RESEARCH ARTICLE

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The Effect of Eyestalk Extract on Vitellogenin Levels in the Haemolymph of the Giant Tiger Prawn *Penaeus monodon*

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ABSTRACT: Competitive ELISA using a combination of monoclonal antibodies specific to vitellin subunits was used to monitor the fluctuation of haemolymph vitellogenin levels in *Penaeus monodon* during ovarian development induced by bilateral eyestalk-ablation and to monitor the effect of eyestalk extract injection in prawn with developing ovary. The haemolymph vitellogenin levels were undetectable in the prawn with ovary at the resting stage but elevated sharply when the ovary began to develop, remained high during the ovary developing into ripe stage then fell to the low levels before spawning and spent stages. The result from injection of eyestalk extract into prawn with developing ovary revealed that haemolymph vitellogenin levels elevated sharply within 2 hr, reached the maximal levels and remained high during 4-10 hr, then declined slightly at 24 hr. This response directly depended on the amount of injected eyestalk extract and the response was species specific. The application of eyestalk extract from *Metapenaeus affinis* demonstrated the same stimulatory effect but was less potent whereas the eyestalk extract from *Macrobrachium rosenbergii* did not cause any changes in haemolymph vitellogenin levels. Therefore, the assay is specific and provides an indicator to monitor the activity of the putative gonad inhibiting hormone by assaying the alteration of vitellogenin levels.

KEYWORDS: eyestalk, gonad inhibiting hormone, ovarian development, *Penaeus monodon*, vitellogenin.

INTRODUCTION

Ovarian maturation in crustaceans is under sophisticated controls of the endocrine system which are mostly unknown. A hypothesis about its regulation is based on the principle stating that ovarian development is achieved through the balance of antagonistic actions of two groups of hormones; namely gonad stimulating hormone (GSH) and gonad inhibiting hormone (GIH).[1] The roles of GIH on ovarian development in various crustaceans have been firmly established in that destroying the eyestalk using various means induces precocious gonadal development in almost all crustaceans.[2-4] The endocrine nature of this response was confirmed by implantation of sinus glands or injection of eyestalk extract into the eyestalk ablated animals which resulted in suppression of gonadal development.[2,5] The chemical characterization of GIH has been reported in the American lobster *Homarus americanus* and Norway lobster *Nephrops nornygicus* as a peptide consisting of 77 amino acid residues. This peptide was shown to inhibit the onset of vitellogenesis (yolk accumulation processes); therefore, it was named vitellogenesis inhibiting hormone (VIH).[5] The gene encoded this peptide was identified and found to be expressed only in the eyestalk.[6] In the Norway lobster, GIH mRNA was expressed mainly in male and female eyestalks but was also found in supraesophageal ganglia.[7] The characterization of GIH in other species are scarcely reported due in part to the difficulties in the purification and the bioassay of this hormone which is not feasible in most species of interest.

An alternative method of GIH assay is to determine the inhibitory effects on vitellogenin synthesis by incubating the ovarian tissue with radioactive-labeled amino acid and determining the inhibition efficiency of eyestalk products upon incorporation of radioactive-labeled amino acid into ovarian protein.[8] In those assays, the antibody specific to vitellin is

required to identify the labeled vitellin. However, many nonspecific factors may affect incorporation of radioactive-labeled amino acid into yolk protein. Therefore, the assay system must be performed with caution by including appropriate controls.

During the reproductive cycle, vitellin accumulates in the developing oocyte to provide a primary source of nutrition through embryogenesis and early larval development. Vitellogenin, a haemolymph yolk protein precursor, is immunologically and electrophoretically indistinguishable from vitellin as demonstrated in several decapod species.[9] Vitellogenin is synthesized and delivered to the ovary via haemolymph circulation, then transported into the developing oocyte and incorporated into yolk protein. Vitellin synthesis in decapod crustaceans has been shown in several organs including ovary[8], hepatopancreas[10] and adipose tissues.[11] The changing levels of vitellogenin in the haemolymph were observed during reproductive cycle.[12-14] It has been hypothesized that vitellogenin levels are controlled by GIH or VIH[5, 15] and GSH or VSH.[1] Therefore , a determination of haemolymph vitellogenin levels could be used as an indicator for the action of hormone instead of determination of ovarian growth and incorporation of amino acid during protein synthesis in the hormonal assays.

Penaeus monodon, an economically important prawn, has been widely cultivated in Thailand and in many Southeast Asian countries. Therefore, this study analyzing mechanisms of ovarian maturation would be of benefit to the fry production industry and reduce the demand for brood stock caught from the sea. Monoclonal antibodies specific to various subunits of vitellin of *P. monodon* have been successfully generated in order to characterize the nature of yolk protein and to develop an immunoassay for quantitative analysis of vitellogenin levels in the haemolymph.[9, 16] This study aims at the quantitative analysis of the haemolymph vitellogenin levels in order to correlate them with ovarian development and the determination of the effect of eyestalk extract upon haemolymph vitellogen in levels in order to develop the GIH assay.

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 $3 \times 3 \times 0.6$ m^3, rectangular concrete tanks with natural pond water (25 ppt) at ambient temperature (26-28 °C) and natural photoperiod. Squid was presented during the morning and evening and half of water was changed every two days. After three days of acclimation, the prawns were used for the designed experiments.

Haemolymph Collection for Quantitation of Vitellogenin

Haemolymph was collected via arthrodial membrane of the fourth walking leg for 50 to 100 µl and stored immediately at –20°C. Before use, the haemolymph was centrifuged at 5,000 g and the supernatant was used for vitellogenin analysis.

