CHAPTER IV

RESULTS

4.1 Protein preparation

4.1.1 Vitellogenin and Vitellin preparation

The protein extracted from the egg yolk of Greenback Mullet was positively detected to all staining techniques indicating the glycophospholipoprotein which was the characteristic of VTG. In SDS-PAGE analysis, purified VTG and vitellin was detected the majority 3 denatured bands was approximately 109, 88, and 74 kDa. The size of 109 kDa gave positive results to all stainings (Fig 4.1).

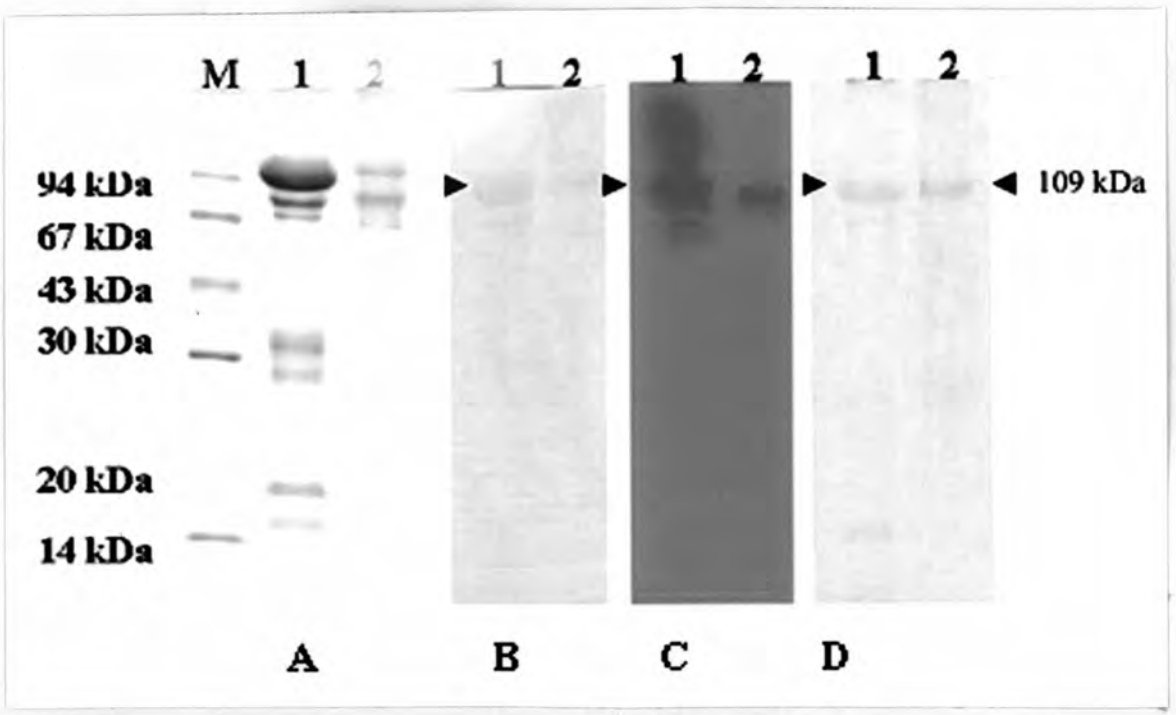

Figure 4.1 SDS-PAGE of vitellin extract from the egg yolk of Greenback Mullet (*Liza subviridis*)(1) and purified VTG(2) stainning with Coomassie blue(A); the far left lane contain molecular standard protein (M), Periodic acid schiff reagent(B), Phosphoprotein staining(C), Sudan black B(D). Protein content was 20 μg/lane (1) and 10 μg/lane (2) for all stainings.

4.1.2 Zona radiata protein preparation

ZRP were visualized by total protein and glycoprotein staining and appeared as 68, 60, and 48 kDa major proteins. Protein band at 60 kDa was observed from both Coomassie blue and Periodic acid schiff reagent stainings. (Fig 4.2)

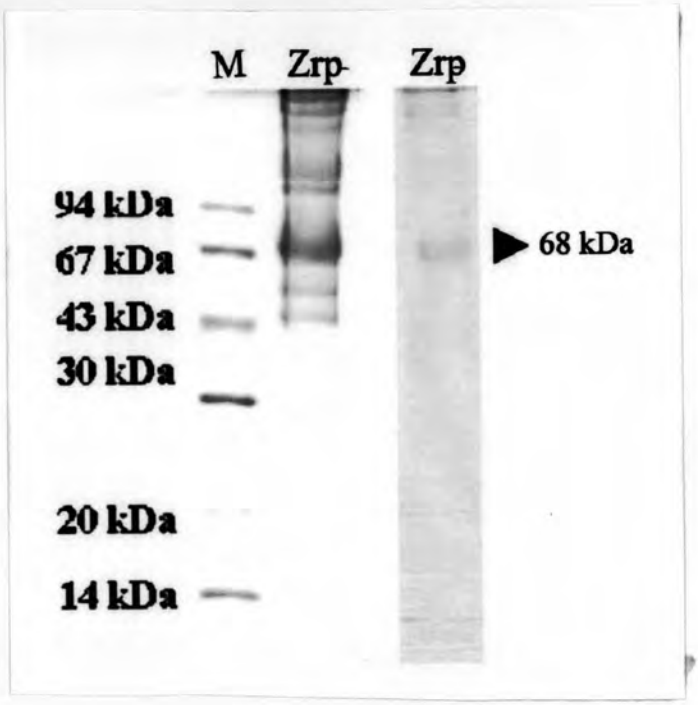

Figure 4.2 SDS-PAGE of ZRP extract from the eggshell of Greenback Mullet (*Liza subviridis*) stainning with Coomassie blue(A) and Periodic acid schiff reagent (B). Protein content was 20 μg/lane for all stainings.

4.2 Immunization

4.2.1 Vitellin Immunization

After three immunizations with vitellin native form and denatured form which was treated with SDS, 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 (Fig 4.3). All mice

showed similar responses; one of these mice was used as the spleen cell donor for hybridoma production.

