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# Is the Unique Negatively Charged Polypeptide of Crayfish Yolk HDL a Component of Crustacean Vitellin?

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*ABSTRACT* The yolk protein of *Cherax quadricarinatus* contains six major high-density lipoprotein (HDL) subunits with the approximate molecular masses of 177, 155, 106, 95, 86, and 75 kDa, of which only the 106-kDa polypeptide is negatively charged. On the basis of their molecular weights, time of appearance and disappearance, their floating density and susceptibility to enzyme degradation (by a serine proteinase), these six HDL polypeptides were classified into two subgroups. One group comprises the higher-molecular-weight compounds above 106 kDa, and the other includes the lower-molecular-weight compounds up to 95 kDa. Other than being different from the lower-molecular-weight polypeptides, the negatively charged 106-kDa polypeptide was significantly different from members of its higher-molecular-weight group belonging to a different, less abundant, yolk protein as shown by HPLC separation. Immunological studies and peptide mapping in which the 106-kDa polypeptide did not show similarity to any of the other HDL components confirmed these differences. Moreover, the amino acid composition of the 106-kDa polypeptide was different from that of known vitellin from other crustacean species. This unique negatively charged polypeptide presents an enigma as it is known to be a secondary vitellogenic-related HDL polypeptide, immunolocalized in yolk globules; however, it is different to all the other HDL polypeptides, thus presenting the question whether it is indeed a component of "classical" crustacean vitellin. *J. Exp. Zool. 290:218–226, 2001.* 

The most important source of nutrients for the developing crustacean embryo is yolk protein (Lee et al., '97). The major fraction of this protein consist of vitellin, a high-density lipoprotein (HDL) having a carotenoid prosthetic group and a carbohydrate moiety (Wallace et al., '67; Fyffe and O'Connor, '74; Lee and Puppione, '88; Komatsu and Ando, '92; Lee and Walker, '95; Pateraki and Stratakis, '97). In the crayfish *Cherax quadricarinatus*, a number of yolk polypeptides have been identified (Abdu et al., 2000) but neither the yolk HDL nor the vitellin in this species has been completely characterized. It is, however, known that with *C. quadricarinatus* there is a gradual change in the ovarian polypeptide profile throughout the course of the vitellogenic process (Abdu et al., 2000). In the primary–vitellogenic ovary, the predominant polypeptides are those with relatively low molecular masses (65–95 kDa) but later in the vitellogenic process, an additional group of polypeptides having relatively high molecular masses (177, 155, and 106 kDa) appears (Abdu et al., 2000). These higher-molecular-weight subunits, including a negatively charged (Sagi et al., '99) 106-kDa polypeptide, are present exclusively during secondary vitellogenesis, as shown previously by the induction of secondary vitellogenesis (via the removal of the androgenic gland) in intersex individuals (Khalaila et al., '99).

Abbreviations used: BSA, bovine serum albumin; ECL, enhanced chemiluminescence; EDTA, ethylendiamine tetraacetic acid; DTT, DL-dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′, N′-tetraacetic acid; GC/MS, gas chromatography-mass spectrometry; HDL, high-density lipoprotein; HRP, Horseradish Peroxidase; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE; sodium dodecyl sulfate-polyacrylamide gel electrophoresis. TFA, trifluoroacetic acid.

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In this study, yolk HDL of *C. quadricarinatus* was classified into two major groups of polypeptides on the basis of their time of appearance, their molecular weights, and their susceptibility to enzyme degradation. Particular emphasis was placed on characterizing the 106-kDa compound, which exhibits a wide range of differences compared to the two other components of the higher-molecular-weight group and all the other components of the "vitellin." The question whether the above described yolk HDL components fit the conventional definition of crustacean vitellin is discussed.

## MATERIALS AND METHODS

### *Animals*

*C. quadricarinatus* females were raised in the facility at the Ben-Gurion University of the Negev. All animals were kept in 100-l freshwater tanks at 27 ± 2°C, with a photoperiod of 14L:10D. Water quality was assured by circulating the water through a gravel biofilter. Pelleted feed and vegetables were supplied ad libitum.

### *Morphological and anatomical observations*

The crayfish were examined externally for endopod morphology (Sagi et al., '96) and carapace length. For anatomical studies, the animals were anesthetized in ice-cold water, and the gonads were dissected out and weighed (±0.001 g). The gonadosomatic index (GSI) was calculated as the percentage of gonad to body weight. Oocyte diameter was measured under a light microscope, and mean oocyte diameter (±SE) was calculated from a sample of 15 oocytes (from the large oocyte population) per ovary. The ovarian stage in the reproductive cycle was determined in terms of the following parameters: endopod morphology, GSI, mean oocyte diameter, and color of the gonad (Sagi et al., '96).