Determination of Vitellogenin in Haemolymph

Indirect competitive ELISA for determination of vitellogenin content in haemolymph was performed as described previously [9] using a combination of 4 monoclonal antibodies specific to all vitellin subunits; PMVS-93, 109, 140 and 158 antibodies[16] at the dilution of 1:3000. The intra- and inter-assay variations were tested using 2 haemolymph samples at a low concentration (0.8 mg/ml) and a high concentration (4.5 mg/ml). A monoclonal antibody, PMVS-106, specific to ovarian specific protein (215 kD) was also used to determine whether the ovarian specific protein was released into the haemolymph in the same manner.

Preparation of Eyestalk Extract

Eyestalks were excised from live adult female prawns with scissors, frozen immediately on dry ice and stored at –70°C until use. Eyestalks were dissected to remove the outer shell while partially thawed out, then homogenized immediately in cold saline[17] with a glass homogenizer. After centrifugation at 10,000 g at 4°C for 30 min, the pellet was re-extracted with the saline. Supernatants from both extractions were pooled and the volume adjusted with saline to 1 eyestalk/ 100 µl, divided into small aliquots and stored frozen at –70°C.

The Relationship between Haemolymph Vitellogenin Levels and Ovarian Development

Ovarian development was induced by bilateral eyestalk-ablation. Two separate experiments were performed to determine the relationship between ovarian development and vitellogenin levels. The first experiment was performed by sampling the prawns every two days for 12 days after eye-ablation. After the haemolymph was collected for vitellogenin analysis, the prawns were sacrificed in cold water and their body weight and ovarian weight were determined. The gonado-somatic index (GSI) or ovarian index (OI) was calculated by using the formula as follow :

$$\text{OI (\%)} = \frac{\text{Ovarian weight}}{\text{Body weight}} \times 100$$

The stages of ovarian development were determined and divided into five stages as described previously by Tan-Fermin and Pudadera (1989).[18]

The second experiment was performed by collecting haemolymph for vitellogenin analysis from individual prawn everyday from day 2 to day 12 after eyestalk-ablation. On the last day, prawns were sacrificed for determination of OI and stage of ovarian development as described above.

The Effect of Eyestalk Extract on Haemolymph Vitellogenin Levels

Three to four days after induction of ovarian growth by bilateral eyestalk-ablation, individual prawn with developing ovary was injected with eyestalk extract approximately 3 eyestalks/prawn. The haemolymph was collected at 0, 2, 4, 6, 10, 14 and 24 hr after injection and then stored at –20°C for vitellogenin analysis. The changes of vitellogenin levels after injection of eyestalk extract were compared with vitellogenin level at 0 hr. The control experiment was performed by injecting the prawns with 300 µl of saline then processed in a similar manner. Different doses of eyestalk extract were also tested in the same fashion and the haemolymph from individual prawn was collected from 0 to 10 hr. At the end of the experiment, all the prawns were sacrificed for determination of OI and stages of ovarian development.

The Effect of Eyestalk Extracts from Other Prawn Species

Eyestalk extracts from another penaeid prawn *Metapenaeus affinis* and a palaemonid prawn *Macrobrachium rosenbergii* were prepared in the same manner as those of *P. monodon* and were used to inject into *P. monodon* approximately 3 eyestalks/300 µl / prawn. Determinations of the changes of vitellogenin levels were performed as described above.

Western Blot Analysis of Vitellogenin Subunits

In order to monitor the changes of vitellogenin subunits, some sets of haemolymph samples were separated in 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using mini-PROTEIN II electrophoresis apparatus (Bio-Rad). The proteins were transferred from gel to nitrocellulose sheets using a Transblot apparatus (Bio-Rad) at 50 V for 3 hr. The nitrocellulose sheet was quenched in 5% blotto and processed for Western blotting to localize vitellogenin subunits and ovarian specific protein using combination of specific monoclonal antibodies for each protein subunit as described previously.[16] All the blots were reprobed with a very dilute PMVS-22 (the monoclonal antibody specific to haemocyanin) as a reference band.

Statistical Analysis

Data were expressed as the mean ± standard error. Means were compared by analysis of variance followed by Duncan's multiple range test and two-way ANOVA.

Results

Specificities of Monoclonal Antibodies

Indirect competitive ELISA using a combination of four monoclonal antibodies gave similar results to experiments that used only one monoclonal antibody.[9] The curves generated using either ovarian vitellin or female haemolymph were superimposable (Fig 1). This result demonstrated that vitellogenin in female haemolymph is as effective as vitellin in inhibiting monoclonal antibody binding to vitellin. In contrast, male haemolymph had no competitive effect in the ELISA assay (Fig. 1). The range of vitellogenin that can be measured by this method was 5 to 200 µg/ml. The intra-assay variation was in the range of 5 to 10% and inter-assay variation was 2 to 3 %.

Correlation between Vitellogenin Levels and Stages of Ovarian Development

Measuring haemolymph vitellogenin levels at different stages of ovarian development, using ovarian index as an indicator, demonstrated that there was no direct correlation between ovarian index and haemolymph vitellogenin. The ovarian index of the ovaries at stage IV showed high level of variation (ranging from 3 to 8%). Similarly, haemolymph vitellogenin levels in prawns at stages II and III also showed a high range of variation as well (Fig 2). At resting stage, the haemolymph vitellogenin level was undetectable. After the ovaries began to develop, the haemolymph vitellogenin levels elevated to the highest concentration at stage III then dropped to lower levels at ripe (IV) and spent (V) stages; that was similar to the vitellogenin level at stage II. The increase in OI was associated with increasing haemolymph vitellogenin levels except in stage IV, indicated that the vitellogenin levels dropped before the reduction of OI resulted from spawning (Fig 3).