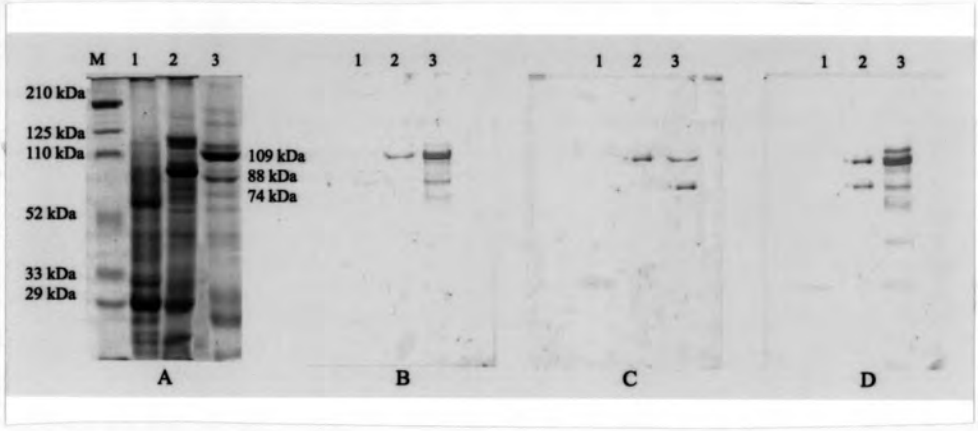

Figure 4.3 SDS –PAGE and immunoblot analysis of vitellin extract (3), female serum (2) and male control serum (1). Preparations were stained with Coomassie blue (A); the far left lane contain molecular standard protein (M), western blotting with mouse anti-vitellin antiserum from 3 mice (B, C, and D are mouse 1, 2, and 3, respectively). Protein content was 10 μg/lane (1, 2, 3) for Coomassie blue staining and 5 μg/lane (1, 2, 3) for Western blotting.

4.2.2 Zona radiata protein Immunization

After 3 immunizations with ZRP and denatured vitellin, mouse antisera from 4 mice produced strong precipitation bands to ZRP antigens and to female serum (VTG), but not to male serum (Fig 4.4). All mice showed similar responses. One of these mice was used as the spleen cell donor for hybridoma production.

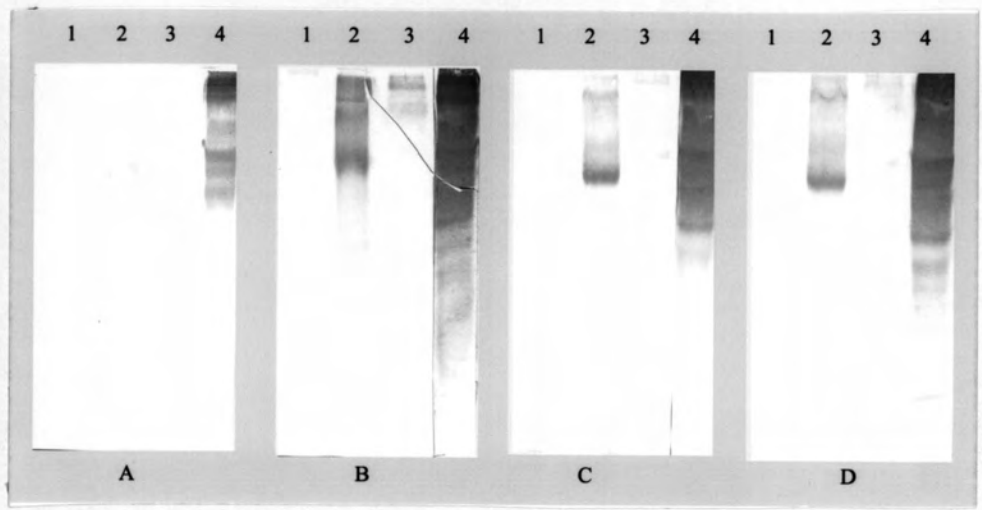

Figure 4.4 SDS –PAGE and immunoblot analysis of ZRP extract (4), vitellin extract (3), female serum (2) and male control serum (1). Preparations were stained with Coomassie blue (A), western blotting with mouse anti-vitellin antiserum from 4 mice. A to C are mouse number 1 to 4, respectively.

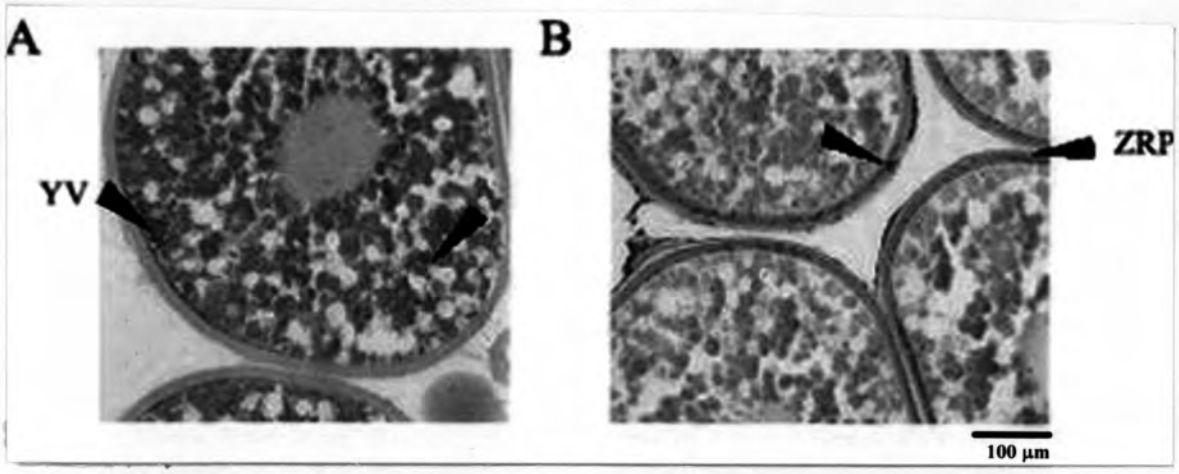

Figure 4.5 Immunohistochemical staining of Greenback Mullet (*Liza subviridis*) oocyte.(A) Section of whole oocyte, probed with mouse antisera to Greenback Mullet VTG, with cross-reactivity to yolk vesicles (yv). (B) Section of whole oocyte, probed with mouse antisera to Greenback Mullet ZRP, with no cross-reactivity to yolk vesicles.

4.3 Hybridoma production

4.3.1 Hybridoma production of Vitellogenin

From fusion, the trial with 30 microculture plates yielded approximately 800 culture wells. The first screening by dot-blot against vitellin native and denatured forms yielded over 200 positive wells, only about 90 wells containing a few hybridoma colonies were further screened by dot-blotting against viellin using both native and denatured froms, female and male serum, Western blot and immunohistochemical analysis. Eleven hybridoma clones showing strong

immunoreactivities were re-cloned successfully and grouped into 6 categories according to their binding capabilities (Table 4.2, Table 4.3).

