

## **Yolk proteins from mature ovaries of mud crabs, *Scylla serrata*: purification, characterization and quantification**

### **Abstract**

The present study was aimed to purify, characterize and quantify vitellin from the mature ovaries of the mud crabs, *Scylla serrata*. Vitellin was purified using saturated ammonium sulfate method followed by dialysis. Analysis on SDS-PAGE revealed two subunits of 105 kDa and 85 kDa. To analyse the peptide profile of vitellin, the bands obtained were digested with trypsin and the tryptic peptides were sequenced by liquid chromatography-mass spectroscopy and MALDI-TOF analysis. The peptides identified from 105 kDa were spread along the entire sequence of vitellogenin corresponding to the full length of the protein. Purified vitellin was used to raise polyclonal antisera with which an enzyme linked immunosorbent assay method was developed. Typical standard curve using ELISA showed a detectable vitellin sensitivity range between 5.0 ng and 1.0 µg (linear regression analysis:  $R^2=0.991$ ). Quantification of vitellogenin during natural reproductive cycle of mud crabs, revealed a significant increase in vitellogenin levels as compared to crabs at immature stage. To conclude, we demonstrated the isolation, purification, characterization and quantification of ovarian yolk protein from mud crabs, *S. serrata* which could be used as an aquaculture tool to identify and assess the signalling molecules that promote seed in hatchery industry.

## Introduction

Crustacean aquaculture industry is one of the revenue spinning industries because of its economic value and also protein rich content (Jayasankar et al., 2020). However, due to limited availability of seed the development of hatchery industry including culturing of mud crabs is hampered and not expanded as expected. In order to promote seed in hatchery industry, several researchers are focusing on the identification of signalling molecules that can able to induce ovarian maturation thereby seed. One of the important aspects of ovarian maturation is induction of a process known as vitellogenesis. Therefore, development of suitable technology to promote vitellogenesis thereby ovarian growth is an emerging area of crustacean endocrinology. To accomplish this task, isolation, purification and characterization of vitellogenin associated with its quantification is a promising tool during assessment of signalling molecules that can able to promote ovarian maturation in crustaceans. It is well known that the developing oocyte is nourished via sequestering of vitellogenin protein into the ovaries from the circulation during ovarian development (Jayasankar et al., 2020). This process is known as vitellogenesis. During vitellogenesis, high-density lipoproteins, including carotenoid pigments and yolk are synthesized and stored in oocytes. Vitellin, a subunit of vitellogenin, provides nourishment to developing oocytes during ovarian maturation in crustaceans (Tsukumura, 2001). Vitellogenin also acts as a carrier protein for carotenoids, retinoids, thyroxine, and riboflavin in fishes and vertebrates (Metzler and Sandell, 2016). On the other hand, accumulation of carotenoids and detection of retinoids accompanied by elevated levels of vitellogenin during ovarian maturation in crustaceans could reflect the transporter role of vitellin to channel carotenoids and retinoids (Wade et al., 2015; Venkaiah et al., 2019). Hence, analysis of vitellogenin levels in crustacean's ovary is used as a reproductive marker to assess the vitellogenesis process (Vazquez Boucard et al., 2002).

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Herein, we report the methodology of isolation, purification, characterization and quantification of vitellogenin using ELISA from the ovaries of mud crabs, *Scylla serrata*. This technology might be useful as a tool to identify signalling molecules that can promote seed in hatchery industry.

## Materials and methods

Among the edible crustaceans, the mud crab, *Scylla serrata* is popularly known as poor man's protein.

### Collection and maintenance of animals

Uninjured female mud crabs (body weight: 140 to 150 g; carapace width:  $11 \pm 2$  cm), *S. serrata* at intermolt (C4) stage were collected from the Nellore coast, Andhra Pradesh, India and brought to the laboratory. Ovaries from crabs at mature stage III (Venkaiah et al., 2019) were immediately dissected and cleaned from adhesive tissues and weighed to its nearest milligram. They were stored at  $-80^{\circ}\text{C}$  until further analysis.