### *Ovarian HDL analysis*

Females of different reproductive stages were anesthetized on ice. Ovarian samples were dissected out and homogenized in 0.05 M Tris-HCl buffer, pH 7.4, supplemented with 0.25 M NaCl and the following protease inhibitors: 4 mM EDTA, 10 mM leupeptin, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). The samples were centrifuged (10,000*g*, 15 min at 4°C), and sodium bromide was added to the supernatants to a final concentration of 1.22 or 1.25 g/ml. These supernatants were then centrifuged again at 120,000 *g* for 48 hr at 12°C, and the orange floating layers (containing the lipoprotein) were collected and dialyzed against 0.25 M NaCl–10 mM phosphate buffer (pH 7.4). The isolated lipoprotein was subjected to 7% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, '70), for which the following high-molecular-mass markers were used: 200, 116.5, 97.4, 66.2, 45, and 31 kDa (Sigma Chemical Co., St. Louis, MO) . The gel was stained with Coomassie brilliant blue R-250 for general protein staining and with "Stains all" to detect negatively charged proteins (King and Morrison, '76).

### *Chromatography*

HDL samples were subjected to high-performance liquid chromatography (HPLC) performed on a DEAE (anion-exchange) column (Merck), equilibrated with 0.01 M $NaH_2PO_4$ (Tris) buffer, pH 7.4. Fractions were eluted with a NaCl gradient (0–1 M) at a flow rate of 1 ml/min. Absorbance was measured at 280 nm for protein. Fractions of 1 ml were collected, dialyzed, and kept at –20°C. Polypeptide profiles of the HPLC-separated fractions from several runs were pooled together, and the volume was reduced to approximately 200 μl by means of a speed-Vac (Freeze Dry System, LABCONCO). Samples were mixed with sample buffer (3:1, respectively), heated (100°C, 3 min) and separated by SDS-PAGE.

### *Preparation of polyclonal antisera*

For the production of a polyclonal antisera, the HDL fraction from secondary–vitellogenic female ovaries was separated on 7% SDS-PAGE, as described above. After staining, the gels were soaked for few days in doubly distilled water to free them of methanol and SDS. The 95-kDa polypeptide bands were cut from the gel and then repeatedly transferred, first through a 300-μm mesh and then through an 80-μm mesh, using phosphate-buffered saline (PBS, 2:1 v/v). The final mash was used as the antigen and the anti-immune sera were produced in rabbits according to the method of Harlow and Lane, '88. The anti-106-kDa polyclonal antiserum was the same that was used by Sagi et al. ('99).

### *Enzyme assay*

The HDL of density 1.25 g/ml isolated from secondary–vitellogenic ovaries, which included the 177-, 155-, 106-, 95-, 86-, and 75 kDa polypeptides, was used as the substrate for the enzyme assay. Endogenous proteinase activity was screened in eggs of different developmental stages (from newly laid eggs up to 4 days after laying) and different

pH (pH 2.0, HCl; pH 3.0, sodium formate; pH 4.0–5.0 sodium acetate; pH 7.0–8.0 Tris-HCl) as follows. The eggs were homogenized (1:10, w/v) in 0.05 M Tris-HCl, pH 7.4, containing 0.25 M NaCl. For each experiment, 45 μl of reaction mixture, containing 20 μg of HDL, 5 μg of egg protein homogenate, and 3 vol of 0.1 M buffer solution of various pH, was incubated for 1 hr at 37°C. To raise or lower the pH, a small amount of 0.1 M HCl or 0.1 M NaOH was added as necessary. Since endogenous proteinase activity was found at pH of 7.0–8.0, the protease inhibitor experiment was conducted at pH 7.4 with egg homogenate 3–4 days after laying (when enzymatic activity was first detected). The effect of the following specific proteinase inhibitors was studied by adding each to a sample of the reaction mixture: 20 μM aprotonin, 5 mM EDTA, 7 μg/ml of pepstatin A, 3 mM PMSF, 200 μg/ml soybean trypsin inhibitor, 10 μM E-64, and 50 μg/ml of leupeptin. An aliquot (15 μl) of each reaction mixture containing an inhibitor was subjected to SDS-PAGE.

### *Western blot analysis*

HDL samples from newly laid eggs were separated on 7% SDS-PAGE and electrobloted onto a nitrocellulose membrane. Western blot analysis was performed using the ECL method with either polyclonal anti-95 (1:7,500) or anti-106 (1:8,000) for 1 hr as the first antibody. The second antibody applied was anti-rabbit horseradish peroxidase (HRP) conjugate (whole antibody) for luminol-based detection. The chemiluminescence signal resulting from the HRP-induced breakdown of luminol, using ECL reagents (RPN-2108), is detected on high-performance chemiluminescence film (Amersham Pharmacia Biotech).