For the results of characterization of the monoclonal antibodies, Several groups of monoclonal antibodies bound to both native and denatured antigens (VTG-319, 149, 64, 90, 183, 144, 231, 483, 496) while one group of them (VTG-313, 530) bound to only native antigen (Fig 4.6).

In Western blot analysis of ovarian extract and female serum separated by SDS-PAGE, 4 groups of monoclonal antibodies were specific to vitellin and vitellogenin while one group of them (VTG-319, 149) bound to vitellin specific protein (88 kDa) (Fig 4.7). 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 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 (Table 4.2).

Sensitivity of these antibodies could be used to detect vitellin by means of dot blot assay with different dilution of native and denatured vitellin extract from eggs of Green Mullet (*Liza subviridis*). The sensitivity ranging that could be detected from 0.04-2.5 ng/µl (Table 4.3).

For the results of epitope determination of monoclonal antibodies, the culture fluids of high sensitivity MAbs were determined whether they bound to overlapping epitope by indirect ELISA using mixture of 2 MAbs at excess amount of antibodies in each well coated with minimal amount of vitellin extract (10 ng/well). The averages OD of the selected MAbs from 2 replications were showed in the middle row of Table 4.1.

The MAb VTG-496 revealed highest sensitivity with vitellin when compared with OD value of other MAbs. The group 1 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. Similar results of group 2 (VTG-496) and group 3(VTG-64) indicated that group 2 and group 3 might bind to different epitope with other group of MAbs. For group 4, 5, and 6, combination of 2 MAbs among the same group did not show improvement in OD values indicating that MAbs in the same group of group 4, 5, and 6 bound the overlapping position in the antigen.

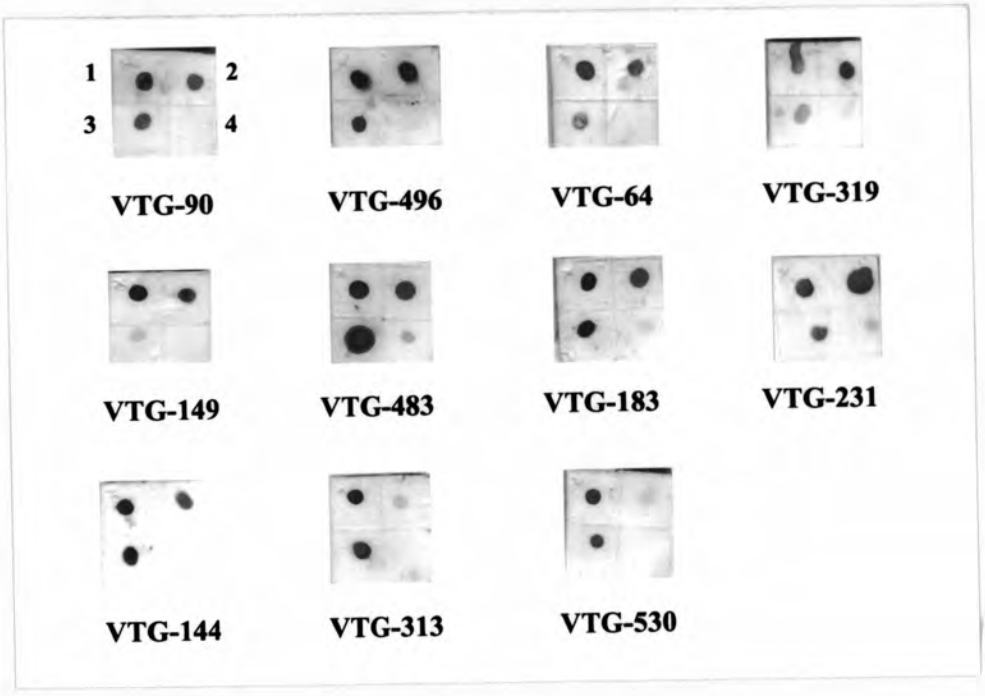

Figure 4.6 Screening results of 11 monoclonal antibodies by dot-blot against native and denatured antigens; native VTG from eggs extract (1), denature VTG from eggs extract (2), VTG from serum (3), serun of juvenile control (4)

