Chapter 2

Materials and Methods

Materials

1. Banana shrimp, *Penaeus* (*Fenneropenaeus*) *merguiensis*

Live mature female and male *P. merguiensis* were collected from Trang Coastal Aquaculture Station and Nakhon Si Thammarat province. They were 10-14 cm long and weighed between 30-40 g. Ovarian development in female shrimps was classified into 4 stages on the basis of the color and size of the ovary observed through the external carapace according to Auttarat, *et al*. (2006) as follows:

Stage 1 (undeveloped or immature): The ovary is thin, transparent, unpigmented and not visible through the dorsal exoskeleton. Ovaries in stage 1 and stage 5 (spent) often cannot be distinguished from each other.

Stage 2 (early maturation): The ovary is visible through the exoskeleton as a thick opaque line along the dorsal central axis in a white to olive green color.

Stage 3 (late maturation): The anterior portion of the ovary is thick and expanded, and the color is a deep or dark green which clearly visible through the exoskeleton.

Stage 4 (mature): This stage begins when the ovaries are classified as ripe and have expanded throughout the exoskeleton of the first abdominal segment. The ovaries appear dark olive green.

The ovary, hepatopancreas, muscle, intestine and heart were dissected out from each shrimp and stored at -20°C for purification Vt or stored at -80°C for molecular and proteomic analysis.

In the present study, the different stages of ovarian development have been defined as follows: stage 1 as previtellogenic; stage 2 as early vitellogenic; and stage 3-4 as vitellogenic. Additionally, the gonadosomatic index (GSI) was calculated as ovarian weight/body weight x 100.

2. Chemicals

2.1 Analytical grade

Chemicals used were of analytical grade and purchased from the following companies.

Chemical	**Company**
Acetic acid	Merck
Acetonitrile	Fisher
Acrylamide	Fluka
Agarose	Sigma
Ammonium persulphate	Merck
Ammonium sulphate	Fluka
Beta-Mercaptoethanol	Fluka
Bisacrylamide (N,N' methylenediacrylamide)	Fluka
Bovine serum albumin	Sigma
Broad range molecular weight marker	Promega
Bromophenol blue	Merck
Calcium chloride	Ajex
Citric acid	Ajex
Coomassie Brilliant Blue R-250	Sigma
Coomassie plus protein assay reagent kit	Pierce
DEAE-Sephacel	Sigma
Ethylenediaminetetraacetic acid	Fluka
Ethanol	Scharlau
Glycerol	Sigma
Glycine	Fluka
Hydrochloric acid	Merck
L-Glutamic acid	Sigma
L-Lysine	Sigma
Magnesium chloride (six hydrate)	Sigma
Methanol	Merck
N,N,N',N'Tetramethylethylenediamine	Fluka
Phenylmethylsulphonylfluoride	Sigma

Chemical	**Company**
Potassium bromide	Merck
Potassium dihydrogen phosphate	Merck
Sephadex G-200	Sigma
Silver staining kit	Bio-Rad
Sodium chloride	Sigma
Sodium dodecyl sulphate	Riedel de-Haen
Sodium metabisulphite	Mallinckrodt
Standard bovine serum albumin	Pierce
Standard pI markers	Sigma
Trichloroacetic acid	Carlo Erba
Tris (hydroxymethyl) aminomethane	Sigma
Triton X-100	Merck

2.2 Molecular biology grade

All chemicals used below were molecular biology grade and purchased from the following companies.

Chemical	Company
Agar	BD Bioscience
Agarose	Promega
Ampicillin	Sigma
100 Base pair DNA ladder	Biolabs
5-Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside	USB Corporation
High fidelity platinum Taq DNA polymerase	Invitrogen
Isopropylthiogalactoside	USB Corporation
Luria Bertani broth	USB Corporation
ProteinaseK	Viogene

2.3 Plasmid vectors

Plasmid vectors used in this study are listed as below and the physical maps of the vectors pDrive and pCR 4-TOPO are shown in Fig. 18 and Fig. 19.

Plasmids	Description of use	Antibiotic	Company
pCR 4-TOPO	Cloning PCR products	Ampicillin	Invitrogen
pDrive	Cloning PCR products	Ampicillin	Qiagen

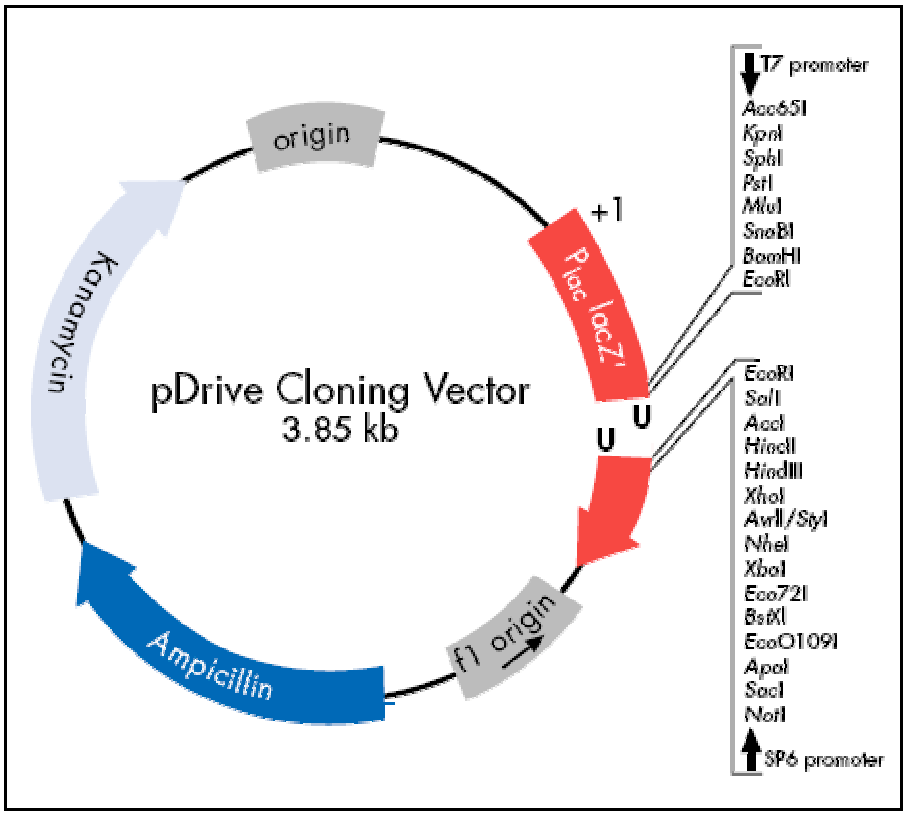

Fig. 18 The physical map of pDrive cloning vector.

The linearized vector has U overhangs and the restriction endonuclease recognition sites of the cloning site are listed.

