

High-density lipoprotein associated with secondary vitellogenesis in the hemolymph of the crayfish *Cherax quadricarinatus*

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Abstract

The high-density lipoproteins LPI and LPII were isolated from the hemolymph of the crayfish *Cherax quadricarinatus* by gradient ultracentrifugation and high-performance liquid chromatography (HPLC). Both lipoproteins contained a carotenoid moiety. LPI is comprised of a single polypeptide with an approximate molecular mass of 96 kDa. LPII was composed of two similar native components, LPIIa and LPIIb, both having polypeptides of 80 and 177 kDa. Both under natural conditions and after endocrine manipulations, LPI was present in males and in females, regardless of the female reproductive stage. LPII was present only in secondary-vitellogenic females, but not during the winter reproductive arrest period. LPII was also absent from young females that had received androgenic gland implants. LPII also appeared in the hemolymph of intersex individuals from which the androgenic gland had been removed. It is therefore suggested that LPII serves as a marker indicating the onset of secondary vitellogenesis in *C. quadricarinatus* females. © 2000 Elsevier Science Inc. All rights reserved.

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1. Introduction

In crustaceans, secondary vitellogenesis is accompanied by the accumulation of yolk (Charniaux-Cotton and Payen, 1988), which is composed of lipids, carbohydrates and proteins (Adiyodi and Subramoniam, 1983), the main protein being a high-density lipoprotein (HDL) known as vitellin (Adiyodi and Subramoniam, 1983; Meusy and Payen, 1988). Both ovarian and extra-ovarian sites have been proposed for yolk protein synthe-

sis in decapods (Lui and O'Connor, 1976; Eastman-Reks and Fingerma, 1985; Paulus and Laufer, 1987; Tom et al., 1987; Yano and Chinzei, 1987; Fainzilber et al., 1992). The extra-ovarian hemolymphatic precursor of vitellin is the lipoglycocarotenoprotein vitellogenin, which has similar biochemical and immunological characteristics to vitellin (Kerr, 1969; Meusy, 1980; Derelle et al., 1986; Chang et al., 1994; Lee and Watson, 1994). A decapod species for which the composition and site(s) of synthesis of vitellin and vitellogenin have not been fully elucidated is the red-claw crayfish *Cherax quadricarinatus*, although the processes of ovarian development and vitellogenesis in this species have been investigated in detail (Sagi et al., 1996a; Abdu et al., 2000).

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The presence of HDLs, usually glycoproteins with a carotenoid moiety (Lee and Puppione, 1988; Komatsu et al., 1993; Tom et al., 1993), in the hemolymph of many crustacean species is, however, not necessarily associated with ovarian development. One such protein, designated LPI, appears in both male and female animals, whereas a second lipoprotein, termed LPII, is specific to the hemolymph of females undergoing vitellogenesis (Lee and Puppione, 1988; Komatsu et al., 1993). In most decapod species, LPI is comprised of one to three polypeptides, one of which usually has an approximate molecular mass of 100 kDa (Lee and Puppione, 1988; Komatsu et al., 1993; Lubzens et al., 1995). The female-specific protein is composed of at least two polypeptides, usually having subunits of approximately 80 and 180 kDa, as has been shown for many decapod species (Lee and Puppione, 1988; Komatsu et al., 1993; Chang and Jeng, 1995; Lubzens et al., 1995). In some species, LPI and LPII share polypeptides having similar molecular weights (Komatsu et al., 1993). In *C. quadricarinatus*, LPI and LPII have not yet been identified and characterized, although it is known that the hemolymph of both the male and the female contains a polypeptide of approximately 96 kDa and that the hemolymph of the secondary-vitellogenic female contains four specific polypeptides, having approximate molecular masses of 80, 196, 177 and 208 kDa (Abdu et al., 2000).

Since the appearance of the female-specific lipoprotein in the hemolymph is related to secondary vitellogenesis, it is thought that this polypeptide could serve as a marker for the onset of this process. The search for such a marker in *C. quadricarinatus* was thus the objective of this study. Two approaches were taken to fulfill this objective: In the first, a correlation was sought between HDL content of the hemolymph and the transition between seasons of reproduction and reproductive arrest (Jones, 1990; Barki et al., 1997). The second approach was based on the effect of the androgenic gland on sexual differentiation, gonad maturation (Charniaux-Cotton, 1964; Charniaux-Cotton and Payen, 1988; Sagi et al., 1997), and the balance between 'maleness' and 'femaleness' in intersex individuals (Charniaux-Cotton and Payen, 1988; Sagi et al., 1996b; Khalaila et al., 1999): implantation of an androgenic gland into a female animal represses ovarian development (Meusy and Payen, 1988; Taketomi and Nishikawa, 1996), while removal of the androgenic gland from a male or

intersex individual results in female differentiation (Charniaux-Cotton, 1964; Sagi et al., 1990). Endocrine manipulation, via implantation of the androgenic gland into females or removal of the gland from intersex individuals (Sagi et al., 1996b; Khalaila et al., 1999), was thus used as a means to investigate changes in hemolymph characteristics during sexually plastic processes and the onset of secondary vitellogenesis in *C. quadricarinatus*.

