The hepatopancreas as a site of yolk protein synthesis in the prawn *Macrobrachium rosenbergii*

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Summary

Our previous study failed to show vitellin synthesis in the ovary of the prawn *Macrobrachium rosenbergii* (Sagi et al., 1995); thus the role of the hepatopancreas as a possible site of synthesis was evaluated. Extracts of hepatopancreas and hemolymph of a secondary-vitellogenic female exhibited higher levels of yolk protein than those from a primary-vitellogenic female. Clear vitellin immuno-cross-reactivity was observed in hepatopancreas sections from a secondary-vitellogenic female while no such reaction was found in a male hepatopancreas. Furthermore, vitellin-immuno-cross-reactive polypeptides released into the culture medium of the hepatopancreas of a secondary-vitellogenic female were similar to those found in the hemolymph and ovary (92 and 105 kDa). The most prominent immuno-reactive polypeptide in the hepatopancreas extract was a relatively low-molecular-weight species (42 kDa). *De novo* synthesis of cross-reactive-vitellin polypeptides (34, 38 and 42 kDa) was detected in the hepatopancreas of a secondary-vitellogenic female. Synthesis of these polypeptides were not detected in the secondary-vitellogenic ovary or in the male hepatopancreas. The appearance of similar polypeptides following incubation of a secondary-vitellogenic ovarian extract with a glycosidase suggests that these polypeptides could be subunits of a core protein of vitellogenin, which was synthesized in the hepatopancreas and then released to the hemolymph following post-translational modifications. Our findings thus suggest the hepatopancreas to be a likely site of synthesis of a yolk protein precursor in *M. rosenbergii*.

Key words: Vitellin, vitellogenin, *Macrobrachium rosenbergii*, yolk proteins, biosynthesis, hepatopancreas

Introduction

There has long been debate in the literature over the site of yolk protein synthesis in crustaceans. A number of studies have suggested the ovary to be the main source of yolk protein. In the blue crab *Callinectes* *sapidus* and in the shrimp *Penaeus japonicus*, for example, *in vitro* synthesis of vitellin, the main component of the yolk, was detected solely in the ovary (Lee and Watson, 1995; Yano and Chinzei, 1987). Browdy et al. (1990) claimed that 40–60% of

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the protein synthesized *in vitro* in the ovary of *P. semisulcatus* to be immunologically identical with vitellin. Less conclusive was the study of Fainzilber et al. (1992) on the same species, which suggested that there was some contribution to vitellogenin bio-synthesis from extraovarian sources, although it was low compared to that from intraovarian synthesis.

In contrast to the studies suggesting that vitellin synthesis takes place exclusively or almost exclusively in the ovary, our previous study on the prawn *Macrobrachium rosenbergii* showed that there was no incorporation of labeled amino acids into ovarian vitellin *in vitro* (Sagi et al., 1995). There is supporting evidence in the literature for extraovarian vitellin synthesis in a number of crustacean species; for example, in *M. nipponense* and *M. rosenbergii*, vitellogenin, the precursor of vitellin, was found in the hemolymph where it began to increase in the early stages of ovarian development and then, after reaching a maximal level, fell abruptly close to oviposition (Chang and Shih, 1995; Okumura et al., 1992). Extraovarian synthesis is also supported by the isolation of a vitellogenin receptor from the ovarian membrane of crayfish and lobster (Jugan and Van Herp, 1989; Laverdure and Soyez, 1988). The hepatopancreas and the subepidermal adipose tissue have been suggested as possible extraovarian sites of yolk protein synthesis. Vitellogenin was indeed detected in the hepatopancreas and the subepidermal tissue of vitellogenic *M. nipponense* females (Han et al., 1994). Furthermore, *in vitro* experiments showed a low amount of vitellogenin synthesis in the hepatopancreas of penaeid shrimps and prawns (Quackenbush, 1989a; Quackenbush, 1989b; Soroka et al., 1995). Based upon immunolabeling of electron microscope sections, Yano et al. (1996) suggested that in *P. japonicus* yolk protein is transferred to the surface of yolk-stage oocytes through spaces between the neighboring follicle cells; the yolk protein is then incorporated into the ooplasm by pinocytosis through the microvilli and subsequently aggregated to form yolk bodies.

Vitellogenin was recently purified and character-ized in the hemolymph of a vitellogenic *M. rosenbergii* female (Chen and Kuo, 1998). It was identified as a lipoglycocarotenoprotein with an apparent molecular mass of 700 kDa (Chang et al., 1993; Lee et al., 1997). In the denatured state, *M. rosenbergii* vitellogenin appeared to be dissociated into three subunits (~180, ~104, and ~90 kDa), while denatured vitellin showed only two subunits (~104 and ~90 kDa) (Chang et al., 1993; Lee et al., 1997; Chen and Kuo, 1998). A strong

similarity was found in the amino acid composition of vitellin and vitellogenin, i.e., eight amino acids from the N-terminal of each of the 104 and 90 kDa subunits were identical in the two proteins (Lee et al., 1997). This correlation strengthens the likelihood of extra-ovarian synthesis, the products of the synthesis moving through the hemolymph to accumulate in the ovary.

