydrate, trypsin, L-3,4-dihydroxyphenylalanine, L-cysteine, bovine serum albumin, sodium hexametaphosphate, gentamicin sulfate, potassium chloride, sodium dihydrogen phosphate dehydrate, hematoxylin crystals, potassium aluminium sulfate, chloral hydrate, eosin Y and sodium cacodylate trihydrate from Sigma Chemical Co. Ltd., USA. Calcium chloride dihydrate, sodium chloride and citric acid from BDH Chemicals Ltd., England. Isopropyl alcohol, xylene and citric acid from Farmitalia Carlo Erba Company, Germany. MEM essential amino acid with L-glutamine and Hepes buffer from Gibco Chemical Co., USA. Trisodium citrate and sodium hydrogen carbonate from Fisher Chemicals, United Kingdom. Sodium iodate from Fluka, Switzerland. Barford protein assay kits from Bio-Rad Laboratories Ltd., USA. Hydrochloric acid (36.5-38.0%) solution from Mallinckrodt Baker Inc., USA. Water quality kits were obtained from NASA Lab Co. Ltd., Thailand.

2. Equipments

1. Rotary evaporator (Büchi, Rotavapor R-220 and R-200, Switzerland)
2. Vacuum pump (Büchi, Vac®V-1000, Switzerland)
3. Vacuum pump (SIBATA, WJ-20, Japan)
4. Recirculating chiller (Büchi, B-740/14, Switzerland)
5. Recirculating chiller (Boss Tech., CB-1, Thailand)
6. Filter set (BRITISH PORTACEL®, CRB, Thailand)
7. Electric Pump (GRUNDFOS®X, MQ3-45 A-O-A-BVBP, Italy)
8. Hot plate (E.G.O., 931-12607, Germany)
9. pH Meter (Mettler Toledo, Seven Easy, Switzerland)
10. Pipetting aid (Eppendorf, Easypet 4420, Germany)
11. Hot air Oven (Memmert, UL40, Germany)
12. Hot air Oven (YEO HENG Co. Ltd., Capacity 50 kg., Thailand)
13. Viscometer (Brookfield, LVDV-I$^+$, USA)
14. Electric Balance (Mettler Toledo, PL602-5, Switzerland)
15. Spectrophotometer (Spectronic®, GENESYS^{TM}5, USA)
16. Refrigerator (Bio Advance, SY-200, Thailand)
17. Upright Freezer (Sandent, ID974, Thailand)
18. Microplate reader (Molecular Devices, VERSAmax, USA)
19. High speed centrifuge (Hettich zentrifugen, Universal 32R, Germany)
20. High Intensity Ultrasonic Processor (Sonics & Material Inc., VCX 750, USA)
21. Microscopy (Nikon, AFX-IIA, Japan)
22. Tissue embedding centre (Cambridge instruments Company, 8041, USA)
23. Vortex (Scientific industries Inc., Vortex-2 Genic® G-560E, USA)
24. Hand refractometer (ATAGO, S-10E, Japan)
25. Hematocytometer (BLAUBRAND®, Neubauer Improved bright-line, Germany)
26. Automatic tissue processor (Cambridge instruments Co., Histokinette 2000, USA)
27. Inverted microscope with phase contrast (Zeiss, Axiovert 135, Germany)

3. Methods

3.1 Preparation of polysaccharide gel (PG) from durian fruit-hulls

3.1.1 Isolation of polysaccharide gel (PG)

A polysaccharide gel was isolated from dried fruit-hulls of durian (*Durio zibeıhinus* L.). Waste of fresh durian fruit-hills was collected, washed in water, blended and dried in hot air oven at 60 °C. Samples were stored in cold place. A process of PG isolation was performed based on the method previously described by Pongsamart and Panmuang (1998). The durian polysaccharide gel (PG) was isolated by extracting with boiling deionized water and precipitating the polysaccharide gel in acidic alcohol solution. A crude extract of PG was dried and blended, pale brown powder was obtained.