2. Materials and methods

2.1. Animals

C. quadricarinatus males, females and intersex individuals were transferred from earthen ponds at the Aquaculture Research Station, Dor, Israel to the facilities of Ben-Gurion University of the Negev. The animals were held in 100-l freshwater tanks, at $27 \pm 2^\circ\text{C}$ and a photoperiod of 14 h light, 10 h dark. Water was circulated through a gravel biofilter, and nitrite and ammonium levels were monitored. Food, consisting of wheat grains, fresh vegetables and fish pellets, was given ad libitum three times a week. The sex of the animals was determined by external examination (Sagi et al., 1996a). Females were anesthetized in ice cold water before dissection, and reproductive stages were assessed as described previously (Sagi et al., 1996b; Abdu et al., 2000). Hemolymph samples of less than 400 μl were withdrawn from the sinuses at the base of the fifth walking legs of males, females and intersex animals (Sagi et al., 1996b; Abdu et al., 2000) by means of a syringe, and each sample was transferred into a tube, containing a final concentration of 1% EDTA, and kept on ice. Samples were pooled into groups according to the female reproductive stage, and the HDL fraction was isolated as described previously (Sagi et al., 1996b; Abdu et al., 2000). Newly laid eggs for isolation of HDL were collected from berried females with the spermatophores still attached to their abdomens. Total protein was determined according to Bradford (1976).

2.2. Experimental procedures

2.2.1. Removal of the androgenic gland from intersex animals

Twelve 9-month-old intersex animals were used for the experiment. Six animals were

andrectomized by cauterization as described by Khalaila et al. (1999), and the remaining intact crayfish served as the control animals. Two months later, the reproductive parameters of the animals were determined, and hemolymph samples were taken for isolation of HDL.

2.2.2. Implantation of the androgenic gland into young females

Thirty-two immature females, each weighing 5 ± 3 g and having a carapace length of 20.8 ± 3.0 mm, were divided into two groups. Each female in the first group received an implant of a hypertrophied androgenic gland from a destalked male (Khalaila et al., 1998). Each crayfish in the second treatment group received an implant of a fragment of a sperm duct. Each female was held in an individual compartment (Khalaila et al., 1999). Six months later, after 90% of the females receiving the androgenic gland implants had developed a red-patch (a male secondary sexual character) on the propodus (Khalaila et al., 1998), samples were taken for isolation of HDL, the animals were dissected, and reproductive parameters were determined.

2.3. Changes in the HDL fraction of mature females during the winter reproductive arrest period

A population of previously spawned *C. quadricarinatus* females (Sagi et al., 1996a) was collected on 18 September 1997 from earthen ponds at the Aquaculture Research Station, Dor, Israel. On the same day, hemolymph samples were taken for HDL isolation, as described above. A similar population of 150 females, each weighing 40–60 g, was collected in the autumn of 1998 (beginning of September). The animals were held until the spring (March 1999) in the facilities of Ben-Gurion University. The females were kept in groups of 20 in 500-l rearing tanks at $27 \pm 2^\circ\text{C}$ and a photoperiod of 14 h light, 10 h dark. On five occasions in this 11-week period, hemolymph samples of about 500 μl were withdrawn from 15 females chosen at random and pooled for HDL isolation.

2.4. Electrophoresis

Separation of HDL from hemolymph was performed on mini-polyacrylamide 5% PAGE and 7%

SDS-PAGE slab gels (Laemmli, 1970). A 50% sucrose solution was added to the native PAGE samples (at a ratio of 1:3) before they were loaded onto the gel. For SDS-PAGE separation, high molecular mass standards of 200, 116.5, 97.4, 66.2, 45 and 31 kDa (Sigma, St Louis, MO) were used. After electrophoresis, gels were stained with Coomassie brilliant blue R-250 for proteins.

2.5. Chromatography

Hemolymph HDL samples (100 μl) were subjected to high-performance liquid chromatography (HPLC) on a DEAE anion-exchange column (Merck). The column was 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 and 470 nm for carotenoids. Fractions of 1 ml were collected, dialyzed, and kept at -20°C . Polypeptide profiles of HPLC-separated fractions from a number of male or female samples were pooled together, and the volume was reduced to approximately 200 μl by means of Speed-Vac (Freeze Dry System, LABCONCO). Samples were mixed with sample buffer (3:1), heated (100°C , 3 min), and separated on SDS-PAGE.

