CHAPTER III

Monoclonal antibodies production against vitellin of the giant tiger prawn *Penaeus monodon*

Introduction

This study was a preliminary study on a production of monoclonal antibody against vitellin using ovarian extract from female *Penaeus monodon* as an immunogen. After the hybridomas were obtained, selection for the clones producing antibodies that can bind to vitellin and vitellogenin in both native and denatured forms were performed by dot-blotting. The monoclonal antibodies were characterized for their specificities by Western blot, identified isotype and subisotype of antibodies by sandwich ELISA, and determined for sensitivity in quantitative analyses of vitellogenin levels in the haemolymph samples. This study also demonstrated the feasibility of monoclonal antibody technique that is not required pure antigen for immunization, then the hybridoma were obtained, the desired antibody can be selected for specific requirement of each experiment and the antibody can be obtained virtually unlimited amount.

Materials and Methods

Source, animal handling and initial preparations

Adult female *P. monodon* (80-120 g) were obtained from local fishermen in Cholburi Province, the Gulf of Thailand. They were held in 1 X 5 X 1 m^3, rectangular concrete tanks with full-strength seawater (30 $^{o}/_{oo}$) at ambient photoperiod. Commercial shrimp pellet diet was presented during the morning and evening. Water temperature was 26-28 °C and water was changed every morning. After acclimatizing for 3 days, prawns were bilaterally, eye ablated to induce ovarian development. Five to seven days after eye-ablation, prawns with gravid ovaries were anesthetized using cold water (4 °C) and haemolymph was collected via the arthrodial membrane of the fourth walking leg. Ovaries were removed by dissection and washed in cold, 0.15 M phosphate buffered saline pH 7.4 (PBS). Haemolymph was also collected from adult male *P. monodon* (60-80 g). Haemolymph and ovaries were frozen on dry ice and stored at -70°C.

Ovarian extract preparation

Individual ovaries were homogenized for 5 min in 0.5 mM EDTA in PBS (0.5 g/ml). The pellet and lipid layer were eliminated after centrifugation at 10,000g and 4°C for 30 min. The protein content of the extract was determined by Bradford reagent (Bradford, 1976), adjusted to 10 mg/ml with PBS, then divided into 1 ml aliquots and stored at -70°C.

Immunization

Five BALB/c mice were injected with 0.5 mg ovarian extract 1:1 mixed with complete Freund's adjuvant. At 2-week intervals, they were re-injected with ovarian extract mixed with incomplete Freund's adjuvant for the second injection, or with the

extract without adjuvant for the following injections. One week after the fourth injection, mouse anti-vitellin antisera were collected and tested against ovarian extract, and female and male haemolymph by double immunodiffusion. The best performing mouse was boosted 1 week before hybridoma production. BALB/c mice were kindly supplied by the Animal House of the Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand.

Hybridoma production

A cell fusion procedure was adapted from the method developed by Köhler and Milstein (1976), with modification described by Mosmann et al. (1979). A P3X myeloma cell line was used as the fusion partner. The P3X myeloma cell line was obtained from the Department of Virology, AFRIMS, Bangkok, Thailand. Fusion products from one mouse were plated on 16 microculture plates (96 wells). After identifying wells containing desired clones by the screening methods described below, cells were re-cloned at least twice by the limiting dilution method (Eshhar, 1985).

Screening methods

ELISA

Hybridoma cell lines were first screened by ELISA against ovarian extract. The ovarian extract (1 μg/well protein) was plated on Maxisorb microtiter plates (NUNC). Blotto (5% or 0.5% nonfat dry milk in PBS, Johnson et al., 1984) was used as blocking solution, antibody diluent, and washing solution. Antibody binding to plates was detected using horseradish peroxidase labeled goat anti-mouse IgG heavy and light chain specific (GAM-HRP; Biorad) at 1:1,000 dilution (Fig 3.2). Positive wells were further screened by dot-blotting.

Dot-Blotting

Selected antigens, ovarian extract (1 mg/ml), female and male haemolymph in native forms, and SDS-mercaptoethanol treated forms were used for the second step of screening. All antigens (1 µl/spot) were applied to pieces of nitrocellulose membrane. After the membrane was baked at 60°C for 10 min, each piece was incubated in hybridoma conditioned media from each clone (1:20 in 5% Blotto) for 2 hr. After extensive washing in diluted Blotto, the membrane was incubated in GAM-HRP (1:1,000 in 5% Blotto with 40% male prawn haemolymph) for 2 hr. The membrane was then washed as before and applied to substrate mixture (0.03% diaminobenzidine, 0.006% hydrogen peroxide, 0.05% cobalt chloride in PBS) for 5 min (Fig 3.3).

Selected hybridoma clones that bound with ovarian extract and with female haemolymph, but not binding with male haemolymph, were re-cloned and cryo-preserved for further characterization. Some hybridoma clones were injected into pristane primed mice for ascites fluid production.

Monoclonal antibodies characterization

Polyacrylamide gel electrophoresis and immunoblotting

Polyacrylamide gel electrophoresis (PAGE) or sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in slab gels in a mini-PROTEIN II electrophoresis apparatus (Bio-Rad) to identify vitellin and vitellogenin in the extract. Ovarian extract or haemolymph were applied to 5% gel for PAGE or 7.5% gel for SDS-PAGE according to Bio-Rad Manual. Parts of the gel were cut off and visualized by staining with 0.1% Coomassie brilliant blue R250 for proteins, with Sudan black B for lipoproteins, or with periodic acid Schiff reagent (PAS) for glycoprotein (Modified from Humason, 1979). Proteins in other parts of the gel were transferred to nitrocellulose sheet using Transblot apparatus (Bio-Rad) at

50 V for 2 hr. The nitrocellulose sheet was then separated from the gel and quenched in 5% Blotto, cut into strips and assayed for antibody binding as described for dot-blotting assay (Fig 3.4).

Vitellin isolation from PAGE

To confirm the number of subunits of vitellin, glycolipoprotein bands from PAGE were cut off and homogenized in equal volume of 0.01% SDS, allowing the protein to dissolve for 12 hr. The dissolved protein was separated from the gel by centrifugation and the gel pellet was re-extracted with the same solution for 12 hr. Both supernatants were combined and concentrated by vacuum concentrator (Savants) and reapplied in SDS-PAGE as described above.

Vitellin and vitellogenin immunoprecipitation

Due to very high haemocyanin content and low proportion of vitellogenin in the haemolymph, the proportion of vitellogenin in the sample was increased by (immunoprecipitation) using female haemolymph mixed with pooled mouse anti-vitellin antiserum (4:1) collected previously before the fusion. After incubation at 4°C overnight, the precipitate of antibody antigen complex was collected by centrifugation (5000g), and washed twice with PBS. The ovarian extract was prepared in the same manner as a control. Precipitates were then dissolved in treatment buffer and subjected to SDS-PAGE as described above.

Detectability of vitellin and vitellogenin by monoclonal antibodies specific to vitellin and vitellogenin

The range of vitellin concentrations that could be measured by these monoclonal antibodies was determined by competitive ELISA (Fig 3.5) using the lowest concentration of each monoclonal antibody that gave maximal absorbance

mixed with various dilutions of ovarian extract (initial concentration is 10 mg/ml) or female haemolymph, or male haemolymph, then processed as described in ELISA.

Isotype and subisotype determination

The isotype and subisotype of the mouse immunoglobulin produced by hybridomas were determined by sandwich ELISA (Fig 3.6) using a subisotyping kit, mouse (Calbiochem).