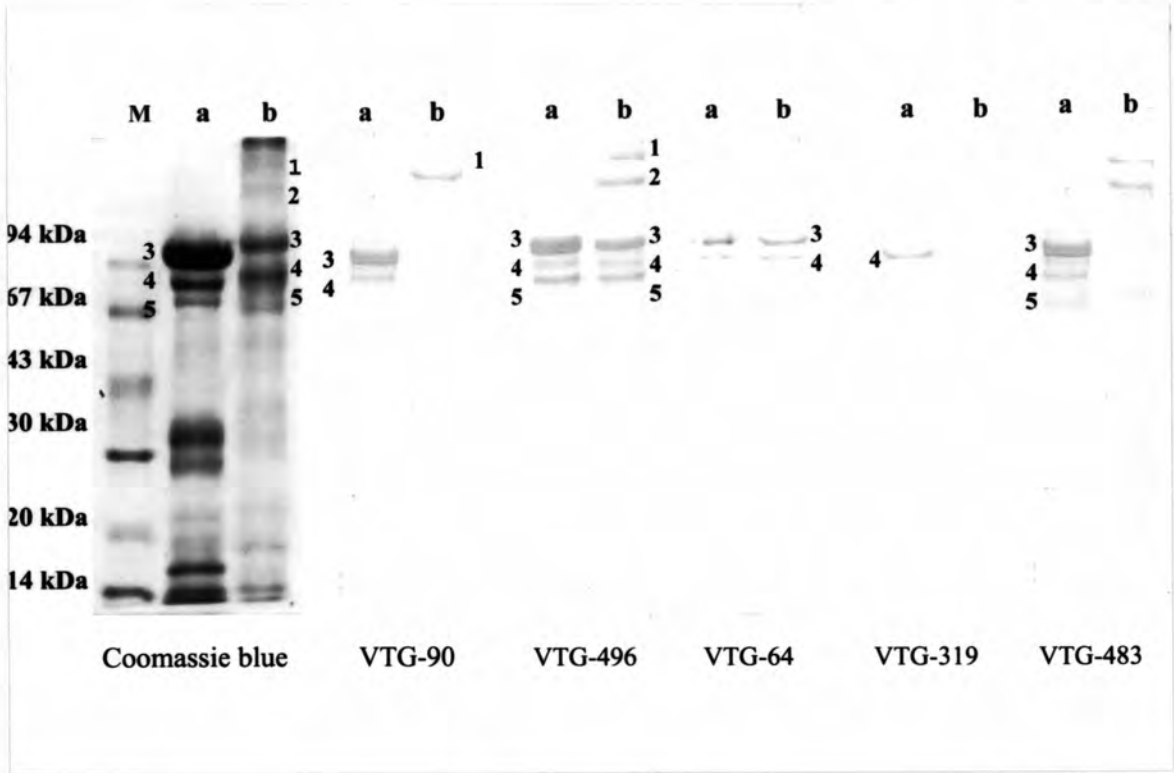

Figure 4.7 SDS-PAGE and immunoblot analysis of vitellin extract (a) and female serum (b). Preparations were stained with Coomassie blue; the far left lane contain molecular standard protein (M). The protein were transferred to a nitrocellulose membrane and immunoblotted with various monoclonal antibodies. The numbers on the left side are the molecular weights of the marker proteins. The number (1-5) between lane are molecular mass of 204, 160, 109, 88, 74 kDa, respectively. Protein content was 20 μg/lane (a) and 10 μg/lane (b) for Coomassie blue staining, and 5 μg/lane (a, b) for Western blotting.

Table 4.1 ELISA OD values of various MAbs to determine epitope of MAbs.

group1 group2 group3 group 4 group 5 group 6

MAbs	1 VTG90	2 VTG496	3 VTG64	4 VTG149	5 VTG313	6 VTG144	7 VTG483	8 VTG183	9 VTG231	10 VTG530	11 VTG313
1 VTG90	1.654	2.531	1.762	2.044	2.200	2.343	2.480	2.663	2.576	2.440	2.177
2 VTG496		2.076	2.085	2.663	2.304	2.825	2.831	2.443	2.549	2.154	2.650
3 VTG64			0.516	0.713	0.646	1.424	1.460	1.376	1.993	1.310	0.903
4 VTG149				0.203	0.186	1.057	1.201	1.033	1.550	1.110	0.556
5 VTG319					0.232	1.272	1.399	1.057	1.913	0.996	0.579
6 VTG144						1.568	1.511	1.285	1.901	1.331	1.089
7 VTG183							1.563	1.399	1.751	1.394	1.271
8 VTG231								1.482	1.890	1.215	1.165
9 VTG483									2.008	1.977	1.862
10 VTG530										0.139	1.121
11 VTG313											0.671

Table 4.2 Characterization of monoclonal antibodies specific to vitellin and vitellogenin tested by western blot analysis and subtype

Monoclonal antibodies obtained from mice	**Western blot analysis (kDa)**							**Isotype & Subisotype**
	Vitellogenin				**Vitellin**			
	204	**160**	**109**	**88**	**109**	**88**	**74**	
1) VTG-90	+	-	-	-	+	+	-	IgG1
2) VTG-496	+	+	+	+	+	+	+	IgG1
3) VTG-64	-	-	+	+	+	+	-	IgG1
4) VTG-319, 149	-	-	-	-	-	+	-	IgG1, IgG2(VTG-313)
5) VTG-231, 144, 183, 483	+	+	-	-	+	+	+	IgG1
6) VTG-313, 530	-	-	-	-	-	-	-	IgG1

+ = Antibody can bind to the proteins

- = Antibody can not bind to the proteins

Table 4.3 Characterization of monoclonal antibodies specific to vitellin and vitellogenin tested by dot blot and immunohistochemistry. The underlined clone is the representative MAb used in other tests.

Monoclonal antibodies obtained from mice	**Dot-Blot analysis**			**Sensitivity of Dot blotting (ng/µl)**	**IHC**
	Female serum	**Vitellin**			
		Native	**Denature**		
Denatured vitellin					
1) VTG-90	+	+	+	0.2	-
2) VTG-496	+	+	+	0.04	-
3) VTG-64	+	+	+	0.2	-
4) VTG-319, 149	+	+	+	1.2	-
5) VTG-483, 144, 183, 231	+	+	+	1.2	+++
Native vitellin					
6) VTG-313, 530	+	+	-	2.5	+

+ = Antibody can bind to the proteins

- = Antibody can not bind to the proteins

4.3.2 Hybridoma production of Zona radiata protein

From fusion, every well contained hybridoma colonies was ranged from 1-4 colonies/well in 30 microculture plates. The first screening by dot-blot against ZRP and vitellin denatured form yielded over 500 positive wells with vary immunoreactivity. Since there were so may positive wells containing several colonies, only about 90 wells containing a few hybridoma colonies were further screened by dot-blotting against ZRP, denatured vitellin, female serum, and male serum, Western blot and immunohistochemical analysis. Eleven hybridoma clones showing strong immunoreactivities were re-cloned successfully and grouped into 4 catagories according to their binding capabilities (Table 4.5). All monoclonal antibodies belonged to the IgG_1 isotype.

Several groups 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 antigen but not to native antigen in serum. In Western blot analysis of ZRP extract and female serum separated by PAGE, one group (ZRP 30, 72, 109) bound all 68, 60, and 48 kDa major proteins. The group of ZRP-102, 113 bound to 68 and 48 kDa and the group of ZRP-68, 110, 125 can bound to 68 and 60 kDa major proteins.

Sensitivity of these antibodies could 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 could be detected from 0.01-0.08 ng/µl (Table 4.6).

In immunohistochemical staining of the zona radiata protein from Greenback Mullet (*Liza subviridis*), all groups showed strong immunoreactivities. For the results of epitope determination of monoclonal antibodies, the culture fluids of high

sensitivity MAbs were determined whether they bound 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 the selected MAbs from 2 replicated were showed in the middle row of Table 4.4.

The MAb ZRP-21 revealed highest sensitivity with ZRP extract when compared with OD value of other MAbs. All group combination of 2 MAbs among the same group did not show improvement in OD values indicating that MAbs in the same group of group1, 2, 3 and 4 (Table 4.4) bound the overlapping position in the antigen.

