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Structure and stability of crustacean lipovitellin: Influence of lipid content and composition

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ABSTRACT

Lipovitellin (LV) is essential in crustacean eggs for embryo viability and development. Two LV were isolated from eggs of *Macrobrachium borellii*. corresponding to early (LVe) and late (LVl) embryo developing stages. They differ in lipid composition but not in lipid/protein ratio or apoprotein composition. Structural information was obtained by fluorescence spectroscopy, far-UV circular dichroism, partial trypsinolysis and electron microscopy applied to LVe and LVl and two partially delipidated forms of LVe generated by phospholipase A2 (LVp) or Triton X-100 (LVt) treatment. All LV forms contained two apoprotein subunits of 94 and 112 kDa, being the 112 kDa subunit more accessible to trypsinolysis in all. Only in LVp, different cleavage sites appeared. Secondary structure was similar in LVe and LVl, but LVp and LVt showed a small increase in β-sheet at expense of α-helix. Electron microscopy revealed a spheroidal morphology in all LV and a decreased size in LVp. Delipidated LVs were more resistant to denaturation with guanidinium-HCl. Acrylamide quenching of tryptophan fluorescence was more efficient in delipidated LVs, probably due to apolipoprotein rearrangement, as reinforced by fluorescence anisotropy. It is concluded that LV stability, shape, and apoprotein conformation is not largely affected by the changes in lipid composition that take place during embryogenesis.

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1. Introduction

Proteins, lipids and carbohydrates present in the vitellus of eggs are important energy and material sources for the embryo development in oviparous species.

Eggs from the decapod crustacean *Macrobrachium borellii* have only one lipovitellin (LV), whose protein and lipid composition have been characterized (Garcia et al., 2004). Native LV has a molecular mass of 440 kDa, similar to the vitellogenin of the same species (Garcia et al., 2002). It is composed by two subunits of 94 kDa and one of 112 kDa forming a heterotrimeric structure (Garcia et al., 2006). When isolated from eggs at different stages of embryo development, two LV were obtained which differed in their lipid composition but not in the lipid/protein ratio or apoprotein composition (named LVe and LVl, corresponding to early and late embryo development stages respectively) (Garcia et al., 2004).

Lipovitellin is related to other lipoproteins. By using sequence alignment, Baker has determined in 1988 that vitellogenins of some invertebrates (nematodes and insects) contain homologous domains with human apo B-100 and lipase (Baker 1988). Recently, a comprehensive study using different structural and phylogenetic data indicated that the vitellus precursor protein of decapod crustacean is close related to insect apoLp-II/I and to vertebrate apoB (Avarre et al., 2007).

Lipoprotein lipid composition may alter the apolipoprotein conformation, and this may influence the ability of LV to interact with proteins or cellular receptors. This was well studied in human lipoproteins by Tricerri et al. (1998). It has also been suggested that the lack of phospholipids in insect lipoproteins might lead to structural changes in the apolipoprotein which could affect the stability of the lipoprotein particle (Rimoldi et al., 1996).

In contrast, Maya-Monteiro et al. (1997) did not find any change in the tryptophan fluorescence properties after removal of all the phospholipid from the lipoprotein of *Rhodnius prolixus*. These authors reported a greater resistance of the lipoprotein devoid of phospholipids than that of the native particle to denaturation by chaotropics agents and hydrostatic pressure.

In the present study, several study models (LVe, LVI, lipovitellin treated with Triton X-100 (LVt) and lipovitellin treated with phospholipase A2 (LVp)) were studied in order to determine for the first time in crustacean lipoproteins whether different lipid contents and compositions alter the structure and stability of this lipoprotein. For this purpose four LV forms differing in lipid composition were analyzed by far-UV spectroscopy, circular dichroism, partial trypsinolysis and electronic microscopy.

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2. Materials and methods

2.1. Lipoprotein isolation

Eggs from 10 females at early stage of the embryonic development (less than 26 days), were collected. This stage was identified according to previous reports describing the stages of *M. borellii* embryos along embryogenesis where an abundant vitellus (over 70% egg) was observed under a magnifying glass (Lavarias et al., 2002). Eggs from 10 females were homogenized in 3 mL 50 mM phosphate buffer, pH 7.4, in a Potter type homogenizer (Thomas Sci, Swedesvoro, NJ, USA), and sequentially ultracentrifuged at 10,000*g* for 20 min, and then at 100,000*g* for 60 min. Lipovitellins were isolated from the final supernatant (cytosolic fraction).