3.1.2 Preparation of PG solution for microbiological test

Powder of polysaccharide gel was dissolved in sterile distilled water to make a series of two fold dilution of various concentrations of polysaccharide gel, each dilution was added on the agar plates and broth media to make the desired concentration (3.2, 6.3, 12.5, 25.0 and 50.0 mg/ml) for microbiological test.

3.2 Preparation of microorganisms and media

3.2.1 Agar and broth media

The agar and both media were prepared in distilled water with 1% NaCl and then sterilized in autoclave for15 min at 15 pounds pressure (121 °C). Tryptic soy broth (TSB) and tryptic soy agar (TSA) with 1% NaCl was used for stock microorganism culture. Thiosulfate citrate salt sucrose agar (TCBSA) with 1% NaCl was used for diagnostic test of bacterium. Mueller Hinton agar (MHA) with 1% NaCl was

used for agar diffusion test. Mueller Hinton both (MHB) with 1% NaCl was used for broth macrodilution test.

3.2.2 White spot syndrome virus media

Lobster hemolymph medium (LHM) was a broth medium used for white spot syndrome virus (WSSV) challenge test and total hemocytes count test. The powder medium was dissolved in sterile distilled water, adjusted the solution to pH 7.4 with 7.5% $NaHCO_3$ and adjusted volume to 1,000 ml with distilled water in volumemetric flak. This medium was sterilized by filtration through a 0.45 μm pore diameter of membrane filter.

3.2.3 Preparation of microorganisms

3.2.3.1 Bacteria

The luminescent bacterium, *Vibrio harveyi* 1526, was obtained from the Shrimp Culture Research Center, Charoen Pokphand Foods public company limited. The bacterium was inoculated in tryptic soy broth (TSB) with 1% NaCl in shaking flasks for a challenge test, and the inoculated broth was streaked on tryptic soy agar (TSA) slant with 1% NaCl and then incubated overnight at 30 °C for 16 hours for using in the determination of antimicrobial activity of PG. The bacteria were collected from surface of agar slant and then transfered into sterile normal saline solution (NSS), adjusted to match turbidity of standard Mcfarland no. 0.5 before used as a tested bacterial suspension.

3.2.3.2 Virus

The WSSV was obtained from the Shrimp Culture Research Center, Charoen Pokphand Foods public company limited. The WSSV stock solution was prepared by the modified method of Wu et al. (2002). The hemolymph of

moribund shrimps was withdrawn from the WSSV infected *Penaeus monodon* juvenile shrimp, using a 26-gauche needle and a 5 ml syringe filled with a 4 fold volume of LHM pH 7.6, 5% L-cysteine was used as an anticoagulant and stored at -80 °C. The virulence or concentration of virus in the stored hemolymph was quantitated by two-step WSSV PCR method before stored in a freezer and used in challenge test. The WSSV stock solution was thawed and centrifuged at 1500X g at 4 °C for 10 min. The supernatant fluid was diluted to the required concentrations and injected intramuscularly into shrimp.

3.3 Determination of antimicrobial activity of PG

3.3.1 Agar diffusion test

Agar diffusion test was determined following the standard guideline technique (Lorian, 1991) and modified method of Brock et al. (1994). Agar diffusion test was performed as follows: serial two-fold dilutions of various concentrations of PG 50.0, 25.0, 12.5, 6.3 and 3.2 mg/ml, in distilled water were freshly prepared.

Petri dishes with internal diameter of 100 mm containing 25 ml of enriched agar medium MHA with 1% NaCl were inoculated with 1% bacterial suspension by seed over the solidified base layer of the medium in Petri dishes. Sterile stainless steel cups (6 mm internal diameter and 10 mm high) were placed over the surface of seeded media. The various concentrations of PG were filled into the cups (300 µl per cup) and the plates were left for pre-diffusion in room temperature for 1 hour and incubated at 30 °C for 16 hours. The results of clear inhibition zones were observed after incubation then the diameter of the clear zones was measured. The sterile normal saline filled cups were used as control and the determination was carried out in triplicate.