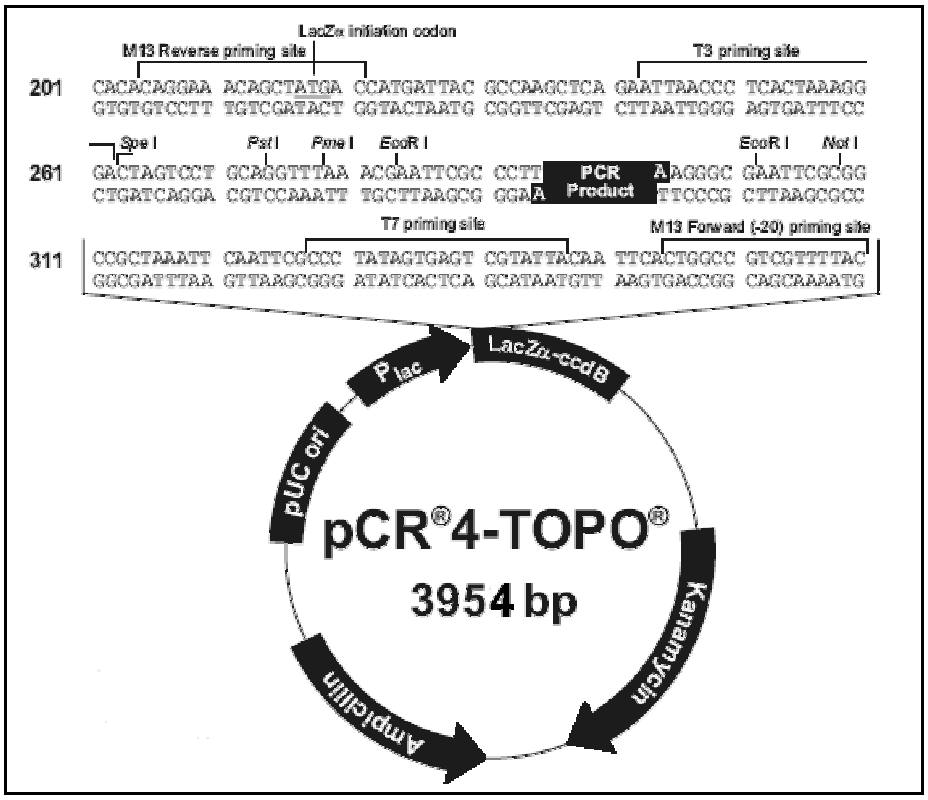

Fig. 19 The physical map of pCR 4-TOPO vector.

The linearized vector has T overhangs and the restriction endonuclease recognition sites of the cloning site are listed.

2.4 Bacterial strains

Two strains of *Escherichia coli* were used and purchased from Invitrogen and Qiagen as follows.

Invitrogen : *E. coli* strain TOP10 [F^- *mcr* A Δ(*mrr-hsd*RMS-*mcr*BC) Φ 80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G],

Qiagen : *E. coli* strain Qiagen EZ [F'::Tn*10*(Tc^r) *pro*A^+B^+ *lacI*^{q}ZΔM15] *recA1 end A1 hsdR 17*($r_{K12}^- m_{K12}^+$) *lac glnV44thi-1 gyrA96 relA1*.

2.5 Primers

The oligonucleotide primers were custom-ordered from Qiagen and Life Technologies or Bio Service Unit, National Science and Technology Development Agency (NSTDA), Thailand.

2.6 Enzymes

Restriction enzymes and other modification enzymes were purchased from New England Biolabs, Invitrogen and Promega.

3. Instruments

Instrument	Model	Company
Amino acid analyzer	Pico-Tag	Waters
Autoclave	ES-315	Tomy
Automated DNA sequencer	ABI PRISM 3100 or 377	Applied Biosystems
Bacteria incubator	1510E	Shel Lab
Balance (4 digits)	AB204-S	Mettler
Balance (3 digits)	GT410	Ohaus
Balance (2 digits)	PG5002-S	Mettler
Capillary LC system		Waters
Centrifuge	5804R	Eppendorf
Centrifuge	5415C	Eppendorf
Gel document	BioDoc-It™System	UVP
Glass-teflon homogenizer		Thomas
Gradient makers	SG 15	Hoefer
Heat box	AccuBlock	Labnet
Imaging densitometer	GS-700	Bio-Rad
Mini IEF cell	111	Bio-Rad
Mighty small™transphor	TE22	Hoefer, Pharmacia
Microcentrifuge	SD220	Clover
Micropipettes		Gilson, Brand, Eppendorf
Oven		Binder
Orbital shaker		GallenKamp
pH meter	713	Metrohm
Protein/peptide sequencer	ABI473A	Applied Biosystems
Protein/peptide sequencer	Precise492HT	Perkin-Elmer
Power supply	1000/500	Bio-Rad
PCR	Master Cycler	Eppendorf
Q-TOF mass spectrometer		Micromass
Real-Time PCR system	7500	Applied Biosystems
Slab gel electrophoresis	AE-6530	Atto
Submarine electrophoresis system	Mupid-ex	Advance
UV-VIS Spectrophotometer	160A	Shimadzu

Instrument	Model	Company
Vortex-mixer	G-560E	Scientific Industries
Water bath	EcoTempTW20	Julabo

Methods

1. Preparation of ovaries

Ovaries from vitellogenic female shrimp were freshly dissected out and washed three times with 50 mM Tris-HCl, pH 7.5 containing 0.15 M NaCl and 1 mM PMSF. The tissues were then homogenized using a hand held glass-teflon homogenizer (10 cm^3, Thomas, PHILA. USA.) in the 25 mM Tris-HCl, pH 7.5 containing 0.15 M NaCl and 1 mM PMSF (TBSP). The homogenate was centrifuged at 2,500 x g for 30 min at 4^oC and the supernatant was further purified. The floating fatty layer and the precipitate from the ovary were discarded.

2. Purification of vitellin

Vt was purified from ovaries in stages 3-4 as reported in Auttarat, *et al*. (2006) with a change in the matrix of the gel chromatography column. The ovarian extract was dialyzed against 25 mM Tris-HCl, pH 7.5-1 mM PMSF (TBP) and then isolated by ion exchange chromatography on a diethylaminoethyl (DEAE)-Sephacel column (1.25 x 15 cm) previously equilibrated with TBP. The column was washed with equilibrating buffer and absorbed Vt was eluted with a linear gradient 0 to 0.5 M of NaCl in the TBP buffer at flow rate of 20 ml/h. Fractions 1.5 ml each were collected and the optical density determined at 280 ($O.D._{280}$) and 474 nm ($O.D._{474}$) for measuring proteins and carotenoid. Fractions with high $O.D._{474}$ were analyzed by 4-10% nondenaturing polyacrylamide gel electrophoresis (nondenaturing PAGE) to determine the fractions that contain Vt without hemocyanin. Selected fractions were pooled and dialyzed against TBP at 4^oC for 12 h and then concentrated by Carboxymethyl (CM)–Cellulose. They were further purified by chromatography on a Sephadex G-200 column (0.8 x 84 cm). The column was eluted with TBSP with a flow rate of 0.1 ml/min. Fractions (0.5 ml each) were collected and measured for $O.D._{280}$ and $O.D._{474}$. The fractions with a high $O.D._{474}$ were pooled, concentrated, dialyzed and tested for purity by 4-10% nondenaturing PAGE.

3. Determination of protein

Protein was determined by the method of Bradford (1976). Twenty µl of each sample was mixed with 1 ml Coomassie plus protein assay reagent kit for 2-3 min and the $O.D._{595}$ was determined using the UV-Vis spectrophotometer 160A. The calibration graph was prepared from 1, 3, 5, 7 and 9 µg of the standard protein (BSA, bovine serum albumin) assay using the same procedure.

4. Polyacrylamide gel electrophoresis (PAGE)

4.1 Nondenaturing PAGE

A (10 x 10 x 0.1 cm) slab gel composed of stacking gel (3 cm) and separating gel (6 cm) was used. Nondenaturing 4-10% gradient PAGE was performed according to the method of Davis (1964). Gel compositions were as below.

Composition	Stacking gel	Separating gel	
	3% (5 ml)	4% (3 ml)	10% (3 ml)
30% Acrylamide-0.8% Bisacrylamide	0.50 ml	0.40 ml	1.00 ml
0.5 M Tris-HCl, pH 6.8	0.63 ml	-	-
1.5 M Tris-HCl, pH 8.8	-	1.50 ml	1.50 ml
10% Ammonium Persulphate	50 µl	30 µl	30 µl
TEMED	5 µl	3 µl	3 µl
Distilled water	3.82 ml	1.07 ml	0.47 ml

Samples were mixed with a sample buffer (0.2 M Tris-HCl, pH 6.8, 8 mM EDTA, 40% glycerol and 0.4% bromophenol blue) in a ratio of sample per dye as 3:1. They were then electrophoresed in the electrode buffer (25 mM Tris - 0.192 M glycine buffer, pH 8.3) at a constant current of 15 mA for 2 h.