The vitellogenin levels from individual prawn after induction of ovarian growth displayed a similar correlation (Fig 3). The vitellogenin concentration of the prawn with ovary at resting stage (I) was undetectable. On the second day after eyestalk-ablation (early development = stage II) haemolymph vitellogenin levels rose sharply and reached maximal levels from 2-4 days after eyestalk-ablation (developing ovary at stage III). When the ovary reached maturity (IV), the vitellogenin levels abruptly dropped to very low levels a day before spawning. After spawning the vitellogenin levels began to increase and the second cycle of ovarian development began. In the second cycle of ovarian development, the vitellogenin levels

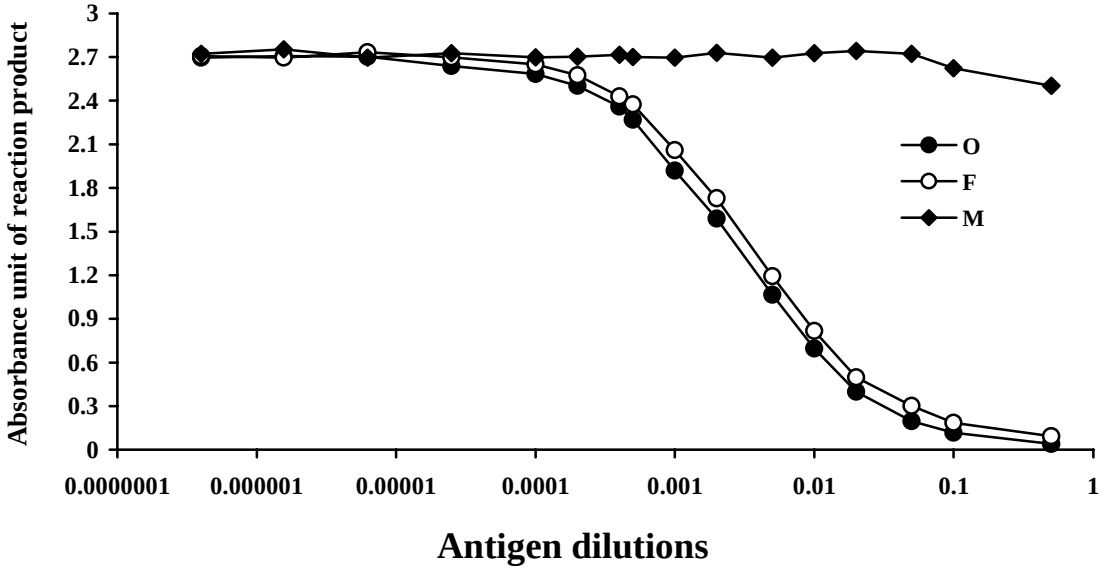

Fig 1. Validation of specificity and sensitivity of competitive ELISA for determination of vitellogenin using combination of four monoclonal antibodies specific to each vitellin subunit. Various dilutions of ovarian vitellin (O) with initial concentration of 10 mg/ml were compared to a similar regime of dilutions of haemolymph from adult male (M) and female with developing ovary (F).

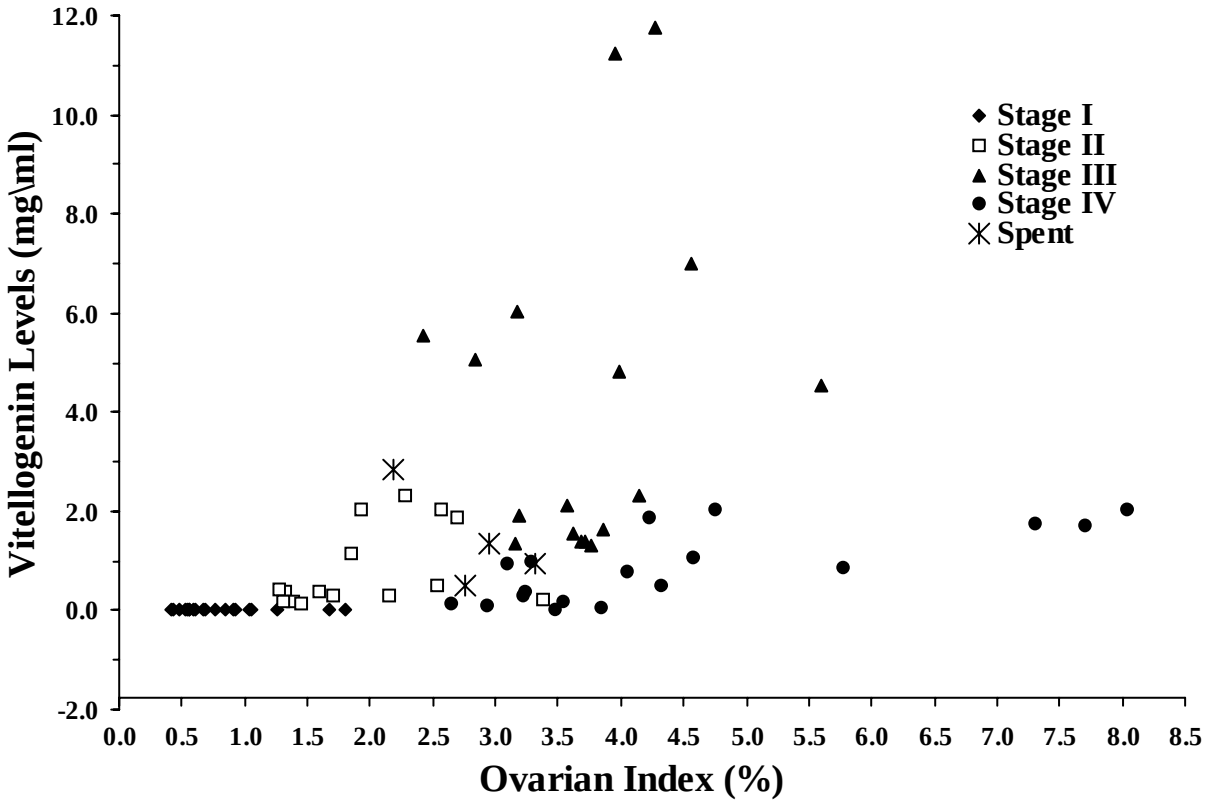

Fig 2. Haemolymph vitellogenin levels from individual prawn at different stages of ovarian development were compared with ovarian index. Each spot represents the data from individual prawn.

