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Characterization of Yolk Proteins in the Freshwater Crab *Travancoriana schirnerae*

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Abstract

Vitellogenesis, the most important part of the reproductive process, involves the synthesis of yolk proteins and their deposition in oocytes. The objective of this study was to characterize yolk proteins of the freshwater crab *Travancoriana schirnerae* using sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The results revealed that the hemolymph yolk protein, vitellogenin is composed of two high molecular weight polypeptide subunits 153.53 and 166.73 kDa. The ovarian yolk protein, vitellin is composed of four polypeptide subunits with molecular weights 60.25, 69.25, 82.68 and 115 kDa. The high molecular weight vitellin polypeptides, 82.68 and 115 kDa appeared in early primary vitellogenic ovary. The vitellin fraction of late primary and secondary vitellogenic ovaries contained a low molecular weight subunit, 69.25 kDa besides the 82.68 and 115 kDa fractions. All the four polypeptide fractions were detected in the tertiary vitellogenic ovary and freshly oviposited eggs. Vitellin polypeptides were not demonstrated in previtellogenic or spent ovaries. Biochemical analyses revealed that vitellin is a lipoglycoprotein as it is stainable with Sudan Black B and periodic acid Schiff's reagent. It was concluded that the two vitellogenin subunits may possibly undergo proteolytic cleavage to give rise to four vitellin subunits of the ovary.

Keywords: Hemolymph, ovary, *Travancoriana schirnerae*, vitellin, vitellogenin

Introduction

Vitellogenesis, the synthesis of yolk proteins and their subsequent deposition in ovarian oocytes, is an important reproductive process in all oviparous animals. Vitellogenesis in crustaceans is associated with several complex processes that include the synthesis of vitellogenin and the uptake of it from the hemolymph into oocytes accompanied by the accumulation of yolk globules (Meusy & Payen, 1988; Okumura & Aida, 2000). Substantial quantities of yolk accumulate within the developing oocytes, causing a rapid increase in the oocyte diameter and size. Yolk formation in most crustaceans occurs in a biphasic manner - an initial intraoocytic autosynthesis, where oocyte machinery is used for yolk synthesis followed by a phase of heterosynthesis, where yolk is constituted from a lipoprotein complex, vitellogenin, present in the hemolymph and sequestered by the oocytes (Meusy & Payen, 1988). In the second phase, the oocytes acquire relatively larger size due to rapid accumulation of yolk. Within the oocytes, vitellogenin undergoes proteolytic cleavage to become the major egg yolk protein, vitellin (Avarre et al., 2003). The vitellin, is a high density lipoglycoprotein, which serves as the source of nutrients in the eggs during embryonic development (Chen et al., 2004).The complexity and heterogeneity of their structure among crustaceans are still disputable.

In 1969, Kerr demonstrated in the blue crab *Callinectes sapidus*, the occurrence of a blood-borne protein, present only in females with developing oocytes, which was serologically identical to the oocyte vitellin and named vitellogenin. Among crustaceans, vitellogenin and vitellin are well characterized in prawns, shrimps and in marine crabs (Chen & Chen, 1993; Chen & Kuo, 1998; Avarre et al., 2003; Yang et al., 2005; Zmora et al., 2007). Vitellogenin is reported to be synthesized in the hepatopancreas of the mud shrimp *Upogebia major*, secreted to the hemolymph, taken up by developing oocytes and intra cellularly processed to generate vitellin (Kang et al., 2008). Vitellogenin and vitellin are described from the hepatopancreas, hemolymph and ovary of the freshwater prawn *Macrobrachium*

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malcolmsonii (Shanju & Geraldine, 2010). Zmora et al. (2007) reported the hemolymph vitellogenin profiles of females during ovarian development in *C. sapidus*. Vitellin has been purified and characterized in other marine crabs such as *Portunus trituberculatus* (Xiu, 2007) and *Charybdis japonica* (Xie et al., 2008).