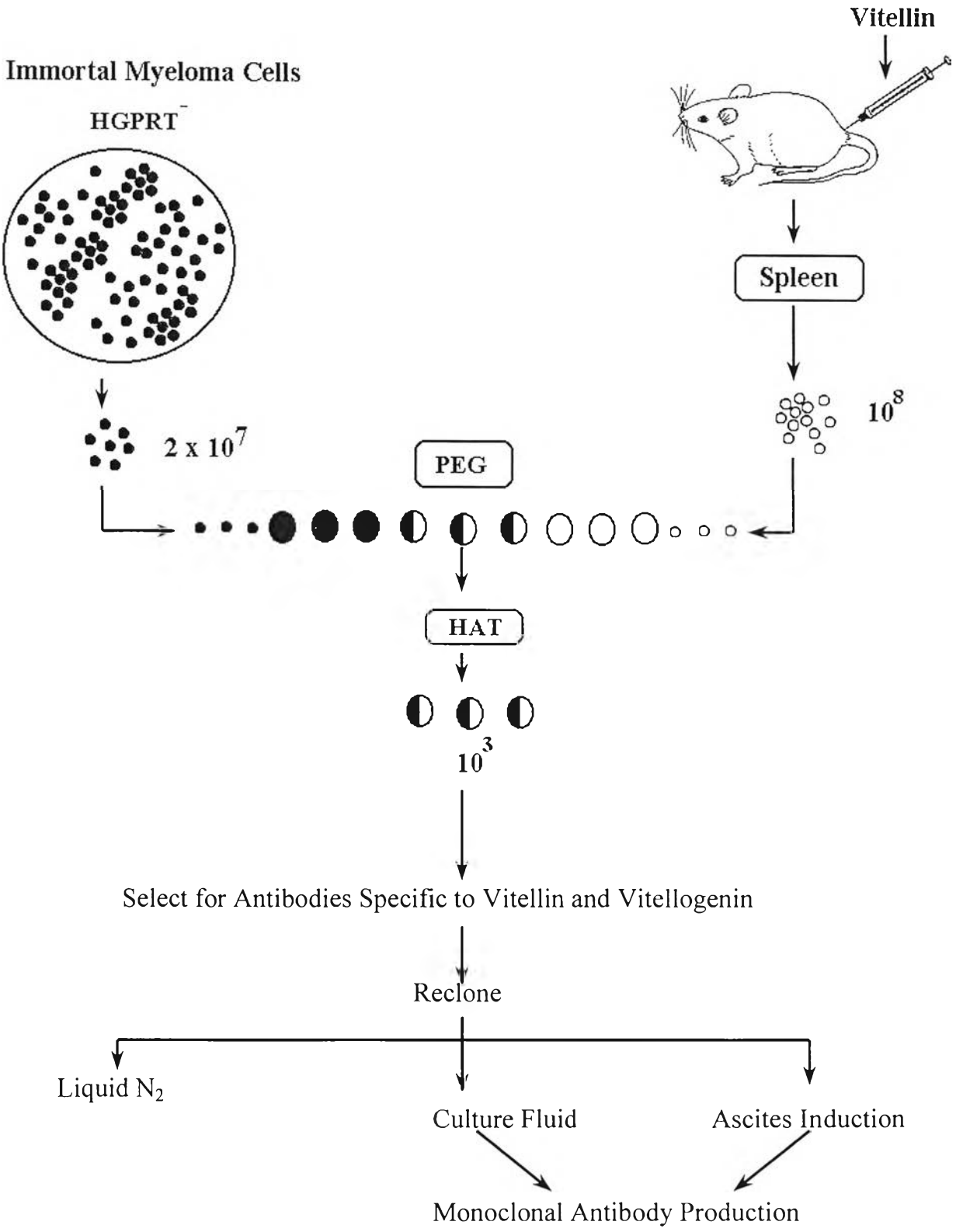

Figure 3.1 Diagram of hybridoma production.

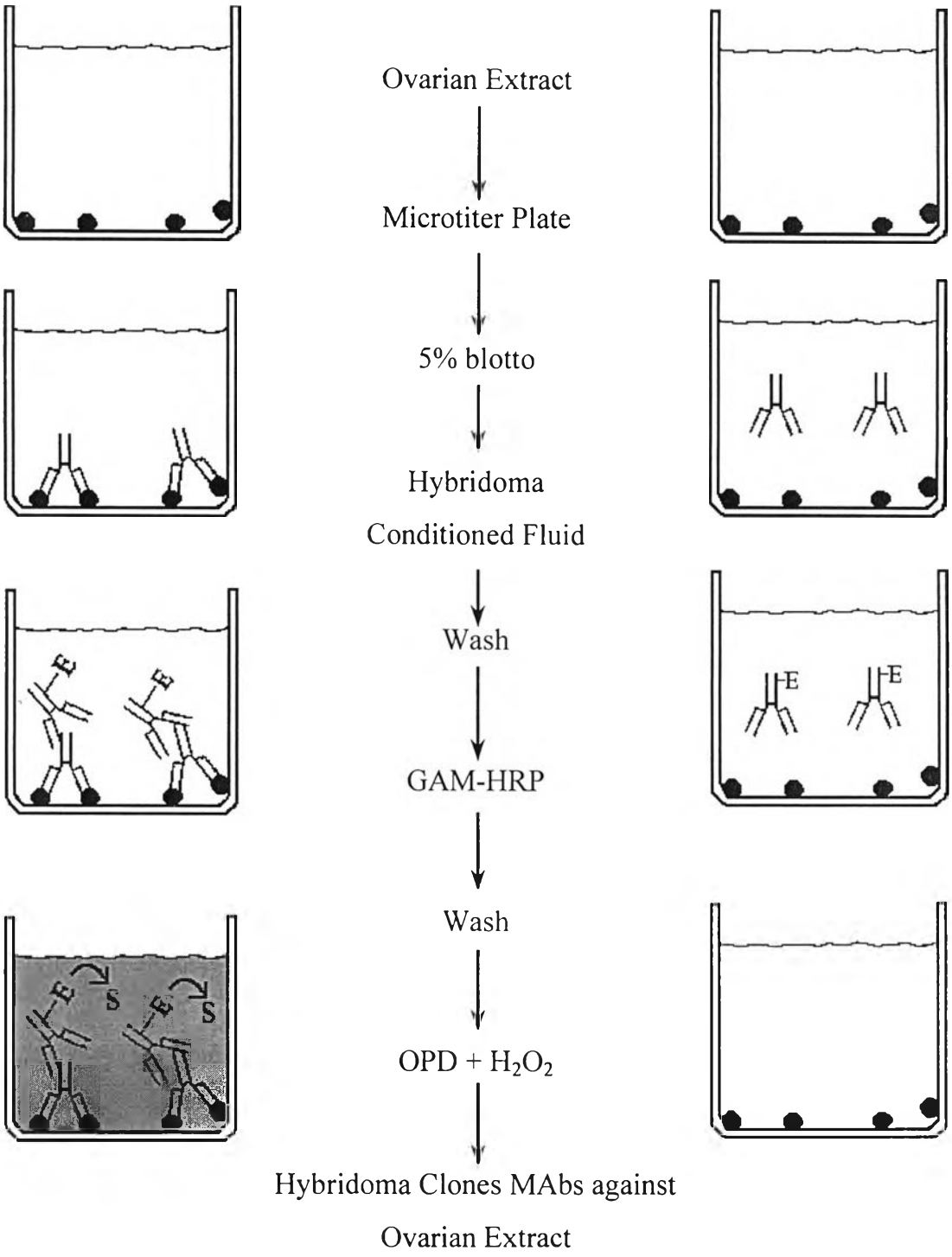

Figure 3.2 Diagram of ELISA (Enzyme-Linked Immunosorbent Assay) for screening method.

