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Ovarian protein synthesis in the prawn *Macrobrachium rosenbergii*: Does ovarian vitellin synthesis exist?

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Summary

During the reproductive cycle oocytes of *Macrobrachium rosenbergii* females grow in diameter from 20 μm to 650 μm, while the gonado-somatic index increases from 0.2 to 8.0. SDS-PAGE polypeptide profiles of the cytosolic fraction from ovaries in various stages were studied. Specific vitellin bands were identified by immunoblotting. *In vitro* synthesis of protein was measured by TCA precipitation and autoradiography of SDS-PAGE separated polypeptides. Incorporation of [^{35}S]-methionine and cysteine was highest in animals having an oocyte diameter of 50–150 μm and lower in pre-vitellogenic ovaries (20–50 μm). The lowest synthesis was found in late-vitellogenic ovaries (300–500 μm). Most of the incorporation was found in non-vitellin polypeptide bands. These results suggest the existence of an extraovarian source of vitellogenin in *M. rosenbergii*.

Key words: Vitellin biosynthesis, *Macrobrachium rosenbergii*, ovarian polypeptides, Decapoda

Introduction

In nature, *Macrobrachium rosenbergii* females may lay eggs three or four times a year (Ling, 1969). Embryogenesis in eggs carried by the female and the vitellogenesis in its ovary may overlap under optimal conditions (O'Donovan et al., 1984). The oogenesis is characterized by a rapid deposition of yolk and other proteins in the oocyte, which results in a fast increase in oocyte diameter. The yolk contains proteins, lipids, and carbohydrates (Adiyodi and Subramoniam, 1983; Adiyodi, 1985). The major lipoglycoprotein in the yolk is the vitellin that is accumulated in the oocyte cytoplasm and used later as a nutritional source for the developing embryo.

Vitellogenin is the precursor to egg yolk protein and is one of two lipoproteins known in crustacean hemolymph (Kerr, 1969; Lee, 1990; Quackenbush, 1991). Its concentration in the hemolymph is correlated with yolk accumulation in the oocyte (Quackenbush, 1989a; Lee, 1990; Okumura et al., 1992). A

controversy exists regarding the significance of extraovarian sources of vitellogenin synthesis such as the hepatopancreas and adipose tissue (Fainzilber et al., 1989). Some studies suggest that most of the vitellin in vitellogenic penaeid females is produced in the ovary and only a small amount is produced in extraovarian tissue (Fainzilber et al., 1992; Eastman-Reks and Fingerman, 1985; Lui and O'Connor, 1976a, 1976b). Furthermore, *in vitro* experiments show a low amount of vitellogenin synthesis in the hepatopancreas of penaeid shrimp and crabs (Quackenbush and Keeley, 1988; Quackenbush, 1989a, 1989b). Other researchers have been unable to identify vitellogenin synthesis in any extraovarian tissue of penaeid shrimp (Yano and Chinzei, 1987; Rankin et al., 1989). On the other hand, there is evidence that extraovarian sites do produce yolk proteins in crabs (Paulus and Laufer, 1987; lee and Puppione, 1988). Shafir et al. (1992a) demonstrated a role for the hemolymph in transporting vitellogenin between its processing and target sites. The latter was supported by the isolation of vitellogenin receptors from ovarian membrane of crayfish and lobster (Jugan and Van Herp, 1989; Laverdure and Soyez, 1988). The above studies suggest that vitellogenin enters the ovary by endocytosis, apparently through vitellogenin receptors. In this study we investigate the significance of ovarian vitellin synthesis in *M. rosenbergii*.

In vitro gonad culture has been established for many crustacean species (Lui and O'Connor, 1976a, 1976b; Rankin et al., 1989; Quackenbush, 1989a, 1989b; Franzilber et al., 1989). Here we described the gradual changes in protein composition which take place in the developing ovary of *M. rosenbergii* during the reproductive cycle, including the identification of vitellinic polypeptides by immunoblot. We used an organ culture of ovaries to show which polypeptide was synthesized *in vitro* during each stage of ovarian maturation.