### *Amino acid composition*

HDL samples were separated on SDS-PAGE as described above and then electrobloted onto a PVDF membrane. The membrane was stained with Coomassie brilliant blue R-250, and the 106-kDa band was cut out. The excised piece was hydrolyzed in vacuo at 110°C in 6 M HCl for 24 hr and analyzed with a Beckman 6300 amino acid analyzer.

### *Immunohistochemical localization of the 106-kDa polypeptide*

Ovarian tissue from females at different reproductive stages was removed and fixed in Bouin's solution. Ovaries with a GSI of up to 3 were fixed for 24 hr, dehydrated in an alcohol series, and embedded in Paraplast (melting point 60–61°C). Ovaries with a GSI of 3–5 were fixed in Bouin's solution for 48 hr, dehydrated in an ethanol series, and embedded in Paraplast. Five-micron sections were stained with hematoxylin and eosin for general histology. After determination of the ovarian stage, oocyte diameter was measured under a light microscope in sections passing through the nuclei, and the mean oocyte diameter (±SE) was calculated from a sample of 15 oocytes from each stage. Sections were blocked with 3% bovine serum albumin (BSA) in PBS for 60 min, followed by incubation for 30 min with 106-kDa antiserum (pre-absorbed with male hemolymph) or with pre-immune serum as the negative control. After three additional washes with PBS, the sections were incubated for 30 min with alkaline-phosphatase-conjugated goat anti-rabbit IgG (Sigma). After another three washes with PBS, sections were incubated with the alkaline phosphatase substrate: nitro blue tetrazolium (NBT) plus 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

### *Peptide similarity*

HDL samples were separated on SDS-PAGE as described above. The gel was then stained with Coomassie blue followed by destaining, first with 50% methanol and 10% acetic acid for 1 hr and then with 5% methanol and 7% acetic acid. Thereafter, the polypeptide bands were cut out of the gel. Isolated gel sections were then washed overnight with doubly distilled water. The polypeptides were carboxymethylated "in gel" with 100 mM iodoacetamide, further destained in 50% acetonitrile (ACN) with 100 mM ammonium bicarbonate, cut into little pieces, and dried. The gel pieces were rehydrated with 100 mM ammonium bicarbonate, pH 7.4, containing modified trypsin (Promega). After an overnight incubation at 37°C, the resulting peptides were eluted from the gel pieces with 60% ACN in 0.1% trifluoroacetic acid (TFA) and analyzed by liquid chromatography mass spectrometry (LC-MS) as described below. The peptides were resolved by HPLC on a 1 × 150mm Vydac C-18 column, eluted with a linear gradient of 4%–65% ACN in 0.05% TFA at 1% ACN/min and a flow rate of 40 μl/min. While most of each eluting peptide was collected by hand after the HPLC, about 20% of the sample was microsprayed directly from the HPLC column into an electrospray ion trap mass spectrometer (LCQ, Finnigan). The analysis was performed in the positive ion mode, using a full mass spectrometry (MS) scan followed by a mass spectrometry/mass spec-

trometry (MS/MS) (collision-induced fragmentation) on the dominant ion selected from the first MS scan. These two scan events were repeated throughout the run. The MS and MS/MS data from the run were compared with the simulated proteolysis and fragmentation of the proteins in the Owl Database by means of the Sequest software (University of Washington). The comparison between the polypeptides was based on the MS and MS/MS data of the different tryptic digested peptides. A peptide was considered to be identical if its mass, retention time and MS/MS spectrum were the same in all samples, and it did not appear in the blank.

## RESULTS

During the ovarian vitellogenic process and the development of the embryo, there was a sharp change in the SDS-PAGE polypeptide profile of yolk HDL (Fig. 1). Three polypeptides with approximate molecular masses of 95, 86, and 75 kDa were the only polypeptides detected in early secondary-vitellogenic ovaries (Fig. 1A, lane 1). The 95- and 86-kDa polypeptides were also detected in late secondary-vitellogenic ovaries and in newly laid eggs, along with three new polypeptides of approximate molecular masses 177, 155, and 106 kDa (Fig. 1A, lanes 2 and 3). Four to five days after the eggs had been laid, only the 95-, 86-, and 75-kDa polypeptides were present, and the higher-molecular-weight polypeptides could no longer be detected (Fig. 1A, lane 4). Figure 1B (lanes 2 and 3) shows that among the relatively high-molecular-weight secondary–vitellogenic polypeptides, a polypeptide with an approximate molecular mass of 106-kDa was stained blue with "Stains all," indicating that it was negatively charged. The finding of two subclasses of HDL appearing at different times in secondary–vitellogenic ovaries was confirmed by a floating density ultracentrifugation separation (Fig. 2). At a floating density of < 1.22 g/ml, only the three relatively low-molecular-mass apolipopolypeptides (95, 86, and 75 kDa) were detected (Fig. 2B), whereas at a floating density of < 1.25 g/ml, the 177-, 155-, and 106-kDa were also present along with the low molecular components (Fig. 2A) showing a different ratio of protein to lipid in each of the two groups. The presence of the 75 kDa in lane A was confirmed; however, due to its relatively low abundance it is not clearly seen in the figure.