Yolk lipoprotein was isolated by density gradient ultracentrifugation. 1 mL of cytosolic fraction was overlayered on 3 mL NaBr solution (density 1.26 g/mL) containing 0.01% sodium azide, and centrifuged at 178,000*g* at 10 °C for 24 h in a Beckman L8 70 M centrifuge, using a SW 60 Ti rotor. Saline solution of the same density as that of samples was centrifuged in parallel to determine relative densities, and to check the correct gradient formation. Density was determined in a Bausch and Lomb refractometer. The total volume of the tubes was fractionated from top to bottom into 0.2 mL aliquots, and the protein content of each fraction was monitored spectrophotometrically at 280 nm. The aliquots containing the lipovitellin were pooled as one fraction.

2.2. Treatment of lipoprotein with phospholipase A2 and Triton X-100

LVe was completely depleted of phospholipids by incubation with phospholipase A2 from *Crotalus durissus* (Sigma-Aldrich Co.) resuspended in 0.1 M Tris–HCl buffer (pH 8.6) 0.2 U/mg of LV protein. The enzyme reaction was performed at 37 °C for 30 min. The phospholipids were eliminated using macroreticular adsorbent beads (Bio-Beads SM-2, Bio-Rad Laboratories, CA) 1 g/g protein ratio, with constant shaking at room temperature for 1 h. The beads were separated by filtration on lipid-free glass fiber.

LVe was delipidated using Triton X-100 (Sigma) 0.02 g/g protein. The mixture was incubated with shaking at room temperature for 1 h and, the detergent was removed as described above (Mangum et al., 1987).

2.3. Lipid analysis

In order to determine the amount of lipids remaining after the treatment of lipoproteins with phospholipase and Triton, samples were extracted by following the method of Folch et al., 1957. Quantitative determinations of lipid classes were performed by TLC coupled to an FID in an Iatroscan apparatus Model TH-10, after separation on Chromarod SIII, using a triple development solvent system as described previously Cunningham and Pollero (1996). All lipid classes were quantified using monoacylglycerol as internal standard.

2.4. Native and SDS PAGE

Lipovitellin preparation (LVe, LVl, LVt and LVp) were analyzed by native and dissociating electrophoresis. Analyses were performed using a 4–23% acrylamide PAGE (Laemmli, 1970). The gels were stained with Coomassie Brilliant Blue R-250 (Sigma). Molecular weight standards (Pharmacia, Uppsala, Sweden, and Marker kit, Sigma) were run in parallel lines.

2.5. Electron microscopy

Samples of lipovitellin were dialyzed in 50 mM potassium phosphate buffer (KPB), pH 7.4 and negatively stained with 2% phosphotungstate (Forte and Nordhausen, 1986) for its examination by electron microscopy. Preparations were made at least in triplicate, viewed on a JEOL 100 S electron microscope (JEOL Co., Japan) at 100 kV and photographed at a magnification of 60,000×. Final magnification was 120,000×. Approximately 100 free-standing lipoprotein particles were measured on multiple photographs taken from different areas of the grid.

2.6. Trypsin treatment

One μg/μL of LVe, LVl, LVt and LVp were incubated with trypsin (sequencing grade, Promega, Madison, WI) 0.01 μg/μL in 5 mM ammonium bicarbonate pH 8, at 37 °C for 30 min. Proteolysis was stopped with ice. Samples for SDS-PAGE were boiled for 5 min immediately after the addition of sample loading buffer (Laemmli, 1970) to prevent the reactivation of trypsin that occurs in the sample buffer.