Materials and Methods

Animals

Adult female *M. rosenbergii* were obtained from earthen ponds at Ginossar research station, Kibbutz Hazaorea and Mevo-Hama, Israel. The prawns were acclimated in a 500 gal freshwater tank for at least 1 week before experiments were begun. The water was recirculated through a gravel biofilter and temperature was kept at 25±3°C. The prawns were fed daily on frozen *Daphnia* and commercial prawn pellets. Females were selected as ovary donors according to their reproductive state: Females possessing mature ovaries (late-vitellogenic) were distinguished by their orange carapace color (Sagi and Ra'anan, 1985). Pre- to early-vitellogenic ovaries were selected according to their dark carapace coloration or the color of the eggs carried by the female (O'Donovan et al., 1984). Molt stage (Peebles, 1977) and oocyte diameter (O'Donovan et al., 1984) were determined for each dissected female. The latter was measured by means of objective micrometer. At least 30 oocytes were counted and measured per ovary. The results are given as minimal and maximal oocyte diameter found in the field.

Gonad culture

The ovaries were dissected into a sterile Petri dish containing phosphate buffered saline (PBS), adjusted to the osmolarity of *M. rosenbergii* hemolymph (Stern, 1985) at pH 7.4. Both lobes of the ovary were sliced into 2–4 mm fragments. The fragments were incubated in Dulbeco's modified Eagle's medium (DMEM) adjusted to *M. rosenbergii* osmolarity (Sagi et al., 1991), supplemented with 400 units penicillin per ml, 1% BSA, [^{35}S]-cysteine (3 μCi/ml). For autoradiography, fragments were incubated with a mixture of [^{35}S]-cysteine and [^{35}S]-methionine (60 μCi/ml) at 30°C with gentle shaking in an oxygen-enriched atmosphere (Fainzilber et al., 1989). At the end of the incubation period, the tissue fragments were removed and homogenized on ice with 0.5 ml of 0.05 M Tris-HCl buffer, pH 7.2, supplemented with 4 mM EDTA, 1 mM benzamidine, 1 mM ε-amino caproic acid and the protease inhibitors: 10 μg/ml leupeptine, 0.2 mM PMSF (in DMSO), 10 μg/ml and pepstatin (from DMSO dissolved stock solution). The homogenates were centrifuged at 18,000×g for 10 min at 4°C. The supernatant was separated and kept at −20°C. Total protein precipitation was performed by cold 5% TCA on Whatman 3 MM paper discs. The discs were washed with 500 ml 5% TCA (×3), 500 ml acetone and 500 ml of petrol ether, dried and submerged in scintillation fluid (80% toluene, 20% Lumax) and counted. The level of incorporation was expressed in cpm/μg total protein and normalized to the relative size of the ovary by multiplying by GSI. Total protein in each sample was determined by a mini-assay based on the Lowry method (Lowry et al., 1951) in a 96-well plate using an ELISA reader (Model EL 309, Bio-Tek Instruments, USA).

For autoradiography, samples from the supernatants were taken for SDS-PAGE and separated on 7% acrylamide gel (15 μg protein per lane) (Laemmli, 1970). After staining and destaining the gels were incubated for 30 min in Amplify (Amersham), dried and exposed in −80°C to X-ray film.

For immunoblot, ovaries were dissected, homogenized in the above homogenization buffer and centrifuged as above. A sample of the supernatant was used for SDS-PAGE as described above. Part of each gel was stained with Coomassie blue and used for the study of patterns of polypeptide distribution. The molecular weights were determined from a standard calibration curve of log molecular weight versus relative mobility of commercial protein markers (Sigma). The remaining parts of each gel were electro-transferred onto nitrocellulose paper (2 h 200 mA) in transfer buffer (12.5 mM Tris, 192 mM glycine, pH 8.3 containing 10% methanol). The nitrocellulose paper was stained by Ponceau S to assess the efficiency of electrotransfer. The relative stain intensity of the transferred polypeptides was similar to the original Coomassie blue stained profile. The nitrocellulose was blocked for 1 h in 5% Marvel's milk powder solution in PBS containing 0.1% Tween 20, then 2 h with 1000 fold diluted anti-vitellin serum raised in rabbit (antibodies against *M. rosenbergii* vitellin were generated, tested for specificity and supplied by Dr. Hans Laufer, University of Connecticut). Excess antibodies were washed five times for 5 min each in PBS and blocked again for 30 min without blocking buffer. The nitrocellulose was then incubated with [^{125}I]-protein A (30 mCi/mg protein A, Amersham, UK) for 1 h. Excess protein A was washed five times for 5 min each with PBS containing 0.1% Tween 20. Finally the nitrocellulose was dried and exposed to X-ray film at −80°C.

