

Characterisation of vitellogenin and vitellin of *Fenneropenaeus merguensis* (De Man)

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ABSTRACT

Purification and characterisation of vitellogenin and vitellin from haemolymph and mature ovaries respectively of *Fenneropenaeus merguensis* were conducted using ammonium sulphate precipitation technique. The vitellogenin level in the haemolymph was low in the early stages of development, but increased significantly in the late maturing stages (Stage III) and fell abruptly in spent females. Purified vitellogenin had an approximate molecular weight of 350kDa as determined by PAGE and was characterised as lipoglycoprotein with calcium affinity on the basis of staining data. Two forms of purified vitellin (Vn1 and Vn2) of molecular weights 550 and 300kDa were also isolated. Vn1 was characterised as a lipo-glyco-calcium complex while Vn2 was glycoprotein in nature based on staining data. SDS PAGE analysis revealed 6 subunits in purified vitellogenin and 5 subunits in vitellin.

Introduction

Vitellogenesis is a crucial period in the female reproductive cycle of Crustacea characterised by a rapid yolk (vitellin) deposition in the developing oocytes and a corresponding appearance of vitellogenin in haemolymph. It has been intensively studied in lower vertebrates and in various groups of crustaceans, but due to the diverse assemblage in the group, penaeids have received little attention. But of late, there has been an increasing interest in crustacean vitellogenesis, due to their growing commercial importance in aquaculture.

Vitellogenin (female specific protein), a possible precursor of major yolk protein vitellin, is generally

detected as a prominent fraction of low electrophoretic mobility in the serum of vitellogenic females and characterised as a lipo-glyco-phosphoprotein. It is mostly absent or present in very small amounts in males. It has been demonstrated to be immunologically and electrophoretically related to vitellin in the gonad during maturation (Chang *et al.*, 1994; Chang and Jeng, 1995a, b; Lubzens *et al.*, 1997; Lee *et al.*, 1997; Longyant *et al.*, 2000; Okumaura and Aida, 2000). Vitellogenin levels in the haemolymph increase in expression with advancement in vitellogenesis, with the highest concentration prior to maximum accumulation of yolk in the developing oocytes (Quackenbush, 1989b; Qunitio *et al.*, 1989; Laxmilatha, 1991; Shafir *et al.*, 1992; Lee *et al.*, 1997), thus forming

a good indicator of the vitellogenic state of the animal.

Vitellin (Vn), accumulates within the ovary during the process of vitellogenesis in crustaceans and forms the nutritive material necessary for the successful development of an embryo independent of its mother (Meusy and Payen, 1988). Vitellin has been purified and characterised in several penaeid shrimps namely *P. japonicus* (Yano and Chinzei, 1987), *P. vannamaei* (Quackenbush, 1989a; Tom *et al.*, 1992), *P. semisulcatus* (Khayat *et al.*, 1994; Lubzens *et al.*, 1997), *P. chinensis* (Chang *et al.*, 1996), *P. monodon* (Chen and Chen, 1993; Longyant *et al.*, 1999), *Parapenaeus longirostris* (Tom *et al.*, 1987) and *Metapenaeus ensis* (Qiu *et al.*, 1997). The molecular weight of native vitellin in most penaeids is about 300kDa and is cleaved to 2 – 11 subunits by SDS PAGE in various crustacean species and reported to contain sugar, lipid and carotenoid moieties (lipo-glyco-carotenoprotein) (Charniaux-Cotton, 1985).

The present study was aimed to describe the changes observed in the serum and ovarian protein profile (vitellogenin and vitellin respectively) of *Fenneropenaeus (Penaeus) merguensis*, an important species in fisheries and aquaculture, during maturation. Partial purification and characterisation of vitellin from the ovaries and vitellogenin from haemolymph were attempted to elucidate their characteristics and relationship between them.