2.7. Circular dichroism and fluorescence measurements

Circular dichroism spectra of LVe, LVl, LVt and LVp in 10 mM ammonium bicarbonate, pH 7.8 at 25 °C were recorded on a Jasco 715 spectropolarimeter using 0.1 cm pathlength cells in the far-UV range (195–260 nm). CD spectra were recorded every 0.5 nm with a 2 s averaged spectra. They were corrected for background, protein concentration and smoothed. Protein was determined by absorption at 280 nm using an extinction coefficient of 1.42 $Lg^{-1}cm^{-1}$. To estimate the composition of the secondary structure, the spectra were analyzed with the program Varselec (Toumadje et al., 1992).

2.8. Solvent exposure of tryptophan residues

Fluorescence spectra were collected on a Perkin-Elmer LS55 Luminescence Spectrometer (Norwalk, CT, USA) using a 200 W Xenon lamp as excitation source. Protein sample was dissolved in a 50 mM phosphate buffer (pH 7.5). The excitation wavelength was 280 nm at which the absorbance of the sample was below 0.05.

2.9. Acrylamide quenching measurements

In fluorescence quenching experiments acrylamide was added to the protein sample from freshly prepared concentrated (2 M) stock solutions.

After corrections for dilution and inner filter, Fo/F ratios were plotted versus acrylamide concentration where Fo and F are the tryptophan fluorescence intensities (excitation wavelength 295 nm, emission wavelength 323 nm) in the absence and in the presence of different concentrations of acrylamide, respectively.

2.10. Unfolding with GdnHCl

For unfolding experiments, lipoprotein samples were incubated with GdnHCl concentrations ranging from 0 to 5 M, in 10 mM Tris–HCl, 0.15 M NaCl, pH 7.4. Fluorescence spectra were obtained after incubation at 4 °C for 12 h and corrected for background contribution of the GdnHCl.

2.11. Steady-state fluorescence anisotropy of Trp, DPH and DPH-PA

Steady-state fluorescence anisotropy (rs) was measured according to Lakowicz et al. (1979) with modifications (Garda et al., 1994; Tricerri et al., 1994). The excitation wavelength was 361 nm (for 1,6 diphenyl-1,3,5-hexatriene (DPH) and 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl) phenylpropionic acid (DPH-PA)) and 295 nm (for Trp), and the emitted light isolated with sharp cut-off filters (below 389 nm for DPH and DPH-PA, and below 320 nm for Trp).

Table 1
Lipid composition of Lipoproteins LVe, LVl, LVt and LVp (% of each lipid class in relation to the whole particle, w/w).

	TAG	FFA	CHO	PE	PC	SM	% lipids in the lipoprotein
LVe	6.1 ± 0.53	1.7 ± 0.17	2.3 ± 0.14	4.7 ± 0.35	12.5 ± 1.07	2.2 ± 0.14	30.0
LVl	4.1 ± 0.36	2.6 ± 0.17	3.4 ± 0.24	4.8 ± 0.88	6.2 ± 1.09	3.5 ± 0.37	25.0
LVt	2.7 ± 0.64	0.7 ± 0.20	1.3 ± 0.60	0.2 ± 0.05	2.9 ± 0.78	2.0 ± 0.06	10.0
LVp	6.1 ± 0.30	1.7 ± 0.10	2.3 ± 0.12	0.0	0.0	2.2 ± 0.11	12.3

(PE), phosphatidylethanolamine; (PC), phosphatidylcholine; (FFA), free fatty acids; (TAG), triacylglycerol; (CHO), cholesterol; (SM), sphingomyelin. Values represent the mean ± SD of three analyses.

2.12. Statistical analyses

At least three separate experiments were performed for each study. The samples being analyzed in triplicate and standard deviations calculated were appropriate. Data were analyzed by Student's *t*-test using Instat v 2.0. Results were considered significant at $P<0.05$ after the comparison of control sample (LVe) and delipidated samples (LVp and LVt).

3. Results

3.1. Analysis of remnant lipids in treated lipoproteins

The lipid compositions of the four LV forms, as determined by TLC-FID, are shown in Table 1. The amount of total lipids remaining in LVp and LVt were 12.7% and 10.0%, respectively. All phospholipids were removed when LV was treated with phospholipase A2. In contrast, treatment with Triton X-100 resulted mainly in the remotion of phosphatidylethanolamine (PE), phosphatidylcholine (PC), free fatty acids (FFA) and triacylglycerol (TAG) (Table 1).