4.2 SDS-PAGE

Samples were electrophoresed in a slab gel (10 x 10 x 0.1 cm), composed of stacking gel (3 cm) and separating gel (6 cm). SDS-PAGE was performed according to the method of Laemmli (1970). Compositions of the gels were as below.

Composition	Stacking gel	Separating gel	
	3% (5 ml)	5% (3 ml)	15% (3 ml)
30% Acrylamide-0.8% Bisacrylamide	0.5 ml	0.5 ml	1.5 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml	-	-
1.5 M Tris-HCl, pH 8.8	-	0.75 ml	0.75 ml
0.2 M EDTA	50 µl	30 µl	30 µl
10% SDS	50 µl	30 µl	30 µl
10% Ammonium Persulphate	50 µl	30 µl	30 µl
TEMED	5 µl	3 µl	3 µl
Distilled water	3.10 ml	1.66 ml	0.36 ml

Samples were mixed with a sample buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 20% glycerol in the presence or absence of 1% β-mercaptoethanol and 0.4% bromophenol blue in a ratio of sample per dye as 3:1. They were then boiled for 2 min. Electrophoresis was carried out in the electrode buffer (25 mM Tris - 0.192 M glycine, 0.1% SDS, pH 8.3) at a constant current of 20 mA for 2 h.

4.3 Protein staining

Protein bands were stained with Coomassie Blue (0.02% Coomassie Brilliant Blue R-250 in 50% methanol-7.5% acetic acid) for 1-2 h. Excess dye was removed and the gel was fixed for 30 min by placing in a solution containing 50% methanol and 7.5% acetic acid. The gel background was destained and stored in a destaining solution containing 7% methanol and 5% acetic acid. Purity of Vt was rechecked by silver staining. The gel was fixed in gel with 40% methanol-10% acetic acid for 15 min and subsequently washed twice with 5% ethanol-10% acetic acid for 15 min with gentle shaking. The gel was then transferred to an oxidizing solution and shaked gently for 3 min then washed with deionized water several times until its background became clear. The gel was then transferred into a silver stain solution for 20 min and washed with deionized water for 15 sec. It was then immersed in a developing solution while shaking gently and the solution was changed several times until protein bands appeared in the gel. This reaction was then stopped by 3% acetic acid.

5. N-Terminal amino acid sequence analysis

Purified Vt was subjected to SDS-PAGE and transferred to a polyvinyl difluoride (PVDF) membrane in 10 mM 3-(cyclohexylamino)-1-propane-sulfonic acid (CAPS) containing 1% methanol at a constant current of 500 mA for 40 min. After Coomassie Blue staining and extensive rinsing in distilled water, the 78 kDa, 87 kDa and 104 kDa bands were cut out separately and subjected to N-terminal sequencing by Edman degradation (Edman, 1950) in a protein/peptide sequencer.

6. Analysis of amino acid composition

The amino acid composition of purified Vt was determined following acid hydrolysis with 6 M HCl vapor in a nitrogen atmosphere at 110^{o}C for 24 h. After hydrolysis, samples were analyzed according to the manufacturer's instructions using a Waters Pico-Tag amino acid analyzer.

7. Determination of pI

Isoelectric pH (pI) of purified Vt was determined by agarose gel isoelectric focusing in a Mini IEF Cell using 2% ampholytes in a continuous gradient of pH ranging from 3.5-10 (a mixture of ampholytes pH 3.5-10 : pH 4-5 in a ratio of 1:5). Three μg of purified Vt was applied to the gel plate and focused sequentially at a constant voltage of 100 V for 15 min, 200 V for 15 min and 450 V for 60 min. Thereafter, the gel was fixed in 30% methanol-5% trichloroacetic acid-3.5% sulfosalicylic acid and then immersed in 95% ethanol. After being dried, the gel was then stained with 0.2% Coomassie Blue and destained by 28% ethanol-14% acetic acid. Finally, the gel was air-dried. The pI value of purified Vt was estimated using standard protein markers (carbonic anhydrase, pI 6.6; β-galactosidase, pI 5.1; glucose oxidase, pI 4.2; amyloglucosidase, pI 3.6).

8. Determination of protease activity

Protease activity of purified Vt was determined on sterile petri dishes containing 15 ml of 2% skim milk - 1.5% agar. Wells 5 mm in diameter were cut into the agar by using a cork borer. One hundred and thirty μg and 260 μg of purified Vt were dispensed into 2 separate wells, and the plate was incubated at 37^{o}C for 6 h to allow for proteolysis of milk protein. Proteolysis is indicated by a change in the agar medium from a translucent white color to transparent clear or faintly white color. Proteinase K 100 μg was used as a positive control to validate the assay.

9. Molecular cloning of cDNA encoding Vg from the ovary

9.1 Primer design

Degenerate primers were designed based on the N-terminal amino acid sequence of the 78 and 87 kDa subunits of *P. merguiensis* Vt and conserved sequences of Vg/Vt from other crustacean species using the Clustal W (1.82) computer program (Thompson *et al*., 1994). Many pairs of degenerate oligonucleotide primers were synthesized as shown in Table 5.

Table 5 Nucleotide sequence of oligonucleotide primers used in this study.

Primer	Oligenucleotide sequences
FVg97	5'-GGAGCRGACGTGCCRAGA-3'
RVg1201	5'-CYCCTGTTGCTCKTCCATT-3'
FVg1150	5'-GGTGCWGTMAAGGTSATGGT-3'
RVg2063	5'-CCYWCAAARCGKGCWCCAAT-3'
FVg1951	5'-AACATCATCTATGCTCCHGG-3'
RVg3004	5'-ATGGAAGGATCTGCYTTYTC-3'
FVg2942	5'-CTGAYGTSTTCCGTGCTAAT-3'
RVg4382	5'-AGGTTGTTGAAGCTDACRTG-3'
FVg4315	5'-GCCCTYATCGAGAACAAG-3'
RVg5706	5'-AGATGGAGTCTTGTGGAASA-3'
FVg5500	5'-AAGGGMACMATYGAACTGGA-3'
RVg6631	5'-CTTCCCTCTTSACATTCTTGAA-3'
FVg6539	5'-GAATGGCAGARACTGGMGA-3'
RVg7666	5'-GGTTRCACCTKGAGCGAAT-3'
5GSPVg477	5'-GGAGTTCTGGAAAGCSGAAGCAACGCC C-3'
5GSPVg1018	5'-GSAGAGCCTTGGCAACCAGAGCAGCRGC-3'
3GSPVg7566	5'-GGACCARCAGTGCTCYACCCCTGAGGTT-3'
FVg112	5'-AGATGCTCCACCGAATGC-3'
RVg711	5'-GATTTCCTGCCTGCACTG-3'
F18s rRNA	5'-GCCTACAATGGCTATCACG-3'
R18s rRNA	5'-AACTACGAGCGTTTCAACCG-3'
FVg24	5'-TCCAACCACCATGACGACCTCAACACTCCT-3'
RVg7786	5'-TGAGAGGAGTGGGAGAAGTTTTCCCTTTGG-3'
R, A/G; Y, C/T; K, G/T; W, A/T; M, A/C; S, C/G; H,A/C/T; D, A/G/T.	

9.2 Total RNA extraction

Total RNA was prepared from the ovary (tissue amounts 30-50 mg) at stage 3-4 of ovarian development by RNeasy Protect Mini Kit (Qiagen), using the protocol of the company for animal tissue with some modifications. In brief, the tissue was disrupted and homogenized in buffer containing guanidine isothiocyanate using syringe and needle. The homogenate was then centrifuged at 8,000 x g for 3 min. The supernatant was transfer to a new tube, and 70% of ethanol was added to precipitate the DNA. The solution mixture was apply to RNeasy column, and centrifuged at 8,000 x g for 15 sec. DNA and contaminate proteins were removed from the column by washing with solutions containing guanidine salt and ethanol. The RNA bound to the silica membrane of the column was eluted in the final step with RNase-free water. Total RNA concentration was determined by spectrophotometer at $O.D._{260}$ ($1O.D._{260}$ RNA = 40 μg/ml) and the purity of RNA was determined using the $O.D._{260}/O.D._{280}$ ratio of 1.8-2.1.