Material and methods

Selection of animals

Live female *F. merguensis* at different stages of maturity were collected from Karwar coast, Karnataka, India. Classification of ovarian

development was done on a five point scale based on their relative size, outline and colour of ovaries as seen through the transparent dorsal exoskeleton (Rao, 1967). Haemolymph was collected from the shrimps through the pericardial cavity using needles rinsed with 0.2M EDTA. Haemolymph from males and immature females was also collected for the purpose of comparative studies. The collected haemolymph was left undisturbed at 4°C overnight to ensure complete clotting. The clot was loosened with a needle and clear serum was obtained by a 10-15 minute centrifugation at 10,000 g. Serum was frozen at -20°C till use. The animals were then sacrificed and ovaries from females in different stages of maturity were dissected out, homogenised and centrifuged at 10,000g, 4°C for 15 minutes. The fat cap was removed before the supernatant was used for electrophoresis or for subsequent purification.

Isolation of vitellogenin fraction

The haemolymph collected from females in the late vitellogenic stage (Stage III) were used for isolation of vitellogenin. Approximately 0.5ml of serum, diluted to 5ml, was subjected to a one step procedure of ammonium sulphate precipitation (Wallace *et al.*, 1967). The bulk of protein precipitated at 60% saturation of ammonium sulphate under ice cold condition was found to contain vitellogenin. The precipitate obtained was centrifuged at 10,000 g for 15 minutes. The pellet thus obtained was dissolved in 200ml of distilled water and its purity was detected electrophoretically.

Isolation of Lipovitellin fraction

Approximately 0.5ml of the supernatant obtained from mature ovarian (Stage IV) homogenate was

subjected to ammonium sulphate precipitation (Wallace *et al.*, 1967). Lipovitellin was precipitated at 50% saturation of ammonium sulphate under ice-cold condition and was centrifuged at 10,000g, 4°C for 15 minutes. The pellet thus obtained was dissolved in 200ml of distilled water and used for further electrophoretic analysis.

Electrophoresis

Haemolymph and ovarian homogenates together with their purified fractions were subjected to a 6.5% Native Polyacrylamide Gel electrophoresis (PAGE) using 1.8M Tris- HCl buffer (pH 8.9) (Davis, 1964). Electrophoresis was conducted at constant current of 65mA at 10°C. Samples were also subjected to a 12% Sodium Dodecyl Sulphate Polyacrylamide Gel electrophoresis (SDS PAGE) using 1.5M Tris HCl buffer (pH 8.8) along with a stacking gel of 6% at a constant voltage of 140V (Laemmli, 1970). The molecular weights of the proteins and their subunits under native and denatured conditions were determined using molecular weight standards run along with the samples. The volumes of samples were adjusted to a concentration of 120µg proteins per well.

Staining the gels

The gels, from both native as well as SDS electrophoresis, were stained for proteins with 0.1% Coomassie brilliant blue R250 according to conventional methods and destained in a solution of 50% methanol containing 10% acetic acid. The native gels were also stained for carbohydrate, lipid and calcium moieties with Periodic acid Schiffs reagent (PAS), Sudan Black B (SBB) and Alizarin Red 'S' respectively. Relative mobilities (R_f) of proteins were estimated and the molecular weights were

determined in comparison with markers.

Results

Identification and characterisation of Vitellogenin

Native PAGE profiles of haemolymph samples from females in different stages of maturity revealed the presence of a specific protein, which increased in intensity with advancement in maturation. This protein, considered as vitellogenin (female specific protein, FSP), was intensively expressed in females in the late vitellogenic stage (Stage III) and was present in low concentration in immature females (Fig. 1). The serum samples were subjected to 60% ammonium sulphate fractionation and subsequent electrophoretic analyses

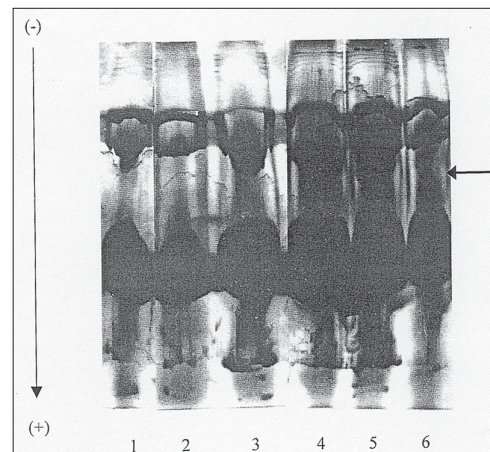


Fig. 1. Native PAGE illustrating the appearance of vitellogenin in haemolymph of females at different stages (Stages I-V) of maturity