3. Results

Electrophoretic profiles of HDL fractions isolated from *C. quadricarinatus* hemolymph and separated on non-denaturing 5% PAGE revealed two hemolymphatic proteins (Fig. 1). One protein, appearing as the lowest band, was present in the hemolymph of both males and females, regardless of the female reproductive stage (Fig. 1B–D). A second protein, was, however, present only in the hemolymph of secondary-vitellogenic females (Fig. 1B). This protein was therefore designated as a ‘secondary-vitellogenic specific protein’ (SVSPr). The relative mobility of the SVSPr was similar to that of one of the proteins in the HDL isolated from newly laid eggs (Fig. 1A).

HPLC analysis of HDL isolated from the hemolymph of *C. quadricarinatus* confirmed the differences among hemolymphatic lipoproteins from male, primary-vitellogenic female and secondary-vitellogenic female animals. The chromatogram of HDL from the male showed two

minor peaks, eluted at approximate retention times of 19 and 50 min, and a major peak eluted at an approximate retention time of 30 min (Fig. 2A). A similar peak was detected in the chromatograms of primary- and secondary-vitellogenic females (Fig. 2B and C, respectively). This non-specific peak was therefore attributed to LPI. Only the chromatogram of HDL from the hemolymph of secondary-vitellogenic female exhibited two additional peaks (presumed to be LPII) at the retention times of 20 and 32 min. These peaks, specific to secondary-vitellogenesis, were therefore designated LPIIa and LPIIb. Absorbance at 470 nm revealed that all three peaks — LPI, LPIIa and LPIIb — contained a carotenoid moiety.

The HPLC-eluted fractions of LPI, LPIIa and LPIIb were subjected to 7% SDS-PAGE. Polypeptides having molecular masses of 80 and 177 kDa (Abdu et al., 2000) were present in the secondary-vitellogenic HDL profile (Fig. 3A) as well as in the profiles of LPIIa and LPIIb (Fig. 3B and C, respectively). Polypeptides with molecular masses lying between 100 and 170 kDa were peculiar to the profiles of LPIIa and LPIIb (Fig. 3B and C, respectively) which differed from one another. A faint band of approximately 208 kDa was present only in the profile of LPIIa, and a faint band of approximately 196 kDa was found in the LPIIb profile (Fig. 3B and C, respectively). A 96-kDa polypeptide appeared in all the LPI and HDL profiles, of both male (Fig. 3E and F, respectively) and female (Fig. 3D and A, respectively). This band was also found in LPIIb (Fig. 3C), probably as a result of incomplete separation

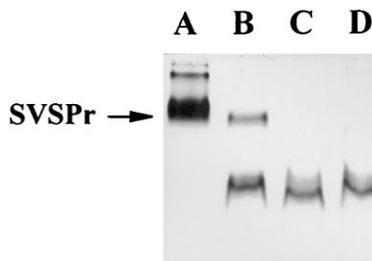


Fig. 1. Non-denaturing PAGE (5%) separation of HDL isolated from *C. quadricarinatus* hemolymph and eggs. (A) Newly laid eggs. (B) Secondary-vitellogenic female hemolymph. (C) Primary-vitellogenic female hemolymph. (D) Male hemolymph. Arrow indicates the secondary-vitellogenic specific protein (SVSPr). The gel was stained with Coomassie brilliant blue.