The protease inhibitor assay was performed to

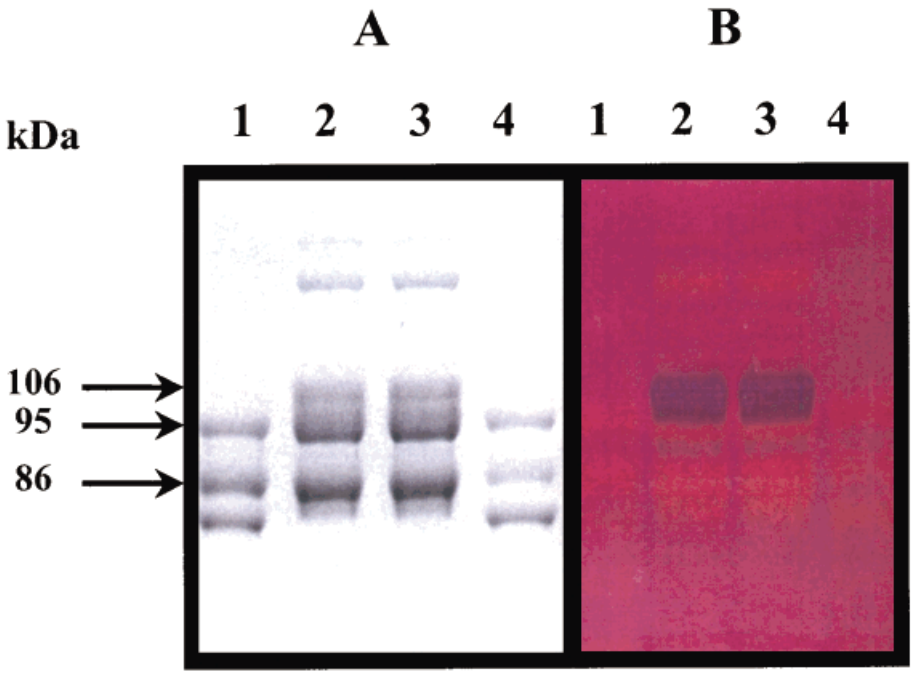


Fig. 1. 7% SDS-PAGE separation of the HDL from secondary–vitellogenic ovaries and from eggs of *C. quadricarinatus* at different stages of embryonic development. (**A**) Stained with Coomassie blue; (**B**) stained with "Stains-all". Lane 1, early secondary–vitellogenic ovary (GSI, 1.4; oocyte diameter, 0.9 ± 0.08 mm); lane 2, late secondary–vitellogenic ovary (GSI, 2.5; oocyte diameter, 1.4 ± 0.3 mm); lane 3, newly laid eggs; lane 4, eyed eggs (developing embryo). Each lane was mounted with 15 μg of protein. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

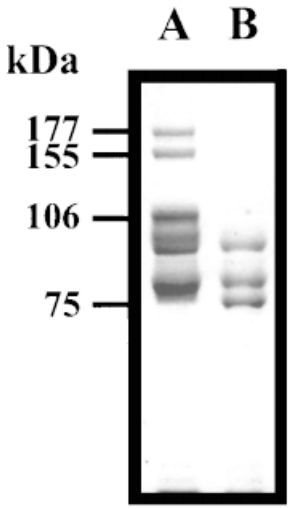


Fig. 2. 7% SDS-PAGE separation of two subclasses of high-density lipoproteins from secondary-vitellogenic ovaries of *C. quadricarinatus*. (**A**) $d < 1.25$ g/ml. (B) $d < 1.22$ g/ml. Each lane was mounted with 15 μg of protein.

characterize the endogenous enzyme that is responsible for digestion of the higher-molecular-weight polypeptides during early embryonic development (Fig. 3). When yolk HDL was subjected to endogenous enzymes from eggs as early as 3–4 days after egg laying at a pH of 7.0–8.0,