The edible freshwater crab, *Travancoriana schirnerae* is a commonly distributed species in the wetlands of Wayanad, Kerala and is also reported from the south Indian states of Karnataka and Tamil Nadu (Bahir & Yeo, 2007). Its meat forms a cheap source of animal protein for the poor, malnourished local tribes. The natives consume the ovary alone during the breeding season. There has been much recent attention on vitellogenin and vitellin characterizations among marine crabs (Zmora et al., 2007; Ravi & Manisseri, 2011). Discernibly fewer studies have examined yolk proteins of freshwater crabs (Pateraki & Stratakis, 2000; Reddy et al., 2006). Knowledge on female reproductive biology is highly essential not only for their sustainable utilization, but also for developing effective strategies for successful aquaculture of a commercially important species. The present study provides the first report on characterization of hemolymph and ovarian yolk proteins in a freshwater crab.

Materials and Methods

Specimens were collected from the paddy fields Mananthavady, Wayanad (Kerala, India) during June 2010-July 2012. The crabs were maintained in cement tanks (area: $1m^2$), fed ad libitum with chopped beef liver and boiled egg. The carapace width (CW), wet weight and moult stages were recorded for all the specimens collected. The ovaries from immature and adult females at different stages of ovarian development (pre-vitellogenic, primary, secondary I, II, III, tertiary vitellogenic and spent) and freshly oviposited eggs were collected. For females, the color, size and wet weight of ovaries were recorded. From each female, one half of the ovary was removed, tore open, hundred oocytes were chosen randomly and their diameter was measured using a calibrated oculometer to determine the stage of development of the ovary.

Hemolymph from adult females (CW 4.0-5.0 cm) at various stages of ovarian development was collected by puncturing the walking legs and placed in sterile centrifuge tubes. The proteins from the hemolymph samples (100 µl) were precipitated with 10% Trichloro acetic acid (TCA). Samples were centrifuged (REMI, India) at 2000 rpm for 10-min. The pellets were washed thrice in distilled water and homogenized in sodium dodecyl sulphate (SDS) sample buffer (10% SDS, glycerol, β-mercaptoethanol, 0.1% bromophenol blue, 0.5 M Tris-HCl [pH 6.8]) at 4°C using an ice-cold hand held homogenizer (Thomson Scientific, USA). The homogenate was denatured in a boiling water bath for 5 min and then cooled immediately. The samples were centrifuged again at 2000 rpm for 30 min at room temperature and the supernatant was used as sample for SDS-PAGE analysis. Hemolymph from males and immature females was also processed by the same method described above, for the purpose of comparative studies. Total protein of the sample was determined by Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

Homogenates for vitellin characterization were prepared according to Yang et al. (2005). Ovaries at different stages of maturation were washed in distilled water to remove the hemolymph content. The samples (10 mg each) were then homogenized in the extraction buffer (0.1 M Tris-HCl, 1M NaCl, 6M urea, pH 7.5) and centrifuged at 2000 rpm for 10 min. The floating fatty layer and the precipitate were discarded. The centrifugation was repeated twice to completely remove the fat cap. Upper fraction was precipitated with TCA and centrifuged at 5000 rpm for 10 min at 20°C. The supernatant was mixed with acetone and centrifuged again at 5000 rpm (10 min) at 20°C. The pellets were collected, washed in distilled water and homogenized in SDS sample buffer (pH 6.8) using an ice-cold hand held homogenizer. The homogenates were denatured in a boiling water bath for 5 min and then cooled immediately. The samples were centrifuged again at 2000 rpm for 30 min (at 20°C) and the supernatant was used as sample for SDS-PAGE analysis. Ovaries of immature females were also processed by the same method described above, for the purpose of comparative studies. Total protein of the samples was determined by Lowry et al. (1951) using BSA as standard.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed on 7% separating gel with 4% stacking gel as described by Laemmli (1970). The samples (10 µl each) were loaded on 4% stacking gel. The gel was stained with 0.1% Coomassie brilliant blue R-250 and destained with

destaining solution. The molecular weights of the subunits were estimated by measuring their relative mobility in SDS-PAGE, in comparison to the standard protein marker (New England Bio Labs, UK) using Alpha Ease FC Software (Alpha Innotech Corporation, USA)