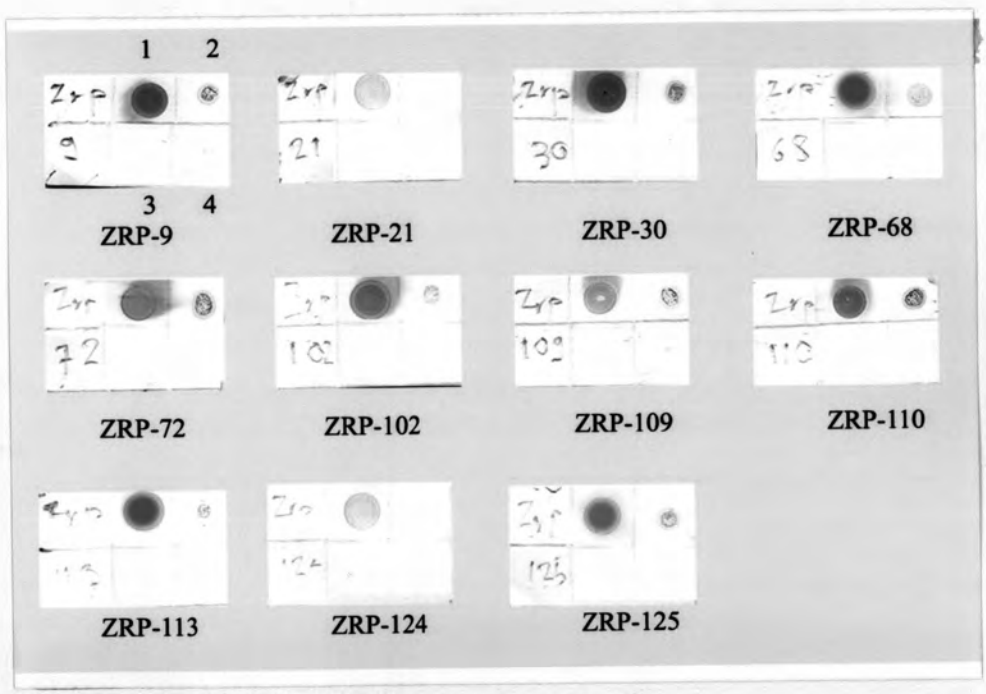

Figure 4.8 Screening results of 11 monoclonal antibodies by dot-blot against ZRP from eggs extract (1), females (a) and ZRP extract female serum (3), serum of juvenile control (4)

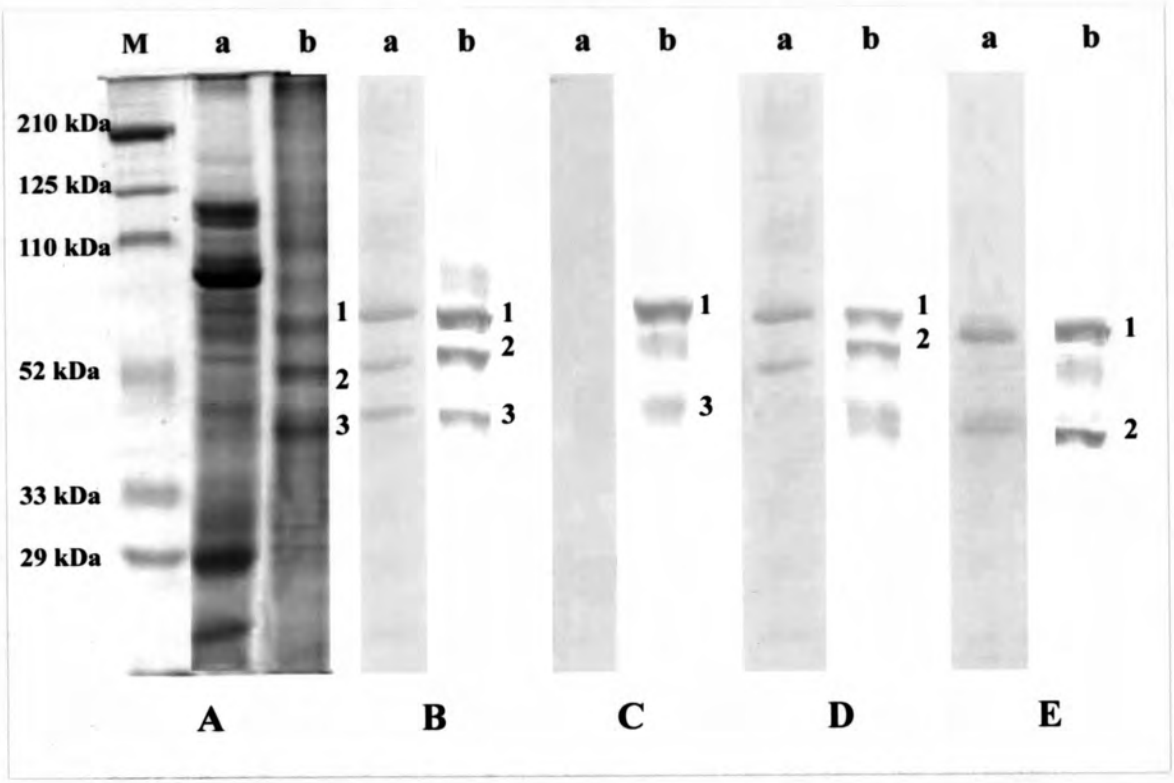

Figure 4.9 SDS-PAGE and immunoblot analysis of female serum (a) and ZRP extract (b). Preparations were stained with Coomassie blue (A); the far left lane contain molecular standard protein (M). The proteins were transferred to a nitrocellulose membrane and immunoblotted with various monoclonal antibodies, ZRP-9 (B), ZRP-124 (C), ZRP-68 (D) and ZRP- 102 (E). The numbers on the left side are the molecular weights of the marker proteins. The number (1-3) between lane are molecular mass of 68, 60, 48 kDa, respectively. Protein content was 20 μg/lane (a) and 10 μg/lane (b) for Coomassie blue staining, and 5 μg/lane (a, b) for Western blotting.

Table 4.4 ELISA OD values of various MAbs to determine epitope of MAbs.

group1 — group2 — group3 — group4

MAbs	1 ZRP9	2 ZRP30	3 ZRP72	4 ZRP109	5 ZRP21	6 ZRP124	7 ZRP68	8 ZRP110	9 ZRP125	10 ZRP102	11 ZRP113
1 ZRP9	1.625	1.581	1.652	1.551	2.340	2.041	1.766	1.589	1.914	1.695	1.492
2 ZRP30		1.905	1.893	1.708	1.766	2.011	1.931	2.013	2.071	1.776	1.960
3 ZRP72			2.068	1.961	2.637	2.874	1.589	2.158	2.352	1.915	2.140
4 ZRP109				1.998	2.478	2.547	1.898	1.866	1.994	1.913	1.971
5 ZRP21					2.460	2.221	2.445	2.434	2.688	2.369	2.526
6 ZRP124						2.364	1.483	2.195	2.468	2.174	2.328
7 ZRP68							1.581	1.562	1.589	1.690	1.665
8 ZRP110								1.614	1.602	1.642	1.789
9 ZRP125									1.927	1.696	1.808
10 ZRP102										1.113	1.064
11 ZRP113											1.139

Table 4.5 Characterization of monoclonal antibodies specific to ZRP tested by tested by western blot analysis and subtype.