### Isolation, purification and characterization of vitellin from the ovary of crabs

#### Purification of vitellin

Isolation and purification of vitellin (VN: subunits of VTG) from the mature ovaries (stage III) of mud crabs was performed in accordance to the previous protocols (Tsukumura et al., 2000; Sainath, 2009). Briefly, 10 g of ovarian tissue was homogenized using ice-cold mortar and pestle (glass-glass) in 100 mL of buffer (pH 7.8) containing NaCl (0.1 M), Tris (0.05 M), EDTA (1 mM) and Tween-20 (0.1 %) with PMSF (10 mg/mL) followed by the centrifugation of the homogenate at  $4000\times g$  for 5 min at  $4^{\circ}\text{C}$  and the resultant supernatant was carefully transferred to another tube and centrifuged at  $20,000\times g$  for 20 min at  $4^{\circ}\text{C}$ . The supernatant was used for further purification by using saturated ammonium sulphate (SAS) to produce 25, 40, 50 and 60 % SAS solution sequentially. In between, the sequential steps from 25 % SAS to 60 % SAS, an incubation step for 1 h at  $4^{\circ}\text{C}$  followed by centrifugation step at

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20,000×g for 10 min at 4 °C was performed. After centrifugation step, the pellet of 60 % SAS solution was suspended in the homogenization buffer and the suspension was subjected to dialysis (thrice; 4 °C for 12 h). The purified ovarian vitellin was stored at 20°C until further use.

#### *Characterization of VN using mass spectroscopy (MALDI-TOF)*

The purified VN was resolved on denatured sodium-dodecyl polyacrylamide gel electrophoresis (Sainath, 2009) to determine the molecular weights of purified vitellin and characterized by mass spectroscopy (MALDI TOF/TOF Ultraflex III, BRUKERS DALTONICS). Individual gel bands were cut into small fragments and placed into individual 1.5 ml eppendorf tubes. A mixture of acetonitrile and 100 mM of ammonium acetate (50:50) in a 50 µL was added to the tube to just cover the gel fragments and the tubes were shaken for 15 min at room temperature. The liquid was removed and discarded accordingly. This was followed by the addition of 50 µL of 10 mM DTT in 100 mM ammonium acetate to the tube and incubated at 55 °C for 1 hr. This step was followed by the addition of 50 µL of 50 mM iodoacetamide in 100 mM ammonium acetate and the tube was completely wrapped in aluminium foil and the tubes were shaken at room temperature in the dark for about 1 hr. The gel pieces were washed twice using 50:50 acetonitrile and 100 mM ammonium acetate, and dried using lyophilizer or speed vac unit. This step was followed by the rehydration of completely dried gel pieces for 10 minutes with 50 mM ammonium acetate solution containing 0.6 µg of trypsin (sequencing grade: Promega) in 6 µL of 0.01% SDS and incubated for overnight at 37°C on an incubator shaker (Thermo) at 400 rpm. After digestion, the supernatant was transferred to a new tube (1.5 mL) and was suspended in 50 µL of 50:50 acetonitrile: 0.1% trifluoroacetic acid (TFA) solution and shaken for 20 minutes. The peptides obtained were mixed with 2 µL of MALDI matrix solution comprising of saturated amount of  $\alpha$ -Cyano-4-hydroxycinnamic acid in 1 mL of 1:2 ratio of acetonitrile: 0.1% TFA. After thorough mixing