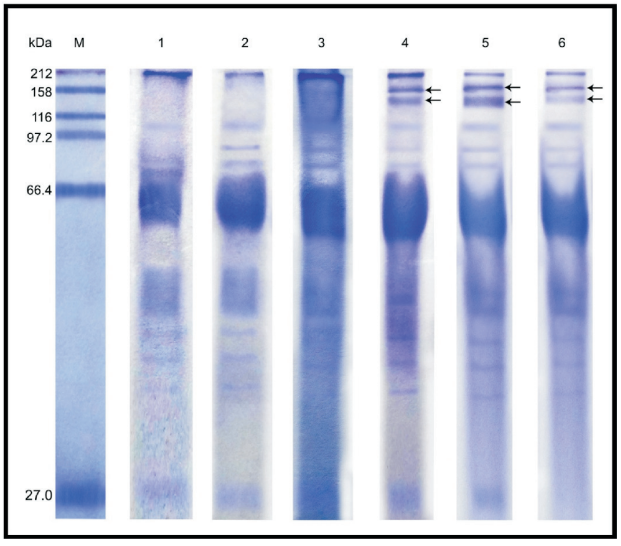
For glycoprotein staining, SDS-PAGE separated gels were transferred to 7% acetic acid, washed in distilled water and fixed in 1% periodic acid for 1 h. Gels were then washed in distilled water, stained in Schiff's reagent, destained in 1% sodium meta bisulphite, stored in the same and photographed. All the staining procedures were carried out inside the refrigerator.

For lipoprotein staining, the gels were incubated in Sudan Black B (500 mg Sudan Black B dissolved in 20 ml acetone followed by the addition of 15 ml acetic acid and 85 ml of distilled water) for 2 h and transferred to the destaining solution (150 ml of acetic acid, 200 ml of acetone, 650 ml of distilled water) (Clinica Chimica Acta 1991). The gels were stored and photographed.

Results and Discussion

The pattern of electrophoretic separation of yolk proteins showed different polypeptide profiles for the hemolymph and ovary at various stages of female reproductive cycle. Under denaturing conditions on SDS-PAGE, the vitellogenin polypeptide profile of hemolymph in primary, secondary and tertiary vitellogenic stages, displayed two high molecular weight fractions (153.53 and 166.73 kDa) (Fig. 1). Of the two vitellogenin subunits, the 153.53 kDa was the major fraction and 166.73 kDa was the minor fraction. Both these bands showed an increase in staining intensity from primary to secondary vitellogenic stages while lighter bands were noted in tertiary vitellogenic stage. These polypeptide bands were not detected in the hemolymph of males, previtellogenic and immature females, indicating these fractions were specific to vitellogenic females (Fig. 1).

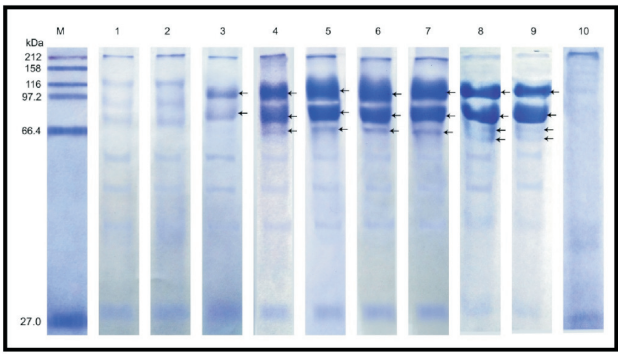
Vitellin protein from vitellogenic ovaries was separated into four polypeptides of molecular weights 60.25, 69.25, 82.68 and 115 kDa respectively. Of the four polypeptide subunits, the 82.68 and 115 kDa polypeptides were major bands and the 60.25 and 69.25 kDa appeared as minor bands (Fig. 2). Throughout the course of vitellogenic process, there was a gradual change in the vitellin polypeptide profile as shown by SDS-PAGE. The polypeptide profile of early primary vitellogenic ovary revealed two moderately stained polypeptide fractions of molecular weights 82.68 and 115 kDa (Fig. 2). In the late primary and secondary (I, II and III) vitellogenic stages, three polypeptide subunits (69.25, 82.68 and 115 kDa) were apparent in the ovary. The high molecular weight polypeptides 82.68 and 115 kDa were darkly stained whereas the low molecular weight polypeptide subunit 69.25 kDa-appeared faintly stained in late primary vitellogenic ovary. The 82.68 and 115 kDa bands were more darkly and intensely stained in the secondary vitellogenic stages (I-III) (Fig. 2). Four polypeptide fractions were evident (60.25, 69.25, 82.68 and 115kDa) in the tertiary vitellogenic stage ovary and in freshly oviposited eggs. The 82.68 and 115 kDa polypeptides from tertiary vitellogenic ovary and freshly oviposited eggs, stained more intensely than those of secondary vitellogenic stages and the 60.25 and 69.25 kDa subunits appeared as faint bands (Fig. 2). The major vitellin subunits appeared heavily stained (82.68 and 115 kDa) in secondary (I-III) and tertiary vitellogenic ovaries, indicating the accumulation of these polypeptides was greatest during these stages. The vitellin polypeptide fractions were not perceptible in immature females, previtellogenic and spent ova-