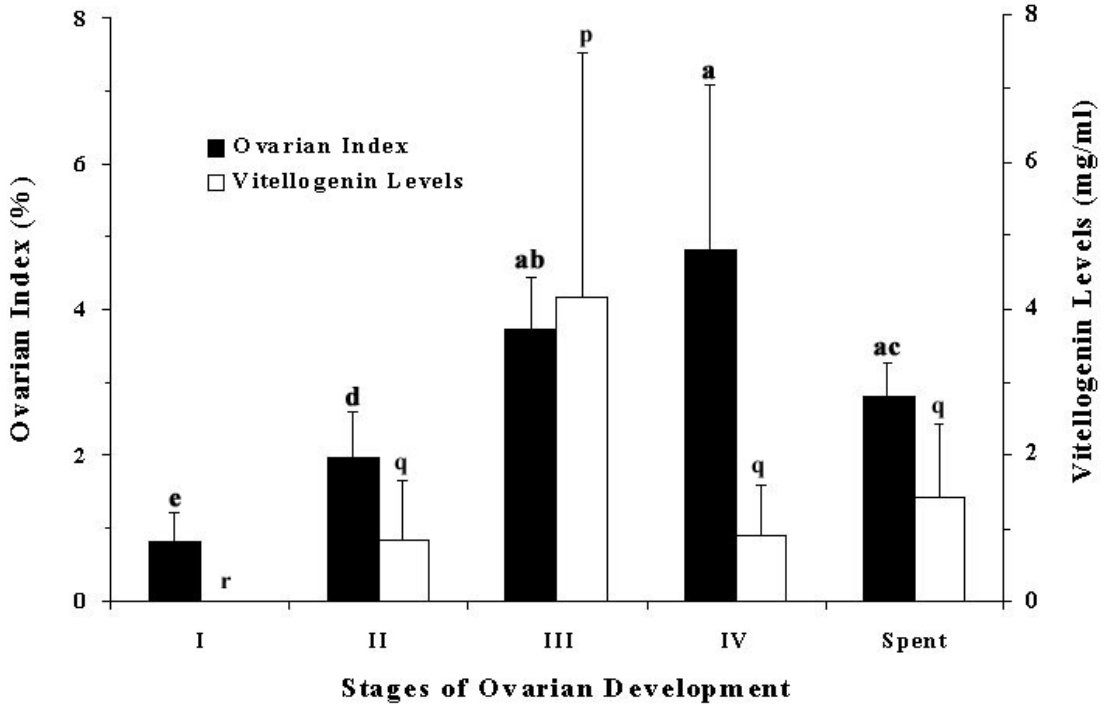

Fig 3. Comparison of average ovarian index and haemolymph vitellogenin levels at different stages of ovarian development. All data were derived from Fig 1. Bars are standard error. Different letters (a to e for ovarian index and p to r for vitellogenin levels) above the bars indicate significant difference ($p < 0.05$).

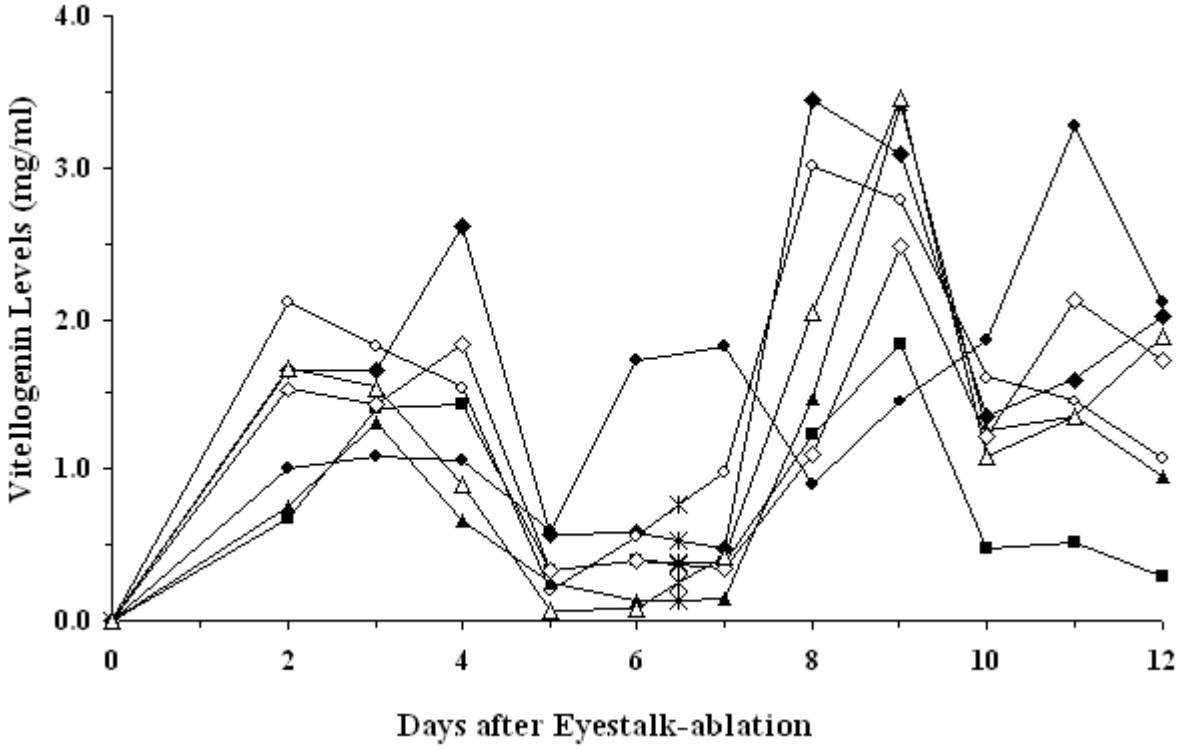

Fig 4. The relationship between haemolymph vitellogenin levels and ovarian development indicated by time after eyestalk-ablation. The asterisk (*) represents observable spawning. Each line represents data from individual prawn. Only 7 out of 12 individuals with almost synchronized vitellogenin levels were displayed to prevent confusion from too many lines.