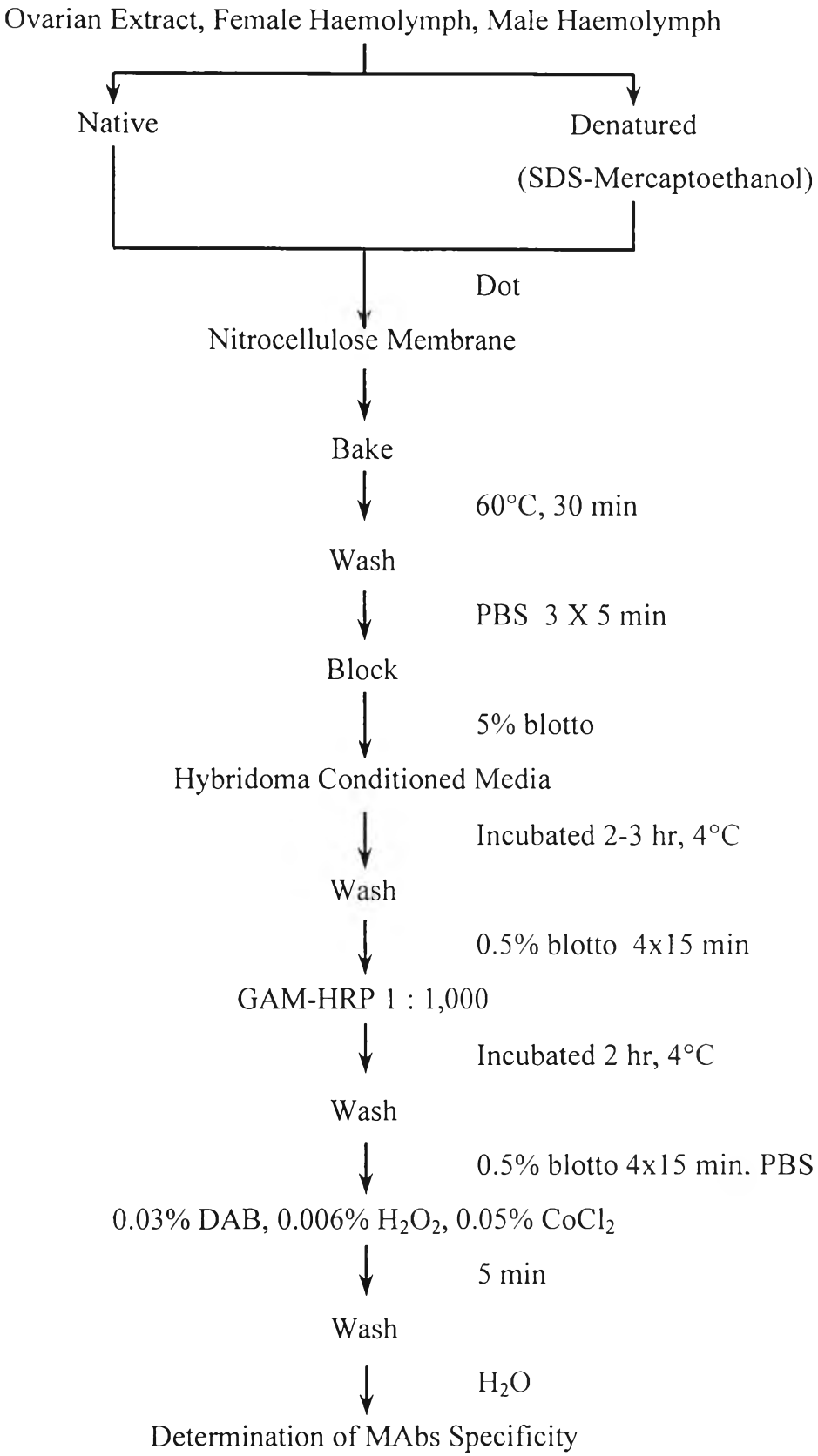

Figure 3.3 Diagram of dot-blotting for screening method.

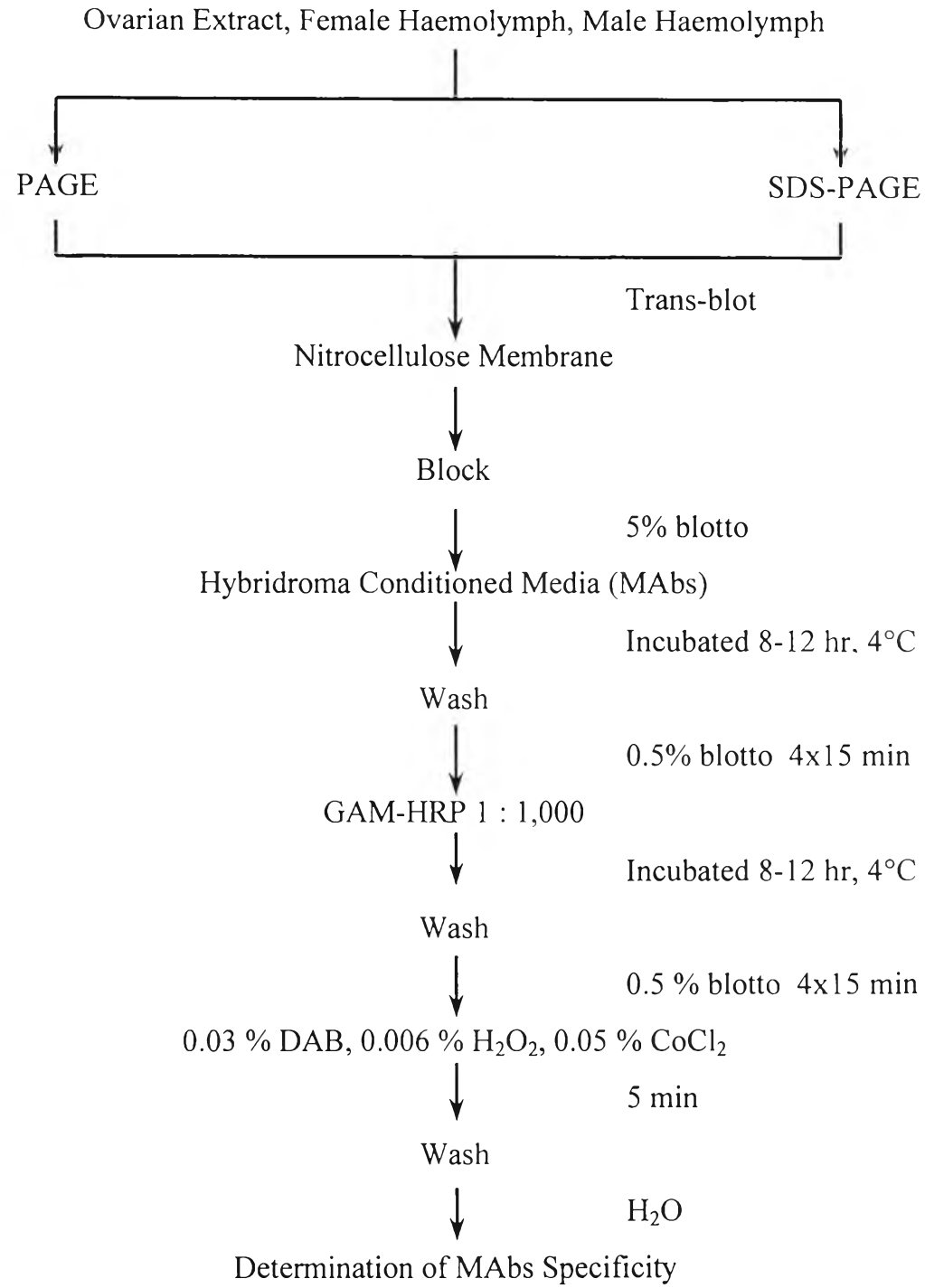

Figure 3.4 Diagram of Western blot analysis for characterization of monoclonal antibodies.