In the present study we investigated the incorporation of labeled amino acids into newly synthesized polypeptides in the hepatopancreas of *M. rosenbergii*. In the vitellogenic female, these newly synthesized polypeptides reacted immunologically with an anti-vitellin-antibody, which suggested that the hepatopancreas is indeed an extraovarian site of yolk protein synthesis in this species.

Materials and Methods

Animals

Adult *M. rosenbergii* were obtained from earthen ponds at Kibbutz Neve-Ur, Israel. The prawns were acclimated in a 500-gal freshwater tank for at least 1 week before the experiments were begun. The water was recirculated through a gravel biofilter, and the temperature was kept at $25 \pm 3\,°C$. The prawns were fed daily on frozen *Daphnia* and commercial prawn pellets. Females were selected as ovary donors according to their reproductive state: animals possessing mature ovaries (secondary-vitellogenic specimens) were distinguished by the orange color observed through the carapace (Sagi and Ra'anan, 1985). Primary- to early-vitellogenic females were selected according to the dark coloration of the ovary through the carapace. Molt stage (Peebles, 1977) and oocyte diameter were determined for each dissected female. Oocyte diameter was determined under a light microscope by means of an objective micrometer. A sample of at least 20 oocytes per ovary was measured, and the average oocyte diameter was calculated.

ELISA

ELISA was performed according to the method of Derelle et al. (1986). Cytosolic extracts of hepato-pancreas and hemolymph were diluted in Na_2CO_3 (0.1 M, pH 9.6) and allowed to bind in ELISA 96 plates overnight at 4 °C. The immunoreaction was performed with rabbit polyclonal anti-vitellin (*M. rosenbergii*) antisera (supplied by Prof. H. Laufer, University of Connecticut). The antiserum was

prepared against ovarian protein purified by the following purification cascade: saturated ammonium sulfate precipitation; NaBr gradient; Sepadex G-200 column; PAGE separation followed by elution of the separated band, dialysis; injection into rabbit with complete Freund's adjuvent (Laufer, personal communication). The antiserum was confirmed to be specific by an immunodiffusion test, and further removal of nonspecific hemolymphatic components was done by precipitation with 10% (v/v) male hemolymph. The secondary antibody was goat anti-rabbit conjugated with alkaline phosphatase. The amount of vitellin cross-reactive material was determined by monitoring the color change reaction of *p*-nitrophenyl phosphate — as the substrate — to *p*-nitrophenol. The color change was read at 405 nm with an ELISA reader (Bio-Tek Instruments). A cytosolic extract of newly laid unfertilized eggs (assuming that it contains mostly yolk protein) was used to construct a standard curve. Newly laid eggs were collected, homogenized in homogenization buffer (0.05M Tris-HCl buffer, pH 7.2) and centrifuged (15,000 g, 10 min). The amount of protein was determined in the supernatant, which was then used for calibration.

Histology and immunofluorescence

The hepatopancreas of a vitellogenic female and that of an adult male were dissected out and sliced into fragments, which were fixed in Bouin's solution for 24 h, dehydrated in five steps from 30% to 100% ethanol, and embedded in Paraplast. Six-micrometer sections were cut and mounted on glass slides. Tissue sections were stained with hematoxylin and eosin and observed under a light microscope.

Localization of vitellogenin was performed in the hepatopancreas of a secondary-vitellogenic female prawn [gonadosomatic index (GSI) = 0.7] and in that of a male specimen. The tissue was fixed in 3% paraformaldehyde for 2 h at room temperature, frozen in liquid nitrogen, sectioned with a cryostat to a width of 6 μm, and mounted on glass slides. The sections were blocked in blocking buffer [10% milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20] for 1 h, following exposure to anti-vitellogenin antibody in the same buffer overnight at 4°C. Excess antibody was washed off with PBS. The samples were incubated with a secondary anti-rabbit antibody conjugated with fluorescein isothiocyanate (FITC) and analyzed by a confocal fluorescence microscope (Bio-Rad, MRC1024).

Gonad organ culture

The organ — ovary or hepatopancreas — was dissected into a sterile Petri dish containing Dulbecco's modified Eagle's medium (DMEM), pH 7.4, adjusted to *M. rosenbergii* osmolarity (Sagi et al., 1991; Stern, 1985). The tissue was sliced into 2- to 4-mm fragments, weighed, and incubated for 8 h at 28°C with gentle shaking in an oxygen-enriched atmosphere in DMEM supplemented with penicillin, 400 units/ml, 1% BSA, and [^{14}C] amino acid (F,V,G) mixture (Amersham), 50 μCi/ml (Sagi et al., 1995). At the end of the incubation period, the tissue fragments were removed and homogenized on ice in an homogenization buffer (0.05 M Tris-HCl buffer, pH 7.2, containing 4 mM EDTA, 1 mM benzamidine, 1 mM ε-amino caproic acid and the following protease inhibitors: 10 μg/ml leupeptine, 0.2 mM PMSF and 10 μg/ml pepstatin). The homogenates were centrifuged at 15,000 g for 15 min at 4°C. The supernatant was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 8.5% acrylamide gel) normalized per equal amount of wet weight and used for autoradiography.