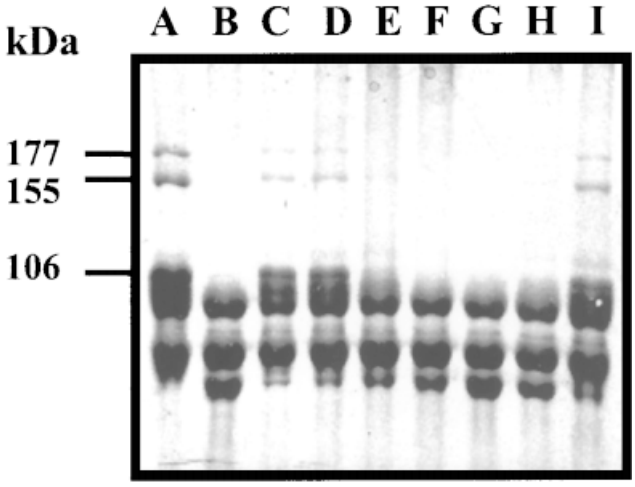


Fig. 3. 7% SDS-PAGE separation of HDL ($d < 1.25$ g/ml) from secondary–vitellogenic ovaries of *C. quadricarinatus* treated with endogenous proteases and protease inhibitors. (**A**) Without endogenous enzyme, (**B**) with endogenous enzyme, (**C**) with endogenous enzyme and aprotonin, (**D**) with endogenous enzyme and leupeptin, (**E**) with endogenous enzyme and PMSF, (**F**) with endogenous enzyme and pepstatin A, (**G**) with endogenous enzyme and E-64, (**H**) with endogenous enzyme and EDTA, (**I**) with endogenous enzyme and trypsin inhibitor.

all the higher-molecular-mass polypeptides (177, 155, and 106 kDa) were digested (Fig. 3, lane B). The endogenous enzyme activity was inhibited by aprotonin, leupeptin, and trypsin inhibitor (Fig. 3, lanes, C, D, and I, respectively), but not by PMSF, pepstatin A, E-64, or EDTA (Fig. 3, lanes E, F, G, and H, respectively). Moreover, a new lower molecular band at the approximate molecular weight of 70 kDa was found in lanes where the endogenous enzyme activity was not inhibited, suggesting that it is a degradation product (Fig. 3, lanes B, E, F, G, and H). These results suggest that all the high-molecular-weight yolk HDL components are susceptible to degradation by serine proteinase.

The first evidence of a difference among the three high-molecular-weight polypeptides (177, 155, and 106-kDa) was provided by native separation of the HDL fraction on an HPLC equipped with a DEAE column. This separation gave three peaks, which eluted at 22.6, 25.5, and 30.7 min (Fig. 4). The 106-kDa polypeptide was found only in the smaller peak, eluted at 30.7 min. This finding was confirmed by a "Stains all" test (data not shown). On the other hand, the most abundant 22.6-min peak was composed of the 177- and 155-kDa polypeptides, along with the low-molecular-mass group polypeptides (95, 86, and 75 kDa). Also, this peak contained another 115-kDa polypeptide, which is usually found in relative low abundance in the HDL. The 25.5-min peak was similar in its polypeptide profile to the prominent 22.6-min peak.

Antibodies raised against the 95-kDa and the 106-kDa polypeptides were used to detect immune cross-reactivity with the HDL components (Fig. 5). Antibodies against the 95-kDa polypeptide recognized the 95-kDa polypeptide and showed cross-reactivity with the 177- and 155-kDa polypeptides but not with the 106-kDa (Fig. 5, lane B). Whereas antibodies against the 106-kDa polypeptide recognized the 106-kDa polypeptide, they did not show any cross-reactivity with other HDL polypeptides (Fig. 5, lane C). A peptide mapping comparison between the 95-kDa and the high-molecular-weight HDL polypeptides was performed on the basis of these immunological results. The analysis revealed similarities between the 177-kDa and the 155-kDa polypeptides (56%), between the 177- and the 95-kDa polypeptides (30%), and between the 155-kDa and the 95-kDa polypeptides (44%). There was, however, no similarity between the 106-kDa polypeptide and any of the other mapped polypeptides (177, 155, and 95 kDa).

