CHAPTER IV

Characterization of vitellin and vitellogenin of giant tiger prawn *Penaeus monodon* using monoclonal antibodies specific to vitellin subunits

Introduction

From the preliminary study in chapter III, the result of our study on characterization of vitellin subunits was slightly different from previous reports (Quinitio et al., 1990; Chang et al., 1993, 1994; Chen and Chen, 1993, 1994). These differences in vitellin characterization may relate to differences in ovarian maturity and/or methodologies for vitellin purification used by different researchers (Qui et al., 1997). Most of the monoclonal antibodies obtained in previous study can bind to native proteins and therefore, can not be used for characterization of all of vitellin and vitellogenin subunits. In order to obtain antibodies that can bind to all denatured vitellin subunits, therefore in this chapter, the ovarian extract was denatured with sodium dodecyl sulfate before immunization and the screening was designed to search for monoclonal antibodies specific for all vitellin subunits. We were able to produce monoclonal antibodies specific to each subunit of vitellin as well as to another oocyte specific protein and haemocyanin. These antibodies were used for characterization of vitellin and vitellogenin subunits and hopefully they will be useful for studying of vitellogenesis processes in future.

Materials and Methods

Source, animal handling and initial preparations

Adult female *P. monodon* (80-120 g) were obtained from local farms around Bangkok. They were held in 5000 liter round tanks with sea water (30 $^{o}/_{oo}$) at 26-28 ^{o}C and ambient photoperiod. The prawns were fed with squid or small mussels twice daily. Three days after molting, female prawns were bilaterally eye-ablated to induce ovarian development. Seven to ten days after eye-ablation, 5 prawns with gravid ovaries (gonado-somatic index > 4% with dark grayish green color) were anesthetized using cold sea 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 (PBS) pH 7.4. Haemolymph was also collected, in the same manner, from normal adult male *P. monodon* (60-80 g). Haemolymph and ovaries were frozen on dry ice and stored at -70 ^{o}C.

In order to determine the presence of various vitellin subunits at different stages of ovarian development, ovaries and haemolymph from 12 individual prawns with ovaries at different developmental stages were also collected for SDS-PAGE and Western blot analysis of vitellin subunits as described below. The results were from 5 different representative prawns. The haemolymph vitellogenin levels were determined by competitive ELISA as described previously (Longyant et al., 1999).

Ovarian extract preparation

Pooled ovaries from 5 prawns 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 ^{o}C for 30 min. The protein concentration of the extract was determined by Bradford reagent (Bradford, 1976), adjusted to 10 mg/ml with PBS, then the extract was divided into 1 ml aliquots and stored at -70 ^{o}C.

Immunization

Ovarian extract was denatured by mixing 1:1 with 2X treatment buffer (for SDS-PAGE), boiled for 1 minute and dialysed against 4 changes of PBS for 48 hrs. Four Swiss albino mice were injected with 0.5 mg SDS-treated ovarian extract 1:1 (V:V) mixed with complete Freund's adjuvant. At two 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 subsequent 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 mouse producing strongest precipition band was boosted one week before hybridoma production.

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 Department of Virology, AFRIMS, Bangkok, Thailand. Fusion products from one mouse were plated on 23 microculture plates (96 wells, NUNC). 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 1:1000 dilution. 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 the same piece 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 (1:1000 in 5% blotto with 40% male prawn haemolymph) for 2 hr. The membrane was then washed as described previously and exposed to the substrate mixture (0.03% diaminobenzidine, 0.006% hydrogen peroxide, 0.05% cobalt chloride in PBS) for 5 min.

Selected hybridoma clones were further screened by dot-blotting against 5 vitellin subunits and the 215 kD protein eluted from ovarian extract separated by SDS-PAGE (as described below) then processed in the same manner (dot-blotting) as described above.

Selected hybridoma clones were re-cloned and cryopreserved. Some hybridoma clones were injected into pristane primed mice for ascites fluid production.

Monoclonal antibody 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 the 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 glycoproteins (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 3 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 the dot-blotting assay.

Isolation of ovarian proteins from SDS-PAGE

After ovarian extracts were separated by SDS-PAGE and stained with Coomassie blue, the desired protein bands were cut off, destained and then homogenized in an equal volume of 0.01% SDS, allowing the protein to be dissolved 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 in a vacuum concentrator (Savants). The vitellin subunits and oocyte specific protein from SDS-PAGE were used for screening hybridoma clones using dot-blotting as described above.

Isotype and subisotype determination

The Isotype and subisotype of the mouse immunoglobulin produced by hybridomas were determined by sandwich ELISA using a subisotyping kit for mouse (Zymed Laboratories).

Results

After the fourth immunization with *P. monodon* ovarian extract treated with SDS, the mouse anti-vitellin antisera from four mice produced strong precipitation bands to antigens in *P. monodon* ovarian extracts and to female haemolymph, but not to antigens in male haemolymph. All antisera bound to the denatured ovarian extract better than to the normal ovarian extract. Precipitation bands from ovarian extract and female haemolymph showed reaction of identity with all mouse anti-vitellin antisera (not shown). All mice showed similar responses; one of these mice was used as the spleen cell donor for hybridoma production.