of the prawns appeared higher than those in the first cycle and revealed higher variation and unsynchronization (Fig 4). Western blot analysis of vitellogenin subunits in the haemolymph from individual prawn showed that vitellogenin level was not detected before eye-ablation. During the first cycle of ovarian development vitellogenin precursor (200 kD) and the 74 kD protein were prominently observed. The vitellogenin levels dropped on the fifth day after eye-ablation. During the second cycle of ovarian development, the 200 and 74 kD proteins reappeared with higher levels than the first cycle and the 104 and 83 kD proteins were found at high levels on the eighth to ninth day.

Alteration of Haemolymph Vitellogenin Levels in Bilateral Eyestalk-Ablated Prawn After Injection of Eyestalk Extract

Three to four days after eye-ablation in prawns with developing ovaries (stage III), the vitellogenin levels in the prawn injected with eyestalk extract increased sharply and reached at the maximal levels at 4 hr after injection, and remained relatively unchanged through 10 hr., then decreased slightly but remained high through 24 hr. In the control group injected with saline, the vitellogenin levels slightly increased throughout 24 hr (Fig 5). Control group values were significantly different from the eyestalk injection group ($p < 0.01$).

Western Blot Analysis of Vitellogenin Subunits from Individual Prawn

In Western blot analysis of vitellogenin subunits from individual prawn, control prawns injected with saline did not show any change in vitellogenin subunits (200 and 74 kD) for up to 10 hrs. In contrast, the haemolymph of prawns at 4, 6, 10 hrs after injection of eyestalk extract showed a prominent accumulation of the 200 and 74 kD proteins along with the appearance of the 104 and 83 kD (Fig 6).

Dose-Dependent Response

When the amount of injected eyestalk extract was decreased, the changes of vitellogenin levels were decreased in a dose-dependent manner from 2-10 hr. The minimal amount of eyestalk extract with measurable effect was about of an eyestalk (Fig 7).

Cross-Reactivity of Eyestalk Extract

Cross-reactivity of eyestalk extract from different

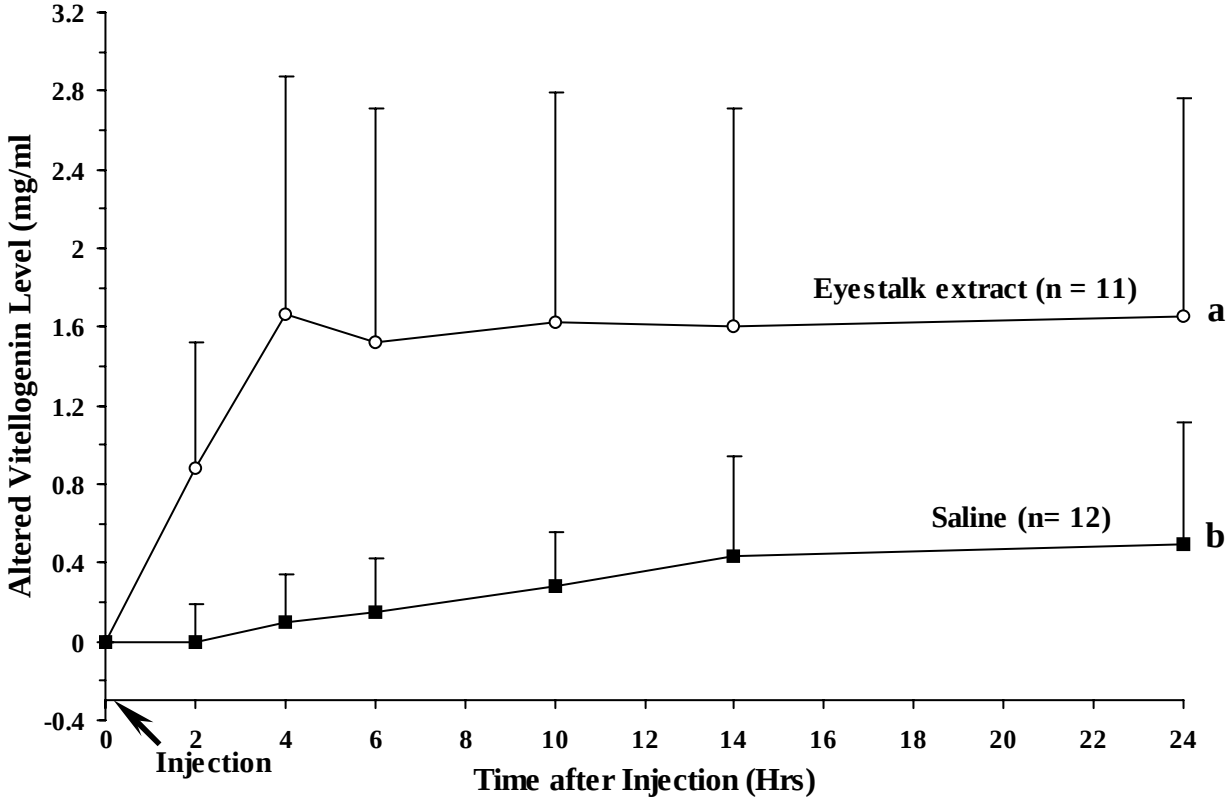

Fig 5. Alteration of haemolymph vitellogenin levels during 0 to 24 hr after injection of eyestalk extract (3 eyestalks/prawn) or saline (n = number of sample). Different letters (a and b) indicate the significant difference ($p < 0.01$). Bars are standard error.