9.3 Removal of contaminated DNA with deoxynucleotidase (DNase)

Although the company claims that silica-membrane technology of the RNeasy kit efficiently removed most of the DNA, additional DNA removal is necessary for certain RNA application such as RT-PCR and real-time PCR which is sensitive to very small amounts of DNA. In order to remove residual DNA, the digestion with DNase I was performed prior to RT-PCR or reverse transcription, using the basic protocol recommended by the company with some modifications. The 20 μl reaction mixture was composed of 2 μg of total RNA, DNase I buffer (40 mM Tris-HCl, pH 8.4, 4 mM $MgCl_2$, 100 mM KCl), 40 U of RNaseOut (RNase inhibitor) and 2 U of DNase I. Digestion of the DNA was allowed to occur at room temperature for 15 min before 1 μl of 25 mM EDTA solution was added and the mixture was incubated at 65^{o}C for 10 min to inactivate the DNase. The reaction mixture was kept at -20^{o}C, or immediately used for cDNA synthesis.

9.4 Synthesis and amplification of cDNA fragment by One-Step RT-PCR

Reverse transcription (RT) and Polymerase Chain Reaction (PCR) was performed using the One-Step RT-PCR Kit (Qiagen). Amplifications were carried out by using the pairs of degenerate primers shown in Table 5. The PCR conditions were as follows: reverse transcription at 50^{o}C for 30 min, initial PCR activation step at 95^{o}C for 15 min, followed by 30 cycles of 94^{o}C for 1 min, 55^{o}C for 1 min and 72^{o}C for 2 min, and then a final extension step was performed at

72°C for 7 min. Each PCR reaction was conducted in a Mastercycler (Eppendorf), in a 50 µl reaction mixture using 1 µg of total RNA, 400 µM of each dNTP, RT-PCR buffer (Tris-HCl, KCl, $(NH_4)_2SO_4$, DTT, 2.5 mM $MgCl_2$ pH 8.7), 10 mM forward and reverse primers, and 2 µl of One-Step RT-PCR enzyme mix (including Omniscript™ and Sensiscript™ Reverse Transcriptase and HotStarTaqDNA Polymerase). The PCR products was kept at -20°C, or immediately used for electrophoresis.

9.5 Agarose gel electrophoresis

To determine the size of interesting DNA fragments, agarose gel electrophoresis was performed. The concentration of agarose depended on the size of DNA to be separated. Agarose was melted in TAE buffer (40 mM Tris-acetate buffer, pH 8, 1 mM EDTA). The DNA samples were mixed with 1/6th volume of loading dye [0.25% (w/v) Bromophenol Blue, 4% (w/v) sucrose] before loading into the gel slots that were submerged in TAE solution in an electrophoresis chamber. Electrophoresis was carried out with constant voltage; the running time depended on the size of the DNA to be fractionated. After electrophoresis, the gel was stained with a staining solution containing 0.2 µg/ml of ethidium bromide solution for 5 min and destained with water for 10-15 min. The DNA patterns were observed under UV light.

9.6 Isolation and purification of DNA from agarose by using QIAquick Gel Extraction kit (QIAGEN)

DNA fragments were excised from agarose gels and transferred to a microcentrifuge tube. Three volumes of buffer QG (Guanidine thiocyanate, pH $\leq$ 7.5) were added to 1 volume of the gel (e.g. 100 mg of gel was mixed with 300 µl of buffer QG) and the mixture was incubated at 50°C for 10 min and mixed by vortexing every 3 min during incubation. If the gel slice contained < 500 bp or > 4 kb DNA fragment, 1 gel volume of isopropanol was added to the mixture before transfer to a QIAquick column inserted in a 2 ml collection tube. The column was centrifuged at 8,000 x g for 1 min at room temperature. The flow-through solution was discarded and the column was washed with 0.75 ml of PE buffer (containing ethanol) followed by centrifugation at 8,000 x g for 1 min. The flow-through was discarded and the column was centrifuged for an additional 1 min. In order to collect the eluted DNA, the column was placed on a 1.5 ml microcentrifuge tube and 30 µl of EB buffer (10 mM Tris-HCl, pH 8.5) was applied to

the column. Then the column was left at room temperature for 1 min before centrifugation at 8,000 x g for 1 min. The eluted DNA was stored at 4°C until used.

9.7 Cloning of DNA fragments using Qiagen® PCR cloning kits

The ligation reaction was performed by mixing the following components: 4 µl of eluted DNA, 1 µl of pDrive cloning vector, and 5 µl of 2 x Ligation Master Mix. The reaction was briefly mixed and incubated at 4°C for 30 min. Then the ligation mixture was used to transform competent cells.

9.8 Transformation of *E. coli*, chemically competent EZ cells

A volume of 100 µl of *E. coli* competent EZ cells (Qiagen) was mixed gently with 2 µl of ligation mixture. The mixture was left on ice for 30 min, heat shocked at 42°C for 30 sec, and immediately transferred to ice. The resulting cells were mixed with 250 µl of room temperature SOC medium and incubated at 37°C for 1 h with shaking. The transformed culture was spread on Luria Bertani (LB) agar plates containing 100 µg/ml of ampicillin, 50 µM isopropylthiogalactoside (IPTG), 80 µg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) and incubated at 37°C for 16 h.

9.9 Plasmid DNA extraction

9.9.1 Plasmid DNA extraction using QIAprep Spin Miniprep kit

E. coli plasmids were prepared following the alkaline lysis procedure published by Birnboim and Doly (1979), using the QIAprep Spin kit (Qiagen). An overnight culture of the *E. coli* was prepared in the presence of antibiotic (100 µg/ml culture of ampicillin). The cells were collected by centrifugation, and suspended in buffer P1 (50 mM Tris-HCl, 10 mM EDTA, pH 8.0) containing RNase A. Then, the bacterial membrane was broken down with a lysis buffer for 5 min at room temperature. Thereafter, the lysate was neutralized and adjusted to be in a chaotropic salt (1.6 M guanidine hydrochloride) for binding to silica with neutralizing buffer consisting of 4 M guanidine HCl, 0.5 M potassium acetate, pH 4.2. The precipitated chromosomal DNA and cell debris were removed by centrifugation. The supernatant containing the plasmid was purified by adsorption of the DNA to the surface of a silica filter. Bound plasmids were then freed from salts, proteins and other cellular impurities by washing with 20 mM NaCl and 2 mM Tris-HCl, pH 7.5 in 80% methanol. The plasmid was finally eluted with 10 mM Tris-HCl, pH 8.5. The concentration of DNA was determined by spectrophotometer at $O.D._{260}$ (1 $O.D._{260}$ DNA = 50 µg/ml) and the purity of isolated

DNA was judged by $O.D._{260}/O.D._{280}$ (pure DNA solutions have an $O.D._{260}/O.D._{280}$ ratio of 1.9-2.0), and stored at -20°C until used.

9.9.2 Plasmid DNA extraction by alkaline lysis with SDS

An overnight bacterial culture was centrifuged at 12,000 x g for 1 min and the cell pellet was completely resuspended in 200 µl of ice-cold 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, (pH 8.0). To lyse cells, 400 µl of 0.2 N NaOH, 1% (w/v) SDS was added and gently mixed by inverting the tube 4-6 times. Tubes were left at room temperature for 5 min. To the cell lysate was added 300 µl of ice-cold 5 M potassium acetate, (pH 5.8) and kept on ice for 10 min. The cell lysate was precipitated by centrifugation at 15,294 x g for 5 min and the supernatant was transferred to a new tube. To obtain DNA, 2 volumes of room temperature isopropanol were added and the mixture was left at room temperature for 2 min. The mixture was centrifuged at 12,000 x g for 5 min and the supernatant was gently removed by decanting. The DNA pellet was washed with 1 ml of 70% ethanol and the tube was inverted several times. To precipitate DNA, the mixture was centrifuged at 17,949 x g for 5 min and the supernatant was removed by gentle aspiration. The DNA pellet was briefly air-dried and dissolved in sterile water. Then the DNA solution was treated by RNase I (BioLab).