M: marker protein; Lane1: male hemolymph; Lane 2: immature female hemolymph; Lane 3: previtellogenic hemolymph, Lane 4: primary vitellogenic hemolymph; Lane 5: secondary vitellogenic hemolymph; Lane 6: tertiary vitellogenic hemolymph; arrow: vitellogenin bands.

Fig. 1. The SDS-PAGE separation of vitellogenin polypeptides of hemolymph in *Travancoriana schirnerae.*

ries, suggesting these fractions were specific to vitellogenic females (Fig. 2).



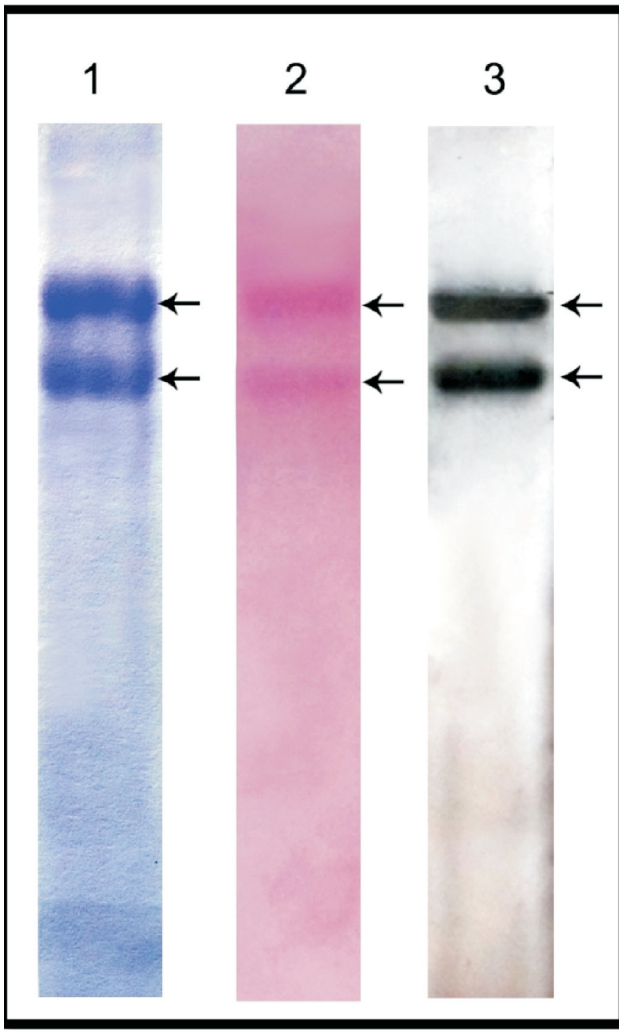
M: marker protein; Lane1: immature female ovary; Lane 2: previtellogenic ovary; Lane 3: early primary vitellogenic ovary, Lane 4: late primary vitellogenic ovary; Lane 5: secondary vitellogenic ovary I; Lane 6: secondary vitellogenic ovary II; Lane 7: secondary vitellogenic ovary III; Lane 8: tertiary vitellogenic ovary; Lane 9: freshly oviposited egg; Lane 10: spent ovary; arrow: vitellin bands.