Immunoprecipitation

The first step was the formation of a complex of the vitellogenin antibody with protein A-agarose (Bio-Rad). Each sample contained 25 μl of protein A-agarose and 3 μl of vitellogenin antiserum in a final volume of 400 μl of HKA buffer, which consisted Hepes 10 mM, pH 7.5; K-acetate, 140 mM; MgCl$_2$, 1 mM; EGTA, 0.1 mM; gelatin 0.1%; and BSA 0.1%. The incubation was carried at 4°C with gentle shaking for 2 h. The complex was precipitated and washed three times with HKA buffer. In parallel, extraction of the sample was performed with 2% Triton X-100 in HKA buffer for 1 h. In the second step, supernatant from the first step (5,000 g for 15 min) was allowed to react with the above-described antibody complex overnight at 4°C with gentle shaking. The final step comprised precipitation the complex (2000 g), followed by three washes with HKA buffer containing 0.2% Triton X-100. The pellet was dissolved in denaturating sample buffer and loaded on a 8.5% acrylamide SDS-PAGE system (Laemmli, 1970).

Immunoblot

Ovaries and hepatopancreases were dissected and sliced into fragments (4 × 3 mm). Each fragment was weighed, homogenized in homogenization buffer (see

above) — 1 mg of tissue per 0.02 ml of homogenization buffer — and centrifuged (15000 g, 15 min). A sample of the supernatant was used for SDS-PAGE (Laemmli, 1970; 8.5% acrylamide) in an Hoefer minigel apparatus. One gel was stained with Coomassie blue and used for the study of polypeptide distribution, while a second gel was electrotransferred onto nitrocellulose paper (1 h, 400 mA) in transfer buffer (12.5 mM Tris, 192 mM glycine, pH 8.3, 10% methanol). The nitrocellulose paper was stained with Ponceau S to assess the efficiency of electrotransfer. The nitrocellulose was blocked for 1 h in blocking buffer (see above) and then incubated for 2 h with anti-vitellin serum. Excess antibody was washed off five times for 5 min each time in PBS. The nitrocellulose was blocked again for 15 min with blocking buffer and then incubated for 1 h with a secondary goat anti-rabbit peroxidase conjugated antibody. Excess secondary antibody was washed off five times for 5 min each time with PBS containing 0.1% Tween 20. Finally, the nitrocellulose was developed by the ECL method (Pollini et al., 1993).

Glycosidation

To study the nature of *de novo* synthesized low-molecular-weight vitellin immunoreactive polypeptides, we incubated secondary-vitellogenic ovarian extracts (1 mg/ml final concentration) with N-glycosidase F (New England Biolab kit) overnight. This enzyme cleaves the innermost GlcNAc and asparagine residues of complex oligosaccharides from N-linked glycoproteins.

Results

Significantly higher levels of vitellin-immunoreactive material ($p < 0.05$) were detected by ELISA in both the hemolymph (Fig. 1A) and hepatopancreas (Fig. 1B) of secondary-vitellogenic females (oocyte diameter >150 μm) than in primary-vitellogenic females (oocyte diameter <50 μm). The average hemolymph concentration of vitellin-immunoreactive material was 2.7 ± 0.9 mg/ml ($n = 31$) and 7.3 ± 1.1 mg/ml ($n = 15$) in primary- and secondary-vitellogenic females, respectively. The average concentration of vitellin equivalent material in the hepatopancreas was 13.1 ± 4.8 ng/μg protein ($n = 16$) and 158 ± 64 ng/μg protein ($n = 8$) in the primary- and secondary-vitellogenic stages, respectively.