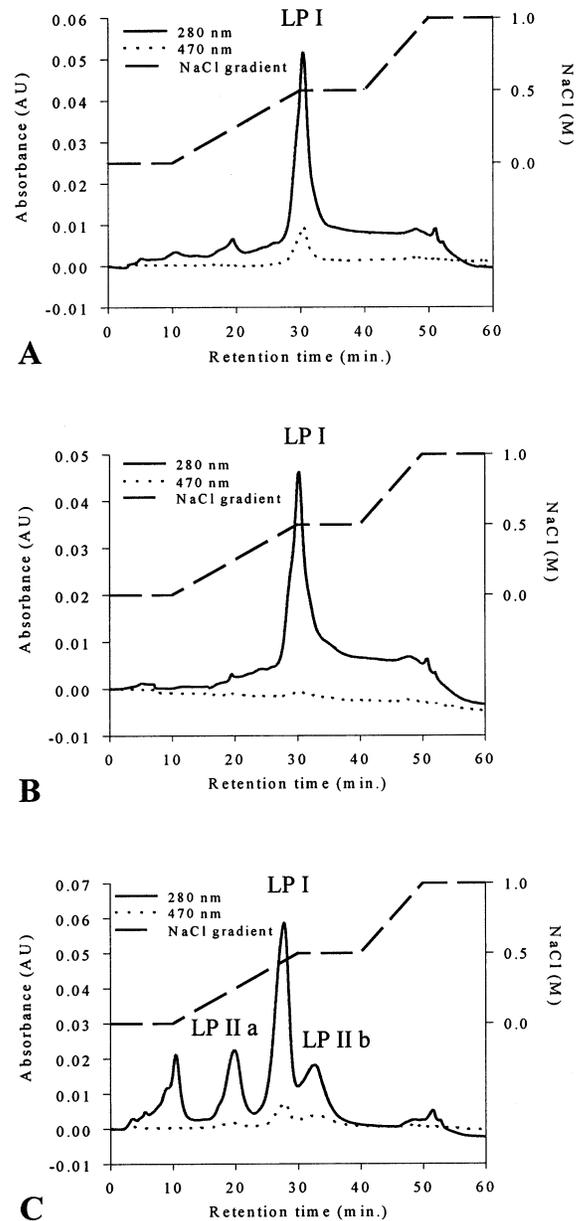


Fig. 2. Representative HPLC chromatogram (on DEAE column) of HDL isolated from *C. quadricarinatus* hemolymph. (A) Male. (B) Primary-vitellogenic female. (C) Secondary-vitellogenic female. Fractions were eluted with a NaCl gradient (0–1 M), represented by a broken line. Solid line represents absorbance at 280 nm. Dotted line represents absorbance at 470 nm.

due to the proximity in elution times of LPI and LPIIb. It is likely that, for the same reason, the LPI peak from the secondary-vitellogenic female was contaminated with the 80-kDa polypeptide found in LPIIb (Fig. 3D).

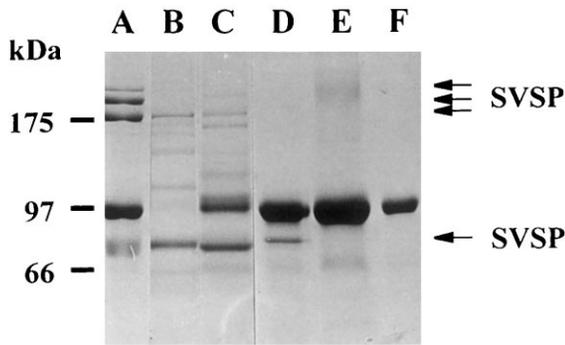


Fig. 3. SDS-PAGE (7%) of HDL from *C. quadricarinatus* hemolymph. (A) HDL from secondary-vitellogenic female hemolymph. (B, C, D) HPLC-separated peaks from secondary-vitellogenic female hemolymph, LPIIa, LPIIb, and LPI, respectively. (E) HPLC-separated LPI from male hemolymph. (F) HDL from male hemolymph. The gel was stained with Coomassie brilliant blue.

The HPLC elution pattern of HDL isolated from the hemolymph of a *C. quadricarinatus* vitellogenic female during the spawning season in the summer (Fig. 4A) differed from that of a female during the winter reproductive arrest period (Fig. 4B). The chromatogram from the reproductively active female showed both the LPI peak (eluted at 30 min) and the LPIIa and LPIIb peaks (eluted at 20 and 32 min, respectively), whereas that of the wintering female almost completely lacked the

two LPII peaks, showing only the single-peak pattern of LPI.

Since preliminary observations (in mid-September 1997) had suggested that the hemolymphatic secondary-vitellogenic specific lipoprotein was absent from females entering the winter arrest period, we designed an experiment to follow the seasonal changes in the hemolymph HDL content. HDL samples were collected at 2- to 5-week intervals (between September 1998 and February 1999) from the hemolymph of mature, previously spawned *C. quadricarinatus* females. Polypeptide profiles of these samples on 7% SDS-PAGE revealed a pattern similar to that detected by HPLC (Fig. 5). At the beginning of autumn (14 September 1998), the profile showed the four secondary-vitellogenic specific polypeptides of 80, 177, 196 and 208 kDa, with the 177-kDa polypeptide being present in the highest concentration (Fig. 5C). Two weeks later (23 September), a relative increase, of almost twofold, in the amounts of the 80-, 177- and 196-kDa polypeptides was observed (Fig. 5D). In addition, polypeptides having molecular masses of 100–170 kDa, not present in the typical secondary-vitellogenic female profile (Fig. 5A), were detected. At the beginning of winter (7 October 1998), the polypeptides of 80, 196 and 208 kDa had almost completely disappeared, and the amount of the 177-kDa polypeptide had significantly decreased (Fig. 5E). The four polypep-