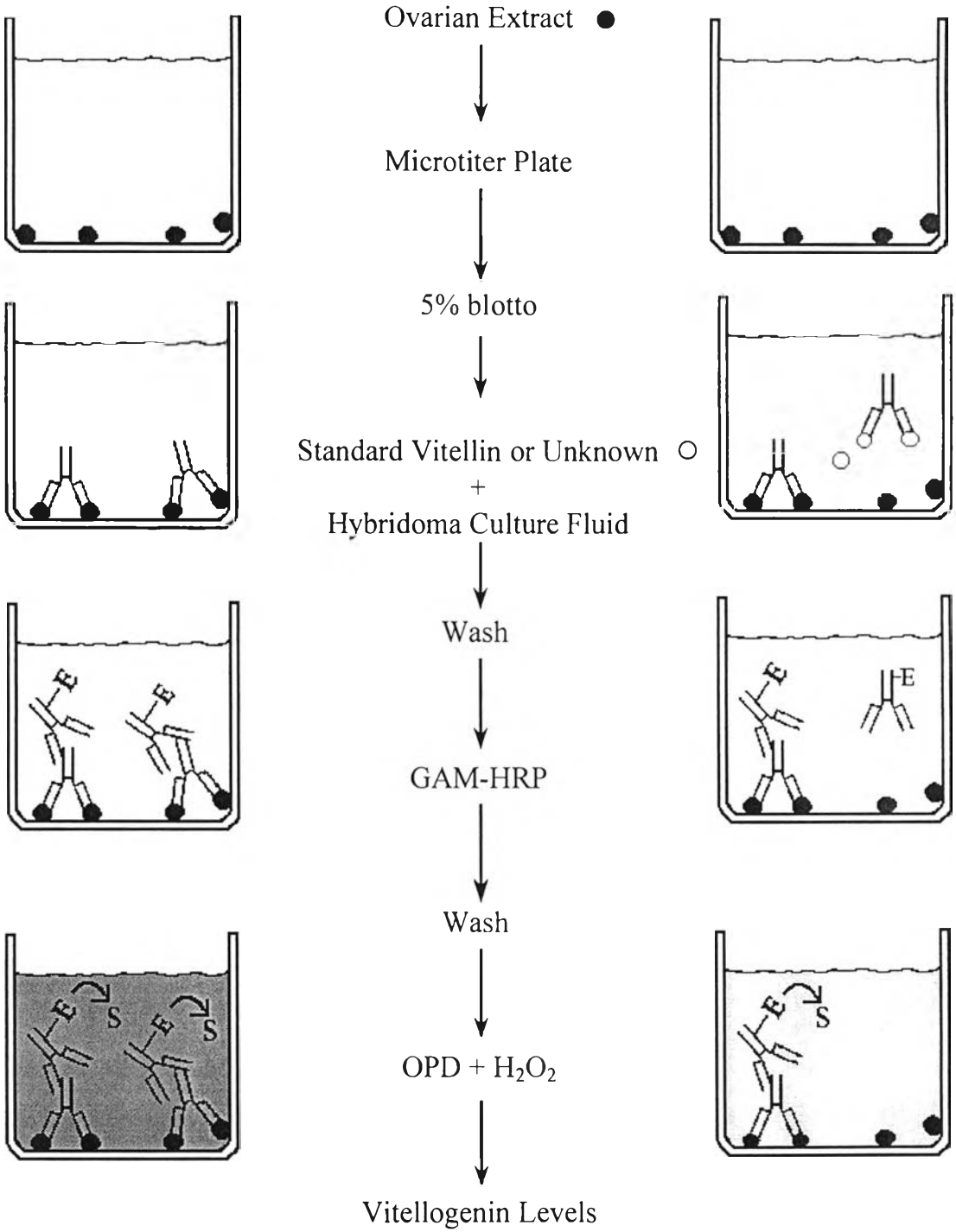

Figure 3.5 Diagram of competitive ELISA for determination of haemolymph vitellogenin levels.

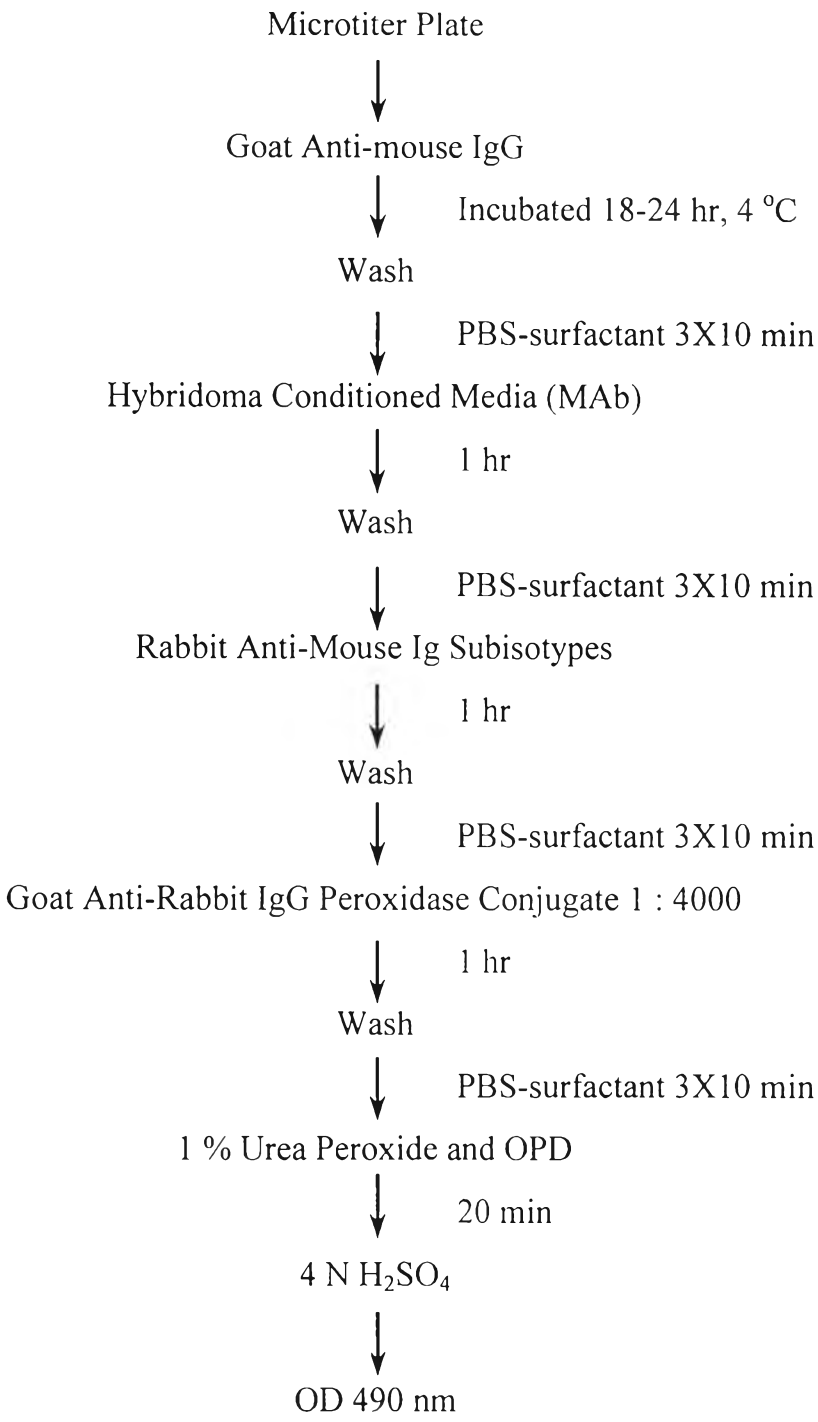

Figure 3.6 Diagram of sandwich ELISA for determination of antibody isotype and subisotype.

Results

After four immunizations with *P. monodon* ovarian extract, mouse antisera from five mice produced strong precipitation band reactions to antigens in *P. monodon* ovarian extract and to female haemolymph, but not to antigens in male haemolymph (Fig. 3.7). Precipitation bands from both antigens showed reaction of identity with all mouse anti-vitellin antisera (not shown). Antiserum from mouse No. 3 seemed to have the fewest numbers of weak precipitation bands with other antigens, so this mouse was used as the spleen cell donor for hybridoma production.

From one fusion, about 700 wells contained hybridoma cells from 16 microculture plates. The first screening on ELISA against ovarian extract yielded 73 positive wells with varying intensities. However, after screening with dot-blotting against ovarian extract, and female and male haemolymphs in both native and SDS-treated antigens, only four hybridoma clones were identified (PMV-11, 15, 22 and 64). These clones produced antibodies that bound both ovarian extract and female haemolymph, but did not bind with male haemolymph. One antibody (PMV-64) bound with both native and denatured antigens (Fig. 3.8, Table 3.1). These four clones and a few other clones that showed different binding were re-cloned and used for further characterization and comparison of monoclonal antibodies by Western blot analysis of native protein (Figs. 3.8 and 3.9). All four monoclonal antibodies specific to vitellin and vitellogenin belonged to IgG1 subisotype (Table 3.1).