From one fusion, every well contains hybridoma colonies ranged from 1-8 colonies/well in 23 microculture plates. The first screening on ELISA against ovarian extract yielded over 300 positive wells with varying immunoreactive intensities. Since there were so many positive wells containing several colonies, only about 200 wells containing a few hybridoma colonies were further screened by dot-blotting against ovarian extract, and female and male haemolymph using both native and SDS-treated antigens (Fig. 4.1). The wells showing strong immunoreactivities (70 wells) were further screened by dot-blotting against vitellin subunits and re-cloned. Twenty hybridoma clones were re-cloned successfully and grouped into 6 categories according to their binding capabilities (Fig. 4.2; Table 4.1). All monoclonal antibodies obtained from this experiment bound to both native and denatured antigens (Fig. 4.1, Table 4.1). In Western blot analysis of ovarian extract separated by PAGE (Fig. 4.3), all four groups of monoclonal antibodies specific to vitellin and vitellogenin bound to the same lipoglycoprotein band while the other monoclonal antibodies (PMVS-106 and other clones) bound to an oocyte specific protein, and PMVS-22 monoclonal antibody showed light staining to a smear of highly negative charged protein.

In Western blot analysis of ovarian extract separated by SDS-PAGE, all four groups of monoclonal antibodies specific to vitellin bound to each vitellin subunit (Fig. 4.4 A-E lane a), 83 kD (PMVS-93), 74 kD (PMVS-140), 104 and 58 kD (PMVS-109), and 104 and 45 kD (PMVS-158). The other monoclonal antibodies (PMVS-106 and other clones; Fig. 4.4 F lane a) bound to the oocyte specific protein molecular mass of 215 kD, and another monoclonal antibody (PMVS-22) bound to both haemocyanin subunits (Fig. 4.4 G lane a).

In Western blot analysis of female haemolymph separated by SDS-PAGE (Fig. 4.4 lane b), a high molecular weight vitellogenin subunit (200 kD), was recognized by PMVS-93, 109 and 158 monoclonal antibodies. These antibodies also recognized the vitellogenin subunits of molecular mass of 83 or 104 kD. The 58 and 45 kD vitellin related proteins were not observed in haemolymph (Fig. 4.4 B, D and E lane b). The PMVS-140 antibody recognized only the vitellogenin of molecular mass of 74 kD (Fig. 4.4 C lane b). The PMVS-106 antibody did not show specific binding to any haemolymph proteins (Fig. 4.4 F lane b) and PMVS-22 antibody bound to both subunits of haemocyanin (Fig. 4.4 G lane b).

Western blot analysis of ovarian extracts prepared from individual prawn at different stages of ovarian development (Fig. 4.5, Table 4.2) revealed that none of the proteins in resting stage ovary reacted with monoclonal antibodies specific to vitellin and oocyte specific protein (Fig. 4.5 B-F lane a). In developing stages, the major vitellin subunits, molecular mass of 200, 104, 83, and 74 kD were recognized while in the ripening stage the 200 kD tended to disappear with the gradual appearance of the 58 and 45 kD subunits. The presence of the 200 kD protein in the ovarian extract seemed to be unrelated to the haemolymph vitellogenin levels but, rather, related to the early development stage of the ovary (Fig. 4.5 A-E lane b-e, Table 4.2).

Isotypes and subisotypes of the monoclonal antibodies mostly (15 out of 20 clones) belonged to the IgG1 isotype (PMVS-10, 11, 18, 43, 50, 52, 53, 72, 93, 97,

122, 137, 140, 157 and 158), only four of them (PMVS-22, 106, 109 and 121) belonged to the IgG2a subisotype and only one (PMVS-42) belonged to the IgG2b subisotype.