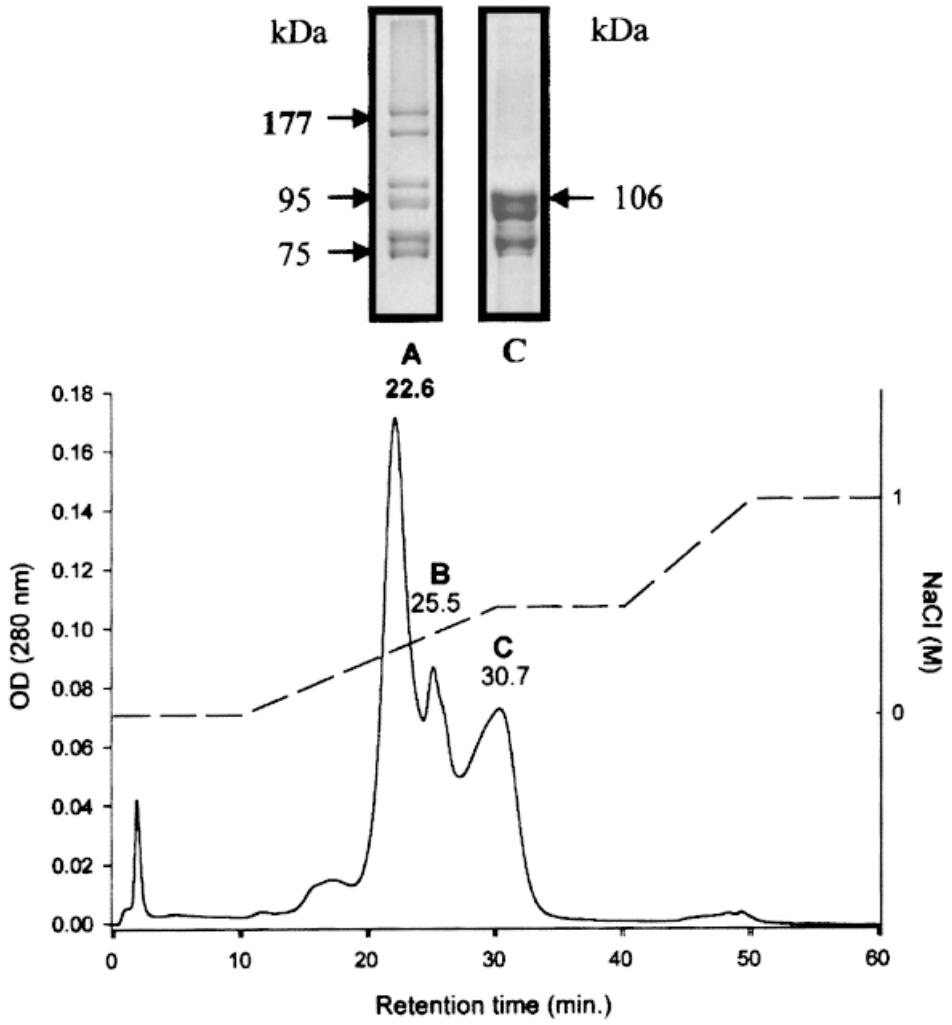


Fig. 4. Separation of the HDL from secondary–vitellogenic ovaries of *C. quadricarinatus* ($d$ < 1.25 g/ml) on an HPLC equipped with a DEAE column (bottom) and 7% SDS-PAGE separation of the three separated peaks (top).

Comparison of the amino acid composition of the 106-, 95-, 86-, and 75-kDa polypeptides showed similarity among the "lower" three subunits of 95, 86, and 75 kDa but differences between these three polypeptides and the 106-kDa polypeptide (Table 1). The amino acid composition of the 95-, 86-, and 75-kDa polypeptides was also similar to that of vitellin from other crustacean species (see Discussion) while the 106-kDa compound did not exhibit similarity to other vitellins. In comparison to the 106-kDa polypeptide, the 95-, 86-, and 75-kDa polypeptides had higher contents of leucine (11.36, 9.72, and 13.46 mol %, respectively), whereas the 106-kDa polypeptide had higher contents of aspartic acid (15.6 mol %), and tyrosine (11.22 mol %). Moreover, the level of isoleucine and histidine in the 106-kDa polypeptide was undetectable whereas the 95-, 86-, and 75-kDa polypeptides had medium level of isoleucine (4–5 mol %) and lower level of histidine (0.75–1.4 mol %). As anticipated from the negatively charged property of the 106-kDa polypeptide, a high level (~30 mol %) of negatively charged amino acids (aspartic and glutamic acid) were found in comparison to the 95-, 86-, and 75-kDa polypeptides (~22 mol % of the two negatively charged amino acids in all three) (Table 1).

Immunolocalization of the 106-kDa polypeptide showed that not only is this polypeptide associated with the HDL, but it is also found in the cytoplasm of secondary vitellogenic oocyte but not in the primary-vitellogenic oocyte (Fig. 6A, eY). Higher magnification of the oocytes at the beginning of secondary vitellogenesis showed that positive cross-reactivity was detected in yolk globules (Fig. 6B, arrows).