3.2. Native and SDS PAGE

The electrophoretic mobility under native and denaturing conditions was similar in the four lipoproteins studied (LVe, LVl, LVt and LVp) (Fig. 1A and B). The molecular mass of the four LVs was 440 kDa under native conditions. All LVs showed two apolipoproteins of 112 kDa and 94 kDa when analyzed by SDS-PAGE.

3.3. Trypsin treatment

The trypsinolysis pattern was similar for LVe, LVl and LVt. However, LVp showed a different trypsinolysis pattern containing 3 peptides not present in the other lipoproteins (Fig. 2). In agreement with previous observations made in LVe (Garcia et al., 2006), the 112 kDa subunit was more accessible to trypsin hydrolysis than the 94 kDa subunits in all LVs tested.

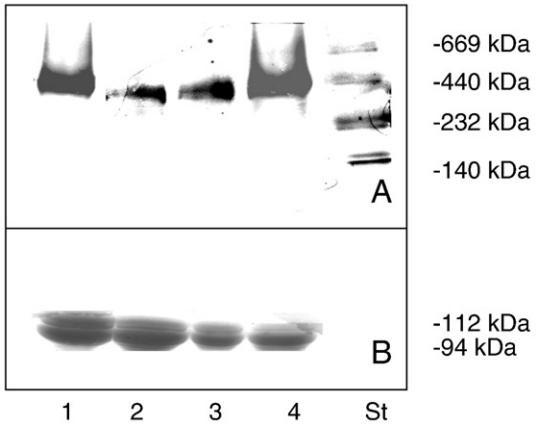

Fig. 1. Native (A) and SDS -PAGE (B) of lipoprotein models in 4–23% acrylamide gradient slab. Line 1 LVe; line 2 LVp; line 3 LVt; line 4 LVl. Proteins were visualized by Coomassie Brilliant Blue.

3.4. Circular dichroism

Apolipoprotein secondary structure was investigated in LVe, LVl, LVt and LVp by far-UV circular dichroism (Fig. 3). Using spectral

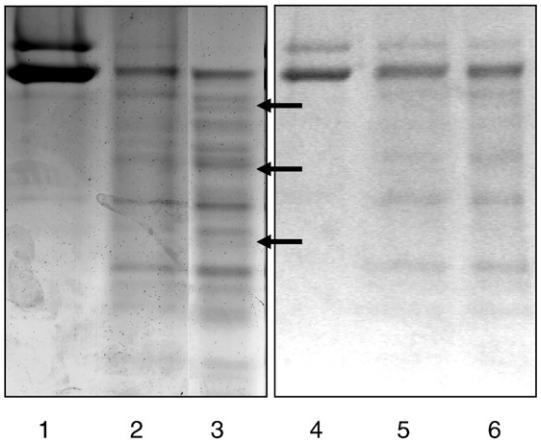

Fig. 2. Trypsin treatment of LV models in SDS-PAGE (4–23% acrylamide gradient slab). Line 1 LVe without trypsin; line 2 LVe with trypsin; line 3 LVp with trypsin; line 4 control LVt; lines 5 and 6 :LVt and LVl with trypsin. Proteins were visualized by Coomassie Brilliant Blue. Arrows indicate three peptides news.