9.10 Determining recombinant clones by enzymatic digestion

The restriction endonuclease *Eco*R I binds and cleaves double stranded DNA at the specific sequence 5'-G↓AATT C-3' with the other strand of the cleavage site being 3'-C TTAA ↑G-5'. The digestion reaction was composed of 1 µg of plasmid DNA, 5 U of *Eco*R I, reaction buffer (50 mM Tris-HCl, pH 8.0 - 10 mM $MgCl_2$ - 100 mM NaCl) and water in a total volume of 10 µl, and was incubated at 37°C for 1 h. The digestion products were analyzed by agarose gel electrophoresis.

9.11 Automated DNA sequencing

All inserted plasmid fragments or PCR products were sequenced on ABI Prism 3100 Genetic DNA Sequencer (Applied Biosystems, Perkin-Elmer) by the Bioservice Unit (BSU), National Science and Technology Development Agency (NSTDA) or were sequenced on ABI Prism 377 Automated DNA Sequencer (Applied Biosystems) by Scientific Equipment Center, Prince of Songkla University (PSU) based on the dideoxynucleotide chain termination method of Sanger *et al* (1977). The plasmid sequencing primers (T7, SP6 and T3), internal primers and a Dye

Terminator Cycle Sequencing Kit (Applied Biosystems) were used to sequence both strands of the insert in the plasmid.

9.12 5' and 3' Rapid amplification of cDNA ends by GeneRacer™ kit

The 5' and 3' ends were generated according to the manufacturer's instructions as described in the GeneRacer™ kit (RLM-RACE) (Invitrogen) protocol. A Vg cDNA was synthesized using total RNA prepared from ovary. First, 5 μg of total RNA was treated with calf intestinal phosphatase (CIP) at 50°C for 1 h to remove the 5' phosphate. The RNA was extracted using phenol extraction and precipitated by ethanol. The 5' cap structure was removed from intact dephosphorylated mRNA by tobacco acid pyrophosphatase (TAP) at 37°C for 1 h and again phenol extraction and ethanol precipitation was performed. The full-length mRNA was ligated to the GeneRacer RNA oligo sequence at the 5' end with T4 RNA ligase at 37°C for 1 h. The ligated mRNA used as template in first-strand cDNA synthesis was reverse-transcribed by SuperScript II RT and using the GeneRacer Oligo dT Primer for 3' ends or random primers for 5' ends at 42°C for 50 min. PCR amplifications were performed using Taq DNA polymerase (Promega) with the reverse primer 5GSPVg1018 (Table 5) and GeneRacer 5' Primer (Invitrogen, 5'-CGACTGGAGCACGAGGACACTGA-3') for 5' RACE while using the forward primer 3GSPVg7566 (Table 5) and GeneRacer 3' Nested Primer (Invitrogen, 5'-CGCTACGTAACGGCATGACAGTG-3') for 3' RACE. The reaction cycle for both 5' and 3' RACE consisted of incubations at 94°C for 1 min, 68°C for 1 min, and 72°C for 2 min. The final elongation step was performed at 72°C for 10 min. A nested PCR amplification was then conducted with a 5 μl reaction mixture of the first 5' RACE (using PCR products as templates) including the reverse primer 5GSPVg477 (Table 5) and GeneRacer 5' Nested Primer (Invitrogen, 5'-GGACACTGACATGGACTGAAGGAGTA-3') in the same condition as for 5' RACE.

9.12.1 Cloning the DNA fragment into pCR 4-TOPO vector

The purified cDNA of interested was ligated with pCR 4-TOPO vector (Invitrogen) according to the manufacturer's instruction. The reaction mixture (6 μl), containing 4 μl PCR product, 1 μl salt solution and 1 μl TOPO vector was briefly mixed and incubated at 22-23°C for 30 min.

9.12.2 Transformation of One Shot TOP 10 competent *E. coli*

A volume of 50 µl of *E. coli* competent cells was mixed gently with 2 µl of ligation mixture. The mixture was left on ice for 30 min, heat shocked at 42°C for 30 sec, and immediately transferred to ice. The resulting cells were mixed with 250 µl of room temperature SOC medium and incubated at 37°C for 1 h with shaking. The transformed culture was spread on LB agar plates containing 100 µg/ml of ampicillin and incubated at 37°C for 16 h. The plasmid was isolated, determined the recombinant clone by cutting with appropriate restriction enzyme and sequenced, as previously described in the method sections 9.9, 9.10 and 9.11.

9.13 Open reading frame (ORF) amplification of Vg cDNA from the ovary

Total RNA from the ovary of vitellogenic females was also extracted with RNeasy Protect Mini Kit and treated with DNase I as described in method sections 9.2 and 9.3. First-strand cDNA was synthesized by reverse transcription using SuperScript III reverse transcriptase (Invitrogen) to transcribe poly(A)$^+$ RNA with oligo-d(T)$_{20}$ as the primer in reaction mixture containing 5 µg of total RNA, 50 mM Tris-HCl, pH 8.4, 75 mM KCl, 3 mM $MgCl_2$, 50 µ M of oligo-d(T)$_{20}$ primer, 20 mM each of dATP, dCTP, dGTP, and dTTP. The reaction mixture was heated to 65°C for 5 min before addition of 400 U of SuperScript III reverse transcriptase. Two µl of the first strand cDNA solution was subjected to PCR with a high fidelity platinum Taq DNA polymerase (Invitrogen). The PCR reactions were initiated at 94°C for 30 sec to denature the template and activate the enzyme, followed by 35 cycles of amplification comprising denaturing at 94°C for 45 sec, annealing at 65°C for 30 sec and extension at 68°C for 10 min. A final extension step was performed at 72°C for 11 min. The forward primer FVg24 and the reverse primer RVg7786 were used to amplify the 5' to 3' region of *P. merguiensis* Vg. Amplification was carried out in a Mastercycler (Eppendorf). PCR products were analyzed, extracted and sequenced as in method sections 9.5, 9.6 and 9.11.

9.14 Full-length cDNA construction

Since the full-length Vg cDNA was too long to sequence completely in a single reaction, many pairs of oligonuclotide primers were designed based on conserved sequences of other shrimp Vg/Vt as shown in Table 5. One-Step RT-PCR was performed with these primers. The clones obtained were again subcloned and nucleotide sequenced. Contiguous cDNA sequence data with individual clones were obtained by primer-walking that repeated the RT-PCR and

cloning steps using a new pair of primers from Table 5. The 5' and 3' ends of cDNA sequences were established using rapid amplification of cDNA ends techniques. After sequencing, overlapping nucleotide sequences were used for reconstruction of a full-length Vg cDNA. The construction of Vg sequence in ovary was confirmed by amplifying and sequencing a large RT-PCR fragment containing the entire ORF of Vg.

10. Bioinformatics analysis

10.1 Primary and secondary structure analysis

The Vg cDNA sequence of *P. merguiensis* (accession number AY499620) was translated to amino acid sequence and used as query for primary, secondary structure and post-translational modification prediction. Signal peptide was predicted using SignalP 3.0 (Bendtsen *et al*., 2004). Post-translational modifications were predicted by several tools and servers. The DictyOGlyc 1.1 (Gupta *et al*., 1999) server was used to predict O-glycosylation sites of GlcNAc (N-acetylglucosamine). Phosphorylation sites for serine, threonine and tyrosine were predicted by NetPhos 2.0 (Blom *et al*., 1999). Sulfinator software (Monigatti, 2002) was used to predict tyrosine sulfation sites. Amino acid composition, theoretical pI and molecular weight were calculated by ProtParam (Gasteiger *et al*., 2005). Secondary structure of Vg precursor was predicted by the Porter program (Pollastri and McLysaght, 2005).