Results

During the reproductive cycle, oocytes in the ovaries from *M. rosenbergii* females grow in diameter from 20 μm up to 650 μm, while the gonado-somatic index increased from 0.2 in pre-vitellogenic ovaries to 8.0 in females with ripe ovaries. The relationship between these two parameters was exponential, as expected from the relationship between area and volume/weight of the oocytes (data not shown).

Incorporation rates of [^{35}S]-cysteine in *in vitro* cultured ovaries with oocyte diameter of 50–150 μm increased exponentially with incubation time (Fig. 1). The increase in the 100–250 μm ovaries seems also to be exponential, although it was less pronounced in the latter. Ovaries with other oocyte diameters showed either minor linear increase or no increase at all (Fig. 1). Fig. 2 shows incorporation levels of labeled cysteine after 8 h of *in vitro* incubation normalized by the gonado somatic index (GSI). Normalization was performed based on GSI in order

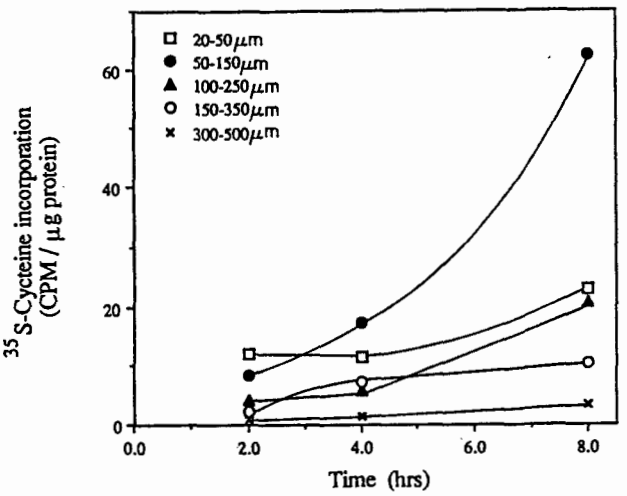

Fig. 1. Kinetics of [^{35}S]-cysteine incorporation (0–8h) into *M. rosenbergii* ovaries in organ culture. The ovaries were dissected from animals with different oocyte diameter (μm).

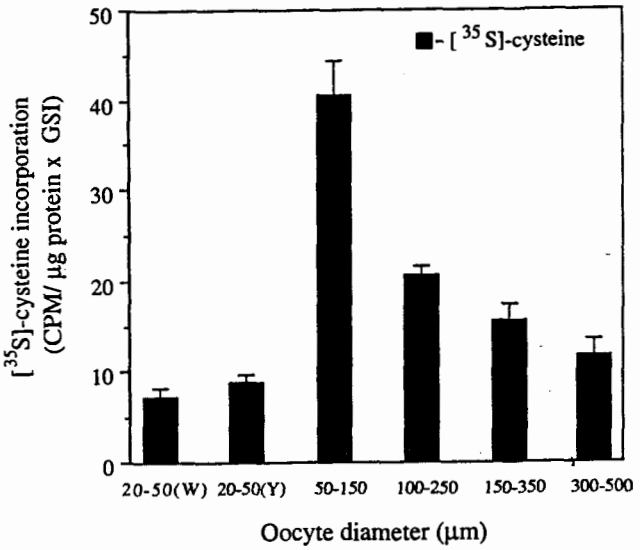

Fig. 2. [^{35}S]-cysteine incorporated into *M. rosenbergii* ovaries in organ culture. The ovaries were dissected from animals with different oocyte diameter (μm). W, white ovary; Y, yellowish ovary.

to correct for the accumulation of bulk non-metabolizing protein which comprises the majority of the protein in the vitellogenic ovary. The highest incorporation was found in the early-vitellogenic ovary with an oocyte diameter of 50–150 μm. The activity in vitellogenic ovaries was gradually reduced in fragments with larger oocyte diameter (100–500 μm). The lowest protein biosynthesis was found in pre-vitellogenic ovaries (20–50 μm).

