CHAPTER V

Discussion

This study was a preliminary study on molecular characterization of VTG from serum and vitellin from unfertilized eggs of Greenback Mullet, *Liza subviridis* by 12% SDS-PAGE stained with Coomassie blue and other staining systems including glycoprotein, lipoprotein and phospholipids staining. The molecular weight of VTG and vitellin were characterized by comparing its relative mobility on SDS-PAGE with standard protein marker. In SDS-PAGE analysis, purified VTG and vitellin extract was detected as the similar major 3 denatured bands at the molecular weight of 109, 88, and 74 kDa. Charles Tyler (1993) studied electrophoretic patterns of yolk protein extract for demonstrate of the relationship between vitellin and VTG and found that the structural of VTG were similar to vitellin. In this study, The 109 kDa band was the majority band that gave a positive result to all staining. This109 kDa band of vitellin and VTG were indicated the characteristic of glycophospholipoprotein.

The ZRP extract were visualized by total protein and glycoprotein staining and appears major band as 68, 60, and 48, at 60 kDa protein was observed both staining system. However, small amount of VTG was also detected contaminating with ZRP extracted from eggshell that may be the few number step of rinsed in 500 mM NaCl.

Vitellin extraction were used for an immunogent to produce the monoclonal antibody against VTG. In previous reports, Kordes and Rieber (2002) isolated vitellin from egg of medaka for production of monoclonal antibodies against VTG. Therefore, the structure of VTG is similar to hat of vitellin. Thus, vitellin was taken to generate

MAbs against VTG. Moreover, this study also demonstrated the feasibility of monoclonal antibody technique that is not required pure antigen for immunization.

After the three immunization, the mouse ani-vitellin antisera from two mice produced strong precipitation bands to antigens in vitellin and to female serum (VTG), but not strong precipitation bands in male serum. Precipitation bands from vitellin and female serum show reaction of identity with all mouse anti-vitellin antisera. All mice showed similar responses; one of these mice was used as the spleen cell donor for hybridoma production. This study the mouse number 3 was chosen as the donor for hybridoma production because the antiserum from this mouse showed strong immunoreactivity by western blot analysis and immunohistochemistry more than other mice. By means of immunohistochemistry, antiserum from the mouse number 3 showed strong specific binding at dilution 1: 2,500.

After the hybridomas were obtained, selection for the clones producing antibodies that can bind to vitellin and vitellogenin in both native and denatured froms were performed by dot-blotting by immunohistochemical, Western blot analysis. The eleven monoclonal antibodies specific to VTG obtained from this experiment were grouped into 6 categories according to their binding capabilities.

The 5 group of (VTG-319, 149, 64, 90, 183, 144, 231, 483, 496) can bind with both native and denatured VTG and vitellin while one group of them (VTG-313, 530) bound to only native antigens. In Western blot analysis of ovarian extract and female serum separated by PAGE, 4 groups of monoclonal antibodies specific to vitellin and vitellogenin while one group of them (VTG-319, 149) bound to vitellin specific protein (109 kDa). In immunohistochemical staining of the oocytes, 2 groups of monoclonal antibodies (group of VTG-313, 530 and group of VTG-183, 144, 483) cross-reacted to all stages of vitellogenic cells. Isotype and sub-isotype of the

monoclonal antibodies identified by sandwich ELISA. The MAbs belonged to the IgG_1 isotype (VTG-64, 90, 144 , 149, 183, 231, 319, 483, 496 and 530) only one of them (VTG-319) belonged to the IgG_2 isotype. After that, the MAbs were chosen to determine for epitope by indirect ELISA using a mixture of 2 MAbs/well in 96 well plates coated with vitellin extract (10 ng/well). The MAb VTG-496 revealed highest sensitivity with vitellin when compare with OD value of other MAbs. The group1 of MAbs VTG-90 may bind to different epitope with other 10 MAbs because when mixed MAbs VTG-90 with 10 MAbs were increased the OD value. Similarity results of group2 (VTG-496) and group3 (VTG-64) which were indicated that group2 and group3 may bind to different epitope with other group of MAbs. For groups 4, 5 and 6, combination of 2 MAbs among same group did not show improvement in OD values indicating that MAbs in same group of these bound the overlapping position in the antigen.

For zona radiata protein, after the three immunization with ZRP and denatured vitellin, the mouse antisera from four mice produced strong precipitation bands to antigens in ZRP and to female serum (VTG), but not strong precipitation bands in vitellin and can not to antigens in male serum. This study aims to produce of monoclonal antibodies against ZRP and denatured vitellin that combination of 2 antigen for immunization, then the desired antibody can be selected for specific requirement of each antigen. However, the mouse antisera from four mice did not bind strong precipitation bands with vitellin. From these results, This study was produced only one of MAb against ZRP. All mice showed similar responses; one of these mice was used as the spleen cell donor for hybridoma production.

Eleven hybridoma clones which showing strong immunoreactivities were re-clones successfully and grouped into 4 catagories according to their binding

capabilities and all monoclonal antibodies belong to the IgG_1 isotype. Several group of monoclonal antibodies bound to both native and denatured antigens (ZRP-9, 30, 72, 109, 110, 168, 125, 102, 113) while one group of them (ZRP21,124) bound to only denatured antigens can not bind to native antigen in serum. In Western blot analysis of ZRP extract and female serum separated by PAGE, one group (ZRP 30, 72, 109) can bind all 68, 60, and 48 kDa major proteins, the group of ZRP-102, 113 can bind 68 and 48 kDa and the group of ZRP-68, 110, 125 can bind 68 and 60 kDa major proteins.