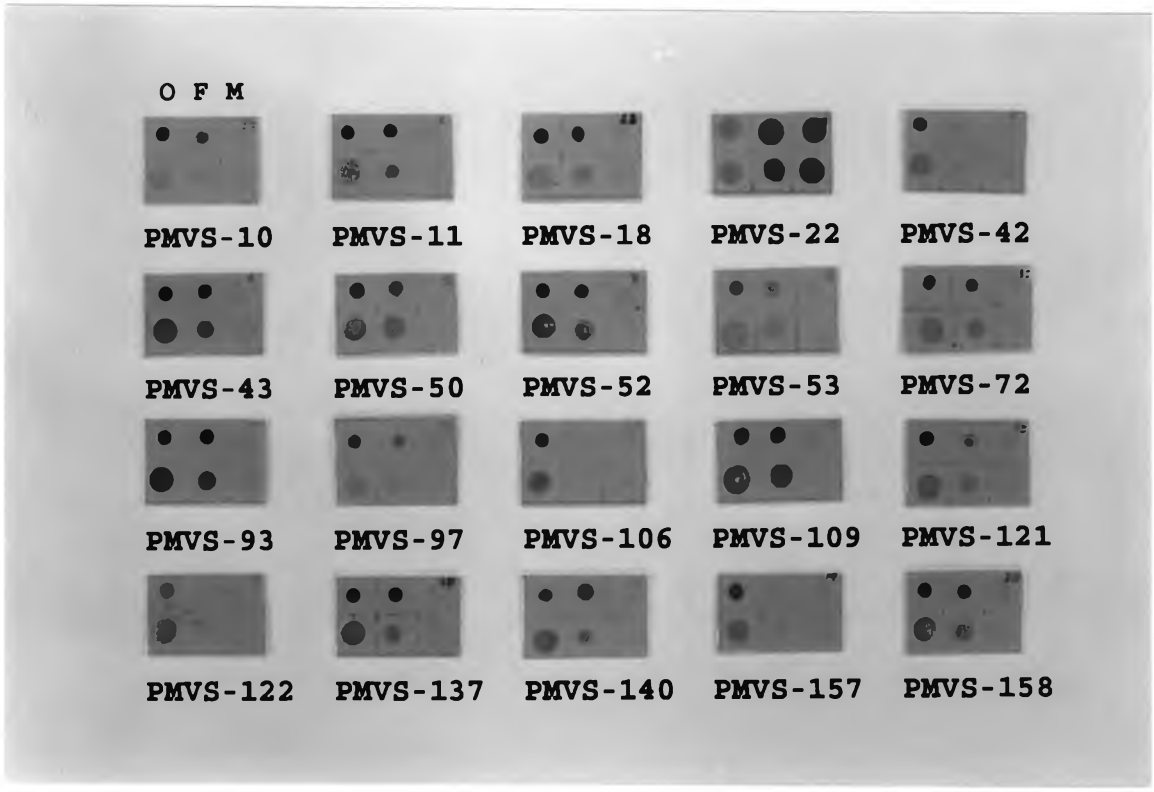

Figure 4.1 Screening results of 20 monoclonal antibodies by dot-blot against native and denatured antigens. Each nitrocellulose sheet was treated with panels of monoclonal antibodies (PMVS-s). Antigens in the vertical columns are *P. monodon*: ovarian extract (O); female (F) and male (M) haemolymph. Proteins in the upper horizontal rows were untreated, while proteins in the lower rows were SDS and mercaptoethanol treated.

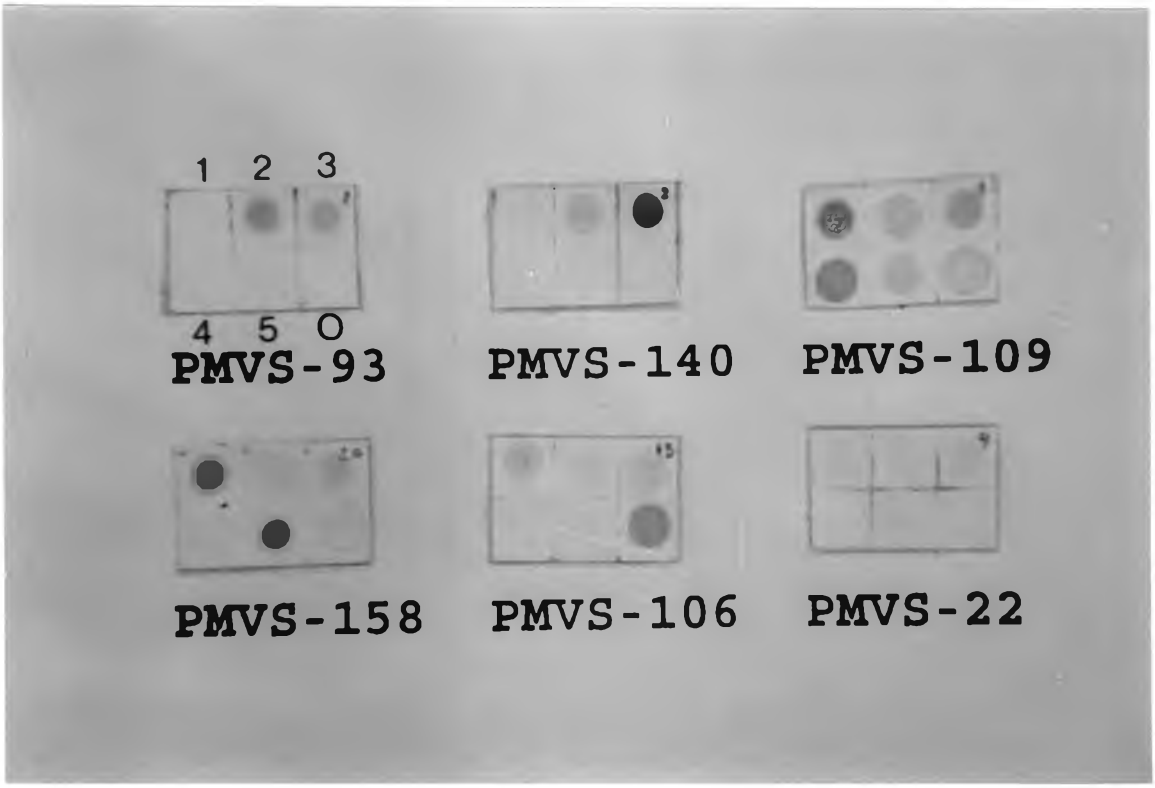

Figure 4.2 Screening results of monoclonal antibodies by dot-blot against each vitellin subunit (1 = 104 kD, 2 = 83 kD, 3 = 74 kD, 4 = 58 kD and 5 = 45 kD subunits) and the oocyte specific protein (0 = 215 kD). The illustrations show only one representative from each group of monoclonal antibodies.

