Protein characterization and fatty acid composition of VHDL subfraction II of the spider Polybotes pythagoricus

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ABSTRACT: VHDL fraction contains hemocyanin as its major apoprotein and transports most of the circulating lipids in the spider Polybotes pythagoricus (Sparassidae). This work shows that subfraction II (the major VHDL component) is composed of a single protein of 420 kDa under native conditions and three subunits, 67, 105 and 121 kDa under denaturing conditions. Circular dichroism indicated that this subfraction contains 20% α-helix, 29% β-sheet, 22.7% turns and 29.7% unordered structures. Comparison of trypsin susceptibility showed that the 105 and 121 kDa subunits were more susceptible indicating that these proteins would be more exposed to the aqueous medium. Peptide mass fingerprinting of the 67 and 105 kDa subunits indicated the 67 kDa subunit is similar to subunit 3 of the spider Cupiennius salei hemocyanin (21% sequence similarity), whereas the 105 kDa subunit is similar to a protein from the mosquito Anopheles gambiae (20% sequence similarity). The N-terminal amino acid sequence from subunit of 121 kDa was also determined. In relation to fatty acids, 16:0, 18:0, 18:1 and 18:2 were found to be the major components. These data provide a better understanding of VHDL subfraction II structure, which is responsible for most lipid transport in the spider P. pythagoricus.

Introduction

Arthropod lipid circulation mechanisms have only been studied in some insects and crustaceans. Insect lipids are mostly carried by the lipophorin lipoprotein in the form of phospholipids and diacylglycerol as energetic lipid (Chino, 1985; Blacklock and Ryan, 1994; Soulagès and Wells, 1994; Gonzalez et al. 1995; Arrese et al. 2001). In crustaceans, high-density lipoproteins (HDL) transport lipids mostly in the form of phospholipids (Lee and Puppione, 1978; Chang and O’Connor, 1983; Lee, 1991; García et al. 2002a; 2002b). Surprisingly, little information is available regarding the characterization of hemolymph lipoproteins for arachnids, where only four species have been studied (see review by Cunningham et al. 2007; Laino et al. 2015). In particular, the hemolymph lipid transport system of Polybotes pythagoricus (Holmberg 1875) (Araneae: Sparassidae) (Fig. 1) is composed of three lipoprotein fractions: one VHDL (very-high-density lipoprotein) (δ = 1.21–1.24 g/mL) and two HDL, named HDL-1 (δ = 1.13 g/mL) and HDL-2 (δ = 1.18–1.20 g/mL), whose hemolymph concentrations were 45.4, 2.3 and 23.6 mg of protein/mL, respectively. Circulating lipids in P. pythagoricus (3.41 mg/mL hemolymph) are therefore carried by VHDL (48.7%), HDL-1 (26.2%), and HDL-2 (25.1%) (Cunningham et al. 1994; Cunningham and Pollero, 1996). VHDL transports mainly phospholipids and triacylglycerols, and presents a bluish coloration given by the high amount of hemocyanin functioning as apolipoprotein (Cunningham and Pollero, 1996). VHDL is a complex lipoprotein isolated by ultracentrifugation and purified by gel permeation analysis and PAGE. It contains three different subfractions. Subfraction II is the major protein and has binding lipids, under denaturing conditions three

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different subunits form subfraction II, one of 67 kDa (described as hemocyanin) and other two of 105 and 121 kDa (Cunningham and Pollero, 1996; Cunningham et al. 1999; Cunningham et al. 2007).

Hemocyanin plays its principal role as oxygen carrier, but it also has other properties or functions such as phenoloxidase activity, antimicrobial activity and lipid transport (Cunningham and Pollero, 1996; Decker and Rimke, 1998; Terwilliger, 1998; Cunningham et al. 1999; Cunningham et al. 2000; Bridges, 2001; Decker and Jaenicke, 2004; Jaenicke and Decker, 2004; Cunningham et al. 2007; Laino et al. 2015 in press). The interaction of hemocyanin with lipids in crustaceans was firstly reported by Zatta (1981), showing the existence of small amounts of phospholipids bound to hemocyanin in the crab *Carcinus maenas* (Portunidae). A significant amount of lipids is found in hemocyanin-containing lipoproteins in the spiders *P. pythagoricus*, *Latrodectus mirabilis* (Theridiidae) and *G. rosea* (Theraphosidae). In fact, though VHDL has a low lipid/protein ratio, in *P. pythagoricus* it is bound to half of hemolymphatic lipids (Cunningham and Pollero, 1996). The HDL-2 of *L. mirabilis* is also bound to lipids though to a lesser extent (Cunningham et al. 2000), and the VHDL of *G. rosea* shows 6.9 % lipids (Laino et al. 2015). In the case of VHDL present in *Eurypelma californicum* shows 16.6% lipids, but unlike the aforementioned, hemocyanin is not shown in the protein structure (Stratakis et al. 1993). Recently, we have shown for the first time that the midgut diverticula of arachnids are a main storage site and a major lipid metabolic centre involved in the uptake and mobilization of lipids (Laino et al. 2009). It was also shown that the VHDL from *P. pythagoricus* innovatively participates in the lipid transport from and toward tissues (Laino et al. 2011) where subfraction II is the main fraction involved in this function (Cunningham et al. 1999).