Fig. 2. The SDS-PAGE separation of vitellin polypeptides of ovary in *Travancoriana schirnerae.*

Biochemical analysis showed that the 82.68 and 115 kDa polypeptide fractions of vitellin stained positively with Sudan Black B and periodic acid Schiff's reagent (PAS), suggesting their lipoglycopolypeptidic nature (Fig. 3).

The electrophoretic study to characterize yolk proteins in the hemolymph and ovary of *T. schirnerae* at different stages of the reproductive cycle revealed two polypeptide fractions in the hemolymph (153.53 and 166.73 kDa) and four fractions in the ovary (60.25, 69.25, 82.68 and 115 kDa). Generally, differences have been noted in the number and molecular masses of vitellogenin and vitellin subunits for several crustaceans (Auttarat et al., 2006). Two subunit composition of vitellogenin had been documented in *C. sapidus* (78.5 kDa and 207.3 kDa) (Zmora et al., 2007) and marine prawn *Penaeus semisulcatus* (74 and 199 kDa) (Avarre et al., 2003), while three subunits of vitellogenin were detected in the freshwater crab *Potamon potamios* (85, 105 and 115 kDa) (Pateraki & Stratakis 1997). Vitellogenin isolated from penaeid shrimp *Fenneropenaeus merguiensis*, by SDS-PAGE resulted in six subunits with molecular masses of 24, 64, 68, 99, 102 and 104 kDa (Zacharia & Kakati, 2004).

Diversity in vitellin components has been reported in several crustaceans. The four subunit composition of vitellin, ranging in molecular weights from



M: marker protein; Lane 1: Coomassie brilliant blue staining; Lane 2: periodic acid Schiff's staining; Lane 3: Sudan Black B staining; arrow: vitellin bands.

Fig. 3. Biochemical characterization of vitellin bands resolved by SDS-PAGE in *Travancoriana schirnerae.*

60.25-115 kDa of *T. schirnerae*, is in agreement with the range described for vitellin in *C. Japonica* (Xie et al., 2008). Four vitellin subunits have been reported in *P. potamios* (Pateraki & Stratakis, 2000) and in *P. monodon* (Chen & Chen, 1993). In contrast, the number of vitellin subunits for litopenaeid shrimps varied from 2-5 (Tom et al., 1992; Chen & Chen, 1994). It is generally accepted that the vitellins of crustaceans are composed of 2-8 subunits with molecular weights ranging from 35-190 kDa (Tom et al., 1992; Chang et al., 1993; Chen & Chen, 1993).

The two major vitellin polypeptides (82.68 and 115 kDa) of *T. schirnerae* were comparable in molecular

weights to the 83.3 and 100 kDa polypeptide subunits of the freshwater rice field crab *Oziotelphusa senex senex* (Reddy et al., 2006) and 85 and 115 kDa subunits of *P. potamios* (Pateraki & Stratakis, 2000). Ravi & Manisseri (2011) demonstrated that the high molecular weight protein, vitellin got split into three subunits of molecular weights 94.2, 98.7 and 103.5 kDa in the blue swimmer crab *P. pelagicus*. In *P. trituberculatus*, Yang et al. (2005) documented three major polypeptides of molecular weights 85,100 and 102 kDa by SDS-PAGE. In general, the numbers of vitellin subunits in brachyurans are estimated in between two and four (Komatsu & Ando, 1992; Lee & Watson, 1995). On the contrary, the vitellin appeared as two polypeptides of molecular weights 89 and 100 kDa in *M. rosenbergii* (Shanju & Geraldine, 2010).