Sensitivity of these antibodies can be used to detect ZRP by means of dot blot assay with different dilution of denatured ZRP extract from eggs of Greenback Mullet (*Liza subviridis*). The sensitivity ranging that can be detected from 0.01-0.08 ng/µl. . In immunohistochemical staining of the oocytes, all groups were showing strong immunoreactivities by immunohistochemical analysis.

For the results of epitope determination of monoclonal antibodies, the culture fluids of high sensitivity MAbs were determination whether they bind to overlapping epitope by indirect ELISA using mixture of 2 MAbs at excess amount of antibodies in each well coated with minimal amount of ZRP extract (10 µg/well). The averages OD of MAb ZRP-21 revealed highest sensitivity with ZRP extract when compare with OD value of other MAbs. The all group combination of 2 MAbs among same group did not show improvement in OD values indicating that MAbs in same group of these bound the overlapping position in the antigen

Finally, these antibodies were determined for sensitivity of VTG levels and ZRP levels in juvenile fish serum, which were injected by various estradiol (E_2) concentration by western blot analysis. The results of VTG synthesis in juvenile greenback mullet induced by E2 injection revealed that significant higher level of

VTG was detected in blood samples of mullet injected with at least 5 mg of E2/kg body weight when compared to control and the results of ZRP synthesis, ZRP was detected in blood samples of E2-treated juvenile that significant higher level at least 2.5 mg of E2/kg body weight when compared to control. The detection of ZRP show that the band of ZRP at 60 kDa is more responsive than 68 and 48 kDa. Induction of an immunoreacting protein band at 60 kDa of ZRP suggests that these protein are synthesized first during zonagenesis. Like Arukwe et al., (1997) investigated the presence of ZRP band in plasma of juvenile Atlantic salmon (*Salmo salar*) exposed to xenoestrogens (nonylphenol) at ZRP-β (55 kDa) is more responsive than ZRP-α, and ZRP-γ (60 and 55 kDa). The results indicated that protein band at the β-position of ZRP are synthesized first during zonagenesis.

Greenback Mullet induced by E2 injection revealed that significant higher level of ZRP was detected in blood samples of mullet injected with at least 2.5 mg of E2/kg body weight when compared to VTG (at least 5 mg/kg body weight). The detection of ZRP and VTG by western blot analysis indicated that ZRP in greenback mullet was a more sensitive biomarkers for the detection of xenoestrogenic effect in water.

Arukwe et al.(1997a) also found higher level of ZRP in blood samples of Atantic salmon (*Salmo salar*) exposed to 4-nonylphenol NP) or to effluent from oil refinery treatment plant when compared to VTG. The sensitivity of ZRP at low E2 dose is probably a result of the physiological function of the eggshell as protector of the growing embryo. It has been demonstrated that the formation of the eggshell envelope precedes the active uptake of VTG during oocyte development (Hyllner & Silversand, 1994) Like Oppen-Berntsen et al.(1994) was also detected ZRP in the

plasma of female salmonids during a normal reproduction cycle several months before VTG is appearing.

The present study was targeted to the development of a competitive ELISA with combination of four MAbs yields high sensitivity that were used to insure the detection of all VTG subunits present in serum. Therefore, this study relied on the structural similarities of VTG and vitellin. The usage of vitellin extract from eggs of green mullet as a reference protein in a competitive ELISA was found to be a suitable alternative to VTG. Kordes et al.(2002) also used lipovitellin extract from eggs of medaka as a reference protein in the sandwich ELISA. There was no evidence that lipovitellin limits the application of ELISA for determination VTG.

The indirect competitive ELISA determination of vitellogenin levels of the fish serum from the individual juvenile after the inductions of various estradiol (E_2) concentrations (0 , 2.5 , 5 and 50 mg of estradiol (E_2)/kg body weight) increased significantly within the induction of 2.5 to 50 mg of estradiol (E_2)/kg body weight ($p < 0.05$) when compared to the control group. The lowest concentration to induce VTG by ELISA were 2.5 mg of estradiol (E_2)/kg body weight indicating the high sensitivity of the method compare with the immunoblot analysis.

For the determination of zona radiata protein in experiment fish, indirect competitive ELISA using combination of three monoclonal antibodies (ZRP-9, 68 and 102) yielded high sensitivity and demonstrated that ZRP in female serum completely inhibited the binding of the antibodies to the fixed ZRP while male serum did not show any inhibitory effect. The ZRP levels of the fish serum from the individual juvenile after the inductions of various estradiol (E_2) concentrations (0 , 2.5 , 5 and 50 mg of estradiol (E_2)/kg body weight) increased significantly within the induction of

2.5 to 50 mg of estradiol (E_2)/kg body weight ($p < 0.05$) when compared to the control group.

The levels of VTG and ZRP were significantly increased in responding to the increase level of E2. At the lowest E2 concentration, ZRP gave higher sensitivity compared to VTG. This indicates that ZRP is more sensitivity than VTG in greenback mullet and can be used these ZRP and VTG as sensitive biomarkers for the detection of xenoestrogenic effect in water.