- | | |
|---------|---------------------------|
| Lane 1. | Male |
| Lane 2. | Immature females |
| Lane 3. | Early vitellogenic female |
| Lane 4. | Late vitellogenic female |
| Lane 5. | Mature female |
| Lane 6. | Spent female |
| —————▶ | Vitellogenin |

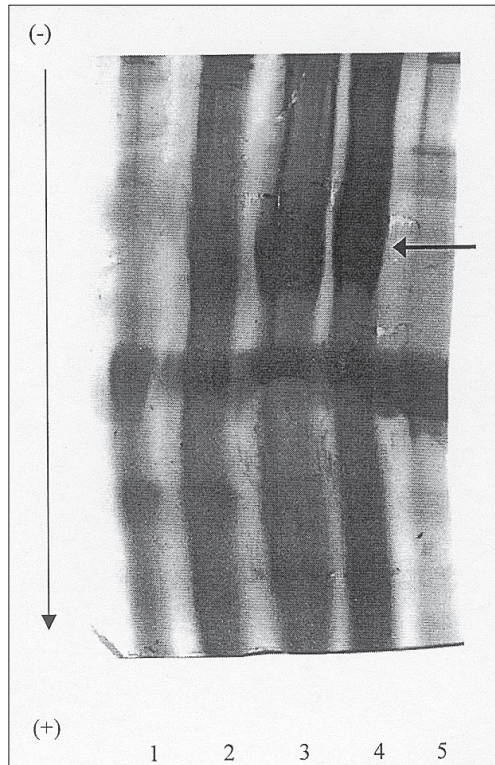


Fig. 2. Native PAGE illustrating the appearance of vitellin in ovaries of females at different stages (Stages I-V) of maturity

- Lane 1. Stage I ovary
- Lane 2. Stage II ovary
- Lane 3. Stage III ovary
- Lane 4. Stage IV ovary
- Lane 5. Stage V ovary
- ▶ Vitellin (Vn1)

revealed a single form of vitellogenin of molecular weight 350kDa, which was stainable with SBB, PAS and Alizarin Red S, indicating the presence of lipid, carbohydrate and calcium moieties in it.

SDS PAGE analysis of immature and mature female serum after ammonium sulphate fractionation showed changes in the polypeptide profile in the course of vitellogenic process. Six polypeptide subunits of

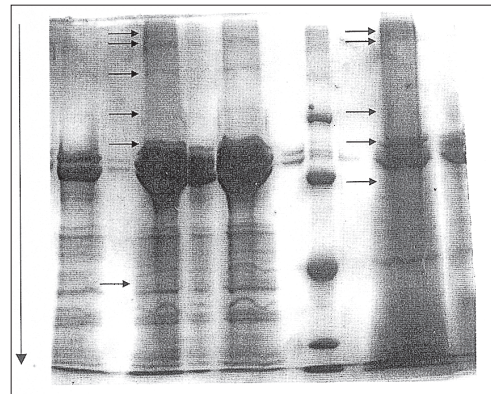


Fig. 3. SDS PAGE analysis of vitellogenin and vitellin from immature and mature females

- Lane 1. Haemolymph from immature females
- Lane 2. Haemolymph from mature females
- Lane 3. Marker
- Lane 4. Mature ovary
- Lane 5. Immature ovary

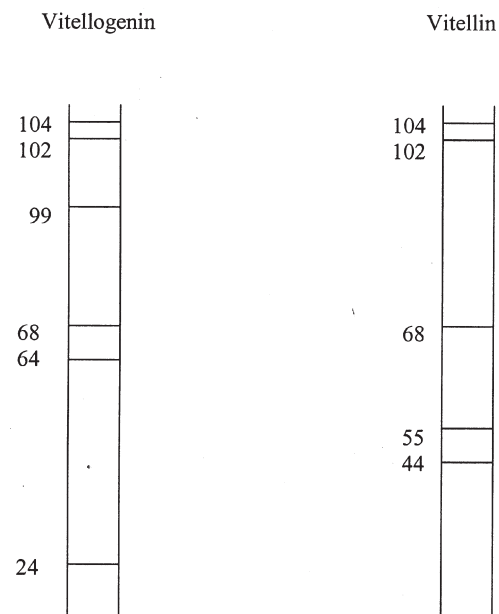


Fig. 4. Comparative electropherograms of vitellogenin and vitellin patterns at vitellogenic stage in *F. merguensis*

molecular weights 104, 102, 99, 68, 64 and 24kDa were detected in purified vitellogenin in SDS-PAGE of which 99kDa was feebly expressed in immature serum (Fig. 3 and 4).