During screening with dot-blotting, we observed substantial nonspecific staining of haemolymph spots with GAM-HRP (Fig. 3.8). This nonspecific staining was, however, considerably reduced by adding male haemolymph to GAM-HRP (40%), but nonspecific staining was not reduced by adding normal goat serum (Fig. 3.8). This background staining is due to nonspecific binding of haemolymph components to HRP, as shown by incubation of samples directly with HRP solution

(0.01 mg/ml in Blotto; Sigma). To reduce nonspecific staining, GAM-HRP mixed with male haemolymph was used in the subsequent tests.

Further characterization of native ovarian extract separated by PAGE using Western blot analysis showed that all four hybridoma clones (PMV-11, 15, 22 and 64) produced antibodies that bound with the same single band of glycolipoprotein (Fig. 3.9 : g, h, i, k) and was also identified by PAS and Sudan black staining (Fig. 3.9 : a, b, c). Since this is the primary constituent of ovarian extracts. It can be presumed to be vitellin. Mouse antiserum and other antibodies from other clones show different staining patterns. PMV-3 antibody and mouse anti-vitellin antiserum bound with the expected vitellin band, but also bound with other proteins as well (Fig. 3.9 : d, e). PMV-10 and PMV-60 antibodies bound with high molecular mass protein (Fig. 3.9 : f, j), while PMV-65 did not bind with any proteins (not shown). Western blot analysis of native female haemolymph was not conducted due to interference caused by excessive amounts of haemocyanin which interfered with movement of other proteins.

Further separation of the glycolipoprotein isolated from PAGE by SDS-PAGE revealed two major subunits with Mw 74 and 83 kD (band No. 3 and 2 ; Fig. 3.11 : Aa), and three light staining subunits with Mw 45, 58 and 104 kD (band No. 5, 4, 1; Fig. 3.5 : Aa). Characterization of SDS-treated vitellin by Western blot was done only with PMV-64 monoclonal antibody since only this antibody can bind with SDS-treated antigens. PMV-64, monoclonal antibody interaction with ovarian extract, revealed a strong reaction at Mw 83 kD, and a lighter band with a smaller Mw 80 kD protein (Fig. 3.10 : Ca). It produced only one band at Mw 83 kD with female haemolymph, and did not bind with any proteins in male haemolymph (Fig. 3.10 : Cb, c). Mouse anti-vitellin antiserum revealed six strong immunoreactive bands Mw 45, 58, 74, 83, 104 and 215 kD in ovarian extract, but did not produce any bands in either male or female haemolymphs (Fig. 3.10 : B), which indicated that the proportion of vitellogenin in female haemolymph is very low beyond the binding capacity of mouse

anti-vitellin antiserum produced from injection of native vitellin. The Mw 215 kD protein is not a vitellin subunit since we did not observe this protein with the SDS-PAGE of glycolipoprotein isolated from PAGE (Fig. 3.11 : Aa, Ba).

Immunoprecipitated ovarian proteins had similar patterns of protein staining as ovarian extract (Fig. 3.11 : Bb). However, with immunoprecipitated female haemolymph, Mw 74, 83 and 200 kD proteins were revealed by the mouse anti-vitellin antiserum, and 83 and 200 kD proteins were recognized by PMV-64 antibody (Fig. 3.11 : Bc, Cc).

Competitive ELISA demonstrated that vitellogenin in female haemolymph can completely inhibit binding of all monoclonal antibodies to fixed vitellin similar to vitellin itself, whereas male haemolymph did not show any affects. The range of vitellin that can be measured by this method is 0.25-200 μg/ml (Table. 3.1, Fig. 3.12).

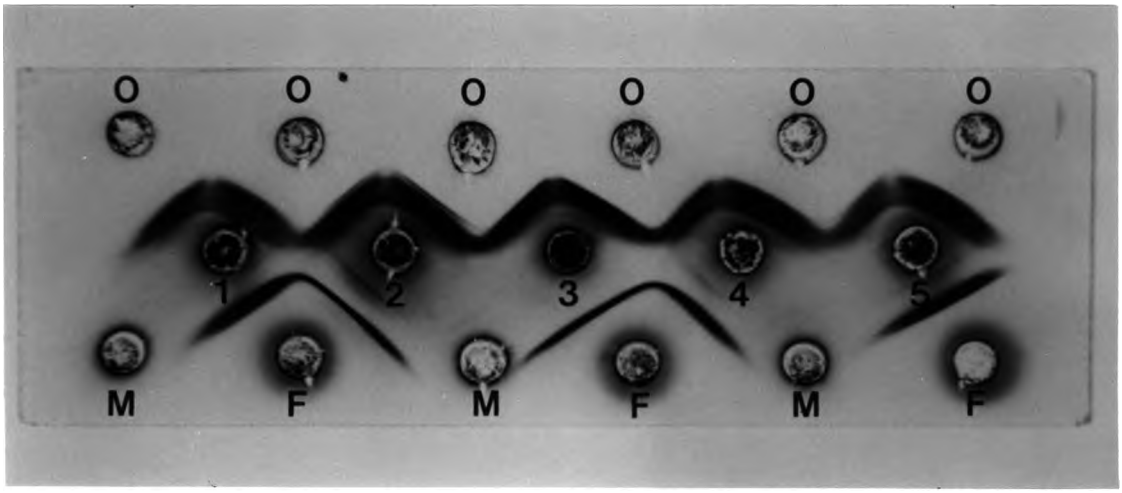

Figure 3.7 Double immunodiffusion of five mouse anti-vitellin antisera (1-5) against *P. monodon* ovarian extract (O), and male (M) and female (F) haemolymph. The most dense precipitation bands occur between all mouse antisera with ovarian extract and with female haemolymph, but not with male haemolymph.