Monoclonal antibodies obtained from mice	Western blot analysis (kDa)						Isotype & Subisotype
	ZRP from female serum			ZRP extract			
	68	60	48	68	60	48	
1) ZRP-9, 30, 72, 109	+	+	+	+	+	+	IgG1
2) ZRP-124, 21	-	-	-	+	+	+	IgG1
3) ZRP-68, 110, 125	+	+	-	+	+	-	IgG1
4) ZRP-102, 113	+	-	+	+	-	+	IgG1

+ = Antibody can bind to the proteins

- = Antibody can not bind to the proteins

Table 4.6 Characterization of monoclonal antibodies specific to ZRP tested by dot blot and immunohistochemistry. The underlined clone is the representative MAb used in other tests.

Monoclonal antibodies obtained from mice	Dot-Blot analysis		Sensitivity of Dot blotting (ng/µl)	IHC
	Female serum	ZRP		
1) ZRP-9, 30, 72, 109	+	+	0.01	+++
2) ZRP-124, 21	-	+	0.02	+++
3) ZRP-68, 110, 125	+	+	0.02	+++
4) ZRP-102, 113	+	+	0.08	+++

+ = Antibody can bind to the proteins

- = Antibody can not bind to the proteins

4.4 Determination of vitellogenin and Zona radiata protein in E2 exposed fish

For the results of determination of vitellogenin in E2 exposed fish, these antibodies were determined for sensitivity of VTG levels in juvenile fish serum, which were injected by various E_2 concentrations. VTG synthesis 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 the

control. This experiment showed that the levels of VTG can be detected by western blot analysis (Fig 4.14)

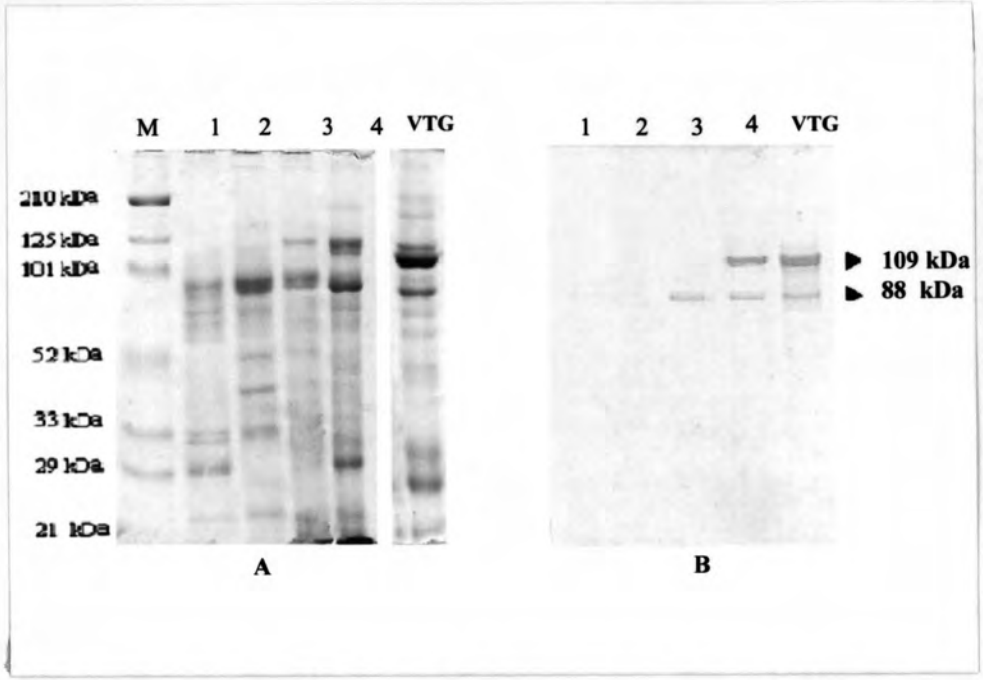

Figure 4.10 SDS-PAGE and Western blot analysis of Greenback Mullet (*Liza subviridis*) juvenile fish serum, which were injected by various estradiol (E2) concentration: lane 1-4; 0, 2.5, 5 and 50 mg of estradiol (E_2)/kg body weight: lane VTG; purify VTG: lane 5, respectively. Preparations were stained with Coomassie blue (A), Western blotting with VTG-496 monoclonal antibody (B). Protein content was 10 μg/lane (1-4, and VTG) for Coomassie blue staining, and 5 μg/lane (1-4, and VTG) for Western blotting.

The results of ZRP synthesis induced by E2 injection revealed that significant higher level of ZRP level was detected in blood samples of mullets injected with merely 2.5 mg of E_2/kg body weight (figure 4.6).