In the course of the vitellogenic process the SDS-PAGE ovarian polypeptide profile (Fig. 3A) changes with the increase in oocyte diameter. Two types of previtellogenic ovaries with an oocyte diameter of 20–50 μm were observed: in one the ovaries were white (lane 1, Fig. 3) and in the other the ovaries were yellowish (lane 2, Fig. 3). Bands 88, 78 and 46 kDa are the most prominent Coomassie blue-stained bands in the white previtellogenic ovary. Bands of 206, 113, 84, 67, 64, 59, 37, 30 and 24 kDa are also present, although these are stained very faintly (lane 1, Fig. 3A). The yellowish pre-vitellogenic ovary (lane 2, Fig. 3A) has the same protein profile, although some additional high molecular weight polypeptide bands such as 113, 105 and 92 kDa are present. Although the oocyte diameter at this stage is identical to pre-vitellogenic ovaries, the yellowish ovary may represent a very early vitellogenic stage. In early-vitellogenic ovaries (lane 3, Fig. 3A), the most prominent bands observed are the 105 and 92 kDa polypeptides (the latter is more prominent by Coomassie blue stain); however, some other polypeptides such as 88, 78 and 45 kDa (barely seen in Fig. 3) are present. Late-vitellogenic phase ovaries (lanes 5 and 6, Fig. 3A) contain the 105 and 92 kDa bands as the main polypeptide of the ovary extract whereas the rest are hardly seen. A minor band of 88 kDa is also apparent (at the bottom of the heavy 92 kDa band).

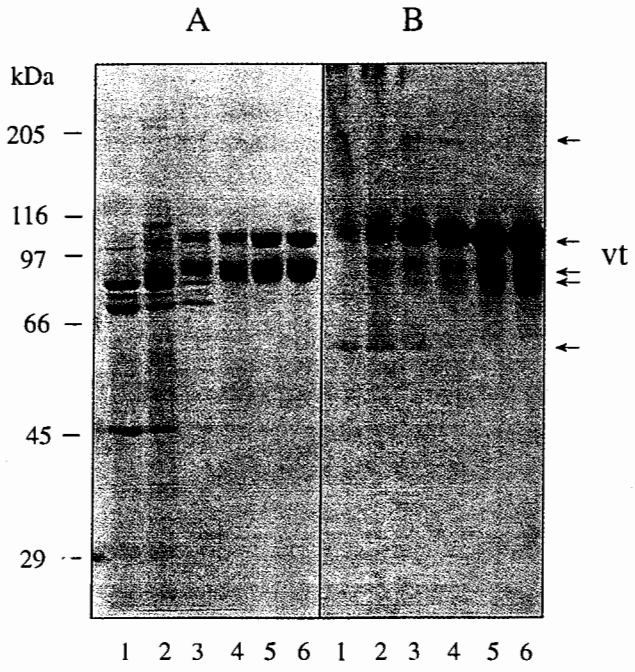

Fig. 3. SDS-PAGE separation of polypeptides from *M. rosenbergii* ovaries with different oocyte diameter. A. Coomassie blue stain of the polypeptide profile. B. Immunoblot blot of the same profile using polyclonal anti-vitellin antibodies, identified in the autoradiogram by [^{125}I]-protein A. 1, 20–50 μm (white); 2, 20–50 μm (yellowish); 3, 150–200 μm; 4, 120–260 μm; 5, 250–300 μm; 6, 500–650 μm.