These results were confirmed by immunolocalization trials. The histology of the hepatopancreas
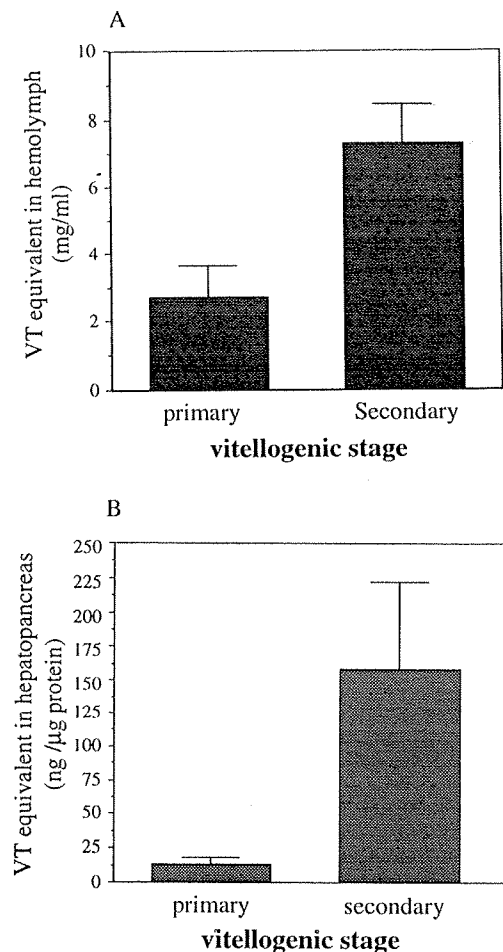
Fig. 1. Concentrations of vitellin cross-reactive material in the hemolymph and hepatopancreas of *M. rosenbergii* females at primary- and secondary-vitellogenic stages. An ELISA was performed with hemolymph samples from primary-vitellogenic females (oocyte diameter <50 μm) and secondary-vitellogenic females (oocyte diameter >150 μm). The differences were significant in both A and B (*t*-test, $\alpha = 0.05$). A, hemolymph; B, hepatopancreas.

from a male and a secondary-vitellogenic female (GSI $= 2.4$; oocyte diameter $= 319 \mu$m) stained with hematoxylin and eosin (Fig. 2A) showed an acinar-like structure, typical of pancreatic tissue (Ross et al., 1995). Immunolocalization with anti-vitellin antibody labeled with FITC showed clear immunoreactivity in the hepatopancreas of the secondary-vitellogenic female in a duct like structure (Fig. 2B). In contrast, there was no vitellin-immuno-reactive material in the male hepatopancreas.

To investigate the possible flow of vitellogenic material, an immunoblot was performed on the SDS-PAGE separated samples of the hepatopancreas, hemolymph and ovary from a secondary-vitellogenic