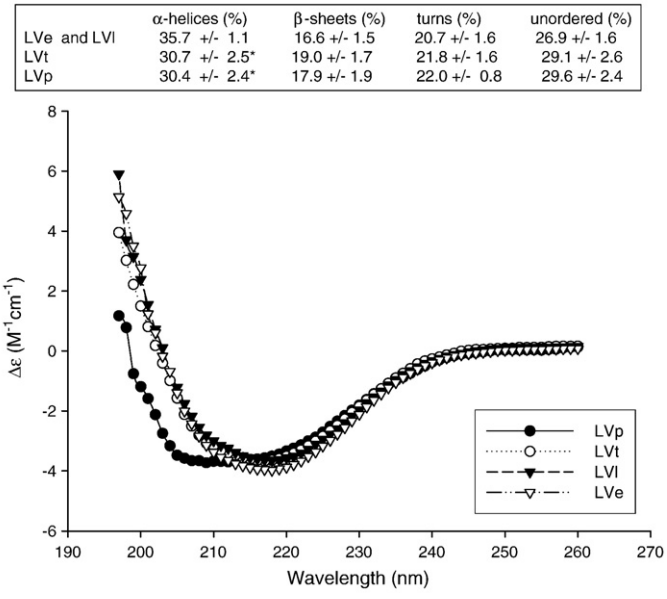

Fig. 3. Far-UV circular dichroism spectrum of Lipoproteins LVe, LVl, LVt and LVp. Inset: Spectral deconvolution of LVe, LVl, LVt and LVp using the program Varselec. Values represent the mean ± SD of three analyses. *: $P<0.05$ calculated by Student's *t*-test comparisons of LVe-LVl with either LVt or LVp.

deconvolution with the program Varselec, it was determined that the secondary structures were similar for LVe and LVl. On the other hand, partially delipidated LVs, LVp and LVt, showed a small increase in β-sheet at the expense of α-helix content (Fig. 3).

3.5. Electron microscopy

Structural integrity of LVt and LVp was confirmed by electron microscopy analysis. The spheroidal morphology previously observed by negative staining electron microscopy for LVe (Garcia et al., 2006) was confirmed. This spheroidal morphology was not affected by the lipid/protein ratio (LVt and LVp) or by the lipid composition (LVl). However, the size of the lipoprotein particles decreased by lipid removal from a diameter of about 18–20 nm in LVl and LVe (Garcia et al., 2006), to a diameter of 11.6–15.0 nm in LVt and 16–20 nm in LVp (micrographs not shown).

3.6. Solvent exposure of tryptophan residues

Fig. 4 shows the tryptophan emission spectra of the four lipoprotein models. Delipidation resulted in an increased quantum yield as indicated by the increased emission intensity. Since no spectral shifts were caused by the removal of lipids, the increase in quantum yields is likely to be due to a conformational change.

In order to obtain further information about the environment of Trp residues, quenching by acrylamide was measured. The Stern–Volmer plots for LVl, LVe, LVp and LVt shown in Fig. 5 indicate that lipid removal resulted in an increased accessibility of Trp residues to this hydrosoluble quencher. This discarded the possibility of a lower degree of Trp exposure to the aqueous medium suggested by the increased quantum yield of delipidated lipovitellins.

3.7. Unfolding of different lipoproteins by guanidine hydrochloride

The effect of this chaotropic agent on LVe, LVl, LVt and LVp was investigated by analyzing the intrinsic fluorescence emission spectra at different GdnHCl concentrations (0, 2.5 and 5 M) (Fig. 6). All the samples were affected by 5 M GdnHCl which produces a red shift and a decrease in Trp fluorescence intensity. However, at 2.5 M GdnHCl only the non delipidated samples were affected showing a decrease of 14 and 36% in the emission intensity of LVe and LVl respectively (Fig. 6A and B). These results indicate that delipidation of lipovitellin results in an increased stability of the protein to denaturation with GdnHCl.

3.8. Fluorescence steady-state anisotropy of Trp, DPH and DPH-PA

The steady-state anisotropy (rs) of Trp fluorescence did not show significant differences between LVe and LVl (0.118 ± 0.004 and

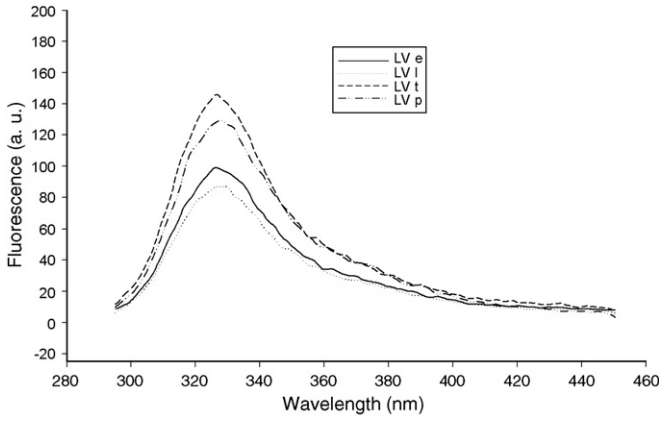

Fig. 4. Intrinsic fluorescence emission spectra of LVe, LVl, LVt, and LVp. The samples were measured in 50 mM phosphate buffer (pH 7.5) after excitation at 280 nm.