Results of the immunoblot using polyclonal anti-vitellin antibodies (Fig. 3B) revealed the following picture: The immunoreactive vitellin bands observed were the 205, 105, 92, 88 and 64 kDa bands (arrows). The strongest immunoreactivity is noted on the prominent band of 105 kDa (in contrast to the Coomassie blue stain, Fig. 3A). This is less noticeable in the earlier stages of ovarian development and increases with the increase in oocyte diameter. The 92 kDa polypeptide seems to be less immunoreactive than the 105 kDa despite its higher Coomassie blue staining (relative abundance). It is completely absent in the white pre-vitellogenic stage (lane 1, Fig. 3B), and much more prominent with the development of the ovary. Immunoreactivity of the low molecular weight vitellin band of 88 kDa is very low. The 64 kDa band is visible in early stages of ovary maturation; both its relative abundance (lanes 1–3, Fig. 3A) and immunoreactivity (lanes 1–3, Fig. 3B) decrease in the process of gonad ripening.

When metabolic labeling of various polypeptides by [^{35}S]-cysteine and [^{35}S]-methionine was studied (Fig. 4), the following phenomena could be observed: About 20 polypeptides were labeled in pre- and early-vitellogenic ovaries (lanes 1–3, Fig. 4B), while almost no polypeptides were labeled in late ovaries at the end of the oocyte development process, including the prominent immunoreactive vitellin bands (lane 4, Fig. 4B). There were very low to negligible amounts of labeling of the vitellin bands (see Fig. 3; bands 64, 88, 92 and 105 kDa) at all stages of ovarian development. The process of diminishing bands' labeling during the maturation process has different patterns in different bands. For example, the labeled 100 kDa polypeptide does not change in the two kinds of pre-vitellogenic ovaries (lanes 1, 2, Fig. 4B). Its labeling decreases sharply

with the progress of the gonad maturation process. The labeled polypeptide 94 kDa (hardly observable by Coomassie blue stain) is diminishing gradually during the maturation process until it disappears completely in the ripe ovaries. The label of 46 kDa and 76 kDa decreases sharply between the two previtellogenic stages (compare lanes 1, 2; Fig. 4) consistently with the relative abundance of both of them in the Coomassie blue-stained profile. The majority of the bands was labeled more or less to the same degree in the pre- and early-vitellogenic ovaries (lanes 1–3, Fig. 4B). The amount of labeled material in the running front of each lane decreases with the progress of the maturation process. This fraction labeling indicates the incorporation of labeled amino acids into a smaller non-resolvable peptide mixture in a 7% acrylamide gel (separation done on denser gels showed that the front is composed of a mixture of low molecular weight peptides; data not shown).

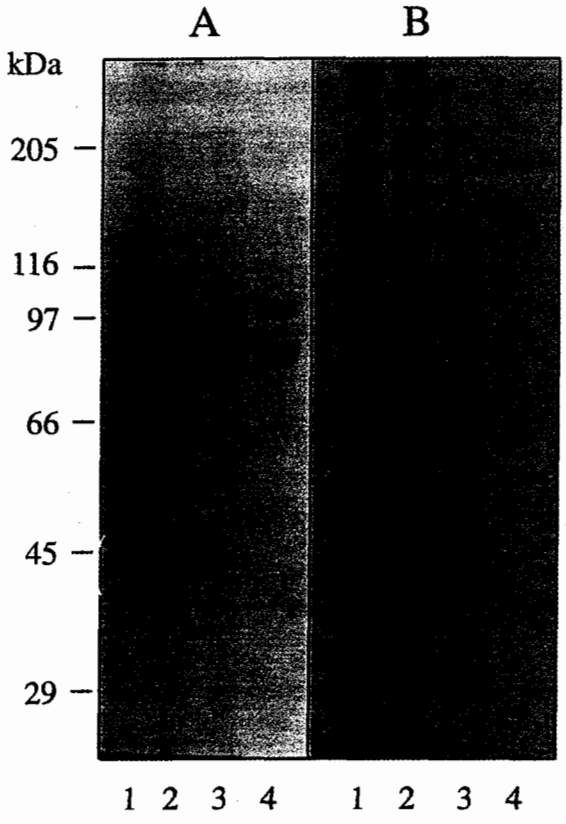

Fig. 4. SDS-PAGE separation of polypeptides from incubated *M. rosenbergii* ovaries with different oocyte diameter. A. Coomassie blue stain of the polypeptide profile. B. Autoradiogram of the same electrophoretogram. Ovarian fragments were incubated for 8 h with [^{35}S]-cysteine and [^{35}S]-methionine. 1, 20–50 μm (white); 2, 20–50 μm (yellowish); 3, 150–210 μm; 4, 500–650 μm.