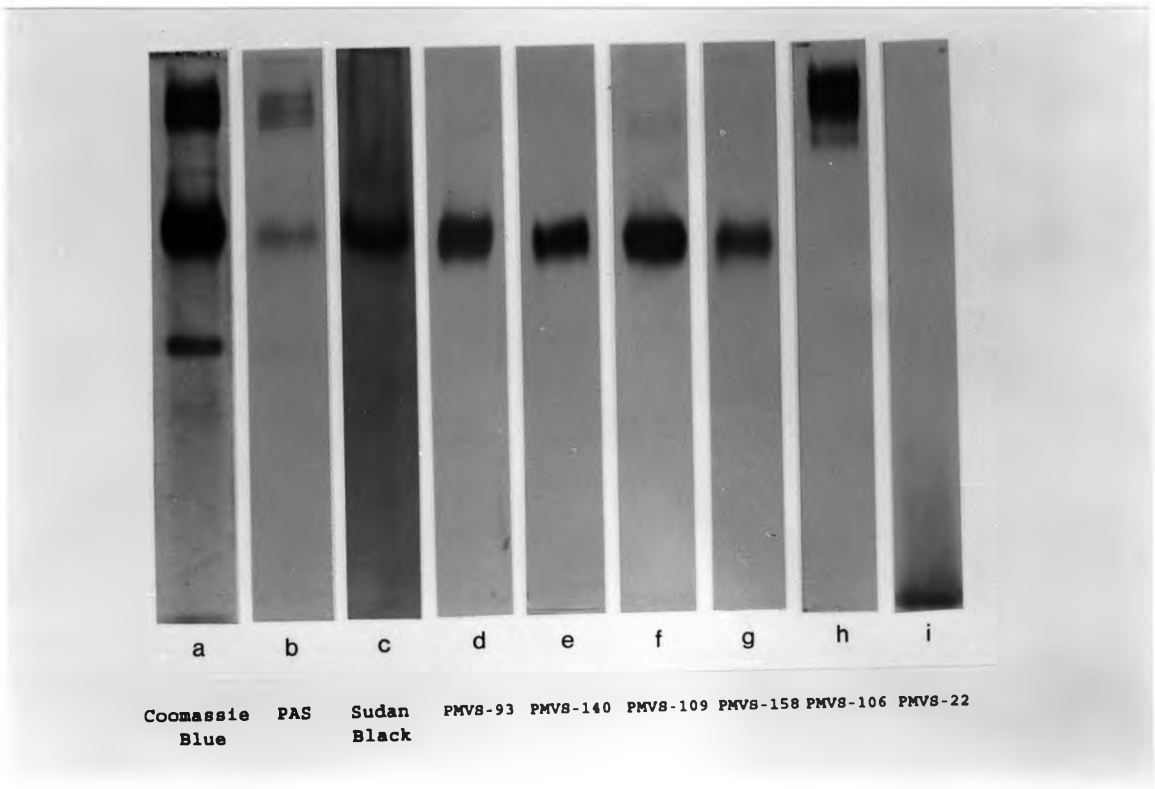

Figure 4.3 PAGE and immunoblot analysis of *P. monodon* ovarian extract. Crude ovarian extract was separated by PAGE and visualized by staining with Coomassie blue (a), periodic acid Schiff (PAS) reagent (b), or Sudan black B (c); or it was transferred to nitrocellulose membrane and treated separately with monoclonal antibodies; PMVS-93 (d), PMVS-140 (e), PMVS-109 (f), PMVS-158 (g), PMVS-106 (h) and PMVS-22 (i). About 20 μg protein/lane was used for glycolipoprotein staining (a-c) and about 2 μg protein/lane was used for immunoblotting (d-i).