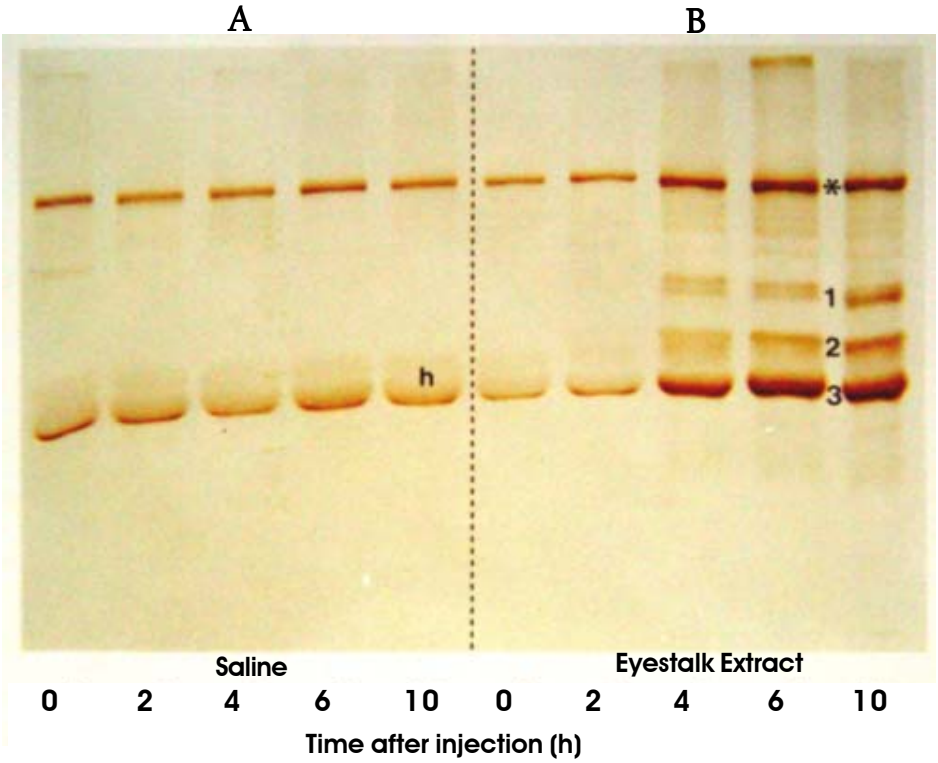

Fig 6. Western blot analysis of haemolymph from 2 representative prawns injected with saline (A) or eyestalk extract (B) at different times and probed with monoclonal antibodies specific to vitellin subunits; 200 (*), 104 (1), 83 (2) and 74 (3) kD proteins. The haemocyanin subunits were also shown (h). Approximately, 0.2 μl of haemolymph was loaded to each lane.

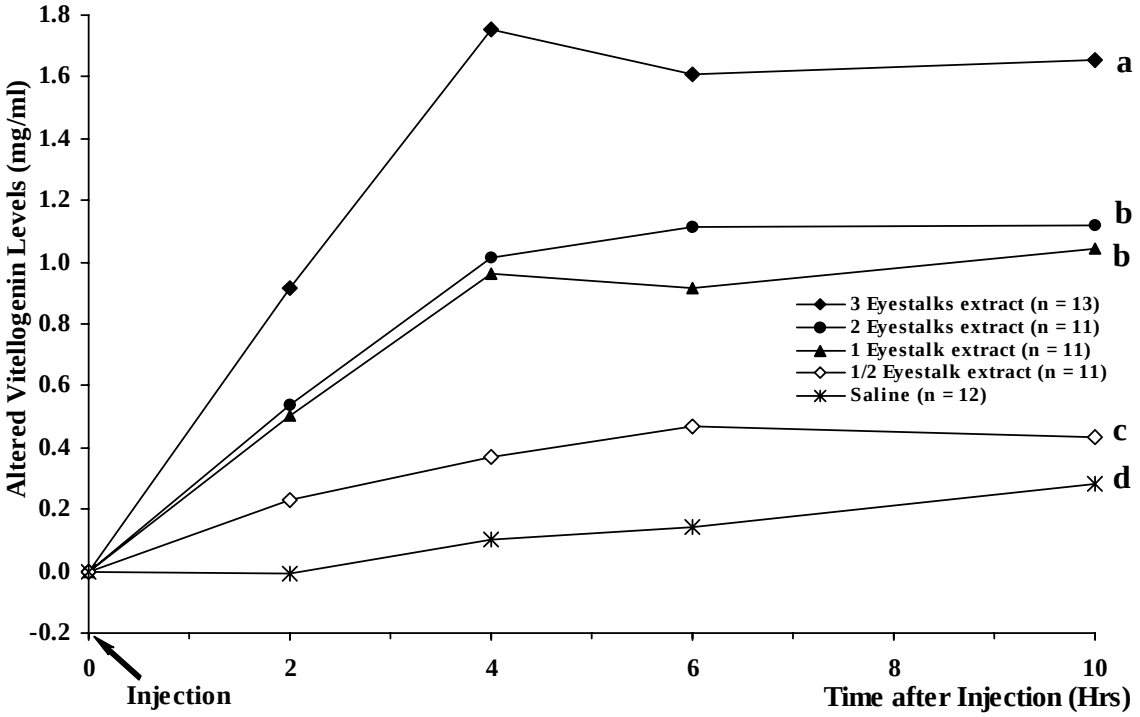

Fig 7. Alteration of haemolymph vitellogenin levels during 0 to 10 hr after injection of eyestalk extract (1/2 to 3 eyestalks/prawn) or saline (n = the number of sample). Different letters indicate the significant difference ($p < 0.01$).

prawn species was also investigated. Eyestalk extract from *Metapenaeus affinis* demonstrated similar results as the extract from *P. monodon* but was a less effective inducer haemolymph vitellogenin levels. In contrast, an eyestalk extract from *Macrobrachium rosenbergii* did not show any effect on haemolymph vitellogenin levels (Fig 8).