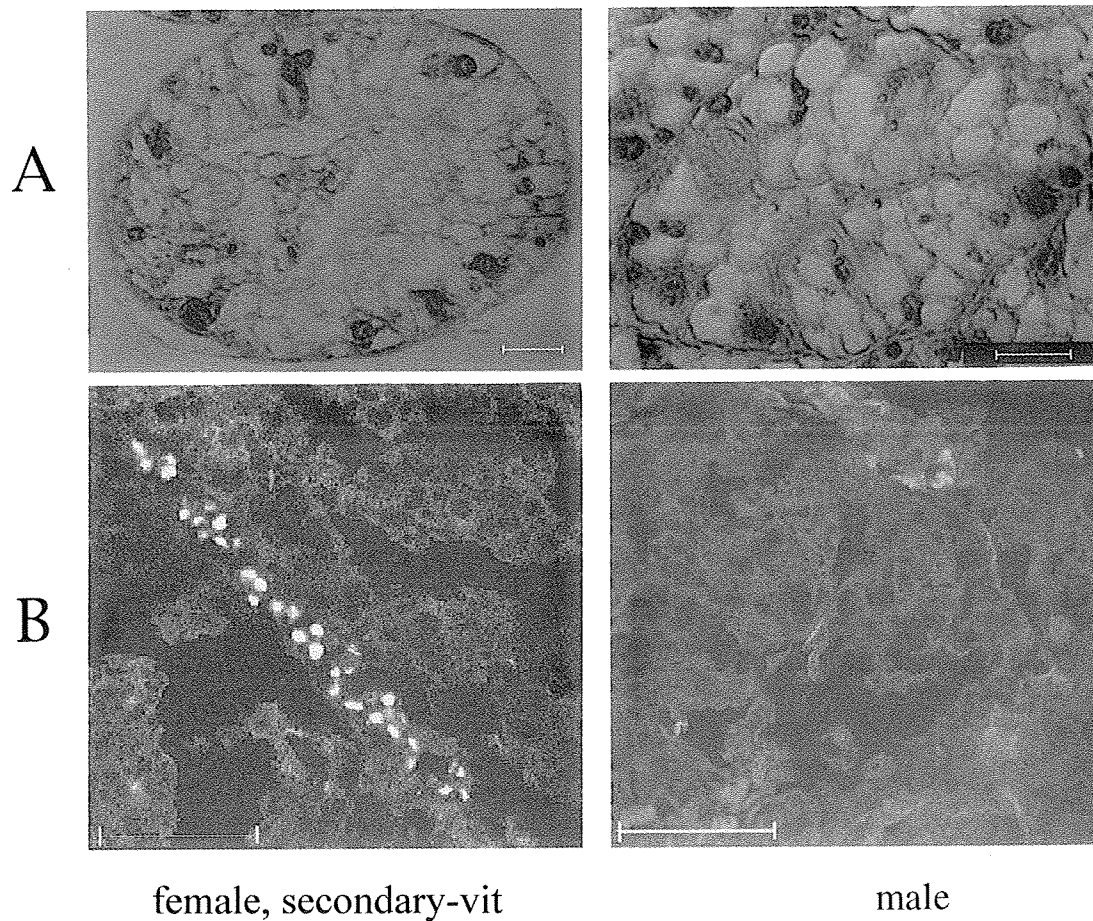female, secondary-vit   male

Fig. 2. Localization of vitellin-immunoreactive material in sections of the hepatopancreas from a *M. rosenbergii* male and a secondary-vitellogenic female. A. Hematoxylin and eosin stain of paraplast section; bars = 25 μm. B Immunofluorescence with FITC-labeled antibody after cryosection. The female had a GSI of 0.7; bars = 50 μm. The male was a mature reproductive specimen.

female (Fig. 3). In the hepatopancreas, the most abundant vitellin-immuno-reactive polypeptide was that of 41 kDa (Fig. 3, HP), while the 105-kDa polypeptide was the predominant cross-reactive polypeptide secreted into the medium during the 8 h of hepatopancreas incubation (Fig. 3, HP MED). A similarity in polypeptide profile was observed for the hemolymph, the ovary, and the culture medium of the hepatopancreas from the same female (all lanes included immunoreactive material with approximate molecular masses of 92 and 105 kDa). In addition, the hemolymph contained a 180 kDa polypeptide and some smaller molecular weight polypeptides showed immunoreactivity in the hemolymph and ovary.

To study *de novo* synthesis of vitellin polypeptides, we performed immunoprecipitation of ovaries and hepatopancreas extracts which were organ cultured in the presence of radiolabeled amino acids. The main ovarian vitellin subunits (92 and 105 kDa) were

immunoprecipitated but were not labeled (Fig. 4, SVO), showing that these polypeptides were present but their synthesis was not detected in the ovary. In the hepatopancreas of a secondary-vitellogenic female, three labeled polypeptides (34, 38 and 42 kDa) were immunoprecipitated, suggesting that they were newly synthesized vitellin-immuno-reactive polypeptides (Fig. 4B, SVH). No such synthesis was detected in the secondary-vitellogenic ovary or in the male hepatopancreas (Fig. 4B, SVO and M, respectively). In the hepatopancreas of the primary-vitellogenic female (Fig. 4B, PVH), only relatively low synthesis of vitellin-immunoreactive polypeptides was observed (39 kDa).

To study the nature of these *de novo* synthesized low-molecular-weight vitellin-immuno-reactive polypeptides, we incubated secondary-vitellogenic ovarian extracts with N-glycosidase F (Fig. 5). This enzyme caused a decrease in the amount of treated 92-kDa
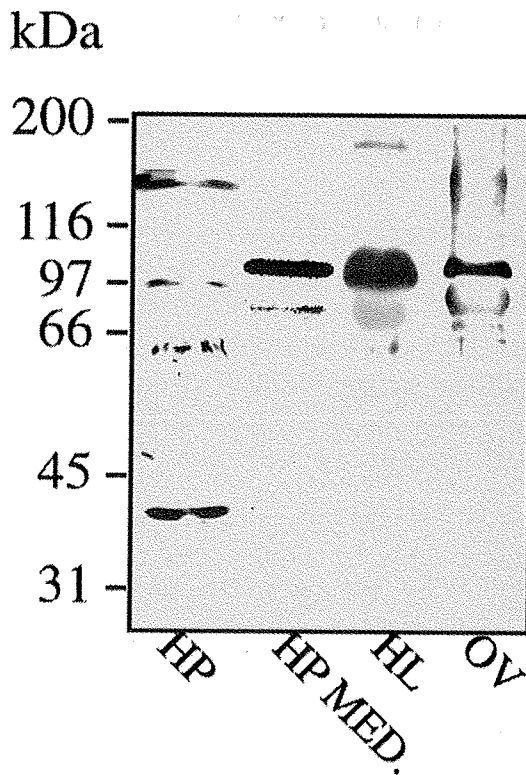
Fig. 3. Vitellin-immunoreactive material in immunoblot of hepatopancreas, culture medium after incubation with hepatopancreas, hemolymph, and ovary from a secondary-vitellogenic *M. rosenbergii* female. HP, hepatopancreas; HP MED, culture medium after 8 h of incubation with hepatopancreas fragments; HL, hemolymph; OV, ovary; the GSI of the female was 2.4; molecular mass markers are indicated in kDa.