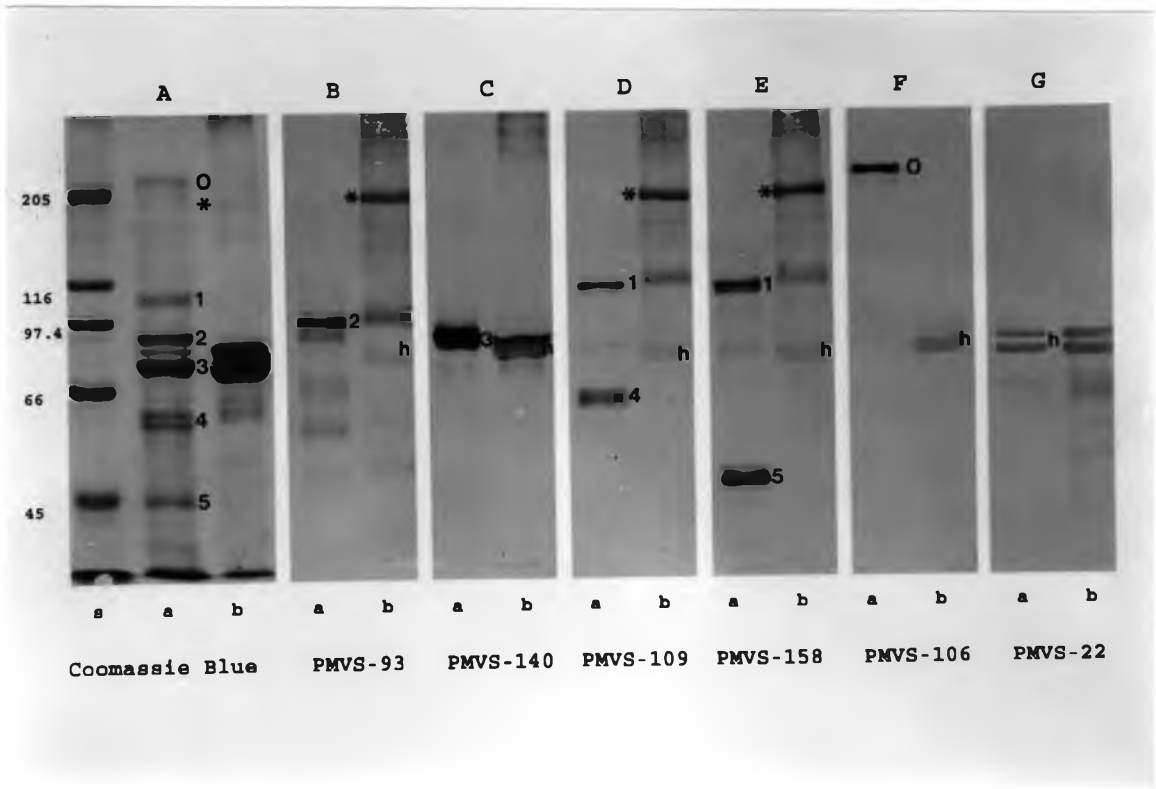

Figure 4.4 SDS-PAGE and immunoblot analysis of *P. monodon* ovarian extract (a) and female haemolymph (b). Preparations were stained with Coomassie blue (A); the far left lane contained molecular standard proteins (s). The proteins were transferred to a nitrocellulose membrane and immunoblotted with various monoclonal antibodies, PMVS-93 (B), PMVS-140 (C), PMVS-109 (D), PMVS-158 (E), PMVS-106 (F), PMVS-22 (G). The numbers on the left side are the molecular weights of the marker proteins. The numbers (0-5) between lane a and b are ovarian specific protein and vitellin subunits (molecular mass of 215, 104, 83, 74, 58, 45 kD, respectively), h is haemocyanin subunits, and * indicates the 200 kD vitellogenin precursor. Protein content was about 20 μg/lane (a) and 50 μg/lane (b) for Coomassie blue staining, and about 2 μg/lane (a) and 5 μg/lane (b; except in G lane b =1 μg/lane) for immunoblotting.