10.2 Sequences comparison and phylogenetic tree

The deduced amino acid sequence of Vg from *P. merguiensis* (Protein ID; AAR88442) was used as query for finding all known Vg from other species, related genes and conserved domains; programs used were PSI-and PHI-BLAST (Position-specific iterated and pattern-hit initiated BLAST) (Altschul *et al*., 1997) network server from NCBI (National Center for Biotechnological Information, Bethesda, MD; http://www.ncbi.nlm.nih.gov) database or Protein Families Data base (Pfam) (Finn *et al*., 2006) for further analysis. Vg sequences were multiply aligned by Clustal W (Thompson *et al*., 1994) or MUSCLE (Edgar, 2004**)** and pairwise aligned by BioEdit program version 7.0.1(Hall, 1999b). A phylogenetic tree was generated with MEGA software version 3.1 (Kumar *et al*., 2004) using the neighbor-joining method (Saito and Nei, 1987). Bootstrap values were calculated by MEGA. The sequences used for constructing the tree depended on conserved regions. Three phylogenetic trees were constructed: one is all deduced

Vg sequences from decapod crustacean; the second tree was constructed from N-terminal regions and the last one was constructed from C-terminal regions. Species names and GenBank accession numbers for the sequences used to generate Vg tree from decapod crustacean are shown in Table 6. The N-terminal (about 600 residues) and C-terminal (about 150 residues) sequence of Vgs and related homologous proteins were used to construct the N- and C-terminal tree. Details of sequences used in the trees are shown in Table 7. The sequence of *Ciona intestinalis* (estchrCiona) was derived from EST Ciona genome (http://genome.jgi-psf.org/Cioin2/ Cioin2.home.html), accession no. 08q1008 (data of access: August 2006).

Table 6 List of Vgs from decapod crustaceans used to construct the phylogenetic tree of full-length sequence.

No	GenBank	Tree label	Species name	Common Name
1	AY499620	*P. merguiensis*	*Penaeus merguiensis*	Banana shrimp
2	AY051318	*P. semisulcatus*	*Penaeus semisulcatus*	Green tiger shrimp
3	AY321153	*L. vannamei*	*Litopenaeus vannamei*	Pacific white shrimp
4	DQ288843	*P. monodon*	*Penaeus monodon*	Black tiger shrimp
5	AB033719	*M. japonicus*	*Marsupenaeus japonicus*	Kuruma shrimp
6	AY103478	*Vg1 M. ensis*	*Metapenaeus ensis*	Greasy back shrimp
7	AF548364	*Vg2 M. ensis*	*Metapenaeus ensis*	Greasy back shrimp
8	AY530205	*Vg3 M. ensis*	*Metapenaeus ensis*	Greasy back shrimp
9	AB117524	*P. hypsinotus*	*Pandalus hypsinotus*	Coonstriped shrimp
10	AB056458	*M. rosenbergii*	*Macrobrachium rosenbergii*	Giant fresh water prawn
11	AF306784	*C. quandricarinatus*	*Cherax quadricarinatus*	Cray fish
12	AY724676	*C. feriatus*	*Charybdis feriatus*	Red Crab
13	DQ000638	*P. triuberculatus*	*Portunus triuberculatus*	Japanese blue crab

Table 7 Details of sequences used to construct phylogenetic trees of N-terminal and C-terminal regions are shown.

No	GenBank	Proteins Name	Species Name	Common Name	Tree Label
1	AY499620	Vitellogenin	*Penaeus merguiensis*	Banana shrimp	VgP.merguiensis
2	AY051318	Vitellogenin	*Penaeus semisulcatus*	Green tiger shrimp	VgP.semisulcatus
3	AY321153	Vitellogenin	*Litopenaeus vannamei*	Pacific white shrimp	VgL.vannamei
4	DQ288843	Vitellogenin	*Penaeus monodon*	Black tiger shrimp	VgP.monodon
5	AB033719	Vitellogenin	*Marsupenaeus japonicus*	Kuruma shrimp	VgM.japonicus
6	AF548364	Vitellogenin	*Metapenaeus ensis*	Greasy back shrimp	VgM.ensis
7	AF306784	Vitellogenin	*Cherax quadricarinatus*	Crayfish	VgC.quandricarinatus
8	AB117524	Vitellogenin	*Pandalus hypsinotus*	Constriped shrimp	VgP.hypsinotus
9	AB056458	Vitellogenin	*Macrobrachium rosenbergii*	Giant fresh water prawn	VgM.rosenbergii
10	DQ000638	Vitellogenin	*Portunus trituberculatus*	Japanese blue crab	VgP.triuberculatus
11	AY724676	Vitellogenin	*Charybdis feriatus*	Red Crab	VgC.feriatus
12	AJ130944	Apolipophorins	*Locusta migratoria*	Migratory locust	ApoLocus
13	U57651	Apolipophorins	*Manduca sexta*	Tobacco hornworm	ApoManduca
14	U62892	Retinoid- and fatty Acid-binding glycoprotein	*Drosophila melanogaster*	Fruit fly	RatinDrosophila
15	NM_019287	Apolipoprotein B	*Rattus norvegicus*	Norway rat	ApobRattus
16	M14162	Apolipoprotein B	*Homo sapiens*	Human	HumanApob100
17	AB179781	Vitellogenin	*Galaxea fascicularis*	Gonochoric coral	VgG.fascicularis

Table 7 (Continued)

No	GenBank	Proteins Name	Species Name	Common Name	Tree Label
18	BC038263	Apolipoprotein B	*Mus musculus*	House mouse	ApobMus musculus
19	XM583646	Apolipoprotein B	*Bos Taurus*	Cattle	ApobB.taurus
20	AB084783	Vitellogenin	*Crassostrea gigas*	Pacific oyster	VgC.gigas
21	XM695885	Apolipoprotein B	*Danio rerio*	Zebrafish	ApobD.rerio
22	U07055	Vitellogenin	*Fundulus heteroclitus*	Killifish	VgF.heteroclitus
23	AB181838	Vitellogenin	*Pagrus major*	Red seabream, fish	VgP.major
24	AY045719	Vitellogenin	*Larus argentatus*	Herring gull, bird	VgL.argentatus
25	AB081299	Vitellogenin	*Sillago japonica*	Fish	VgS.japonica
26	AB181833	Vitellogenin	*Verasper moseri*	Mosquitofish	VgV.moseri
27	AF284034	Vitellogenin	*Melanogrammus aeglefinus*	Haddock fish	VgM.aeglefinus
28	X13607	Vitellogenin	*Gallus gallus*	Chicken	VgG.gallus
29	X92804	Vitellogenin	*Oncorhynchus mykiss*	Rainbow trout	VgO.mykiss
30	1LSHA	Vitellogenin	*Ichthyomyzon unicuspis*	Silver Lamprey, fish	VgI.unicuspis
31	AB092605	Vitellogenin	*Xenopus laevis*	African clawed frog	VgX.laevis
32	U35449	Vitellogenin	*Oscheius brevesophaga*	Nematode	VgO.brevesophaga
33	X56213	Vitellogenin	*Caenorhabditis elegans*	Nematode worm	VgC.elegans
34	XM419979	Apolipoprotein B	*Gallus gallus*	Chicken	ApobG.gallus
35	NM001011578	Vitellogenin	*Apis mellifera*	Honey bee	VgA.melliferal

Table 7 (Continued)