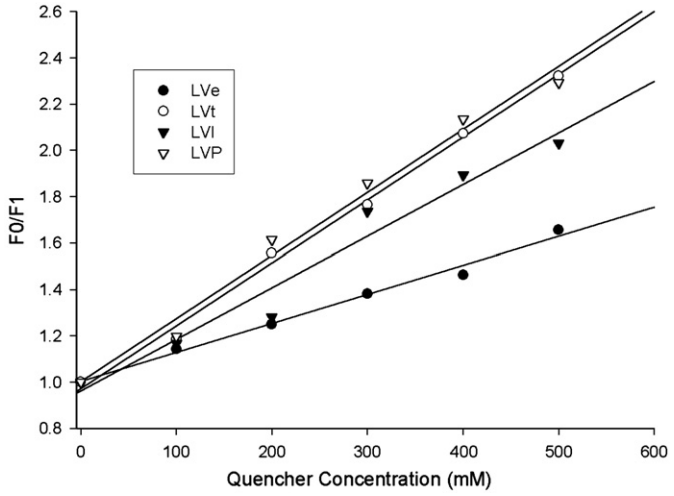

Fig. 5. Stern–Volmer plots for acrylamide quenching of the Trp fluorescence in LVe (solid circles), LVt (open circles), LVl (solid triangles) and LVp (open triangles). Fluorescence intensity was monitored at the emission peak 323 nm.

0.114 ± 0.003, respectively). In contrast, delipidation resulted in a significant increase of anisotropy (0.127 ± 0.002 and 0.136 ± 0.002 for LVt and LVp, respectively). These results indicate that the mobility of Trp is more restricted in delipidated samples (Table 2).

In order to obtain further information on the changes in the physical properties of the lipids remaining after delipidation, the fluorescence intensity and anisotropy of the probes DPH and DPH-PA were determined in native and delipidated LV form. The amphipathic probe DPH-PA locates in a different region than that of DPH, aligning its carboxylate group with the phospholipid's polar head. The fluorescence intensity of DPH is lower in delipidated samples than in LVe or LVl, suggesting that lipid removal results in a higher exposure of these probes to the aqueous environment, though less solubilized DPH in the delipidated LVs should not be discarded. A moderate increase in fluorescence anisotropy was observed in delipidated lipovitellin particles suggesting a more restricted mobility in lipid poor samples (Table 2).

4. Discussion

In the present study, a crustacean lipovitellin was delipidated with Triton X-100 and phospholipase A2 to understand how different compositions and lipid contents may affect the lipoprotein particle structure. It was found that lipovitellin remained stable in an aqueous solution when treated either with Triton X-100 or phospholipase A2. This result is similar to a previous study, where a complete delipidation of a spider VHDL (containing 3% w/w of lipids) caused no loss of stability (Cunningham et al., 1999). For the crustacean LV, however, the lipids removed represented 20% of the lipoprotein mass,. Kawooya et al. (1991) have shown that the insect lipophorin particle remains stable after phospholipid extraction, but that the particle lost stability when DAGs were removed with triacylglycerol lipase. These authors also evaluated the fact that phospholipids could stabilize the lipoprotein throughout the assembly of the lipoprotein particle just before the incorporation of neutral lipids. Thus, neutral lipids could be important to stabilize the lipoprotein particle in the absence of phospholipids. After delipidation of lipovitellin with Triton X-100, the remaining lipids were mainly PC and TAG which are thought to participate in the particle stabilization. In PLA treated LV, LVp, phospholipids were completely removed suggesting that the remaining TAG could sustain the conformation and stability of the apolipoproteins.

Negative staining electron microscopy showed that delipidation with Triton X-100 or phospholipase A2 does not alter the spheroidal morphology of lipovitellin.