3.3.2 Broth microdilution test

3.3.2.1 Determination of MIC

Broth microdilution test was determined following the modified method of Brock et al. (1994). Broth microdilution test was carried out using media containing 100 µl of broth medium MHB with various dilution of PG and inoculated with 0.5 µl of tested bacterial suspension into inoculate each well of a microtitre plate. Media without PG were used as control and the determination was carried out in triplicate.

MIC of PG testing bacterial inhibition was determined. The inoculated medium with various dilution of PG was incubated at 30 °C for 16 hours. MIC was defined as the lowest concentration of PG that inhibited visible growth of bacteria.

3.3.2.2 Determination of MBC

MBC was determined by subculturing from wells of MHB with PG concentrations showing no visible growth of bacteria onto agar plate of PG-free MHA and incubated at 30 °C for 16 hours. MBC was the lowest concentration of PG from wells of non visible growth that showing no growth on agar plate.

3.4 Preparation of shrimp diets

The crude extract of PG was used in preparation of shrimp diet. The durian polysaccharide gel additive shrimp diet in treatment groups was prepared by adding 0.5-2.0 grams PG in 100 grams diet, diet without PG was used in control group. The shrimp diet was prepared by mixing the dry ingredients powder with oil and then adding cold water until the stiff dough was obtained. This dough was then passed through a mincer, and then pass through 2.3 mm die, the resulting strings were dried by using an air-dried at

60 °C for 4 hours. After drying, the string products were broken up. The resulting granules were sieved through 10, 14 and 25 mesh, respectively. The feed preparations were stored in dry place.

3.5 Proximate analyses

Proximate analyses of the diet compositions were performed upon finely ground samples as follows: moisture, protein, fat, fiber, ash, calcium and phosphorus by following standard Association of Official Analytical Chemists (AOAC) official method of analysis (2000). The composition of the diets was determined. The total carbohydrate was calculated by the subtraction of the sum of the crude protein, total fat, moisture, fiber and ash from the total weight of the sample food.

3.6 Shrimp preparation and feeding trials

Shrimp juvenile initial mean body weight 0.36 ± 0.04 grams of the black tiger shrimp *Penaeus monodon*, produced from the Maeklong farm in Samutsongkhram province, was randomly divided into four groups of 400 shrimps. Each group of black tiger shrimps was subdivided into four of 100 shrimps replicate. Each replicate of 25 juveniles was reared in closed-recirculating water system pond for 56 days (8 weeks) by feeding with different concentrations of PG in diet in treated groups except control group fed with shrimp diet without PG. Body weight, total length, survival rate, biomass and feed conversion ratio (FCR) of shrimp were evaluated after 4 weeks and 8 weeks, respectively. FCR is calculated by the amount of feed (kg) need to produce one kg of growth. During the rearing trial the following environmental categories were controlled: salinity = 16-21 ppt, total ammonia nitrogen (TAN) = 0.0-0.2 ppm, nitrite (NO_2) = 0.0-2.0 ppm, total alkalinity = 100-160 ppm, hardness = 2100-3250 ppm, temperature = 29.5-33 °C, dissolved oxygen (DO) = 3.5-6.0 ppm and pH = 7.6-8.2.

3.7 Total hemocyte counts (THC)

The 100 µl of hemolymph was withdrawn from ventral part of the haemocoel at the third walking legs (pereiopods), using a 26-gauche needle and a 1 ml syringe filled with 400 µl of cold lobster hemolymph medium solution, 5% L-cysteine was used as an anticoagulant. The hemocytes were counted using haemacytometer under the 40X microscopy and calculated as number of blood cell per cubic millimeter.