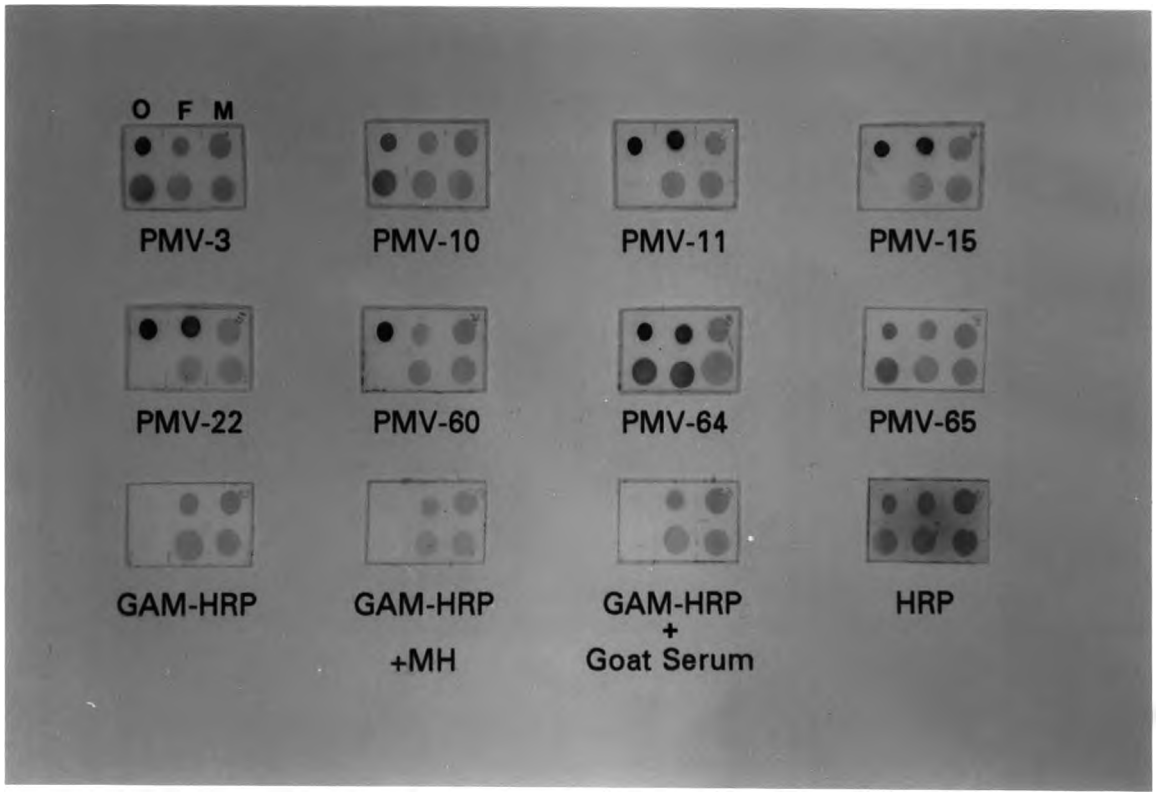

Figure 3.8 Screening results of monoclonal antibodies by dot-blot. Each nitrocellulose sheet was treated with panels of monoclonal antibodies (PMV-s). Antigens in the vertical columns are *P. monodon*: ovarian extract (O), female (F) haemolymph and male (M) haemolymph. Antigens in the upper horizontal rows are untreated proteins, while antigens in the lower rows are SDS and mercaptoethanol treated proteins. Nonspecific binding of goat anti-mouse IgG-horseradish peroxidase conjugate (GAM-HRP) to haemolymph is substantial and cannot be blocked with normal goat serum (GAM-HRP + goat serum), but can be reduced by 40% male haemolymph (GAM-HRP + M). When nitrocellulose sheets were directly incubated with horseradish peroxidase (HRP), the enzyme bound strongly to proteins in all dots.

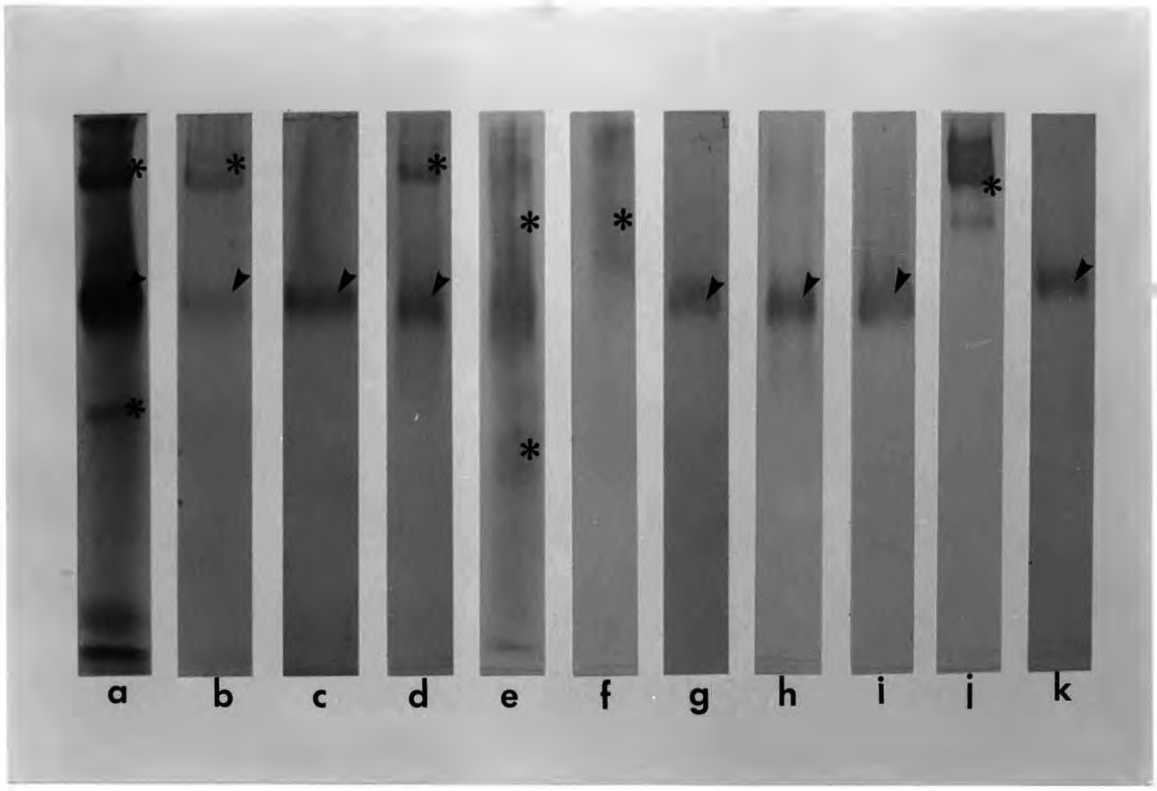

Figure 3.9 PAGE and immunoblot analysis of *P. monodon* ovarian extract. Crude ovarian extract was separated by PAGE and visualized by staining with Coomassie blue (a), periodic acid Schiff (PAS) reagent (b), or Sudan black B (c); or it was transferred to nitrocellulose membrane and treated separately with mouse anti-vitellin antiserum (d), or with monoclonal antibodies; PMV-3 (e), PMV-10 (f), PMV-11 (g), PMV-15 (h), PMV-22, (i), PMV-60 (j), PMV-64 (k). The glycoprotein band (arrow head) was also recognized by PMV-11, 15, 22 and 64 monoclonal antibodies. Mouse anti-vitellin antiserum and other monoclonal antibodies recognized different proteins (asterisk) in the ovarian extract. Protein content was 20 μg/lane for glycolipoprotein staining and 2 μg/lane for immunoblotting.