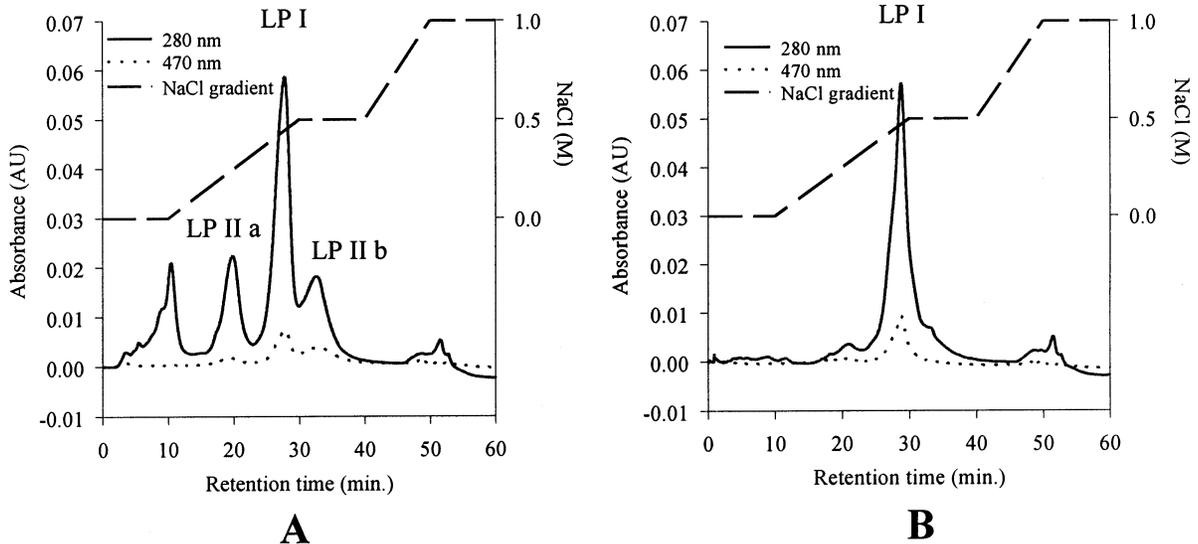


Fig. 4. Representative HPLC chromatogram (on DEAE column) of HDL isolated from the hemolymph of *C. quadricarinatus* reproductively active females (A) and females at their winter reproductive arrest period (B). Fractions were eluted with a NaCl gradient (0–1 M), represented by a broken line. Solid line represents absorbance at 280 nm. Dotted line represents absorbance at 470 nm.

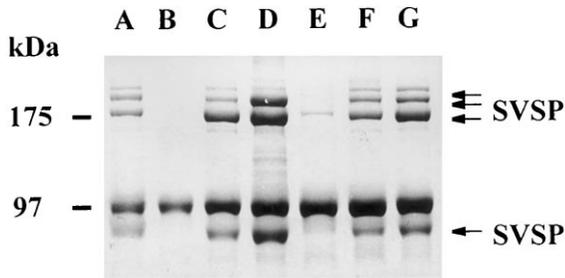


Fig. 5. SDS-PAGE (7%) separation of HDL isolated from the hemolymph of *C. quadricarinatus* males and mature females during their winter reproductive arrest period. (A) Secondary-vitellogenic female. (B) Male. (C) Female, 14 September 1998. (D) Female, 23 September 1998. (E) Female, 7 October 1998. (F) Female, 3 December 1998. (G) Female, 4 February 1999. The gel was stained with Coomassie brilliant blue.

tides (Fig. 5F) re-appeared after the middle of winter (3 December 1998), and their concentrations increased slightly as the winter drew to a close (Fig. 5G, 4 February 1999). The amount of LPI, the sole polypeptide found in male (Fig. 5B), was used for normalization of the amounts of protein loaded onto the gel.

The chromatogram of the HDL isolated from the hemolymph of control (intact) intersex individuals showed a major LPI peak, eluted at about 30 min (Fig. 6A), whereas that from andrectomized intersex individuals showed a similarity

to the pattern for secondary-vitellogenic females, having a marked peak of LPIIa, eluted at about 24 min, and a smaller LPIIb peak, eluted at about 32 min (Fig. 6B).