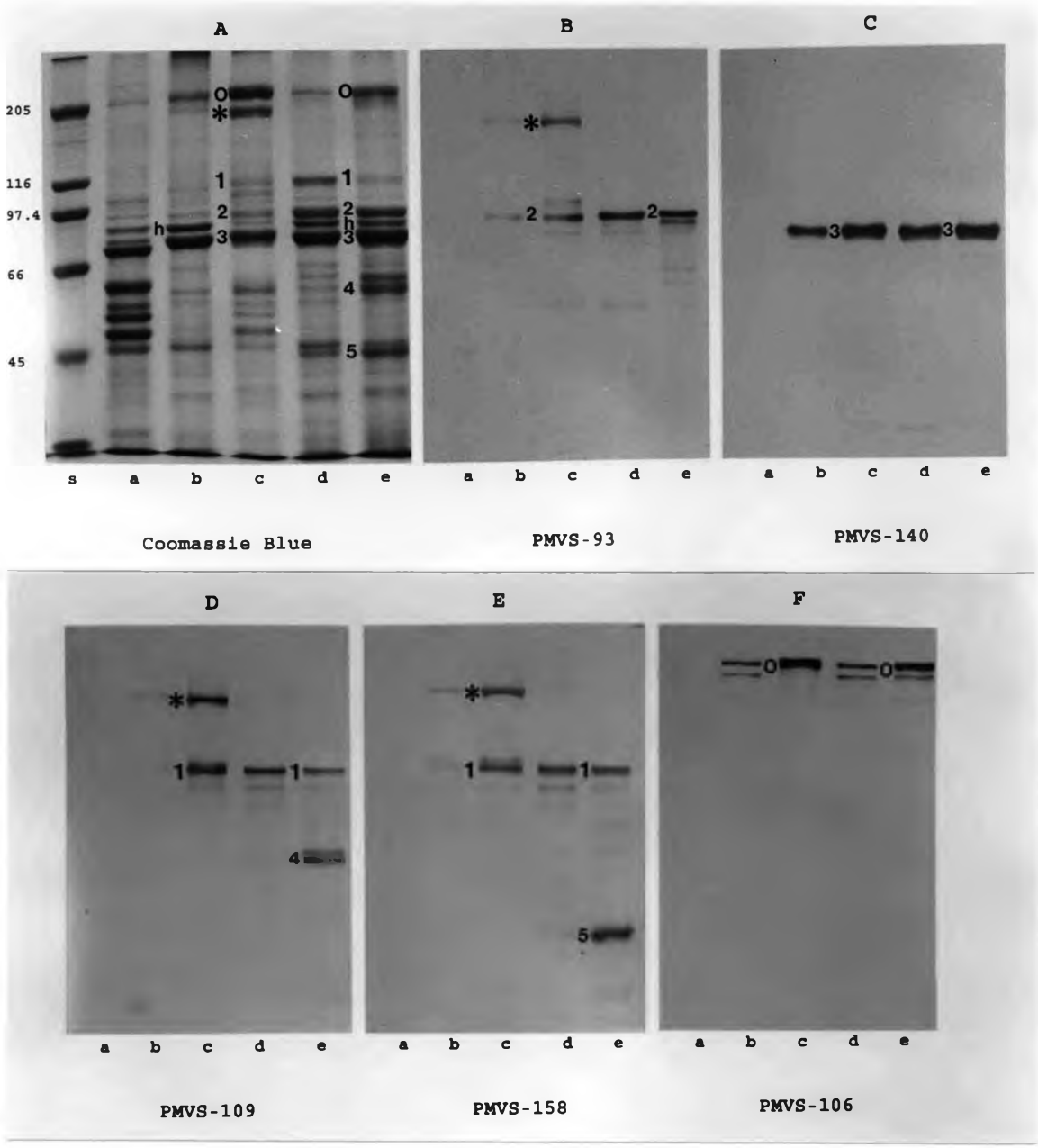

Figure 4.5 SDS-PAGE and immunoblot analysis of *P. monodon* ovarian extract prepared from individual prawn at different stages of development: (a) resting stage, (b) early developing, (c) developing, (d) ripe ovary with a low haemolymph vitellogenin level, (e) ripe ovary with a high vitellogenin level. The preparation was stained with Coomassie blue (A), the far left lane (s) are standard marker proteins. The proteins were transferred and immunoblotted with various monoclonal antibodies: PMVS-93 (B), PMVS-140 (C), PMVS-109 (D), PMVS-158 (E) and PMVS-106 (F). The legends in the figure are the same as in figure 4.4. Protein content was about 25 μg/lane for Coomassie blue staining, and about 1 μg/lane for immunoblotting.

Table 4.1 Specificity of monoclonal antibodies tested by dot-blot and Western blot analyses obtained from this study (b) and compared to previous study (a) (Longyant et al., 1999).

Monoclonal antibodies obtained from mice Immunized with	Dot-blot test				Dot blot test/ Western Blot analysis				
	Ovarian extract		Female haemolymph		Vitellin subunits (kD)				
	Native	Denatured	Native	Denatured	104	83	74	58	45
a. Native vitellin									
PMV-11, 15, 22	+	-	+	-	?	?	?	?	?
PMV-64	+	+	+	+	-	+	-	-	-
b. Denatured vitellin									
PMVS-10, 18, 50, 53, 93, 97	+	+	+	+	-	+	-	-	-
PMVS-11, 43, 72, 121, 137, 140	+	+	+	+	-	-	+	-	-
PMVS-52, 109	+	+	+	+	+	-	-	+	-
PMVS-158	+	+	+	+	+	-	-	-	+
PMVS-42, 106, 122, 157	+	+	-	-	Oocyte specific Protein				
PMVS-22	-	-	+	+	Haemocyanin				

The underlined clones are the representative antibodies from each group that were used for further analysis.

+ = bind, - = not bind and ? = not known

Table 4.2 Vitellin subunits found in individual prawn at various stages of ovarian development.

Stage of development	GSI	Vitellogenin levels	Oocyte specific protein and vitellin subunits (kD)						
	%	(mg/ml)	215	200	104	83	74	58	45
a. Resting	0.54	0	-	-	-	-	-	-	-
b. Early Developing	2.84	5.06	+	±	±	±	++	-	-
c. Developing	3.23	0.37	+++	++	++	++	+++	-	-
d. Ripe	5.78	0.87	+	±	++	++	+++	±	±
e. Ripe	5.59	4.54	++	-	+	++	+++	++	++

The intensity of the immunoreactivity was scored as

- = undetectable, ± = slightly detectable, and

+ = clearly detectable with three levels of intensity.

Table 4.3 Molecular mass of vitellin and vitellogenin subunits reported in six penaeid shrimp species.

Species		Molecular mass (kD)		References
		Holo protein	Vitellin subunits	
P. monodon	Vitellin	540	74^3, 83^2 90, 104^1, 168*	Quinitio et al., 1990.
		ND	74^3, 83^2,104^1, 168*	Chen and Chen, 1993.
		492	35, 45^5, 49, 58^4, 64, 68, 82^2, 91	Chang et al., 1993.
		ND	45^5, 58^4, 74^3, 83^2, 104^1	Longyant et al., 1999.
		ND	**45^5, 58^4, 74^3, 83^2, 104^1, 200***	**This study.**
	Vitellogenin	ND	74^3, 83^2, 104^1, 168*	Chen and Chen 1994.
		ND	82^2, 170*	Chang et al., 1994.
		ND	**74^3, 83^2, 104^1, 200***	**This study.**
P. chinensis	Vitellin	380	40, 58, 78, 85, 105	Chang et al., 1996.
		500	78, 85, 155	
	Vitellogenin	ND	85, 191	Chang and Jeng, 1995.
P. semisulcatus	Vitellin	283	86, 95	Tom et al., 1992.
		ND	50, 63, 80, 90	Browdy et al., 1990.
		ND	80, 96, 158	Khayat et al., 1994.
		ND	80, 120, 200	Lubzens et al., 1997.
	Vitellogenin	ND	80, 120, 200	Lubzens et al., 1997.
P. vannamei	Vitellin	289	61, 69	Tom et al., 1992.
		ND	76, 95, 97, 103	Quackenbush, 1989.
Parapenaeus longirostris				
	Vitellin	ND	45, 66	Tom et al., 1987.
Metapenaeus ensis	Vitellin	350	76, 102	Qiu et al., 1997.