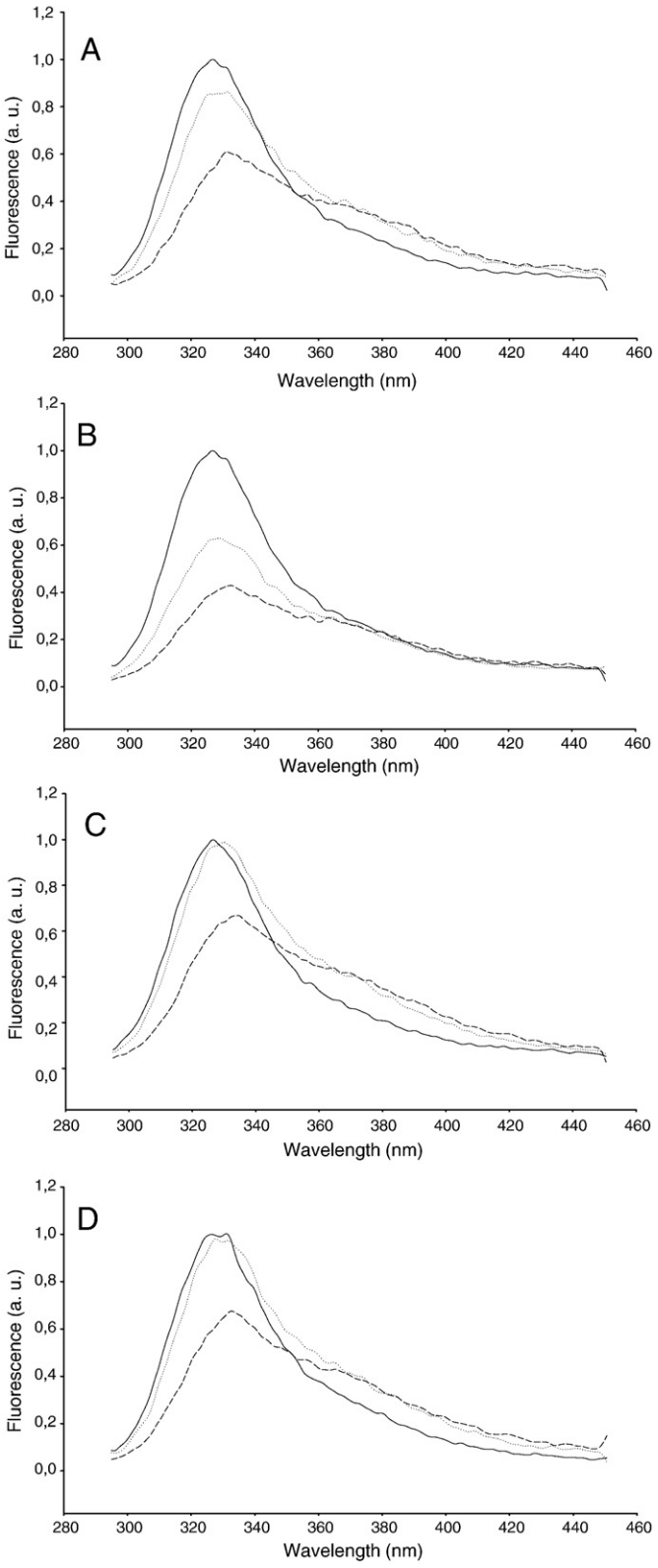

Fig. 6. Denaturation by GdnHCl followed by the fluorescence emission spectra of LVe (panel A), LVl (panel B), LVt (panel C) and LVp (panel D). Samples were incubated in 0 (full line), 2.5 M (dashed line), 5 M (dotted line) GdnHCl.

Lipid extraction from LVe caused a diminution in the lipoprotein size by using both methods. Then, the size of these models was quite similar (15 nm) to the one described for another lipoprotein, the HDL/beta-glucan binding protein of *L. vannamei*. This one unlike the LVs has a discoidal morphology (Morán-Palacio et al., 2006).

Table 2
Fluorescence anisotropy and intensity of Trp, DPH, DPH-PA in LVe, LVl, LVt and LVp.