The influence of the androgenic gland on the presence of secondary-vitellogenic specific lipoproteins in the hemolymph was further investigated by implanting androgenic glands into young females. HPLC analysis of HDL revealed differences between such females and those implanted with fragments of sperm duct (control). The chromatogram of HDL isolated from the hemolymph of control females showed a minor LPIIa peak, eluted at 24 min, and a larger LPIIb peak, eluted at 33 min. The latter peak co-eluted with the LPI peak of 31 min (Fig. 7B). On the other hand, the chromatogram of HDL isolated from the hemolymph of females with androgenic gland implants (Fig. 7A) almost completely lacked the LPIIa and LPIIb peaks, although it did exhibit the LPI peak eluted at the same retention time as that shown in Fig. 6A.

4. Discussion

The two kinds of lipoproteins, belonging to the HDL family, found in the hemolymph of *C. quadricarinatus* appear to be similar to those re-

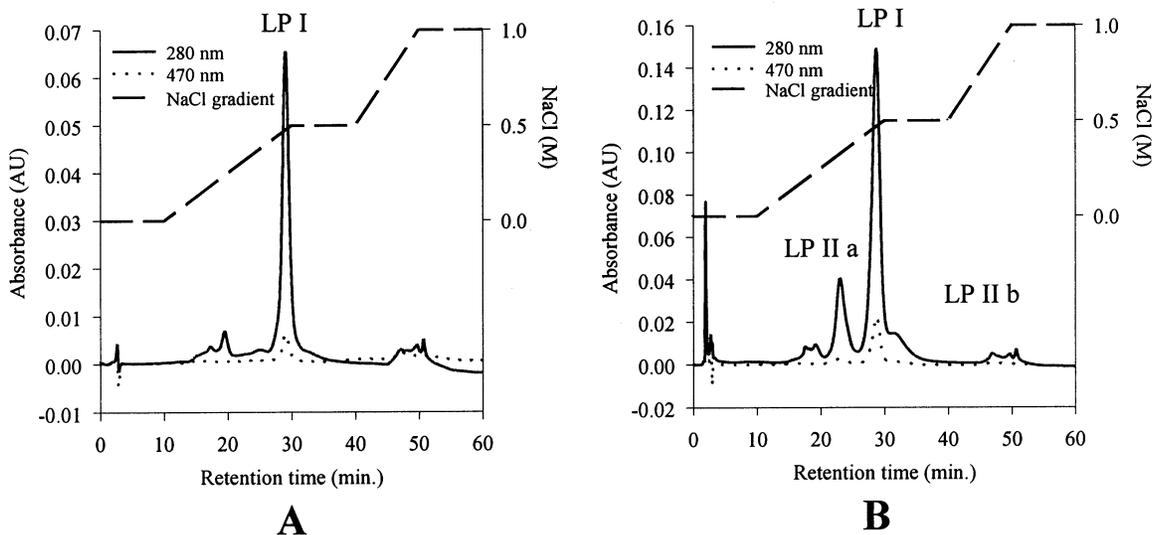


Fig. 6. Representative HPLC chromatogram (on DEAE column) of HDL isolated from the hemolymph of *C. quadricarinatus* intersex individuals. (A) Intact intersex individuals. (B) Andrectomized intersex individuals. Fractions were eluted with a NaCl gradient (0–1 M), represented by a broken line. Solid line represents absorbance at 280 nm. Dotted line represents absorbance at 470 nm.