In *T. schirnerae*, the vitellogenin polypeptides from hemolymph and vitellin polypeptides from ovary were not similar in their molecular weights and vitellin showed higher number of subunits than vitellogenin. A similar trend was noticed in *P. semisulcatus* where the hemolymph vitellogenin was composed of two subunits (74 and 199 kDa) and the ovarian vitellin constituted four subunits (72, 79, 100 and 207 kDa) (Avarre et al., 2003). Differences in the profiles of hemolymph vitellogenin and ovarian vitellin were also reported in *P. monodon* (Chang et al., 1993). In contrast, identical polypeptide fractions were noted in vitellogenin (89, 100 and 170 kDa) and vitellin (89 and 100 kDa) of *M. malcolmsonii* and vitellin consists of lower number of subunits than vitellogenin (Shanju & Geraldine, 2010).

In the present investigation, vitellogenin polypeptides were noticed at the primary, secondary and tertiary vitellogenic stages in the hemolymph of females and not observed in previtellogenic stages. The appearance of vitellogenin subunits in the hemolymph is related to ovarian development and such variations in vitellogenin polypeptides were well correlated with the accumulation of vitellin polypeptides in the ovary. These findings are consistent with those of Pateraki & stratakis (1997), who reported in *P. potamios* that vitellogenin polypeptides were not detected in previtellogenic females and noted only in vitellogenic phases. In contrast, the vitellogenin polypeptides remained at a constant level, irrespective of the stage of the reproductive cycle in *M. malcolmsonii* (Shanju & Geraldine, 2010).

In the present study, the staining intensity of major vitellin polypeptides progressively increased through early primary, late primary, secondary (I-III) and tertiary vitellogenic stages, suggesting an increase in accumulation of vitellin polypeptides. The SDS-PAGE analysis of ovary at different maturation stages revealed that the vitellin polypeptides were not found in spentor previtellogenic females. These results are supported by the findings of Ravi & Manisseri (2011) in *P. pelagicus*, where yolk deposition was noted in the early, late maturing and mature stages and not detected in spent stage. Similarly, in *M. malcolmsonii*, vitellin was noticed in early mature and mature stages of the ovaries and absent in the spent stages (Shanju & Geraldine, 2010). In the speckled shrimp, *Metapenaeus monoceros*, the yolk protein fractions of the ovary showed a steady increase in the level of expression with the maturity stages and vitellin was not detected in spent ovaries (Abraham & Manisseri, 2007). In the present study, vitellin polypeptides were not detected in the immature ovary as reported for *P. pelagicus* (Ravi & Manisseri, 2011) and *M. monoceros* (Abraham & Manisseri, 2007). This could be possibly because vitellin is synthesized only in negligible quantity in the immature stage and is present only in trace amounts in the spent ovary, as reported by Adiyodi & Subramoniam (1983).

In *T. schirnerae,* the 82.68 and 115 kDa subunits of vitellin stained positively with the Sudan Black B and PAS reagent, revealing their lipoglycopolypeptidic nature. These results are supported by the findings of Chen & Kuo (1998) in *M. rosenbergii*, where both vitellin subunits (83.1 and 95 kDa) stained positively with Sudan Black B and PAS reagent. The vitellin in *P. pelagicus* and *M. monoceros* has been identified as a lipoglycocarotenoprotein (Abraham & Manisseri, 2007; Ravi & Manisseri, 2011). By contrast, Shanju & Geraldine (2010) reported that of the two vitellin subunits in *M. malcolmsonii*, only the 89 kDa is a lipoglycopolypeptide whereas the 100 kDa is a lipopolypeptide.

In the light of the results presented here and of the above discussion, we conclude that, the electrophoretic study to characterize yolk proteins in the hemolymph and ovary of *T. schirnerae* revealed two subunits for vitellogenin and four subunits for vitellin. The findings of the present study suggest that the appearance of vitellogenin subunits in the hemolymph is related to ovarian development and

vitellogenin subunits may possibly undergo proteolytic cleavage to form vitellin subunits in the ovary. Further research is needed to better understand these polypeptides and explicate their structure, function and site of synthesis. This will contribute towards a deeper understanding of the commencement of vitellogenesis in freshwater crabs.

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