3.8 Phenoloxidase activity test

The phenoloxidase activity of hemolymph was determined following the modified method of Söderhäll 1983. Treatment and control groups of shrimps at week 8 were tested. The 200 µl of hemolymph was withdrawn using a 26-gauche needle and a 1 ml syringe filled with 400 µl of anticoagulant-1 (AC-1). The hemolymph was centrifuged at 500X g at 4 °C for 10 min. The hemocytes were resuspended in 200 µl of cacodylate buffer (CAC buffer) pH 7.4. The hemocyte lysate supernatant (HLS) was prepared by using a sonicator at 35 amplitudes for 5 second, centrifugation at 1000X g. at 4 °C for 10 min and HLS was collected. Aliquots of 20 µl of 0.1% trypsin (sigma) in CAC buffer was placed in flat-bottomed 96-well microtiter plates and mixed with 20 µl of HLS, added 20 µl of 0.3% L-3, 4-dihydroxyphenyl alanine (L-DOPA, sigma) at 25 °C. The phenoloxidase activity was determined at 490 nm by a microplate reader, CAC buffer was a blank control. Protein concentration of the HLS was determined by Bradford method (1975), BioRad Protein Assay System Kit, with bovine serum albumin was used as the standard. One unit of phenoloxidase enzyme activity was defined by the increasing of 0.001 absorbance per minute per milligram of protein (Söderhäll and Unestam, 1979).

3.9 Challenge test

3.9.1 WSSV challenge test by cohabitation method

The challenge test was conducted on day 28 and 56 of the feeding trial. Each group of black tiger shrimps in each a period of time was subdivided into four of 32-40 shrimps replicates, a replicate control blank and three replication challenge groups, respectively. Each replicate of 8-10 juvenile shrimps was reared in 200 liters aquaria (50x90x50 cm^3) by recycle water system. Cohabitate shrimps were injected intramuscularly at dose of 0.1 ml 10^6x WSSV (dilution 1:100) stock solution, with flow-through seawater to challenge groups. Survival rate of shrimp were evaluated, after 24 hours of injection, for 10 days after challenge or until all moribund shrimps were observed in control group. During the rearing trial the following environmental categories were controlled. The moribund shrimps (challenge and control blank groups) were randomly collected from each replication. The moribund shrimp was stored in Divison fixation and used for the histology assay by stained with hematoxylin and eosin dry. The cumulative mortalities of shrimp were expressed in terms of relative percent survival (RPS) calculated from the following equation.

$$RPS = \left(1 - \frac{\% \text{ Mortality in experimental group}}{\% \text{ Mortality in control group}} \right) \times 100$$

The relative percent survival (RPS) value was estimated with the challenge test at 20-50% mortality in control group, RPS values not lesser than the criterion 60% was acceptable as effective in fish vaccines (Amend, 1981).

3.9.2 *Vibrio harveyi* 1526 challenge test by immersion method

The challenge test was conducted on day 28 and 56 of the feeding trial. Each group of black tiger shrimp in each a period of time was subdivided into four of 32-

40 shrimps replicates, a replicate control blank and three replication challenge groups, respectively. Each replicate of 8-10 juveniles was reared in 35 liters aquaria (50x90x50 cm^3). The shrimp test was immersed with the 10^5-10^7 CFU/ml of bacterium in seawater stocking. Survival rate of shrimp were evaluated after 24 hours of contact, for 10 days after challenge or until all moribund shrimps were observed in control group. During the rearing trial the following environmental categories were controlled. The dead shrimps (challenge and control blank groups) were randomly collected from each replication for the bacteria count on thiosulfate citrate bile salt sucrose agar (TCBSA). The cumulative mortalities of shrimp were expressed in terms of relative percent survival (RPS) calculated from the following equation.

$$RPS = \left(1 - \frac{\% \text{ Mortality in experimental group}}{\% \text{ Mortality in control group}} \right) \times 100$$

The relative percent survival (RPS) value was estimated with the challenge test at 20-50% mortality in control group, RPS values not lesser than the criterion 60% was acceptable as effective in fish vaccines (Amend, 1981).

3.10. Statistical methods

The statistical significance ($P<0.05$) of differences were analyzed using one way analysis of variance (ANOVA). When the ANOVA identified differences among groups, multiple comparisons among means were made with Least Significant Difference (LSD) multiple range tests. Statistical significance was determined by setting the aggregate type I error at 5% ($P<0.05$) for each set of comparisons.