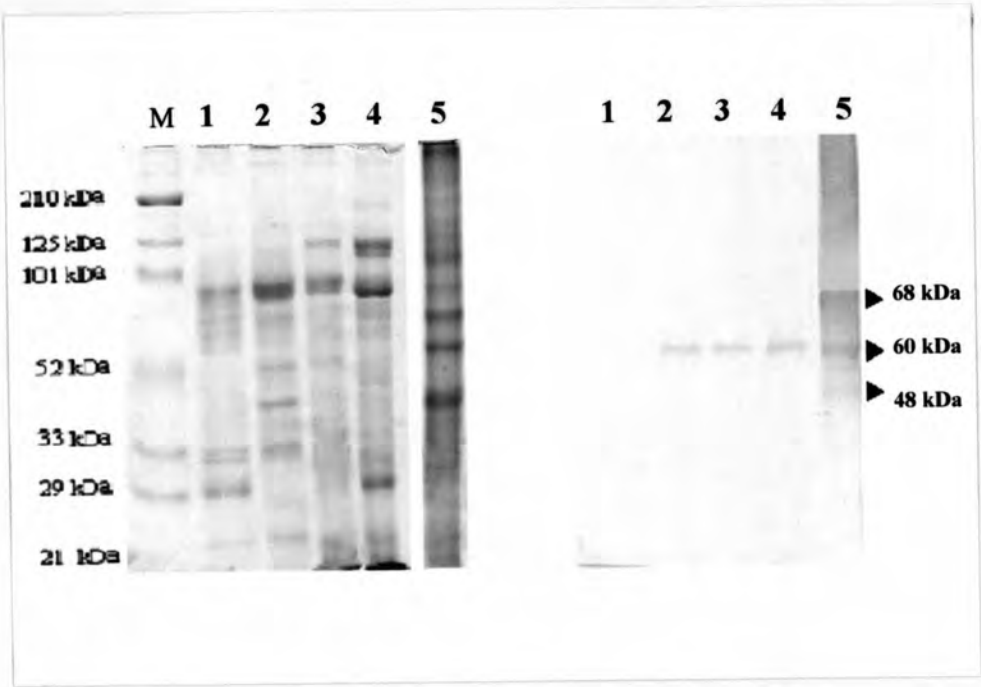

Figure 4.11 SDS-PAGE and Western blot analysis of Greenback Mullet (*Liza subviridis*) juvenile fish serum, which were injected by various estradiol (E2) concentration: lane 1-4; 0, 2.5, 5 and 50 mg of esradiol (E_2)/kg body weight: lane 5; ZRP, respectively. Preparations were stained with Coomassie blue (A), Western blotting with ZRP- 9 monoclonal antibody (B). Protein content was 10 μg/lane (1-4, and ZRP) for Coomassie blue staining, and 5 μg/lane (1-4, and ZRP) for Western blotting.

4.4.2 Competitive ELISA

For the result of determination of vitellogenin indirect competitive ELISA using combination of four monoclonal antibodies (VTG-496, 90, 64 and 486) demonstrated that VTG in female serum completely inhibited the binding of the antibodies to the fixed vitellin which was similar to the binding of vitellin itself while male serum did not show any inhibitory effect. The measurable concentrations of VTG were ranged from 5 to 200 μg/ml (Fig 4.12). The 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 0 mg of estradiol (E_2)/kg body weight (Fig. 4.13, Table 4.7). The intra-assay variation is < 5% (n=12) and the inter assay variation is < 7% (n=4) (98 μg/ml, 2.18 mg/ml)

For the result of determination of zona radiate protein in experiment fish, Indirect competitive ELISA using combination of three monoclonal antibodies (ZRP-9, 68 and 102) 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 measurable concentrations of ZRP were ranged from 1 to 200 μg/ml (Fig. 4.14). 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 0 mg of estradiol (E_2)/kg body weight (Fig. 4.15, Table 4.8). The intra-assay variation is < 7%(n=12) and the inter assay variation is < 10%(n=4).(23 μg/ml, 2.33 mg/ml)

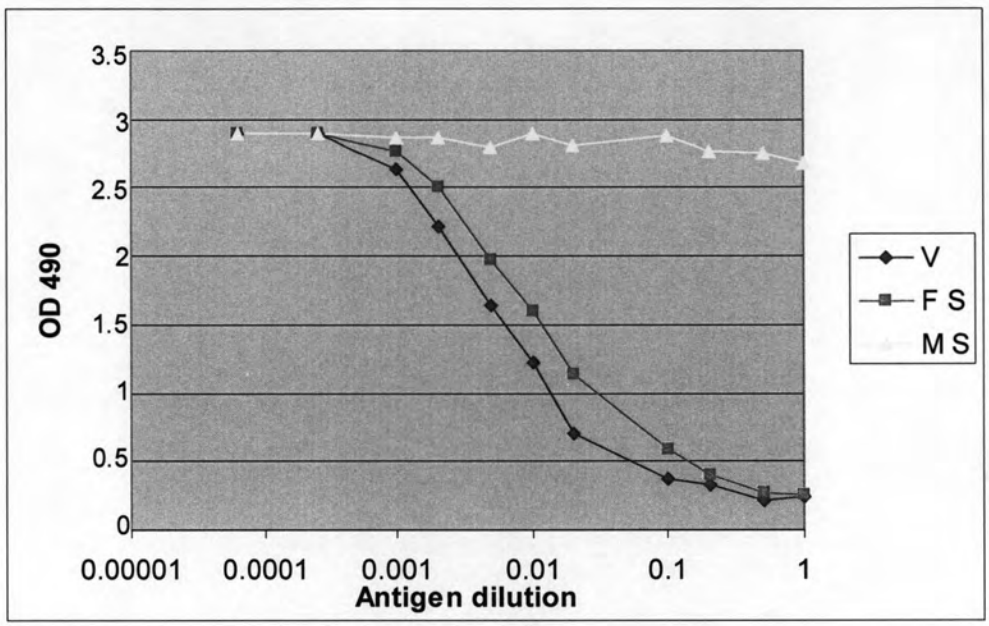

Figure 4.12 Validation of specificity and sensitivity of competitive ELISA for determination of vitellogenin using combination of four monoclonal antibodies specific to each vitellogenin subunits. Various dilution of vitellin (V) with initial concentration of 10 mg/ml were compared to the similar regime of the dilution of serum from male control (M) and female serum (F).

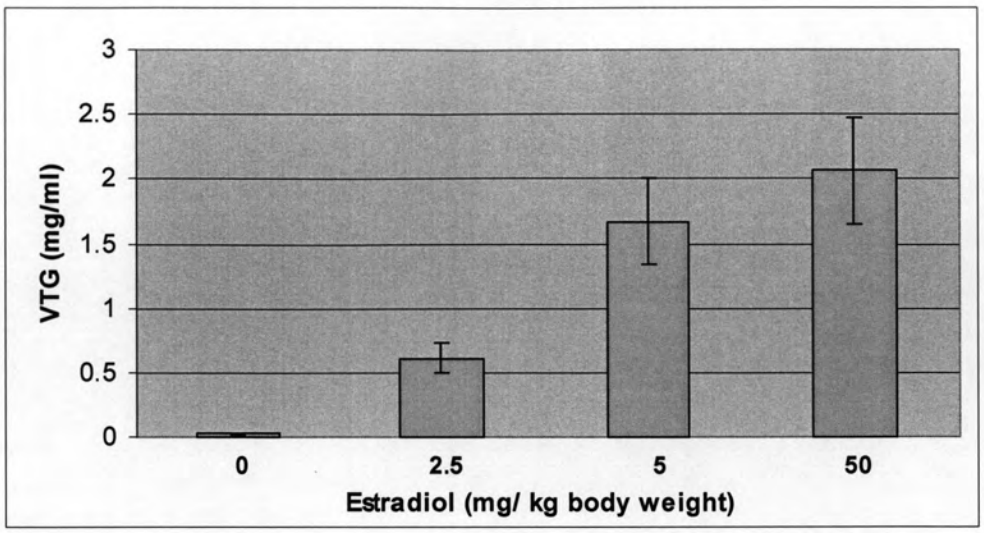

Figure 4.13 Comparison of vitellogenin levels of Greenback mullet (*Liza subviridis*) juvenile fish serum, which were injected by various estradiol (E2) concentration: 1-4; 0 , 2.5 , 5 and 50 mg of estradiol (E_2)/kg body weight respectively.