and centrifugation steps, a 1  $\mu$ L of aliquot of peptide sample was spotted onto the MALDI Anchor plate and allowed to dry. For calibration of peaks a pepmix mixture containing known peptide masses ranging from 1000 to 5000 Da was also spotted on to the MALDI Anchor Plate. The target plate was loaded into the MALDI TOF/TOF Ultraflex III (BRUKERS) mass spectrometer consisting of a laser beam to obtain the Peptide Mass Fingerprint of the proteins. The signal was maximized by adjusting the laser beam around the sample spot. The Flex Control software was used to obtain the spectra with a mass range of 500 to 5000 m/z. The spectra were calibrated using the Pepmix spectra obtained on the software Flex Analysis which includes the baseline correction and smoothening of the peaks. The corrected spectra were then exported on to the Bruker Biotools to perform the search on MASCOT against the database required (Parameters used for MASCOT search: 1. Fixed modification: Carbamidomethyl @ Cysteine; 2. Variable modification: Oxidation @ Methionine; 3. Enzyme: Trypsin; 4. Missed Cleavage: 1).

#### *Quantification of vitellogenin*

After characterization, the purified vitellin was used to raise antibodies in rabbits (Sainath, 2009) and was used for quantification studies. Enzyme linked immunosorbent assay (ELISA) technique was used to estimate the levels of ovarian vitellogenin from crabs during natural and induced conditions. The protocol used in this study was in accordance to Girish et al. (2015) with minor modifications. Briefly, 20  $\mu$ L of dialyzed sample diluted in carbonate buffer (1: 10 ratio) was added to wells and parallel blank samples with carbonate buffer ( $\text{NaCO}_3$ : 15 mM and  $\text{NaHCO}_3$ : 35 mM; pH: 9.6) alone were maintained. The plates were incubated overnight followed a four washing steps with 0.1 M phosphate buffered saline (PBS) containing Tween 20 (0.05%) (pH: 7.2). The primary antibody (raised against the purified vitellin from mature ovaries of crabs, VSIII) was diluted (1: 1000) in PBST buffer containing polyvinylpyrrolidone and ovalbumin (PBST-PO) was added to each well followed by an

incubation of plates at humid environment for 2 h. The plates were washed four times with PBST and 200  $\mu$ L of horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG-HRP) diluted in PBST-PO (1: 1000) was added to each well. The plates were incubated at 37 °C for 1 h followed by three washing steps with PBST. 200  $\mu$ L tetramethyl benzidine substrate was added to each well and kept in dark for 1 h at 37 °C for colour development. The reaction was stopped by adding 1N HCl to each well and the absorbance was measured with ELISA reader at 450 nm. The sensitivity of ELISA was analysed through inter-assay [different concentrations of standard was added to ten different microtitre plates] and intra-assay [same concentration of standard was added ten times to a single microtitre plate] variation experiments. A standard graph was analyzed with KyPlot (Version 6.0.2) by plotting the concentration of VTG on X axis and absorbance values (obtained at 450 nm) on Y-axis. KyPlot is a statistical tool that best fits the standards by determining polynomial equation. The sensitivity of Prior performing VTG-ELISA, several conditions such as dilution of antibodies, incubation time and range of standard curves were optimized. All standards and sample measurements were performed in triplicate.

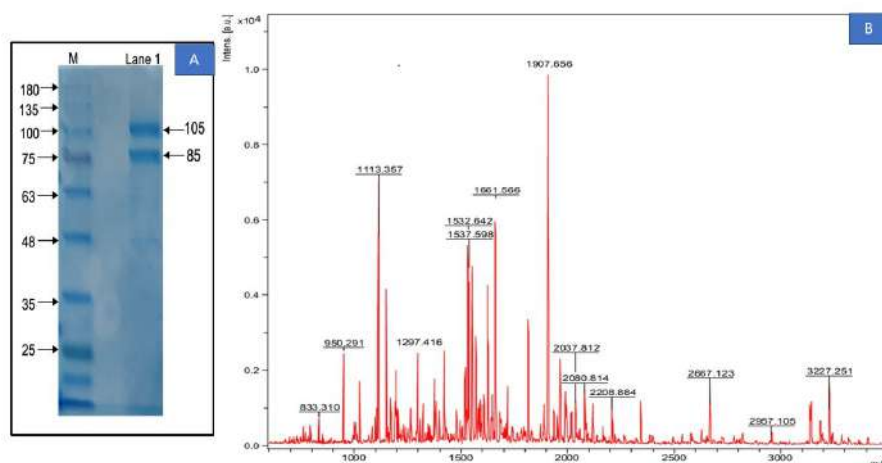
## Results

### *Characterization of ovarian vitellogenin*

The purified ovarian vitellin was separated into two polypeptides on SDS-PAGE gel, having molecular masses of 105 kDa and 85 kDa (**Figs. 1**). Mass spectroscopic analysis was performed using trypsin digestion and Matrix Assisted Laser Desorption/Ionization-Time of