Fig. 4. Incorporation of [^{14}C]-amino acids mixture into immunoreactive vitellin polypeptides in the hepatopancreas and ovary of *M. rosenbergii* female. A. SDS-PAGE polypeptide profile of an immunoprecipitated vitellin in cultured ovaries and hepatopancreas extracts, stained with Coomassie blue; B. Autoradiography of the same gel; SVO, secondary-vitellogenic ovary; PVH, hepatopancreas from primary-vitellogenic female; SVH, hepatopancreas from secondary-vitellogenic female; M, male hepatopancreas. Arrows point to *de novo* synthesized vitellin cross-reactive polypeptides; molecular mass markers are indicated in kDa.

polypeptide (Fig. 5, empty arrow). A prominent 82-kDa band appeared in addition to three low-molecular-weight polypeptides at 35, 42 and 50 kDa (Fig. 5, black arrows), a finding that could indicate at least three sites of asparagine-linked oligosaccharide chains. Among these three, the 35- and 42-kDa low-molecular-weight polypeptides resembled those synthesized in the hepatopancreas of the secondary-vitellogenic female (Fig. 4B, arrows). The amount and the size of the upper 105-kDa subunit remained unchanged.

Fig. 5. Anti-vitellin immunoblot of an ovarian extract and an ovarian extract treated with N-glycosidase F. Dark arrows point to cleaved vitellin polypeptides, the empty arrow points to the 95-kDa subunit of vitellin. Molecular mass markers are indicated in kDa. Control, untreated ovarian extract; ovarian extract treated with N-glycosidase F.

Discussion

Support for the hypothesis of extraovarian yolk protein synthesis in crustaceans comes from our current findings. We showed that vitellogenin concentrations in the hemolymph and in the hepatopancreas of a secondary-vitellogenic
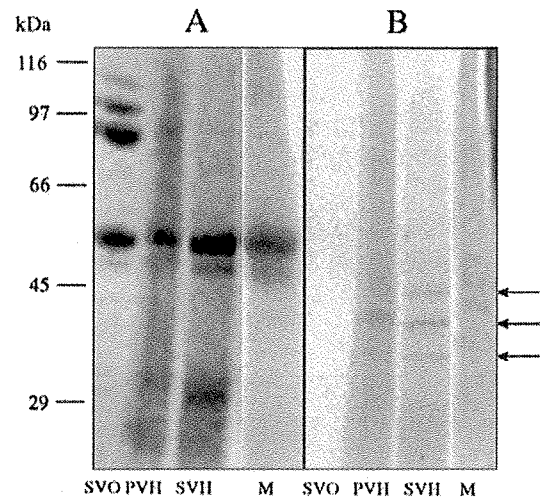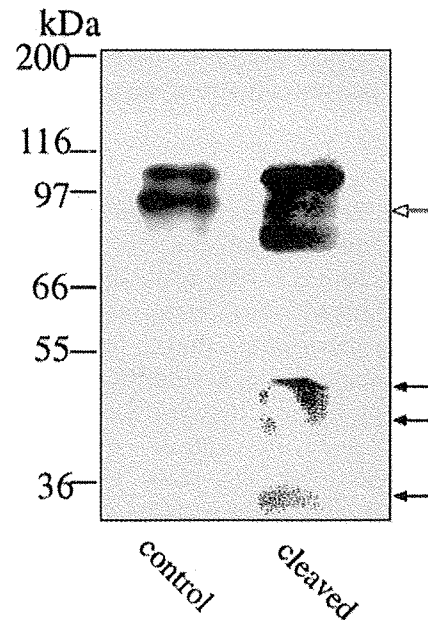
M. rosenbergii female were significantly higher than those in a primary-vitellogenic specimen (Fig. 1). These findings are consistent with previous reports of the presence of vitellogenin in the hepatopancreas and in the hemolymph of *M. nipponense* and *M. rosenbergii* (Han et al., 1994; Lee and Chang, 1997) and of changes in the concentration of vitellogenin in the hemolymph in relation to vitellogenesis in both these species (Okumura et al., 1992; Lee and Chang, 1997). We also showed that in *M. rosenbergii* there are specific sites in the hepatopancreas of secondary-vitellogenic females that exhibited immunoreactivity with anti-vitellin antibody. Furthermore, vitellogenin was synthesized in organ culture of the hepatopancreas and was released into the medium as vitellin-immuno-reactive material in similar molecular weight as in the hemolymph and in the ovary. We also found here two subunits of vitellin in the ovary and three vitellin-immuno-reactive subunits in the hemolymph, in keeping with recent studies of yolk proteins in *M. rosenbergii*. Lee et al. (1997) found a similarity in molecular weights and amino-acid sequences between vitellogenin in the hemolymph and vitellin accumulating in the ovary. Chen and Kuo (1998) demonstrated immuno-cross-reactivity among specific subunits of vitellin and vitellogenin. These authors speculated that the high molecular weight subunit of vitellogenin (~180 kDa) in the hemolymph might be the precursor of the smaller subunits in the ovary. An additional indication of extraovarian synthesis comes from the identification of a receptor to vitellogenin in the oocyte of a decapod (a crayfish; Jugan and Van Herp, 1989). All the above-described evidence suggests extraovarian synthesis of vitellogenin, which moves through the hemolymph to accumulate in the ovary.