No	GenBank	Proteins Name	Species Name	Common Name	Tree Label
36	AF026789	Vitellogenin	*Pimpla nipponica*	Parasitoid wasp	VgP.niponicus
37	AY373377	Vitellogenin	*Aedes aegypti*	Yellow fever mosquito	VgA.aegypti
38	U97277	Vitellogenin	*Riptortus clavatus*	Bean bug	VgR.clavatus
39	AB114859	Vitellogenin	*Daphnia magna*	-	VgD.magna
40	AF089867	Clottable protein	*Penaeus monodon*	Black tiger shrimp	ClotP.monodon
41	AF102268	Clottable protein	*Pacifastacus leniusculus*	Signal crayfish	ClotP.leniusculus
42	AJ005115	Vitellogenin	*Blattella germanica*	German cockroach	VgB.germanica
43	X78567	Microsomal triglyceride	*Bos taurus*	Cattle	MTPB.taurus
44	NM_170767	Vitellogenin	*Danio rerio*	Zebrafish	VgD.rerio
45	NM_000253	Microsomal triglyceride	*Homo sapiens*	Human	MTPHuman
46	X04385	Von Willebrand factor	*Homo sapiens*	Human	vWFHuman
47	U66246	Von Willebrand factor	*Canis familiaris*	Dog	vWFDog
48	AF539800	Von Willebrand factor	*Mus musculus*	House mouse	vWFMouse
49	AB034804	Vitellogenin	*Periplaneta Americana*	American cockroach	VgP.american
50	U35449	Vitellogenin	*Oscheius tipulae*	Nematode	VgO.tipulae
51	M72980	Vitellogenin	*Anthonomus grandis*	Boll weevil	VgA.grandis
52	D13160	Vitellogenin	*Bombyx mori*	Domestic silkworm	VgB.mori
53	U07615	Mucin	*Rattus norvegicus*	Norway rat	MucinRat
54	L21998	Mucin	*Homo sapiens*	Human	MucinHuman

10.3 Tertiary structure modeling of Vg N-terminal region

The entire deduced amino acid sequence of Vg from *P. merguiensis* (Protein ID: AAR88442) was used for finding a template for 3D modeling. The x-ray crystal structure of lamprey lipovitellin [Protein Data Bank (PDB) entry 1LSH_A], refined to an *R*-value of 0.19 at 2.8 A° resolution (Thompson and Banaszak, 2002) was used as a template for the modeling of N-terminal region of *P. merguiensis* Vg from position 42 to 938 or 941 (~900 residues). The homology comparative method was used for tertiary structure modeling by using 3 prediction servers, 3D-JIGSAW (Bates *et al*., 2001), EasyPred (Lambert *et al*., 2002) and Modeller (Sali *et al*., 1995). The results, as pdb files, were visualized by the programs Swiss-Pdb Viewer (Guex and Peitsch, 1997) and RasMol version 2.6 (Sayle, 1995).

11. Tissue specific expression of Vg mRNA

Total RNA from various tissues (hepatopancreas, ovary, muscle, heart and intestine) of vitellogenic *P. merguiensis* females and the hepatopancreas from mature males was extracted and pre-treated with DNAse I for each 1 μg of total RNA as previously described in method sections 9.2 and 9.3. Thereafter, 1 μg of the total RNA from each tissue was used in the One-Step RT-PCR Kit (Qiagen). Amplifications were carried out using Vg specific primers FVg112 and RVg711. RT-PCR conditions were as follows: reverse transcription at 50^{o}C for 30 min, initial PCR activation step at 95^{o}C for 15 min followed by 30 cycles of 94^{o}C for 1 min, 58^{o}C for 1 min and 72^{o}C for 2 min. A final extension step was performed at 72^{o}C for 5 min. PCR products were subsequently analyzed as per section 9.5. RT-PCR amplification of *P. merguiensis* 18s rRNA was performed as internal control. This used F18s rRNA and R18s rRNA primers with the PCR being carried out in the same tube of each corresponding sample.

12. Real-time PCR

12.1 Quantification of Vg mRNA in the hepatopancreas and ovary at various stages of ovarian development

Banana shrimps were acquired and the stage of ovarian development and GSI were calculated as described in section 1. Total RNA was prepared from the ovary and hepatopancreas at differing stages of ovarian development using Rneasy Protect Mini Kit,

determining the concentration of total RNA and pre-treating with DNAse I (1 U/μg of total RNA) as previously described in method sections 9.2 and 9.3. First-strand cDNA synthesis by reverse transcription (RT) was performed using SuperScript III Reverse Transcriptase (Invitrogen) to transcribe Vg with oligo-$d(T)_{12-18}$ as the primer and 18s rRNA with R18s rRNA as the primer. The reaction was incubated for 5 min at 65°C followed by a 2 min incubation on ice, containing 1 μg of total RNA , 500 ng of oligo-$d(T)_{12-18}$ primer or 20 mM of R18s primer, 10 mM each dATP, dCTP, dGTP, dTTP. First-Strand Buffer (50 mM Tris-HCl, pH 8.4, 75 mM KCl, 3 mM $MgCl_2$), 0.1 M dithiothreitol (DTT), 40 U of RNaseOut and 200 U of SuperScript III Reverse Transcriptase (Invitrogen) were added to bring the final reaction volume to 20 μl and then incubated at 52°C for 60 min. The reaction was terminated with a 15 min incubation at 70°C. One μl from 20 μl of the first strand cDNA solution was subjected to real-time PCR. Vg primers and probe were selected from the 3' end region of Vg cDNA of *P. merguiensis* (GenBank accession number AY499620), 18s rRNA probe and primers were designed based on 18s rRNA sequence (GenBank accession number DQ501247). ABI-Primer Express program by PE Applied Biosystems (Perkin Elmer) was used to design primer and probe for both Vg and 18s rRNA. This program was used to select probe and primer sets with optimal melting temperatures, secondary structure, base composition, and amplicon lengths for use in real-time PCR. The set of Vg comprised TaqMan MGB probe (5'-[6FAM]CTGTTGACATGTTTACATTGC [NFQ] phosphate-3', nucleotides 7801-7821), sense primer (5'-GAGAGGAGTGGGAGAAGTTTTCC-3', nucleotides 7758-7780), and antisense primer (5'-CCAAAATCTGAACGAGTCGTCAACT-3', nucleotides 7825-7848). The set of 18s rRNA comprised TaqMan MGB probe (5'-[6FAM]AATACTGTTGCGAGCCCC[NFQ] phosphate-3'), sense primer (5'-GCAGGCGCGCAAA TTACC-3'), and antisense primer (5'-TGCGAGGCCCCGTTC-3'). The quantitative PCR mixture consisted of 1 μl of cDNA sample, 900 nM of each primer, 250 nM probe and 12.5 μl of TaqMan Universal PCR Master Mix consisting of Ampli Taq Gold DNA Polymerase, AmpErase UNG, dNTP with dUTP, passive reference, and optimized buffer components (PE Applied Biosystems) in a final volume of 25 μl. Quantities of mRNA in samples were quantified by comparison with a reference standard curve derived from known amounts of the target gene (Vg or 18s rRNA). The target sequences were ligated into pCR 4-TOPO vector and sub-cloned to OneShot Top10 competence cells (Invitrogen) as described in section 9.12 and the concentrations of the standards were quantified by measuring

the absorbance at 260 nm. The target and the concentrations of the standards were calculated taking into consideration the combined size of the vector and the different target sequences. The standards ranged between $1.2x10^3$-$1.2x10^8$ copies of Vg and between $7.4x10^5$-$7.4x10^9$ copies of 18s rRNA gene. The standard curve was linear over five or six orders of magnitude with the linear correlation between the threshold cycles and 18s rRNA levels or Vg cDNA levels being over 0.99. Amplification was carried out in Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems) under the following conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec, and 60°C for 1 min. Results were analyzed with the Applied Biosystems 7500 Sequence Detection version 1.2.3 software (PE Applied Biosystems).