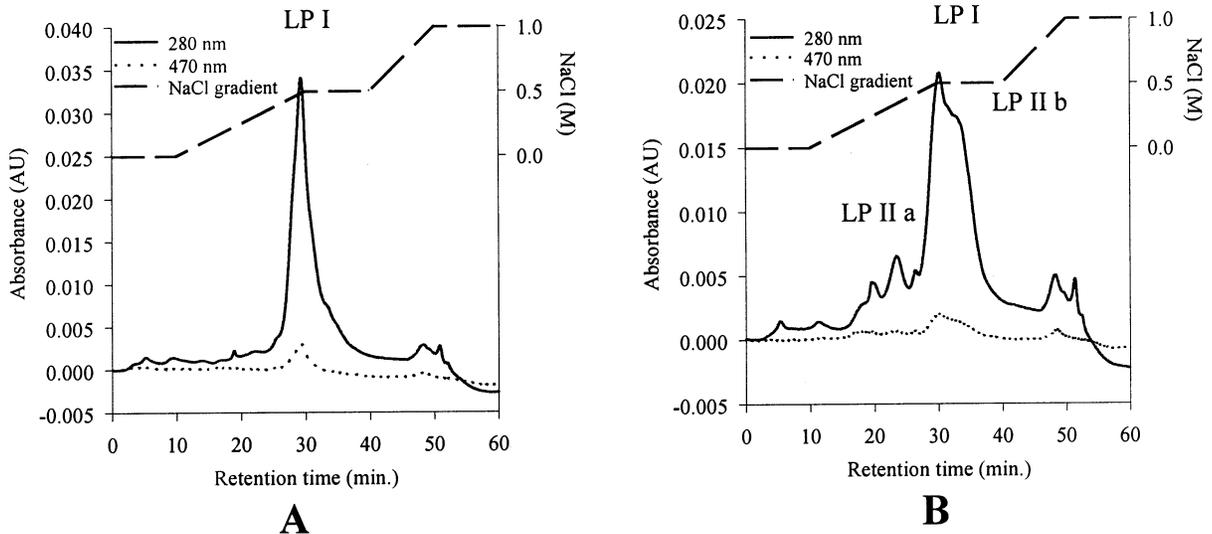


Fig. 7. Representative HPLC chromatogram (on DEAE column) of HDL isolated from the hemolymph of *C. quadricarinatus* females. (A) Sperm-duct-implanted females. (B) Androgenic-gland-implanted females. Fractions were eluted with a NaCl gradient (0–1 M), represented by a broken line. Solid line represents absorbance at 280 nm. Dotted line represents absorbance at 470 nm.

ported in other crustaceans (Kerr, 1969; Lee and Puppione, 1988; Lee, 1990; Komatsu et al., 1993; Lubzens et al., 1997). One of these lipoproteins, which appeared in the hemolymph of both males and females, regardless of the vitellogenic stage, was designated LPI, in keeping with previous reports of such a protein in other species (Lee and Puppione, 1988; Komatsu et al., 1993; Hall et al., 1995). The other lipoprotein, which was specific to the hemolymph of secondary-vitellogenic females, was thus termed LPII. HPLC chromatography showed LPII to be composed of two proteins, termed LPIIa and LPIIb, whereas characterization by PAGE revealed only a single secondary-vitellogenic specific protein. This apparent contradiction may be explained in terms of the inherent characteristics of the two separation methods, suggesting that LPIIa and LPIIb might differ in their polypeptide structure while having similar charge density. Similar cases of more than one form of LPII have been reported for other species, for example, two forms of vitellogenin in the hemolymph of *Homarus americanus* (Nelson et al., 1988) and *Astacus leptodactylus* (Nelson et al., 1988) and four forms of the protein in *Armadillidium vulgare* (Suzuki et al., 1990). However, in other species, such as *Penaeus chinensis* (Chang and Jeng, 1995), *Scylla*

serrata (Rani and Subramoniam, 1997), *Callinectes sapidus* (Lee and Puppione, 1988) and *Macrobrachium rosenbergii* (Lee et al., 1997), native electrophoresis revealed only one form of a secondary-vitellogenic specific lipoprotein.

In different species of Crustacea, the secondary-vitellogenic specific lipoprotein in the hemolymph may be comprised of 2–11 polypeptides, ranging from 75 to 200 kDa (Puppione et al., 1986; Lee and Puppione, 1988; Komatsu et al., 1993; Khayat et al., 1994; Lubzens et al., 1995; Lee et al., 1997; Pateraki and Stratakis, 1997). Our current findings, together with the work of Abdu et al. (2000), suggest that in *C. quadricarinatus* the secondary-vitellogenic specific lipoprotein is comprised of four polypeptides having molecular masses of 80, 177, 196 and 208 kDa. In both male and female animals, LPI consisted of a single polypeptide of 96 kDa. A comparison of the polypeptide composition of LPI and LPII of different species shows that in most of the species the protein denoted LPI includes a subunit of approximately 100 kDa (Table 1). In addition, in other decapod species, including *C. quadricarinatus* but excluding *Cancer antennarius* and *Charybdis feriata*, LPI and LPII do not have polypeptides with the same molecular weights (Komatsu et al.,

1993). However, Lee and Puppione (1988) suggested that in marine crustaceans LPI and LPII are similar (Lee and Puppione, 1988). The differences in the apoprotein between LPI and LPII might imply different origins and functions of these lipoproteins. Another noticeable phenomenon is that in some crustaceans LPII includes two polypeptides having molecular masses of approximately 80 kDa together with a higher molecular mass polypeptide of over 150 kDa. The universality of the 80-kDa polypeptide in LPII, appearing in a diversity of species from marine crabs and shrimps to fresh-water crabs, prawns and crayfish (Table 1), might suggest evolutionary conservation that should be confirmed by amino acid sequence studies. Regarding LPII, *C. quadricarinatus* is rather an exceptional case, since the molecular masses of three of its four polypeptides are higher than 150 kDa.