Discussion

Quantitative analysis of vitellogenin in the haemolymph using indirect competitive ELISA with combination of four monoclonal antibodies yielded high fidelity results since the intra- and inter-assay variations were relatively low. Even though the sensitivity was not different from using one monoclonal antibody[9], four monoclonal antibodies were used to insure the detection of all vitellogenin subunits present in the haemolymph. Similar[19] and different methods of ELISA used for vitellin and vitellogenin determinations have a similar range of sensitivities. [13-14,20-23]

The correlation between haemolymph vitellogenin levels and ovarian index (OI) at various stages of ovarian development was difficult to determined (Fig 2), since the variations of OI and haemolymph vitellogenin levels at each stage of ovarian development from individual prawn were very high especially at stage II, III and IV. In fact, the ovarian development in some prawns were on the second cycle in which most of the nutritional reservoir had been used during the first cycle of ovarian development. Therefore, smaller size and incomplete development of ripe ovaries were observed. Similar results were reported in *M. rosenbergii*[22] and in *P. japonicus*[14] with natural maturation. However, the relationship between haemolymph vitellogenin levels and stages of ovarian development was clearly observed. Even though the ovarian developmental processes are a continuous process, a clear line between each stage can not be identified accurately. The haemolymph vitellogenin levels elevated and fluctuated highly during ovarian development, and dropped sharply when the ovaries were mature and ready to spawn (Figs 3 and 4). Moreover, the same trend is observed when the haemolymph was determined from individual prawn at different times after eyestalk-ablation. Even though the variation among individual prawn was very high,

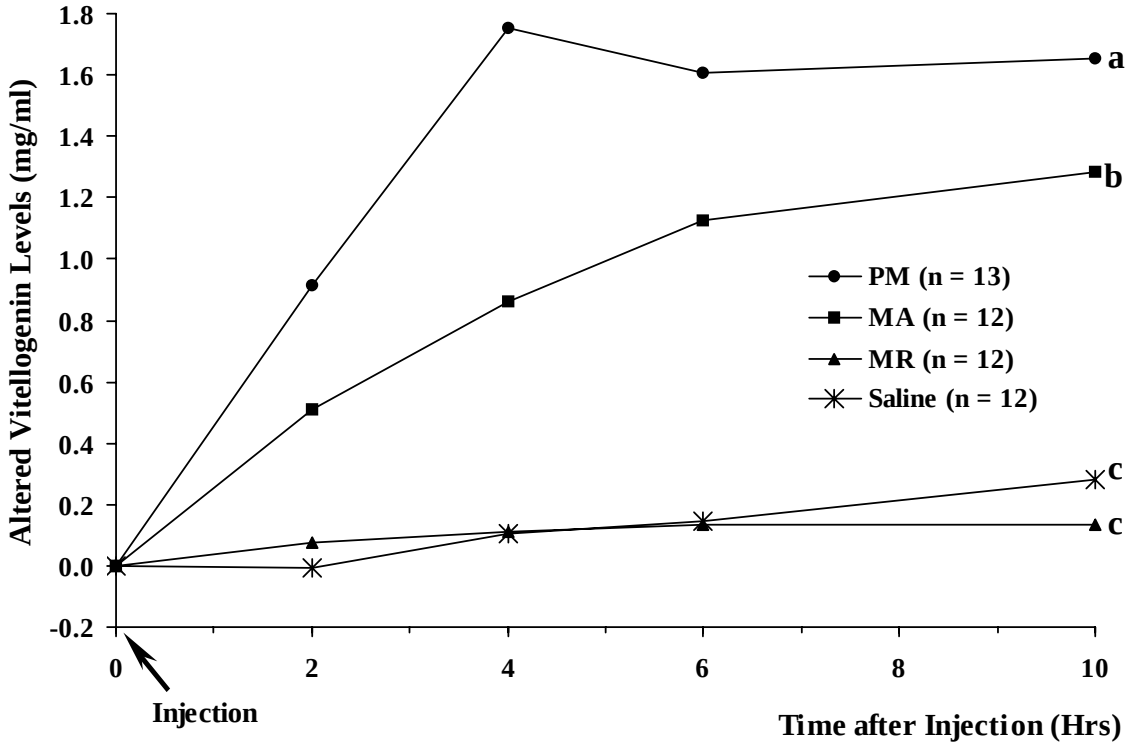

Fig 8. Alteration of haemolymph vitellogenin levels of *Penaeus monodon* during 0-10 hr after injection of eyestalk extracts (3 eyestalks/prawn) from *P. monodon* (PM), *Metapenaeus affinis* (MA), and *Macrobrachium rosenbergii* (MR) or saline. n = number of prawns in each experiment. Different letters indicate the significant difference ($p < 0.01$).

some of the tested prawns had undergone development into two reproductive cycles (Fig 4). Similar experiments conducted in *M. rosenbergii* demonstrated a similar relationship between haemolymph vitello-genin levels and ovarian developmental stages.[13] Since the prawn was subjected to bleeding every day, such a bleeding regime may cause stress and spiking of vitellogenin levels.[13] The increase in vitellogenin levels in the second cycle of ovarian development may be due to the fact that fewer oocytes had undergone development. Therefore, the vitellogenin uptake rate was highly reduced resulting in accumulation of the vitellogenin subunit (74 kD), its precursor (200 kD) and cleavage products (104 and 83 kD).