ND = Not determined

The superscripts indicated the equivalent vitellin or vitellogenin subunits found by other studies.

Discussion

From one fusion of P3X myeloma and spleen cells of a mouse immunized with the denatured ovarian extract of *P. monodon*, we isolated a complete set of monoclonal antibodies specific to vitellin subunits. One group of hybridoma clones produced antibodies that bound to oocyte specific protein and one hybridoma clone produced antibody that bound to both subunits of haemocyanin. All of the monoclonal antibodies could bind to both native and denatured proteins which was not surprised since the immunogens used were denatured proteins; therefore, the production of monoclonal antibodies by immunization with denatured immunogens yields more broadly useful monoclonal antibodies than using native proteins as reported previously (Longyant et al., 1999; Table 4.1).

The monoclonal antibodies specific to various vitellin subunits obtained from this study are comparable to the antisera made against purified vitellin subunits in another previous study (Chen and Chen, 1993). Two groups of monoclonal antibodies (PMVS-93 and 140) recognize each single subunit of vitellin, the 83 and 74 kD subunits respectively. They are equivalent to anti-Ep3 and anti-Ep4 antisera (Chen and Chen, 1993). The other two groups of monoclonal antibodies (PMVS-109 and 158) recognize the same subunit (104 kD) which is equivalent to anti-Ep2 antiserum. However, PMVS-109 and 158 monoclonal antibodies recognize different small subunits (58 and 45 kD), indicating that the two monoclonal antibodies are specific to different epitopes on the 104 kD protein and that the 58 and 45 kD proteins may be derived from the 104 kD protein. The Ep2 and Ep3 antisera crossreact with the 168 kD protein in haemolymph in the same manner as PMVS-93, 109 and 158 monoclonal antibodies in this study crossreact with the 200 kD haemolymph protein. Therefore, it is quite certain that the 168 or 170 kD polypeptides described in other studies (Quinitio et al., 1990; Chen and Chen, 1993, 1994; Chang et al., 1994) are the same as

the 200 kD polypeptide in our study. The difference in size is due to variations in calculation since their studies used a wide range of molecular weight standard proteins (170 and 340 kD). Also, the SDS-PAGE results (Chen and Chen, 1993) clearly shown that the 168 kD polypeptide migrated more slowly than the 170 kD standard protein; therefore, this protein must be bigger than 170 kD. In our study, this protein migrated slightly faster than the 205 kD standard protein (Fig. 4.4 A & 4.5 A). From the immunoreactive crossreactivity (Chen and Chen, 1993), anti Ep2 (104 kD) and anti Ep3 (83 kD) antisera crossreact with the 168 kD protein which indicates that the 104 and 83 kD proteins derived from the cleavage of the 168 kD protein (which is unlikely); thus, this polypeptide must be larger than 168 kD.

In haemolymph from female prawn with gravid ovary, the vitellogenin subunits (vitellin related proteins), molecular mass of 200, 104, 83 and 74 kD (the 104 and 83 kD are slightly larger than the counterpart vitellin subunits) were recognized by PMVS-93, PMVS-140, PMVS-109 and PMVS-158 monoclonal antibodies (Fig. 4.4) which is similar to a previous report on *P. monodon* that haemolymph contained four egg yolk peptides of 168, 104, 83 and 74 kD (Chen and Chen, 1994). The immunoreactive relationships among those vitellin subunits suggest that the 200 kD protein is the precursor and either cleaved to yield the 104 and 83 kD proteins in the haemolymph before or directly after being taken up into the oocyte. Then, in the oocyte, the 104 kD subunit may be further cleaved to yield two smaller 58 and 45 kD subunits which share the same epitopes with the 104 kD subunit. This result shows the superior characteristics of the monoclonal antibodies that can be used to differentiate between different epitopes on the same molecule.

The 74 kD subunit does not share any epitope with the 200 kD protein, in common with the anti-Ep4 antiserum which does not crossreact with the 168 kD polypeptide (Chen and Chen, 1993). This protein may be synthesized, released, and then taken up without or with minor modification (Fig. 4.4 C). This vitellogenin

subunit (74 kD) was not reported in the isolation of haemolymph vitellogenin by Sudan black staining and sucrose density gradient *in P. monodon* (Chang et al., 1994) and *Penaeus chinensis* (Chang and Jeng, 1995). It is possible that, in haemolymph, this subunit may be not associated with the 200 (170), 104 and 83 (82) kD proteins. Moreover, the size of the 74 kD protein is also very close to the size of the high abundant small subunit of haemocyanin, and the co-migration of these two proteins in SDS-PAGE may cause difficulty in identification of this subunit.