Vitellogenin synthesis has also been reported in extraovarian sites other than the hepatopancreas such as the subepidermal tissue in *M. nipponense* (Han et al., 1994) and the subepidermal adipose tissue in *P. semisulcatus* (Fainzilber et al., 1992). Adipose tissue has not been investigated in *M. rosenbergii*. It may thus be assumed that the site of yolk protein synthesis may vary from species to species within the crustaceans.

An explanation is required for the significant difference in molecular weights between the ovarian and hepatopancreatic polypeptides precipitated with anti-vitellin antibody (Fig. 4A "SVO" and Fig. 4B "SVH", arrows, respectively), i.e., the vitellin that had accumulated in the ovary was separated on SDS-PAGE into two subunits of 92 kDa and 105 kDa, while the vitellin-immuno-reactive polypeptides synthesized *de*

novo in the hepatopancreas of the secondary vitellogenic female were much smaller (34, 38, and 42 kDa). These differences in molecular weights could be explained by degradation of vitellin into small polypeptides in the hepatopancreas due to high amounts of proteolytic enzymes in that organ. Alternatively, the relatively small molecular weight polypeptides could constitute the core proteins of vitellogenin, prior to glycosylation and/or incorporation of lipid moieties. The latter possibility is strengthened by the finding, in a vitellogenic *P. semisulcatus* female, of a translation product from purified mRNA from the site of synthesis to yield similar low-molecular-weight polypeptides immuno-reactive to vitellin antibody (Khayat et al., 1994). In addition, Fig. 5 shows that after N-glycosidase F digestion of ovarian vitellin *in vitro*, similar low-molecular-weight polypeptides with immunoreactivity to vitellin appeared. However, the 92-kDa subunit is a glycoprotein, as was shown by the decrease in its molecular weight after digestion with N-glycosidase F, whereas the 105-kDa subunit remained intact. This finding is in agreement with that of Lee et al. (1997), who showed that only the lower-molecular-weight subunit of *M. rosenbergii* vitellin is a glycoprotein. Fig. 3 showed that most of the vitellogenin in the hepatopancreas was of the low-molecular-weight form. This is a possible explanation for the labeling profile of the relatively low molecular weight polypeptides in the hepatopancreas of a secondary-vitellogenic female (Fig. 4B). The N-glycosidase F digestion could support the possibility that the 92-kDa polypeptide is composed from the core protein and some others glycopeptides. However, it does not provide an explanation regarding the 105 kDa.

In light of all the above studies of *M. rosenbergii* vitellogenesis, the following general cascade of synthesis and processing of vitellogenin may be proposed for this species: The core protein is initially synthesized in the hepatopancreas (shown by the presence of low-molecular-weight polypeptides). Glycosylation and incorporation of carotenoids and lipids then takes place to produce vitellogenin, which is secreted rapidly into the hemolymph (as shown by the fact that there is relatively low amount of 92 and 105 kDa in the hepatopancreas, while it is present in high concentrations in the hemolymph or culture medium). Finally, the lipoglycocarotenoprotein is accumulated, with slight structural changes, as vitellin in the ovary.

References

Browdy, C.L., Fainzilber, M., Tom, M., Loya, Y. and Lubzens, E., Vitellin synthesis in relation to oogenesis in *in vitro*-incubated ovaries of *Penaeus semisulcatus* (Crustacea, Decapoda, Penaeidae). J. Exp. Zool., 255 (1990) 205–215.

Chang, C.F., Shih, T.W. and Hong, H.H., Purification and characterization of vitellin from the mature ovaries of prawn, *Macrobrachium rosenbergii*. Comp. Biochem. Physiol. B, 105 (1993) 609–615.

Chang, C.F. and Shih, T.W., Reproductive cycle of ovarian development and vitellogenin profiles in the freshwater prawn, *Macrobrachium rosenbergii*. Invert. Reprod. Develop., 27 (1995) 11–20.

Chen, Y.N. and Kuo, C.M., Purification and characterization of vitellin from the freshwater giant prawn, *Macrobrachium rosenbergii*. Zool. Studies, 37 (1998) 126–136.

Derelle, E., Grosclaude, J., Meusy, J.J., Junera, H. and Martin, M., ELISA titration of vitellogenin and vitellin in the freshwater prawn, *Macrobrachium rosenbergii*, with monoclonal antibody. Comp. Biochem. Physiol., 85B (1986) 1–4.

Fainzilber, M., Browdy, C.L., Tom, M., Lubzens, E. and Applebaum, S.W., Protein synthesis *in vitro* in cultures of the subepidermal adipose tissue and the ovary of the shrimp *Penaeus semisulcatus*. Tissue & Cell, 21 (1989) 911–916.

Fainzilber, M., Tom, M., Shafir, S., Applebaum, S.W. and Lubzens, E., Is there extraovarian synthesis of vitellogenin in penaeid shrimp? Biol. Bull., 183 (1992) 233–241.