Table 4.7 Vitellogenin concentrations of Greenback Mullet (*Liza subviridis*) juvenile fish serum, which were injected by various estradiol (E2) concentration: 1-4; 0 , 2.5 , 5 and 50 mg of estradiol (E_2)/kg body weight respectively. The values are means ± SD.

Estradiol (E2) concentration (mg/kg body weight)	Vitellogenin Levels (mg/ml)	Number of Sample (n)
0	0.021 ± 0.017a	25
2.5	0.406 ± 0.230b	18
5	1.681 ± 0.830c	15
50	2.082 ± 0.153d	25

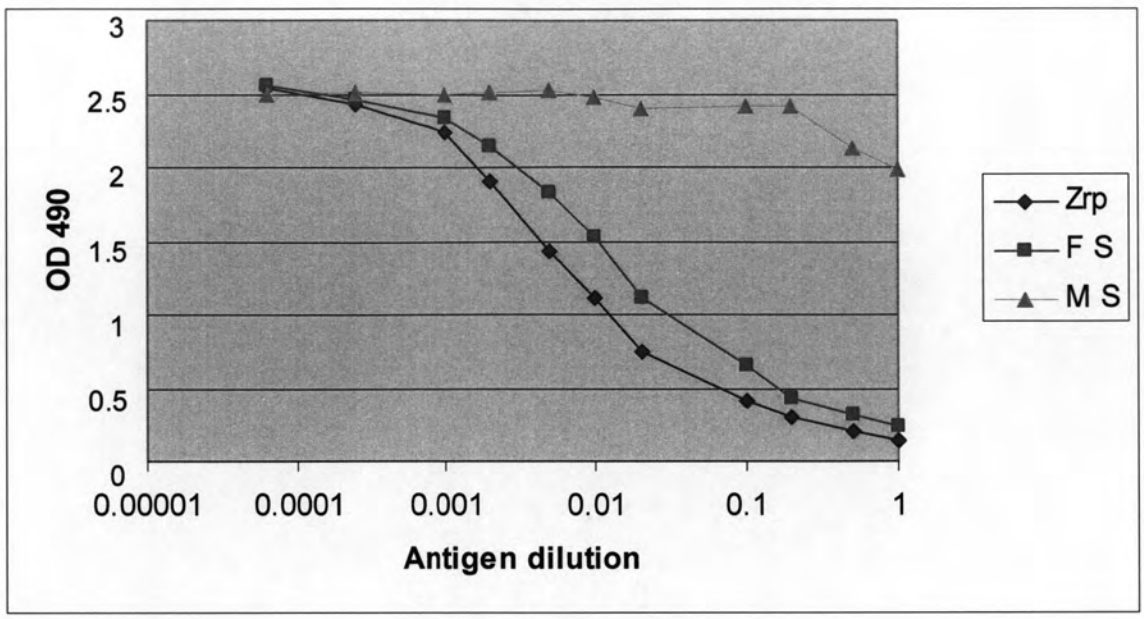

Figure 4.14 Validation of specificity and sensitivity of competitive ELISA for determination of zona radiate protein using combination of four monoclonal antibodies specific to each ZRP subunits. Various dilution of ZRP with initial concentration of 10 mg/ml were compared to the similar regime of the dilution of serum from male control (M) and female serum (F).

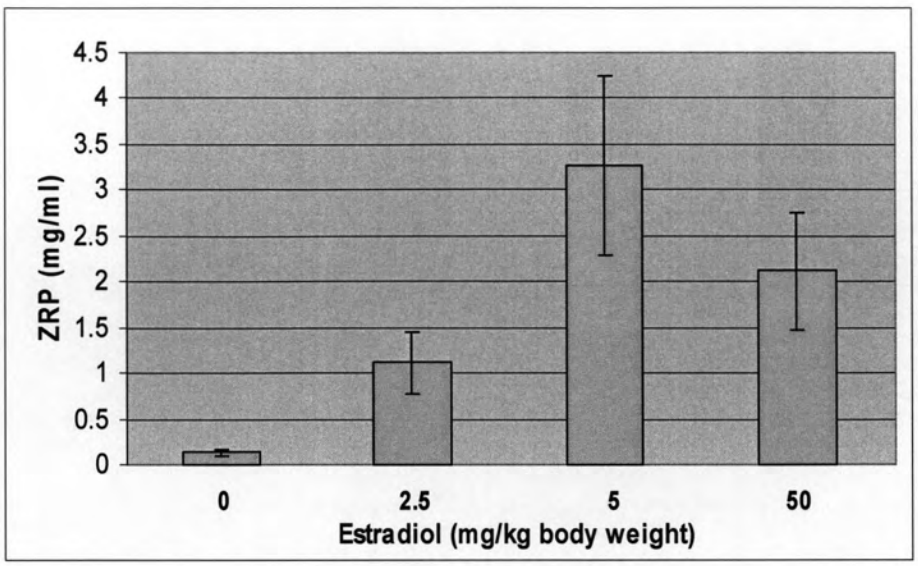

Figure 4.15 Comparison of zona radiate protein levels of Greenback mullet (*Liza subviridis*) juvenile fish serum, which were injected by various estradiol (E2) concentration: 1-4; 0 , 2.5 , 5 and 50 mg of estradiol (E_2)/kg body weight respectively.

Table 4.8 Zona radiate protein concentrations of Greenback Mullet (*Liza subviridis*) juvenile fish serum, which were injected by various estradiol (E2) concentration: 1-4; 0 , 2.5 , 5 and 50 mg of estradiol (E_2)/kg body weight respectively. The values are means ± SD.

Estradiol (E2) concentration (mg/kg body weight)	ZRP Levels (mg/ml)	Number of Sample (n)
0	0.110 ± 0.035a	25
2.5	1.149 ± 1.150b	17
5	3.495 ± 1.080c	15
50	1.860 ± 1.029d	25