	Fluorescence anisotropy of Trp	Intensity
LVe	0.118 ± 0.004	360
LVl	0.114 ± 0.003	323
LVt	0.127 ± 0.002 *	542
LVp	0.136 ± 0.002 *	537
	Fluorescence anisotropy of DPH	**Intensity**
LVe	0.206 ± 0.005	260
LVt	0.230 ± 0.007*	236
	Fluorescence anisotropy of DPH-PA	**Intensity**
LVe	0.268 ± 0.007	190
LVt	0.299 ± 0.014*	215

Values represent the mean ± SD of three analyses.
* $P<0.05$ (calculated by Student's *t*-test comparisons of LVe with either LVt or LVp).

The heterotrimeric structure of all models studied was not affected by lipid depletion, showing the same proportion of subunits and MW. Moreover, as shown by the comparison of LVe and LVl, this heterotrimeric structure remained unaltered along embryogenesis (Garcia et al., 2006).

It is worth noting that the trypsinolysis pattern was different in the LV devoid of phospholipid as compared with the LVe, LVl and LVt models, indicating that if phospholipids are completely removed, new cleavage sites are exposed.

In a previous study we have shown that LV apolipoproteins are selectively degraded during embryo development, being the highest molecular weight subunit the most affected (Garcia et al., 2008). The present study confirmed this result by showing a preferential degradation of the 112 kDa subunit by trypsin.

Though the secondary structure of apolipovitellins is not affected by the changes in lipid composition occurring during embryo development, a small increase in β-sheet at the expense of α-helix is observed after lipid depletion. In contrast, lipid depletion of the insect lipophorin did not produce changes in the secondary structure of its apolipoproteins (Maya-Monteiro et al., 1997).

No spectral shift in the Trp emission was observed upon delipidation of crustacean lipovitellin, indicating that the polarity of the Trp residues environment remained unaltered. However, delipidation produced a significant increment in Trp fluorescence intensity. The decreased tryptophan quantum yield in lipidated lipovitellin could be due to the presence of some lipophilic quencher that would be extracted together with the lipids. However, no detectable absorption in the wavelength region of tryptophan emission is observed in lipovitellin, discarding the possibility of energy transfer to a colored acceptor. Since lipovitellin contains more than 20 trp residues (Garcia et al., 2006), another possibility is that in the lipidated state some trp residues lie close enough to cause autoquenching. A rearrangement of the protein following delipidation could result in the separation of these trp residues eliminating the autoquenching. This rearrangement of the particle is supported by the higher accessibility to acrylamide quenching found in delipidated lipovitellin. Moreover, the increased tryptophan emission anisotropy in the delipidated state could be due to a higher average distance among trp residues and, consequently, to a lower efficiency of Trp–Trp energy homotransfer.

The chaotropic agent (GdnHCl) affected LVe and LVI lipoproteins in a similar way, generating the same degree of denaturation). It must be pointed out that in LVp and LVt the fluorescence emission of tryptophan was not diminished at 2.5 M GdnHCl, presumably due to hidden tryptophan-containing regions protected from the chaotropic effect. A similar effect was observed in the insect dephospholipidized lipoprotein (d-LP) in which the high stability of d-LP in a GdnHCl solution was explained by an increase in protein–protein and/or protein–neutral lipid interactions, which could decrease the guanidine binding to the protein (Maya-Monteiro et al., 1997).

The absence of lipids is likely to produce structural changes in the apolipoprotein; this hypothesis was supported by the increase of tryptophan fluorescence anisotropy in LVt and LVp as compared with LVe and LVl. The increments of the anisotropy values suggest a higher restriction of Trp motion.

In contrast, the mobility of the lipophilic fluorophores DPH and DPH-PA diminished in the delipidated LVe variants, LVp and LVt. This change could be due to the relative increase of apolipoproteins that would preclude lipid movement in the particle. The opposite effect, that is a decrease in the rs, is expected in models totally depleted of the protein such as liposomes, where the fluorophore does not have any mobility restrictions due to proteins. This effect was demonstrated for DPH and DPH-PA added to liposomes prepared with the lipids extracted from LVe and LVl (Garcia et al., 2006).

Overall, the results from the present study support the hypothesis that crustacean LV stability is not affected by changes in the lipid composition, at least under the present experimental conditions. LV in yolk would therefore remain stable throughout embryo development.

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