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Flight (MALDI-TOF) to test the amino acid sequence correspondence to the purified bands 105 kDa.



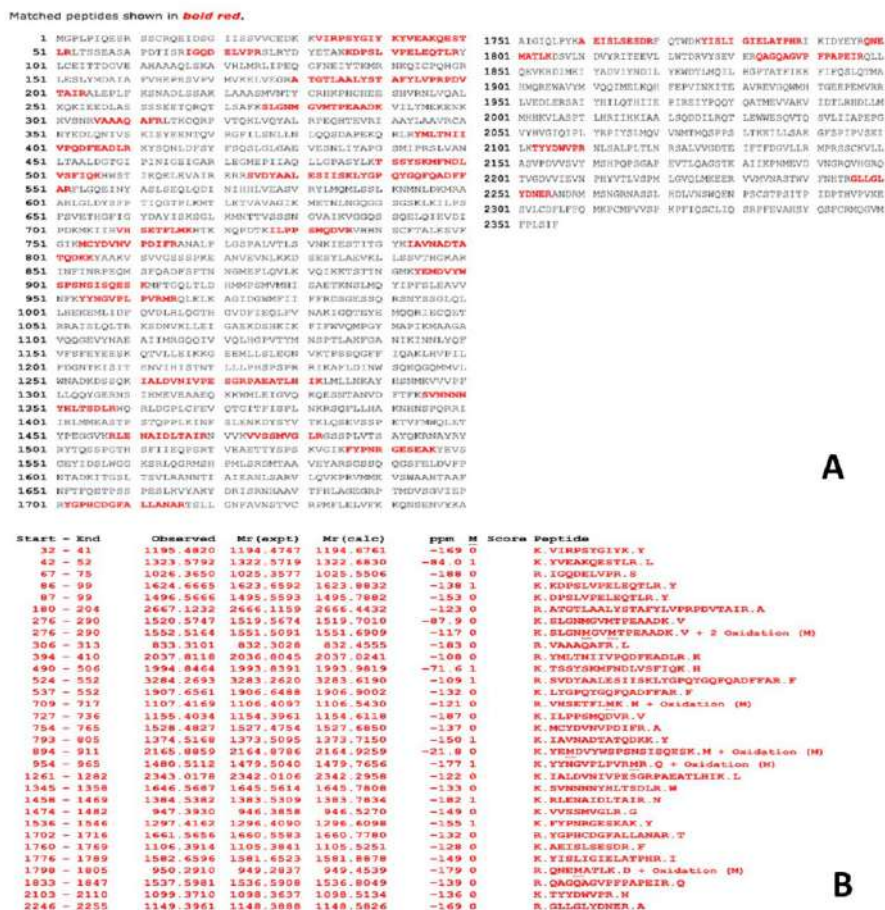
**Figure 1:** A. The SDS-PAGE analysis of purified ovarian vitellogenin from the mud crab *Scylla serrata*. Gel was stained with Coomassie Brilliant Blue R250. Lane M: molecular weight markers; Lane 1: purified ovarian vitellogenin. B. Mass spectroscopic analysis of vitellin peptide fragments from mature ovaries of mud crab *Scylla serrata*