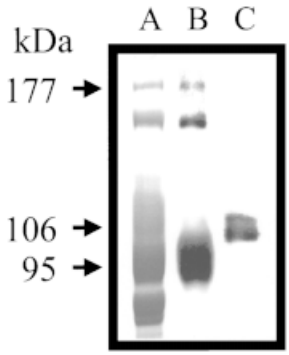


Fig. 5. Immunostaining of 7% SDS-PAGE of HDL ($d$ < 1.25 g/ml) from newly laid eggs of *C. quadricarinatus* by anti-95 kDa and anti-106 kDa-antibodies. (**A**) Stained with Coomassie brilliant blue (10 µg protein), (**B**) Western blot using anti-95 kDa antibodies (5 µg protein), (**C**) Western blot using anti-106 kDa antibodies (5 µg protein).

*TABLE 1. Amino acid composition of the 106-, 95-, 86- and 75-kDa polypeptides from* C. quadricarinatus *egg HDL*

| Amino acid | 106 kDa (mol %) | 95 kDa (mol %) | 86 kDa (mol %) | 75 kDa (mol %) |
|---|---|---|---|---|
| Asp | 15.6 | 7.45 | 8.13 | 8.37 |
| Ser | 9.65 | 7.96 | 8.73 | 10.61 |
| Glu | 15.11 | 15.6 | 13.85 | 13.3 |
| Thr | 8.39 | 5.54 | 5.2 | 6.07 |
| Arg | 8.13 | 8.22 | 8.24 | 8.81 |
| Gly | 7.55 | 4.07 | 6.06 | 4.78 |
| Ala | 6.45 | 5.17 | 6.66 | 4.31 |
| Tyr | 11.22 | 3.75 | 4.64 | 4.76 |
| Pro | 2.87 | 3.71 | 4.44 | 3.92 |
| Met | 1.91 | 0.7 | 0.42 | Not detected |
| Val | 2.67 | 8.45 | 8.88 | 5.77 |
| Phe | 3.66 | 4.5 | 3.4 | 4.32 |
| Ile | Not detected | 4.28 | 4.63 | 5.1 |
| Leu | Not detected | 11.36 | 9.72 | 13.46 |
| His | Not detected | 0.75 | 1.43 | 1.07 |
| Lys | 7.1 | 4.02 | 6.13 | 5.32 |
| Trp | Not done | Not done | Not done | Not done |
| Cyt | Not done | Not done | Not done | Not done |

## DISCUSSION

In crustaceans, vitellin is regarded as the HDL component of egg yolk providing the alimentary needs of the developing embryo (Lee and Puppione, '88; Komatsu and Ando, '92; Lee and Walker, '95; Pateraki and Stratakis, '97). In this study we question the definition of the whole yolk HDL fraction as "vitellin" by characterizing various HDL components from the yolk of the crayfish *C. quadricarinatus* and investigating their similarities. In this species yolk HDL is composed of six major polypeptides which could be classified into two groups, one group containing the relatively-high-molecular-weight polypeptides of 177, 155, and 106 kDa and the other group comprising the relatively lower-molecular-weight polypeptides of 95, 86, and 75 kDa. Among the above, the negatively charged 106-kDa polypeptide presents a unique compound, not similar to the other vitellin subunits, that might represent a functionally different yolk HDL component.

The 106-kDa polypeptide is similar to the other higher-molecular-weight HDL components in its time of appearance and disappearance, its floating density, which is within the HDL sub-class with a density of 1.25 g/ml, and its susceptibility to enzymatic degradation by an enzyme member of the serine proteinase family. This susceptibil-

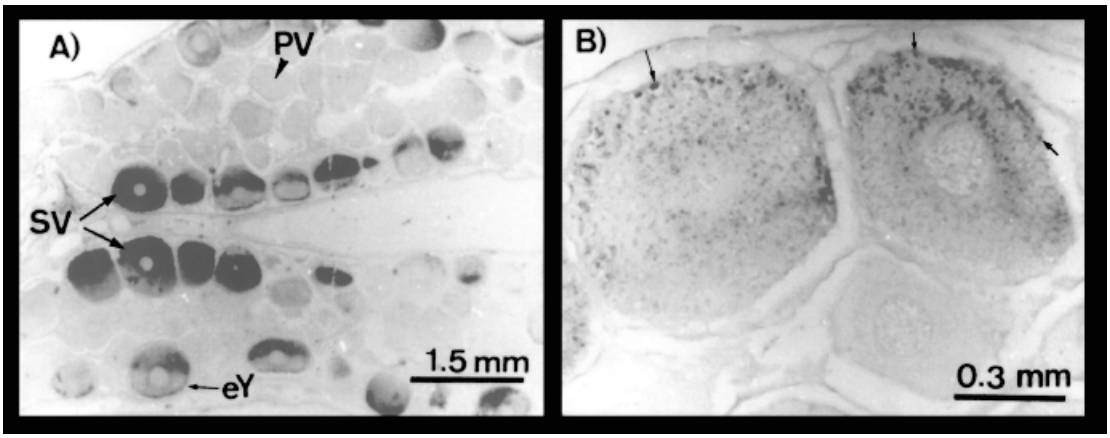


Fig. 6. Immunohistochemical localization of the 106-kDa polypeptide in a *C. quadricarinatus* ovary containing oocytes at different vitellogenic stages. (**A**) General section with primary (PV) and secondary (SV) vitellogenic oocytes showing exclusive reactivity in the latter. Early yolk stage oocytes (eY) are at the beginning of the secondary vitellogenic process. (**B**) Higher magnification of the oocytes at the beginning of secondary vitellogenesis where the positive cross-reactivity is detected in yolk globules (arrows).