Discussion

Polypeptide profiles in different stages of ovarian maturation show a trend of decreasing relative abundance until, at the end of the maturation process, only two predominant polypeptides (82 and 105 kDa) could be seen. Similar results were obtained recently by Chang et al. (1993). These two bands are also immunoreactive when exposed to anti-vitellin antibodies. Studies of vitellogenesis in several crustacean species show that the mobility of vitellin subunits in SDS-PAGE was in the range of 45–200 kDa and includes 2–5 subunits (Vazquez-Boucard et al., 1986; Yano and Chinzei, 1987; Tom et al., 1987; Quackenbush, 1989a, 1989b). Immunoreactive vitellin polypeptides in *M. rosenbergii* show similar patterns (Fig. 3).

Unlike Penaeid shrimps in which the amount of vitellin relative to total protein is reduced during late-vitellogenic stages (Quackenbush, 1991, for review; Shafir et al., 1992b), the proportion of vitellin out of all soluble proteins in *M. rosenbergii* ovaries increases up to the ripening of the gonad (Fig. 4). During the process of gonad maturation in *M. rosenbergii*, there is a massive accumulation of vitellin while the amount of [^{35}S]-amino acids incorporated into total ovarian protein decreases gradually (Fig. 4). These results contradict some studies in Penaeids in which the total incorporation into ovarian protein increases with oocyte maturation (Browdy et al., 1990; Shafir, 1992a). A possible explanation for this apparent contradiction could be the synthesis of cortical crypt proteins before spawning in *P. vannamei* (Bradfield et al., 1989) and the crypt formation stage in *P. semisulcatus*, in which the egg jelly precursor is synthesized (Browdy et al., 1990; Clark et al., 1990). The eggs of *M. rosenbergii* may not contain similar components due to differences in maternal care patterns. Another possible explanation is the difference in the source of vitellin (ovarian vs extraovarian) between these different species of decapods.

In vitro incorporation of [^{35}S]-amino acids took place at the pre- and early-vitellogenic stages. The ovarian 92 kDa and 105 kDa polypeptides that were identified as vitellin subunits were not labeled in the condition of the experiment. However, it may be suggested that the labeled band at the front of the 105 kDa vitellin band represents newly synthesized vitellin in these conditions. These results are surprising if one assumes that vitellin is synthesized in the ovary since during the vitellogenic stages the dominant process in the ovary is the accumulation of

vitellin. Nevertheless, the amount of incorporated radioactivity into suspected vitellin bands is very low in relation to the amount of Coomassie blue stain or immunoreactive vitellin (compare Figs. 3 and 4). These results suggest the possibility of significant extraovarian sources of vitellin synthesis (Paulus and Laufer, 1987; Fainzilber, 1989; Quackenbush, 1989b; Shafir, 1992a). Furthermore, an increase in vitellogenin levels in the hemolymph during the vitellogenic process with a drastic decrease prior to spawning was reported in *M. rosenbergii* (Derelle et al., 1986). This phenomenon, also described by Okumura et al. (1992) in *M. nipponense*, strongly suggests an extraovarian site for vitellogenin synthesis in *M. rosenbergii*. The existence of vitellogenin receptors in oocyte membrane of lobsters (Laverdure and Soyez, 1988) and crayfish (Jugan and Van Herp, 1989) also supports the possibility of the existence of an extraovarian source for yolk protein. Also, in *M. rosenbergii* oocyte endocytosis and exchange systems between hemolymph and vitellogenic oocytes were described (Jugan and Zerbib, 1984; Jugan and Soyez, 1985).

The source of yolk protein may vary in different crustacean species. Similar to crabs and lobsters and unlike penaeid shrimps, this study suggests that there may be a significant extraovarian source for vitellin in *M. rosenbergii*. However, more work is needed to identify the sites of vitellogenin synthesis in these animals.

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