The specificity of LPII to the secondary-vitellogenic state in *C. quadricarinatus* was first confirmed by showing that LPI was present in the crayfish at all times, whereas LPII disappeared around November, when spawning reached the lowest level (Barki et al., 1997), and re-appeared slightly before the end of the reproductive arrest period. Thus, LPII showed a correlation with the seasonal pattern of spawning, which is, in turn, related to reproduction. Similar observations have been reported for *Potamon potamios* (Pateraki and Stratakis, 1997). Likewise, in *Cancer antennarius*, the HDL content in the female

hemolymph peaked at the time most of the specimens were reproductively active (Spaziani, 1988). Further confirmation of the specificity of LPII to secondary vitellogenesis in *C. quadricarinatus* was provided by endocrine manipulation of the onset of the process: Implantation of the androgenic gland into young females inhibited secondary vitellogenesis (Khalaila et al., 1998), whereas its removal from intersex individuals resulted in the onset of secondary vitellogenesis (Khalaila et al., 1999; Sagi et al., 1999). These reports are in keeping with our current findings of the absence of LPII from the hemolymph of females having androgenic gland implants and the appearance of this lipoprotein in the hemolymph of andrectomized intersex individuals. Thus, the presence of LPII in the hemolymph of *C. quadricarinatus* could be used as a marker to monitor reproductive processes in an efficient, non-invasive manner.

In *C. quadricarinatus*, as in other crustaceans, the ovaries accumulate lipids, carotenoids and proteins during vitellogenesis (Harrison, 1990; Khayat et al., 1994; Sagi et al., 1996a; Abdu et al., 2000). It has been proposed that LPI transports lipids from the hepatopancreas to other tissues (Quackenbush, 1989; Lee, 1990). It is also possible that LPII, alone or together with LPI, is the main lipid and/or carotenoid carrier to the ovary (Harrison, 1990; Komatsu et al., 1993; Lubzens et al., 1995). As in the case for the hemolymphatic lipoproteins from other crustaceans (Chang et al.,

Table 1
Polypeptide composition of LPI and LPII from the hemolymph of various crustacean species

Species	LPI subunits (kDa)	LPII subunits (kDa)	Reference
<i>Charybdis feriata</i>	80, 100, 180	80, 100, 180	Komatsu et al., 1993
<i>Eriocheir japonica</i>	100, 340	80, 100, 180	Komatsu et al., 1993
<i>Cancer antennarius</i>	84, 100, 185 108	84, 100, 185 82, 100, 152	Spaziani et al., 1995 Puppione et al., 1986
<i>Penaeus japonicus</i>	80, 100, 180		Komatsu et al., 1993
<i>Penaeus semisulcatus</i>	100 110	80, 96, 158 80, 120, 200	Khayat et al., 1994; Lubzens et al., 1995 Lubzens et al., 1997
<i>Callinectes sapidus</i>	112	78, 107, 190	Lee and Puppione, 1988
<i>Potamon potamios</i>	128	85, 105, 115 and 181 (occasionally)	Stratakis et al., 1992; Pateraki and Stratakis, 1997
<i>Macrobrachium rosenbergii</i>	100, 230	Mainly 85, 95	Komatsu et al., 1993
<i>Cherax quadricarinatus</i>	96	89, 100, 170 80, 177, 196, 208	Lee et al., 1997 Present study

1994; Chang and Jeng, 1995), LPI and LPII in *C. quadricarinatus* were found to contain carotenoid moieties. Furthermore, a strong correlation between oocyte development and astaxanthin levels in the ovary and in the hemolymph of this species was reported by Sagi et al. (1995, 1996a), who suggested that this pigment is incorporated into vitellogenin in an extra-ovarian site and transported via the circulation to the ovary (Sagi et al., 1996a). However, it is also possible that LPII is, in fact, vitellogenin, the precursor of vitellin. Although the function of LPII in *C. quadricarinatus* has not yet been elucidated, the latter possibility is supported by the following evidence: (1) the biochemical and immunological similarities between LPII and yolk components (Sagi et al., 1999; Abdu et al., 2000); (2) the lack of LPII from the hemolymph of spawning females and females that are not in their reproductive season; and (3) the fluctuations in LPII levels in parallel with the ovarian cycle (Kerr, 1969; Byard and Aiken, 1984; Derelle et al., 1986; Komatsu and Ando, 1992; Komatsu et al., 1993). However, we cannot disregard the possibility that the presence of this protein in the hemolymph might be a result of leakage of yolk protein from the ovary as a consequence of re-absorption or as a result of controlled secretion from the ovary (Yano and Chinzei, 1987; Lee et al., 1996). Further investigation is required to determine the precise function of LPII in crayfish reproduction.

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