Han, C.H., Okumura, T., Suzuki, Y., Aida, K. and Hanyu, I., Immunocytochemical identification of the site of vitellogenin synthesis in the freshwater prawn *Macrobrachium nipponense*. Fisheries Science, 60 (1994) 149–154.

Jugan, P. and Van Herp, F., Introductory study of an oocyte membrane protein that specifically binds vitellogenin in the crayfish *Orconectes limosus*. Invert. Reprod. Develop., 16 (1989) 149–154.

Khayat, M., Lubzens, E., Tietz, A. and Funkenstein, B., Cell-free synthesis of vitellin in the shrimp *Penaeus semisulcatus* (de Haan). Gen. Comp. Endocrinol., 93 (1994) 205–213.

Laemmli, U.K., Cleavage of struct⁻   ⁻ring the assembly of the head of bact⁻   ⁻⁻7 (1970) 680–685.

Laverdure, A.M. and Soyez, lobster oocyte m⁺ characterization by a s Reprod. Develop., 13 (.

Lee, C.Y. and Watson, R.D.,   ⌐ᵢ vitellogenesis in the blue crab (*Callinec ⌐ ₛapidus*): Site and control of vitellin synthesis. J. Exp. Zool., 271 (1995) 364–372.

Lee, F.Y. and Chang, C.F., The concentrations of vitellogenin (vitellin) and protein in hemolymph, ovary and hepatopancreas in different ovarian stages of the freshwater prawn, *Macrobrachium rosenbergii*. Comp. Biochem. Physiol. A, 117 (1997) 433–439.

Lee, F.Y., Shih, T.W. and Chang, C.F., Isolation and characterization of the female-specific protein (vitellogenin) in mature female hemolymph of the freshwater prawn, *Macrobrachium rosenbergii*: comparison with ovarian vitellin. Gen. Comp. Endocrinol., 108 (1997) 406–415.

Okumura, T., Han, C.H., Suzuki, Y., Aida, K. and Hanyu, I., Changes in hemolymph vitellogenin and ecdysteroid levels during the reproductive and non-reproductive molt cycles in the freshwater prawn *Macrobrachium nipponense*. Zool. Sci., 9 (1992) 37–45.

Peebles, J.B., A rapid technique for molt staging in live *Macrobrachium rosenbergii*. Aquaculture, 12 (1977) 173–180.

Pollini, C.P., Giunchedi, L. and Credi, R., A chemi-luminescent immunoassay for the diagnosis of grapevine closteroviruses on nitrocellulose membrane. J. Virol. Methods, 42 (1993) 107–116.

Quackenbush, L.S., Vitellogenesis in the shrimp, *Penaeus vannamei*: In vitro studies of the isolated hepatopancreas and ovary. Comp. Biochem. Physiol. B, 94 (1989a) 253–262.

Quackenbush, L.S., Yolk protein production in the marine shrimp *Penaeus vannamei*. J. Crusta. Biol., 9 (1989b) 509–516.

Ross, M.H., Romrell, L.J. and Kaye, G.I.,Histology: a Text and Atlas, 3rd ed., Williams & Wilkins, Baltimore, 1995, p. 79.

Sagi, A., Karp, L., Milner, Y., Cohen, D., Kuris, A.M. and Chang, E., Testicular thymidine incorporation in the prawn *Macrobrachium rosenbergii*: molt cycle variation and ecdysteroid effect *in vitro*. J. Exp. Zool., 259 (1991) 229–237.

Sagi, A. and Ra'anan, Z., Rapid identification of reproductive stage and the reproductive period of females in pond populations of *Macrobrachium rosenbergii* — a new technique. Aquaculture, 48 (1985) 361–367.

Sagi, A., Soroka, Y., Snir, E., Chomsky, O., Calderon, J. and Milner, Y., Ovarian protein synthesis in the prawn *Macrobrachium rosenbergii*: Does ovarian vitellin synthesis exist? Invert. Reprod. Develop., 27 (1995) 41–47.

Soroka, Y., Sagi, A. and Milner, Y., Yolk protein is synthesized in the hepatopancreas of *Macrobrachium rosenbergii*. In: 7th International Congress of Invertebrate Reproduction, 1995, p. 189.

Stern, S., Osmoregulatory mechanisms in the euryhaline crustacean *Macrobrachium rosenbergii* (de Man). PhD Thesis, The Hebrew University of Jerusalem, Israel, 1985, p. 74.

Yano, I. and Chinzei, Y., Ovary is the site of vitellogenin synthesis in kuruma prawn, *Penaeus japonicus*. Comp. Biochem. Physiol., 86B (1987) 213–218.

Yano, I., Krol, R.M., Overstreet, R.M. and Hawkins, W.E., Route of egg yolk protein uptake in the oocytes of kuruma prawn, *Penaeus japonicus*. Mar. Biol. (Berlin), 125 (1996) 773–781.