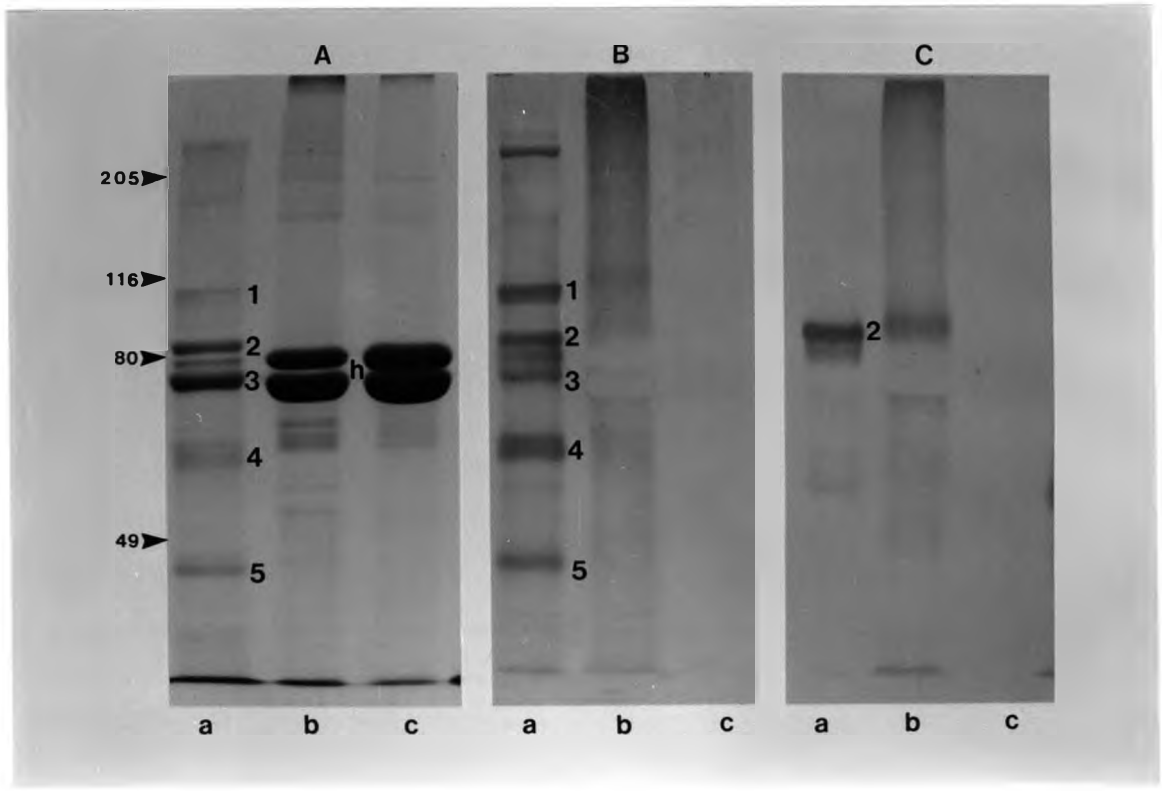

Figure 3.10 SDS-PAGE and immunoblot analysis of *P. monodon* ovarian extract (a), female haemolymph (b) and male haemolymph (c). Preparations were stained with Coomassie blue (A), Western blotting with mouse anti-vitellin antiserum (B), or PMV-64 monoclonal antibody (C). The numbers on the left side are molecular mass of marker proteins. The number 1-5 are presumptive vitellin subunits, h is the haemocyanin subunits. Protein content was 20 μg/lane (a) and 50 μg/lane (b, c) for Coomassie blue staining, and 2 μg/lane (a) and 5 μg/lane (b, c) for Western blotting.

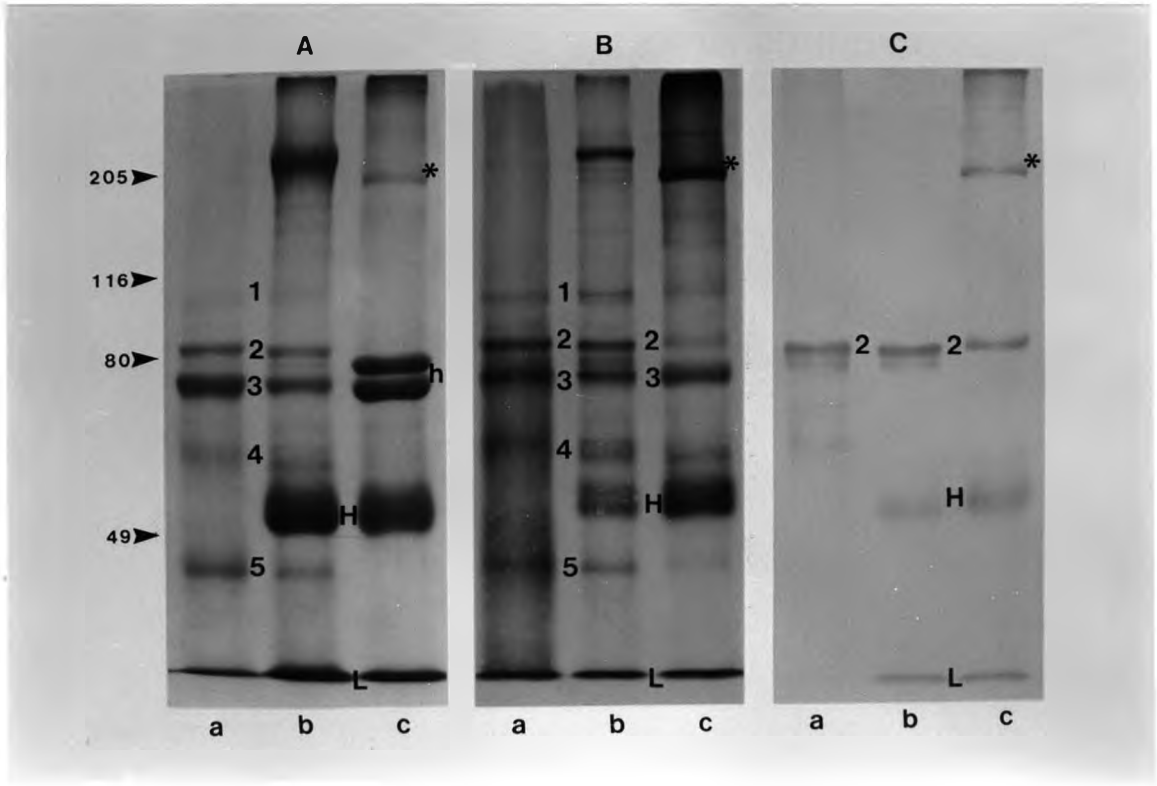

Figure 3.11 SDS-PAGE and immunoblot analysis of isolated proteins from; PAGE-glycolipoprotein band (a), immunoprecipitation of ovarian extract (b), and immunoprecipitation of female haemolymph (c). Preparations were stained with Coomassie blue (A), Western blotting with mouse anti-vitellin antiserum (B), or PMV-64 monoclonal antibody (C). The numbers on the left side are molecular mass of marker proteins. The number 1-5 are presumptive vitellin subunits, h is haemocyanin subunits, H and L are heavy and light chains of the mouse immunoglobulin. * indicates the extravitellin immunoreactive protein which was not found in ovarian extract. Protein content was 20 μg/lane (a) and 50 μg/lane (b, c) for Coomassie blue staining, and 2 μg/lane (a) and 5 μg/lane (b, c) for Western blotting.