ity to enzymatic degradation is responsible for the sharp disappearance of all the higher-molecular-weight HDL components in *C. quadricarinatus* yolk (177, 155, and 106-kDa) during early development of the embryo. It is known that vitellin in stored in membranous organelles, called yolk granules or yolk platelets, until utilized (Raikhel and Dhadialla, '92). Yolk granules contain proteases that degrade their constituent proteins (Xiaodonog et al., '96). Several yolk proteinases, responsible for vitellin degradation, have been identified in fishes (Carnevali et al., '99; Brooks et al., '97) and in insects (Xiaodonog et al., '96; Kageyama and Takahshi, '90; Cho et al., '99). In crustaceans, the sole study on degradation of yolk proteins, which was performed on *Artemia*, suggested the involvement of a lysosomal system in protein degradation (Perona et al., '88). Such susceptibility to enzymatic degradation is responsible for the sharp disappearance of all the higher-molecular-weight HDL components in *C. quadricarinatus* yolk (177, 155, and 106 kDa) during early development of the embryo and for the appearance of a lower molecular weight polypeptide, probably a degradation product. Our study thus presents, for the first time, preliminary characterization of a proteinase that degrades yolk protein. This enzyme was found to be active at a neutral pH; its activity was evident 4–5 days after egg laying; and it was inhibited by aprotonin, leupeptin, and trypsin inhibitor, all these findings suggesting that the enzyme is a serine proteinase.

Other than the above differences from the lower-molecular-weight HDL group, the 106-kDa polypeptide was also significantly different from the members of its higher-molecular-weight group. HPLC separation of yolk HDL showed that it was composed of three proteins, eluted in different peaks. The main peak contained all the polypeptides, excluding the 106-kDa polypeptide, which was part of a different, less abundant protein. Furthermore, immunological studies and peptide mapping showed that the 177-, 155-, and 95-kDa polypeptides exhibit similarities whereas the 106-kDa polypeptide did not show similarity to any of the other HDL components. Analysis of the amino acid composition of the 95-, 86-, and 75-kDa polypeptides showed a significant similarity to the amino acid composition of vitellin from other crustacean species (Chang et al., '94, '96; Lubzens et al., '95; Lee et al., '97), i.e., high concentrations of glutamic acid, aspartic acid, serine, and leucine but low concentrations of cysteine, histidine, and methionine. The amino acid composition of the 106-kDa polypeptide was, however, different from that of the lower-molecular-weight polypeptides and from that of known vitellin from other crustacean species.

On the basis of the findings of this study, we suggest that *C. quadricarinatus* vitellin is composed of polypeptides of molecular masses of 177, 150, 95, 86, and 75 kDa, of which the higher-molecular-weight polypeptides (177 and 155 kDa) may serve as precursors for some of the lower-molecular-weight subunits, particularly for the 95-kDa polypeptide. The 106-kDa polypeptide presents something of an enigma: it is known to be a secondary vitellogenic related polypeptide (Sagi et al., '99, Khalaila et al., '99); it was found in this study to be immunolocalized in association with the yolk; and it seems to be different to all the other HDL polypeptides. In the sense that it is not a part of the "bulk" yolk lipoprotein and is not similar to the other yolk lipoprotein components, it seems that the 106-kDa polypeptide does not constitute a yolk lipoprotein that could classically be termed as "vitellin." On the other hand, it is clearly associated with the yolk HDL and the yolk globules at the end of vitellogenesis and early embryogenesis, which suggests a functional association. The 106-kDa polypeptide might thus constitute a component with a dedicated role in crustacean vitellogenesis.

The results of this study call for further investigation of the physiological function of the 106-kDa polypeptide in comparison to the other HDL components during vitellogenesis and embryogenesis, which will facilitate a sharper definition of crustacean vitellin and its components. To this end, partial internal amino acid sequencing was performed and used in the design of molecular primers. These will be used for future cloning and sequencing of the gene and will enable the study of the molecular conservation of this novel, negatively charged, vitellogenesis-related, subunit of *C. quadricarinatus* yolk protein in comparison with other crustaceans and animals of other taxa.

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