To compare Vg expression in different samples at different stages of ovarian development, the relative abundance of Vg mRNA was normalized against the 18s rRNA as an endogenous reference. The use of ribosomal RNA (28s rRNA and 18s rRNA) is recommended as internal standards for mRNA quantification studies (Thellin *et al*., 1999). The relative value at stage 1 of each tissue (ovary and hepatopancreas) was arbitrarily designated as a calibration sample (abundance set to 1X) to normalize the relative Vg mRNA of the other stages, allowing the fold increase in expression to be determined.

12.2 Statistical analysis

All values are expressed as the mean ± standard error (S.E.) of four samples in each stage of ovarian development. To determine the statistical difference among different stages, multiple group comparisons were performed by one-way analysis of variance (ANOVA). When significant differences between groups were found ($P < 0.05$), multiple comparison tests of Scheffe were used to identify differences between the means by using SPSS (Statistical Package for the Social Science) program version 11.0 software. Values were significantly different at $P < 0.05$ and $P < 0.01$ (at level of 95 and 99% confidence).

13. Molecular cloning of cDNA encoding Vg from the hepatopancreas at the 3'end

13.1 Cloning Vg cDNA from hepatopancreas

Total RNA from the hepatopancreas of vitellogenic female was extracted with RNeasy Protect Mini Kit, and treated with DNase I. Reverse transcription and polymerase chain reaction was performed using One-Step RT-PCR Kit (Qiagen), as previously described in method

sections 9.2, 9.3 and 9.4. A pair of oligonucleotide primers used to amplify Vg of *P. merguiensis* from ovary in the 3' region was used in this experiment. PCR amplification was carried out by using the pair of FVg6539 as forward primer and RVg 7666 as reverse primer. The PCR conditions and reaction was same as described in section 9.4, using 1 μg of total RNA. PCR products were resolved by electrophoresis on a 1% agarose gel, stained, extracted, ligated into the pCR 4-TOPO vector and transformed to OneShot Top10 competent *E. coli* as described in sections 9.5, 9.6, 9.12.1, 9.12.2, respectively. Plasmids were isolated as described in section 9.9 and digested with the appropriate restriction enzyme (*EcoR* I) as described in section 9.10 and then analyzed by 1% agarose gel electrophoresis. The plasmid which containing the PCR insert was subsequently nucleotide sequenced as described in section 9.11.

13.2 Nucleotide-amino acid differences analysis

The same region of Vg sequences from the hepatopancreas and ovary were aligned pairwise and analyzed using the BioEdit version 7.0.1 (Hall, 1999b). The pattern of nucleotide and amino acid differences were analyzed using a custom-writen python program. Given two aligned input sequences, it determines the number of nucleotide differences between them, and then generates a user-specified number of "laboratory error" pseudo-sequences with the same number of nucleotide differences to the first sequence. The output consists of a tally of the number of amino acid differences between the first sequence and each pseudo-sequence (program available at www.acmc.uq.edu.au/DETYA/ibj/). The program was run with the observed ovarian and heptopancreas sequences as input, and set to create 10,000 pseudo-sequences twice.

14. Proteomics analysis at differing GSI values of ovarian development

14.1 Sample preparation

Ovaries at differing GSI values (0.995 ± 0.104, 2.998 ± 0.175 and 8.273 ± 0.092) were dissected out from each shrimp. Ovarian tissues from each GSI value was homogenized separately on ice in three volumes of 25 mM Tris-HCl, pH 7.5 containing 1 mM phenylmethylsulfonylfluoride (PMSF), then were centrifuged for 30 min at 13,000 x g. The supernatant was removed for further experiments and stored at -80^{o}C until analysis. Protein was determined by using the method of Bradford as described in section 3.

14.2 Two-dimensional gel electrophoresis (2-DE)

Proteins from various GSI of ovarian development were prepared for separation in the first dimension by isoelectric focussing. Proteins from each GSI value was desalted freshly and dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 10 mM dithiothreitol (DTT) 0.8% ampholyte pH 3-10, 40 μM bestatin and 15 μM pepstatin A) and rehydration buffer (7 M Urea, 4% CHAPS, DTT, immobilized pH gradients (IPG) buffer 3-10 non-linear (NL)). Samples were applied to in-gel rehydration of 70 mm, nonlinear pH 3-10, IPG gel strips (Amersham Bioscience) overnight. The first dimension of electrophoresis was performed at 100 volt for 30 min and then gradient 200 V to 3,500 V for 1 h at room temperature, using a Pharmacia LKB Multiphor II system.The IPG strips were equilibrated in two steps of equilibration buffer. The first step used 50 mM Tris-HCl buffer, pH 6.8, 6 M urea, 30% glycerol, 1% SDS, and 1% DTT, while 2.5% iodoacetamide replaced DTT in the second step and then applied to the second-dimension 15% SDS polyacrylamide gels (10 x 10.5 x 0.07 cm). Electrophoresis was performed in a Hoefer system at 20 mA for 3 h at room temperature. The protein spots were visualized by staining with Coomassie Blue.

14.3 Tryptic in-gel digestion of 2-DE sports

The protein spots were excised and transferred to 0.5 ml microcentrifuge tubes. Fifty ml of 0.1 M NH_4HCO_3 in 50% acetonitrile was added and then the gel was incubated 3 times for 20 min at 37°C. The solvent was discarded and the gel particles were dried completely by Speed Vac. Reduction and alkylation was performed by swelling the gel pieces in 50 ml buffer solution (0.1 M NH_4HCO_3, 10 mM DTT, and 1 mM ethylamine diamine tetraacetic acid (EDTA) and incubating at 60°C for 45 min. After cooling, the excess liquid was removed and quickly replaced by the same volume of freshly prepared 100 mM iodoacetamide in 0.1 M NH_4HCO_3 solution. The reaction was incubated at room temperature in the dark for 30 min. The iodoacetamide solution was removed and the gel pieces were washed with 50% acetonitrile in water, 3 times for 10 min each, and the gel pieces were completely dried. Aliquots (1 mg trypsin /10 ml 1% acetic acid) of trypsin were prepared and stored at -20^{o}C. Fifty ml of digestion buffer (0.05 M Tris HCl, 10% acetonitrile, 1 mM $CaCl_2$, pH 8.5) and 1 ml of trypsin were added to the gel pieces. After incubating the reaction mixture at 37^{o}C overnight, the digestion buffer was

removed and saved. The gel pieces were then extracted by adding 50 ml of 2% freshly prepared trifluoroacetic acid and incubating for 30 min at 60^{o}C. The extract and the saved digestion buffer were finally pooled and dried.

14.4 Protein identification by LC/MS/MS

Liquid chromatography tandem mass spectrometry (LC/MS/MS) analysis were carried out using a capillary LC system (Waters) coupled to a quadrupole-Time of flight (Q-TOF) mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray ion-source working in the nanoelectrospray mode. Glufibrinopeptide was used to calibrate the instrument in MS/MS mode. The tryptic peptides were concentrated and desalted on a 75 μM ID x 150 mm C18 PepMap column (LC Packings, Amsterdam). Eluents A and B were 0.1% formic acid in 97% water, 3% acetonitrile and 0.1% formic acid in 97% acetonitrile respectively. Six μl of sample was injected into the nanoLC system, and separation was performed using the following gradient: 0 min 7% B, 35 min 50% B, 45 min 80% B, 49 min 80% B, 50 min 7% B, 60 min 7% B. The database search was performed with ProteinLynx 2.1 (Micromass) screening SWISS-PROT and NCBI. For some proteins that were hard to identify, Mascot search tool available on the Matrix Science site screening NCBI-nr was used.

14.5 Two-dimensional gel scanning and image analysis

After electrophoresis, proteins were visualized by Coomassie Brilliant Blue R-250 staining as described in section 4.3. The gels were scanned using an GS-700 Imaging Densitometer (Bio-Rad). The Melanie II system (Bio-Rad) was used for computer analysis.