Identification and characterisation of vitellin

The pale green colour of shrimp yolk was retained, which was used as a marker to follow the yolk during purification. PAGE protein profile of ovarian homogenates showed that the presence of a heavily stained band in stage II - IV ovaries and which was weakly expressed in stage I ovary (Fig. 2). A narrow band, which stained lightly with Coomassie Brilliant Blue, was also visible in mature females. These bands had a molecular weight of 550 and 300kDa respectively and were believed to represent vitellin (Vn1 and Vn2). The deeply stained band (Vn1) was stainable with Sudan black B, PAS and Alizarin Red S, while the narrow band (Vn2) was stainable only with PAS, indicating Vn1 was lipo-glyco-calcium complex while Vn2 was glycoprotein in nature.

12% SDS-PAGE analysis showed that high molecular weight polypeptides were prominent in the ovaries in the late vitellogenesis, which was not discernible at the onset of vitellogenesis. Five bands of molecular weights 104, 102, 68, 55 and 44kDa were prominent in the vitellogenic females of which only 55kDa was feebly expressed in stage I ovary (Fig. 3 and 4). Some or all of these polypeptide bands represented the polypeptides that accumulate to high levels during yolk formation and thus likely to represent vitellin subunits.

Discussion

Vitellogenin, identified as a prominent fraction (molecular weight 350kDa) of low electrophoretic mobility

in the haemolymph of vitellogenic females of *F. merguensis*, was present in low concentration in previtellogenic females. The approximate molecular weight of 350kDa of *F. merguensis* was comparable to that of other species. The protein was characterised as lipo-glyco-calcium-protein based on their staining properties. Purified vitellogenin in *F. merguensis* separated by SDS PAGE had six polypeptide subunits. Purified vitellogenin isolated from *P. monodon* had a molecular weight of at least 263kDa with 2 subunits (82kDa and 170kDa) (Chang *et al.*, 1994). Chang and Jeng (1995a, b) reported two polypeptide subunits (85 and 191kDa) for vitellogenin in *P. chinensis*. Three polypeptide subunits (200, 120 and 80kDa) were reported in *P. semisulcatus* (Lubzens *et al.*, 1997). In the freshwater prawn, *Macrobrachium rosenbergii*, vitellogenin was found to have a molecular weight of 92kDa (Derelle *et al.*, 1986; Lee *et al.*, 1997) with two polypeptide subunits (191 and 85kDa). Thus, crustacean vitellogenin is apparently a composite protein fraction, the production of which is likely to be multi-gene involved and multi-hormone controlled process (Adiyodi and Subramoniam, 1983).

Concentration of vitellogenin in the haemolymph of *F. merguensis* was found to vary during different stages of maturation. It increased dramatically during initial stages of oocyte development and was always highest prior to the maximum accumulation of yolk in the oocytes (Stage III) and the levels decreased markedly after spawning. Thus FSP levels may be used as an index of maturation in *F. merguensis*. A similar trend of vitellogenin expression was also reported in the crab *Cancer antennarius* (Spaziani, 1988) and in shrimps *P.*