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The peptides identified from 105 kDa were spread along the entire sequence of vitellogenin corresponding to the full length of the protein. Mascot peptide mass fingerprint database was used to obtain the corresponded amino acids of the vitellin bands based on the scores using the equation,  $-10 \cdot \log_{10}(P)$ , ( $P$  = absolute probability). The results revealed that the vitellin bands of *S. serrata* showed three matches corresponding to the peptides of *S. paramamosian* with scores 65 (mass value: 266650; e value:  $5.2 \times 10^{-5}$ ; match peptides: 31), 60 (mass value: 266719; e value: 0.00016; match peptides: 30) and 55 (mass value: 289157; e value: 0.00052; match peptides: 30), respectively (Supplementary material: 2). For further comparison studies, the mass fingerprinting peptide score of 65 with an e value  $5.2 \times 10^{-5}$  was

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selected. The corresponding peptides of vitellin (105 kDa) of *S. serrata* against the *S. paramamosian* (31 matches) were shown in the Figs. 2.



**Figure 2:** A. Peaks represent vitellin peptide fragments from mature ovary of crabs, *Scylla serrata* using mass spectroscopy (MALDI-TOF) analysis. The identified peptides are listed according to their amino acids position, sequence, and mass. B. Identified peptides were shown in red colour (bold) against the vitellogenin amino acid sequence of *Scylla paramamosian* (uniprot reference No.: A0A0G2ST52)

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All the mass spectroscopic data was shown in supplementary material. Mass spectroscopic data revealed that disulfide bonds, vitellogenin domain, domain of von Willebrand factor (VWFD) with GLLG motif, one consensus cleavage site (RERR), phosphorylation sites and N-glycosylation sites in the deduced amino acid sequence of ovarian vitellogenin of green mud crab (Jia et al., 2013). From the mass spectroscopic results, peptides related to vitellogenin domain, VWFD, phosphorylated amino acids and N-glycosylated amino acids were found in the 105 kDa of purified ovarian vitellin of *S. serrata*.

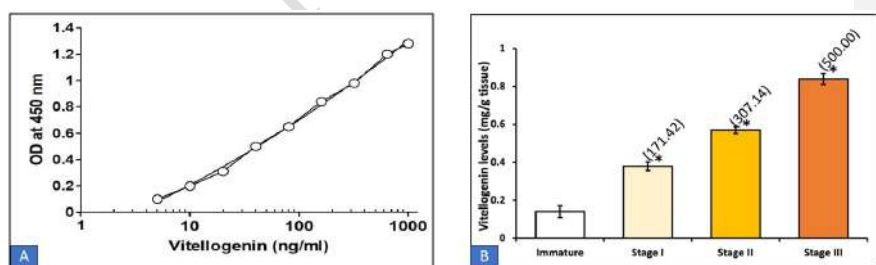
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Figure 3 shows a typical standard curve was observed between 5.0 ng and 1.0 µg of purified VTG (linear regression analysis:  $R^2 = 0.991$ ). The intra-assay variation ranged from 4 to 6% (n=10), and the inter-assay variation ranged from 9 to 10.6% (n=10). The sensitivity of VTG-ELISA was found to be as lowest as 5 ng. As expected, there was a significant elevation in ovarian VTG level as the crab progress from immature stage to mature stage (VSI: 171%; VSII: 307%; VSIII: 500%). As expected, there was a significant elevation in ovarian VTG level as the crab progress from immature stage to mature stage and also in ESX crabs over intact untreated crabs (Figure 3B).

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**Figure 3:** A. A typical standard curve of purified *Scylla serrata* vitellogenin. B. Ovarian vitellogenin levels at different reproductive stages in the mud crab, *Scylla serrata* during the natural reproductive cycle. Stages-I, II and III represent vitellogenic stages I, II and III of ovary, respectively. \*Significant at  $p < 0.01$

## Discussion

In this study, ovarian vitellogenin was purified and characterized using SDS PAGE analysis, revealing two predominant vitellin subunits with molecular weights of 105 kDa and