This study confirms previous findings from SDS-PAGE analysis of the glycolipoprotein isolated from PAGE, revealing that, in gravid *P. monodon* ovary, vitellin consists of 5 major subunits molecular mass of 45, 58, 74, 83 and 104 kD (Longyant et al., 1999) which is similar to the vitellin of *P. chinensis* consisting of 5 major subunits molecular mass of 40, 58, 78, 85, and 105 kD (Chang et al., 1996). The 168 kD protein in the ovarian extracts of other studies (Quinitio et al., 1990; Chang et al., 1993) was not detected in this and the previous studies (Longyant et al., 1999) on *P. monodon.* As discussed above the 168 kD protein may be the same as the 200 kD protein found in the haemolymph in our study and was found during early developmental stages of the ovaries. In studies of vitellogenin synthesis in ovarian tissue *in vitro*; the 168 and 74 kD proteins were secreted into the medium (Chen and Chen, 1994). It is possible that during the highly active stage of ovarian development, the 168 (200) kD protein may be taken up directly and slowly undergo processing to the 104 and 83 kD with maturation progress. The accumulation of the 58 and 45 kD hydrolysis products increased with the reduction of the high molecular weight forms. The other possibility is that the 200 kD protein was a contaminant from the haemolymph surrounding the oocyte follicles in the ovary; this is unlikely since in the gravid ovary with high haemolymph vitellogenin levels the 200 kD protein was not observed in ovarian extract (Fig. 4.5 B, D and E lane d & e, Table 4.2). The immunoreactivity of the PMVS-106 revealed that the 215 kD protein is synthesized in

ovary only during oocyte maturation, not in resting ovary, and that this protein may undergo processing to smaller molecular forms as well since different immunoreactive small molecular forms were also detected in all stages of ovarian development (Fig. 4.5 F). Thus, interpretations of the incorporation of radioactive amino acid using anti-vitellin antiserum must be made with caution, since this protein is synthesized during ovarian development and most antisera against purified vitellin may contain considerable amounts of the antibodies specific to other proteins as shown in previous experiments (Quinitio et al., 1990).

Eight small subunits of vitellin including the 45 and 58 kD polypeptides were observed in another study of this species (Chang et al., 1993). In this case, it is possible that these small subunits may have been generated during sample preparation, since in this preparation the ovaries were homogenized in Tris buffer without EDTA or other protease inhibitors. Then, after homogenization, the ovarian extract was also dialysed without protease inhibitors. Similar confusion in the identification of vitellin subunits has occurred in other species such as *Penaeus semisulcatus* (Browdy et al., 1990; Tom et al., 1992; Khayat et al., 1994; Lubzens et al., 1997: Table 4.3); however, only 2-3 subunits of vitellin has been reported for most penaeid shrimp species (Table 4.3).

From the immunoreactive equivalent of the vitellogenin and vitellin subunits, our results suggested that vitellogenin may be synthesized and released into the haemolymph in two different molecular forms, the 200 and 74 kD proteins, then the 200 kD protein is processed into either the 104 and 83 kD before or after being taken up into the oocyte. Finally, as maturation progresses, the 104 kD fragment is further processed into the 58 and 45 kD subunits. The 74 kD protein was taken up and remained in the oocyte with little or no further modification. However, the relationship between the stage of ovarian development and haemolymph vitellogenin level in the samples shown here seem to be uncorrelated. In the freshwater prawn

Macrobrachium rosenbergii, the vitellogenin level increased during ovarian maturation and then sharply dropped in the ripe ovary (Derelle et al., 1986), however, the haemolymph vitellogenin levels at each gonado-somatic index are highly variated in this species (Lee and Change, 1997). More detailed investigations of the relationship between vitellogenin levels and stage of ovarian development are in progress.

The site of vitellogenin synthesis is still in question. If vitellogenin was synthesized in the ovaries then secreted into haemolymph before being taken up by the developing oocytes as proposed by several authors (Yano and Chinzei, 1987; Lubzens et al., 1997), there are still questions about which cells in the ovary (the oocyte or follicular cell, or other cell) synthesize vitellogenin. Another question is why vitellogenin has to be secreted into the haemolymph before being taken into the oocytes which are in close proximity and are in a very active stage for vitellogenin uptake. In preliminary experiments using various monoclonal antibodies against each subunit for immunocytochemical localization, we were not able to observe any vitellin immunoreactive substance in either follicular cell or hepatopancreas of vitellogenic prawn.

This study showed that the monoclonal antibody technique can generate highly and monospecific antibodies to each of vitellin subunits without the requirement for purification of the antigens. Antibodies with different specificity can be used for characterization of the molecular nature of the molecule while making antisera to each subunit would be a very tedious task (since a slight impurity in the immunogen would cause mis-segregation of the molecules). Monoclonal antibodies specific to each subunit can be used as a tool to investigate the production site of vitellogenin subunits and to follow molecular processing of the vitellin during ovarian development as well as larval development.