indicus (Laxmilatha, 1991), *P. vannamei* (Quackenbush, 1989a, b) and *Pandalus kessleri* (Quinitio *et al.*, 1989). Several studies demonstrated that vitellogenin is sequestered by oocytes during secondary vitellogenesis and constitutes a main precursor of proteinaceous yolk. Thus, haemolymph acts as a conduit for the ovarian polypeptides during ovarian maturation. In fully mature stage, vitellogenin is absorbed by the developing oocytes as lipovitellin, thereby resulting in altered pattern of haemolymph vitellogenin. Since some females were observed to retain a few maturing oocytes after spawning, FSP levels in spent females of *F. merguensis* were higher than in previtellogenic females. However, the high FSP levels in spent females may also indicate that the next vitellogenesis is underway, based on the hypothesis that the source of vitellogenin is outside the ovary and is transported there through the haemolymph (Meusy and Payen, 1988). Similar results were reported in *P. indicus* (Laxmilatha, 1991), *P. japonicus* (Jasmani *et al.*, 2000) and in the fairy shrimp *Streptocephalus dichotomus* (Munuswamy and Subramoniam, 1987). This suggests the possibility of a feedback mechanism that regulates the synthesis and release of vitellogenin into the haemolymph as well as its uptake in the oocytes. Thus vitellogenin concentration in haemolymph may be used as an indirect indicator of regulatory hormone activities.

An increase in the affinity of the female specific protein (vitellogenin) to Alizarin Red S indicates its affinity to calcium. The high calcium content of vitellogenin promotes the solubility of this protein and much of the vitellogenin-bound calcium may be incorporated into the oocytes to meet the needs during embryonic development. Thus

haemolymph serves as a transporting medium for calcium to the developing oocytes. A similar increase in calcium in the haemolymph was reported in *P. indicus* (Laxmilatha, 1991) during the reproductive cycles, which increased from the immature stage to the mature stage.

Vitellin from the mature ovaries of *F. merguensis* was dark-green in colour and identified as lipo-glycoproteins with calcium affinity similar to those of other penaeids (Quinitio *et al.*, 1990; Qiu *et al.*, 1997). Electrophoretic analysis of ovarian samples from *F. merguensis* revealed two forms of vitellin (Vn1 and Vn2) in native PAGE with apparent molecular weights of 550 and 300kDa. Vn1 was the main vitellin as compared to the expression of Vn2 as shown in the results of native PAGE. Vn2 was only a faint narrow band below Vn1 in native-PAGE. Subsequent characterisation of these proteins showed Vn1 and Vn2 to be lipo-glyco-calcium binding and glycoproteins respectively, in *F. merguensis*. Different forms of vitellins were reported in penaeids. Only one intact form of vitellin was detected in *P. japonicus* (Kawazoe *et al.*, 2000), *P. monodon* (Chang *et al.*, 1993), *Pandalus kessleri* (Quinitio *et al.*, 1989) and *Parapenaeus longirostris* (Tom *et al.*, 1987) while two forms of vitellins were purified from *P. chinensis* (Chang *et al.*, 1996), *P. vannamei* (Quackenbush, 1989a), *P. semisulcatus* (Shafir *et al.*, 1992), *P. monodon* (Chang *et al.*, 1994) and *P. chinensis* (Chang and Jeng, 1995a,b) and *Macrobrachium rosenbergii* (Derelle *et al.*, 1986; Lee *et al.*, 1997). The reported average molecular weight of native vitellin in most penaeids appears to be about 300kDa. Vitellins of higher molecular weight have also been reported in other penaeids. The molecular weight of vitellin in shrimps

Pandalus kessleri (Quinitio *et al.*, 1989; Chang *et al.*, 1993) and *P. monodon* (Quinitio *et al.*, 1990; Chang *et al.*, 1993) was 550kDa and 560kDa respectively.

SDS-PAGE analysis showed that vitellogenin and vitellin in *F. merguensis* had six and five major polypeptides respectively. The electrophoretic analysis revealed three polypeptide fractions with molecular weights 104, 102 and 68kDa to be commonly present in both vitellogenin and vitellin. This support the relationship that some subunits of ovarian vitellin were possibly and directly derived from haemolymph vitellogenin suggesting haemolymph polypeptides to be precursors of some yolk proteins. It is suggested that after incorporation of vitellogenin into oocytes, it may get partially digested and transformed into separate subunits forming the dominant components of ovarian vitellin. Similar results were reported in *P. chinensis*, where vitellogenin was shown to have two polypeptide subunits (191 and 85kDa) of which 191kDa was partially digested and transformed into vitellin in combination with 85kDa (Chang and Jeng, 1995a; Chang *et al.*, 1996). Further studies about the relationship of polypeptide subunits of vitellogenin and vitellin are necessary to clarify their association in *F. merguensis*.

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