85 kDa from the ovary of *S. serrata*, whereas two major polypeptides with molecular masses of 105 kDa and 76 kDa was noticed in the ovary of crab, *C. feriatatus* (Mak et al., 2005). On the other hand, three polypeptides with molecular masses of 73.3, 84.3 and 100.0 kDa were observed in the fresh water edible crab, *O. senex senex*. Several researchers also characterized vitellin from ovaries of crustaceans (Jayasankar et al., 2020) and the differences in the occurrence of number and mass of polypeptides could be attributed to the cleavage pattern of vitellogenin (Girish et al., 2014). It has been shown that the first cleavage of vitellogenin takes place in hepatopancreas at the consensus motif, **RXXR** by subtilisin-like protein which leads to the release of two polypeptides into the hemolymph where second cleavage takes place followed by the sequestration of polypeptides/subunits of vitellogenin or vitellin into the ovaries (Subramoniam, 2011). The findings of this study, wherein occurrence of two subunits of ovarian vitellins (105 kDa and 85 kDa) and studies of Thongsaikliang (2020), who showed the occurrence of 90 kDa subunit in the hemolymph of mud crab supports this notion. It was shown that the vitellogenin of *C. feriatatus* finds the first intersection above the sequence, **LYGPQYGGQFQADFFAR** (up to 13 amino acids), which is likely the first cut point that splits the size of the vitellogenin protein into two fragments (Mak et al., 2005) and the occurrence of this peptide sequence in the hemolymph (Thongsaikliang, 2020) and ovaries (this study) of mud crabs could support this notion. Surprisingly, the molecular mass of hemolymph vitellin subunit as per the studies of Thongsaikliang, (2020) seems to be lower than the ovarian vitellin (observed in this study) with a molecular mass of 105 kDa. One possible answer could be attributed to a) post-translational modifications that occur in the ovary of crab, b) reprocessing of the smaller subunits into larger subunit and/or both (Warrier and Subramoniam, 2002; Mak et al., 2005; Jimenez-Gutierrez et al., 2019). Accordingly, several glycosylation sites in the peptides of vitellin were observed in the ovary of *S. serrata* (Jia et al., 2013). Mass spectroscopy was performed to investigate the conserved peptides and cleavage sites in the ovarian

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vitellogenin of *S. Serrata*. Six peptides were identified (peptide 1: VIRPSYGIYKYVEAKQESTLR; peptide 2: IGQDELVPR peptide 3: KDPSLVPELEQTLR; peptide 4: ATGTLAALYSTAFYLVPRPDVTAIR; peptide 4: SLGNMGVMTPEAADK and peptide 6: VAAAQAFR) correspondence to vitellogenin domain, one peptide GLLGLYDNER correspondence to VMFD domain, phosphorylation and N-glycosylation sites To the best of current knowledge, this was the first report indicating the mass spectroscopic analysis of ovarian vitellin subunit (105 kDa) of mud crab, *S. serrata*. Interestingly, studies of Thongsakliang (2020) have shown that mass spectroscopic analysis of hemolymph vitellogenin from the mud crab, *S. serrata* showed a 90 kDa band (characterized on SDS-PAGE: 12.5%) indicated seven peptides: peptide 1: LYGPQYGQFQADFFAR; peptide 2: ELQIEVDIPDKMEIIR; peptide 3: EANVEVNLK; peptide 4: LSSVTHGK; peptide 5: KDSVLNDVYRI; peptide 6: VLASPTLHR; and peptide 7: GLLGLYDNERANDR. In previous studies, it has been shown that different crustacean species have different cleavage sites, because of which different subunits of vitellogenin was obtained in crustaceans. (Jia et al., 2013; Jimenez-Gutierrez et al., 2019). In this study, we determined the ovarian vitellogenin levels during natural conditions using ELISA.

## Conclusion

The results presented in this paper demonstrate that the characterization and quantification of vitellogenin during natural conditions could be used as a valuable tool to identify a variety of signalling molecules that can induce ovarian growth in crabs. Moreover, this assay can be of use to monitor the ecosystem health and in improving aquaculture practices.

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