In contrast to our results, Vincent et al. (2001)[20] determined haemolymph vitellogenin concentrations in female *P. monodon* with ovarian development from stage F0 to F4, and found that the vitellogenin levels increased as the animal advanced to maturation and that there was no reduction at F4 stage. Also at the F0 stage the vitellogenin concentration was 360 μg/ml. In our case, the vitellogenin concentrations in the female with resting ovary was undetectable, increased gradually to the highest point at nearly ripe stage, and then dropped during the ripe stage. The similar patterns of haemolymph vitellogenin concentrations during ovarian development were also reported in various penaeid shrimps, namely *P. japonicus*[14], *P. semisulcatus*[12] *Sicyonia ingentis*.[19] The different results may be due to different criteria used to determine the ovarian stage of development.

An injection of eyestalk extract into eyestalk-ablated prawn with developing ovaries caused a sharp elevation of haemolymph vitellogenin levels within 2 hr and remained at high levels up to 24 hr. This result was unexpected as many studies have demonstrated that eyestalk extract exhibits an inhibitory effect. This inhibition has been assayed by the incorporation of a radio-labeled amino acid into vitellin in ovaries of many shrimps and crabs.[15] A study in *M. rosenbergii* demonstrated that two injections of eyestalk extract induced slow and continuous reduction of vitellogenin in eyestalk-ablated female with developing ovaries, and the effect was more pronounced after the second injection at 24 hr.[24] The *in vivo* inhibition by sinus gland on [^{35}S]-methionine incorporation into ovaries in *Procambarus clarkii,* revealed that the gonad inhibiting activity reached its peak effect between 12 and 24 hr following the sinus gland injection.[25] These studies indicate that the inhibition effect of eyestalk extract on protein synthesis in these species was slow. The injection of the eyestalk extract from *M. rosenbergii* did not demonstrate this effect when injected into *P. monodon* (Fig. 8). However, in many penaeid species including *P. monodon*, it has been well known that when the intact female prawns with developing ovary are reared in captivity, the developing ovaries usually undergo degeneration. In contrast, the ovarian development of *M. rosenbergii* is unaffected by captivity or stress from environmental changes. Therefore, it was possible that the additional inhibitory effect of eyestalk extract in penaeid species was the inhibition of vitellogenin incorporation into oocyte which led to degeneration of the ovary. The supporting evidence was the increased accumulation of the 200 kD vitellogenin precursor, 104, 83 and 74 kD vitellogenin subunits in the haemolymph within 4 hr after injection of eyestalk extract. The similar pattern was also observed in the second cycle of ovarian development. The ovarian specific protein (215 kD) and smaller subunits of vitellin (58 and 45 kD) were not detected in the haemolymph by ELISA and Western blot (results not shown), which indicated that the increase of vitellogenin in haemolymph was not caused by leaking content due to oocyte damaging. Another evidence was that when the eyestalk was abeated in captive prawn with early ovarian development, the degeneration processes stopped and the progress of ovarian development continued.[3,4]

The increase of haemolymph vitellogenin levels induced by eyestalk extract was dose-dependent and species-specific, since the eyestalk extract from closely related penaeid species had less effect while the extract from farther related palaemonid species did not cause any significant change. Similar experiments in *M. affinis* injected with eyestalk extract from *M. affinis*, *P. monodon* and *M. rosenbergii* demonstrated similar results.[4] Even though the eyestalk of *P. monodon* was about 3-4 times larger than the eyestalk of *M. affinis*, the induction of haemolymph vitellogenin concentration was about half the inductive level of the eyestalk extract of *M. affinis*. Therefore, the increase of haemolymph vitellogenin concentration was not due to the high content of the protein from the eyestalk.

In contrast to our results, Vincent et al. (2001)[20] demonstrated that the RP-HPLC purified fractions of eyestalk of *P. monodon* (fraction 20-23) caused a gradual reduction of haemolymph vitellogenin concentration in unilateral eye-ablated prawn within 8 hr. The different result may due to the fact that the purified extract was used which may have excluded other factors that could inhibit vitellogenin uptake; therefore, the reduction of haemolymph vitellogenin concentration was observed.

The correlation of other hormones and vitellogenin levels have been investigated. In *P. monodon*, the progesterone levels in haemolymph were high in prawns with mature ovary, whereas the progesterone levels in prawns with immature ovaries were low or

undetectable and showed high correlation to vitellogenin levels.[23] Another example was explored in the intersex crayfish *Charax quadricarinatus* which has both male and female reproductive systems. The removal of the androgenic gland from intersex individuals led to a significant increase in vitellogenin concentration in haemolymph. In contrast, the implantation of androgenic gland into female crayfish caused a significant lower concentration of haemolymph vitellogenin.[21]

This study demonstrated that haemolymph vitellogenin levels could be used as an indicator for GIH activity, which causes immediate rise of haemolymph vitellogenin levels. The assay for vitellogenin level has several advantages over other previous assay such as suppression of *in vitro* protein synthesis and determination of incorporated radioactive amino acid into ovarian tissue[15] or inhibition of *in vivo* ovarian development using GSI.[5] The assay for vitellogenin requires neither high amount of eyestalk extract nor radioactive substance. Moreover, it can be performed in less time, about 4 hr after injection of half eyestalk extract for determination of GIH activity. In contrast, the suppression of protein synthesis requires 1-2 days and radioactive amino acid as a marker as well as a highly specific anti-vitellin antiserum. In case of determination of GSI, a large amount and several injections of eyestalk extracts over long period of time (6-15 days) are required. High variation of GSI among individual prawn usually contributes to incorrect interpretation of such assay. Therefore, determination of GIH activity by measuring haemolymph vitellogenin levels is an alternative method for further characterization of this hormone.

In conclusion, cyclic pattern of ovarian development in eyestalk-ablated *P. monodon* can be demonstrated by determination of haemolymph vitellogenin levels using competitive ELISA. Application of the eyestalk extract in prawn with developing ovary caused a direct effect on elevation of haemolymph vitellogenin levels, which could be detected during 2-10 hr in a dose-dependent fashion. Therefore, the GIH assay could be established by determination of haemolymph vitellogenin in prawn with developing ovary.

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