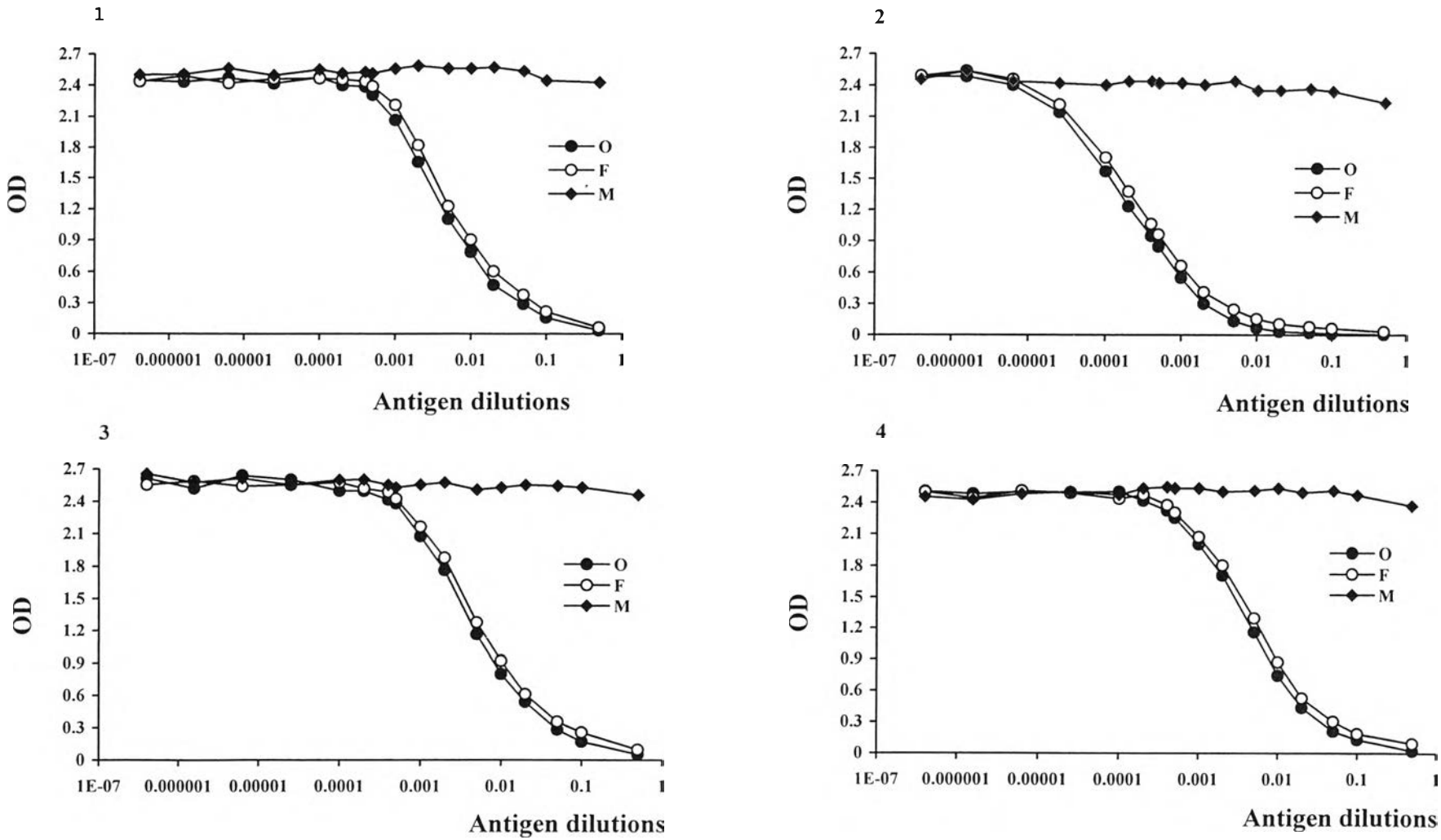

Figure 3.12 Competitive ELISA of PMV-11 (1), 15 (2), 22 (3) and 64 (4) monoclonal antibodies with various antigens; ovarian extract (O, initial protein concentration 10 mg/ml), female haemolymph (F), and male haemolymph (M). The antigens were diluted and evaluated for binding inhibition of antibodies to ovarian extract fixed on the wells. The concentration of the ascites fluid of of PMV-11, 15 and 64 monoclonal antibodies are 1 : 100,000, 1 :45,000 and 1 : 50,000 consecutively. The concentration of culture fluid of PMV-22 monoclonal antibody is 1 : 500.

Table 3.1 Characterization of monoclonal antibodies specific to vitellin and vitellogenin, and range of vitellin concentrations that can be detected by the monoclonal antibodies on competitive ELISA.

Hybridoma Clone	Isotype & Subisotype	Dot-Blot Test				Range of vitellin concentration (μg/ml)
		Ovary Extract		Female haemolymph		
		Native	Denatured	Native	Denatured	
PMV- 3	IgM	+	+	-	-	ND
PMV-10	IgM	+	+	-	-	ND
PMV-11	IgG1	+	-	+	-	4-200
PMV-15	IgG1	+	-	+	-	0.25-20
PMV-22	IgG1	+	-	+	-	4-200
PMV-60	IgG1	+	-	-	-	ND
PMV-64	IgG1	+	+	+	+	4-200
PMV-65	IgM	+	+	-	-	ND

\+ = Antibody can bind to the proteins

\- = Antibody can not bind to the proteins

ND = not done

Table 3.2 Molecular mass of vitellin subunits for six penaeid shrimp species.

Species	Molecular mass (kD)		References
	Vitellin	Vitellin Subunits	
P. monodon	540	74, 83, 90, 104, 168	Quinitio et al., 1990.
	ND	74, 83, 104, 168	Chen and Chen, 1993.
	492	35, 45, 49, 58, 64, 68, 82, 91	Chang et al., 1993.
	ND	**45, 58, 74, 83, 104**	**This study.**
P. semisulcatus	283	86, 95	Tom et al., 1992.
	ND	50, 63, 80, 90	Browdy et al., 1990.
P. vannamei	289	61, 69	Tom et al., 1992.
	ND	76, 95, 97, 103	Quackenbush, 1989.
P. chinensis	380	40, 58, 78, 85, 105	Chang et al., 1996.
	500	78, 85, 155	
Parapenaeus longirostris	ND	45, 66	Tom et al., 1987.
Metapenaeus ensis	350	76, 102	Qiu et al., 1997

ND = Not determined.

Discussion

We isolated four hybridoma clones which produced antibodies that bound with both vitellin and vitellogenin, from fusion of P3X myeloma and spleen cells of a mice immunized with native, *P. monodon* ovarian extract. Only one antibody (PMV-64), however, bound with both native and SDS-treated proteins. This antibody bound with Mw 83 kD protein in ovarian extract and in female haemolymph. A smaller protein (80 kD) in ovarian extract was also recognized by the antibody. This latter protein was not previously reported from *P. monodon*, and it is possible that it may be a degradation product of the Mw 83 kD protein since it was present in minute amounts. With precipitated female haemolymph, PMV-64 antibody recognized an extra protein at Mw 200 kD, but no similar Mw protein in the ovary. This is evidence that this Mw 200 kD protein is a precursor that underwent hydrolysis to Mw 83 kD (Chang et al, 1994) and Mw 74 kD vitellogenin subunits before incorporation into oocytes, since it showed immunological reaction similarity to Mw 83 and 74 kD proteins in female haemolymph and ovarian extract.

SDS-PAGE analysis of the glycolipoprotein isolated from PAGE revealed that this protein consisted of two major subunits of Mw 74 and 83 kD, and three minor subunits Mw 45, 58 and 104 kD. The 168 kD protein was not detected with this preparation. Even though this Mw protein can be visualized slightly in crude ovarian extract, it was not immunoreactive with mouse antiserum (Fig. 3.4). Mouse anti-vitellin antiserum revealed six immunoreactive bands with Mw 215, 104, 83, 74, 58 and 45 kD, but no protein in female and male haemolymph. This occurred because crude ovarian extract in native form was used for immunization, antibody against another cell component (Mw 215 kD protein) was also present, and antibody specific to the denatured form of 83 kD protein was very weak in mouse anti-vitellin antiserum. Consequently, binding of antibodies in mouse anti-vitellin antiserum to

denatured protein was much less pronounced than binding of PMV-64 antibody. With female haemolymph, the vitellogenin content was very low and could not be increased by loading more sample. Vitellogenin detection could, however, be improved by immunoprecipitation of vitellogenin with mouse anti-vitellin antiserum. This result revealed 83 and 200 kD with PMV-64 antibody, and 74, 83 and 200 kD with mouse anti-vitellin antiserum. It is possible that the 83 and 74 kD vitellin subunits may be derived from 200 kD vitellogenin in haemolymph. Neither monoclonal antibodies nor mouse anti-vitellin recognized a 168 kD vitellin subunit (Quinitio, et al., 1990; Chen and Chen, 1993), nor did the SDS-PAGE of protein isolated from lipoglycoprotein of PAGE detect a 168 kD protein. We found vitellin consisting of only five subunits, 45, 58, 74, 83 and 104 kD, in the isolated glycolipoprotein separated by PAGE. Our findings agree in part with other studies (Quinitio et al., 1990; Chen and Chen, 1993 and Chang et al., 1993: Table 3.2). With some penaeid species, two subunits of vitellin were found (Tom et al., 1987, 1992; Qiu et al., 1997), as well as three or four subunits (Quackenbush, 1989; Browdy et al., 1990; Chang et al., 1996; Table 3.2). Monoclonal antibodies specific to other subunits may help resolve this issue and reveal the production site of each vitellin subunit.

Most monoclonal antibodies specific to vitellin and vitellogenin bound with native forms of antigens, which was not surprising since the immunogen used in this study was a native form. Immunization of the SDS-treated antigens is an alternative way of obtaining antibodies which can bind with both native and denatured antigens, and can be used in broader aspects. PMV-64 monoclonal antibody can bind with both native and denatured forms of vitellin and vitellogenin, thus it was more desirable choice for determining vitellogenin concentration by competitive ELISA in our study.

It is noteworthy that native haemocycanin bound with various proteins such as HRP and mouse antibody. This causes substantial, nonspecific staining with the dot-blotting assay. It also occurred with immunoprecipitate of vitellin and vitellogenin

antibody complexes (Fig. 3.5 : Ab), and the precipitate in double immunodiffusion (not shown). Possibly, this abundant protein is not only an oxygen carrier, but also plays a major role in immunity since it can bind to a broad spectrum of alien, organic molecules.