Because of this surprising function of VHDL in *P. pythagoricus*, the purpose of the present paper was to follow up our study on the VHDL subfraction II. We utilized several techniques such as electrophoresis, circular dichroism (CD), partial proteolysis, MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometer) and N-terminal sequence analysis. We also determined the fatty acid composition of VHDL subfraction II by gas-liquid chromatography, being the second analysis (the first one was recently published in the spider *G. rosea*, Laino et al. 2015) of fatty acids of hemocyanin-containing hemolymphatic lipoproteins as apoprotein in the order Araneae.

**Material and Methods**

**Sampling and isolation of VHDL subfraction II**

A total of 15 wild male and female adults of *P. pythagoricus* were caught (Fig. 1) from barks of *Eucalyptus* sp. trees and bled on the same day (Cunningham et al. 1994). For hemolymph collection, spiders were anesthetized by cold exposure and the hemolymph was collected by cardiac puncture.
with a 27G needle on a 1-mL syringe soaked with sodium citrate buffer, pH 4.6, to prevent coagulation. It was immediately centrifuged (15,000 g, 4 °C, 10 min) to remove the cellular fraction. The supernatant was centrifuged at 178,000 g at 4 °C for 22 h (Beckman L8 70 M Ultracentrifuge, using a SW 60 Ti rotor). As plasma density was 1.006 g/ml, a saline solution of the same density was run simultaneously as blank. The total volume of the tubes was fractioned from top to bottom into 0.2 mL aliquots. The protein content of each fraction was monitored spectrophotometrically at 280 nm, and a blue band was separated as a fraction in the very high-density zone (VHDL) of the gradient as previously described (Cunningham and Pollero, 1996; Cunningham et al. 1999; 2007).

The VHDL fraction was separated from the gradient obtained under native conditions by FPLC (Fast Protein Liquid Chromatography) on a Superdex 200 HR 10/30 column (Pharma, Uppsala, Sweden) using 0.1 M Tris-HCl pH 8.0, containing 10 mM CaCl₂ and 50 mM MgCl₂, at a flow rate of 0.4 mL/min. Proteins were detected at 280 nm and the column was equilibrated as described by Cunningham et al. (1996). The purity and integrity of proteins present in subfraction II were analyzed by Native-PAGE (electrophoresis under native conditions using a polyacrylamide gradient) as reported by Cunningham et al. (1996; 2000).

Fluorescence measurements and circular dichroism
Fluorescence spectra were collected on a Perkin-Elmer LS55 Luminescence Spectrometer (Norwalk, CT, USA) using a 200W Xenon lamp as excitation source. The 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.

CD spectra from subfraction II were recorded in 10 mM ammonium bicarbonate at 25 °C on a Jasco 715 spectropolarimeter using 0.1 cm path length cells in the far-UV range (185-260 nm). CD spectra were recorded every 0.5 nm with 2 s averaged spectra, corrected for background, protein concentration, and smoothed. Protein by absorption was determined at 280 nm using an extinction coefficient of 1.42 L·g⁻¹·cm⁻¹. To estimate the composition of the secondary structure the spectra were analyzed with the Varselec program (Toumadje et al. 1992).

**FIGURE 3.** Intrinsic fluorescence emission spectrum (A) and Far-UV circular dichroism spectrum of subfraction II of VHDL fraction from *P. pythagoricus* (B).

**FIGURE 4.** MALDI-TOF MS analysis of 67 kDa subunit of Amino acid sequence of subunit 3 in the spider *Cupiennius salei* (CAC44751). The matched peptides from *P. pythagoricus* are in bold letter. Inset: PAGE-SDS (4-23% acrylamide gradient slab) . Lane 1: subfraction II. Lane 2: MW standards. Arrows indicate the subunit corresponding to spectrum.
MALDI-TOF MS analysis

For peptide mass fingerprinting, the subunits of the subfraction II were separated by SDS-PAGE (electrophoresis under denaturing conditions with a polyacrylamide gradient) using 4-23% polyacrylamide gradient (Laemmli, 1970). Gels were stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). Molecular weight standards were run (HMW, Pharmacia, Uppsla, Sweden) in parallel lines. Bands of 67, 105 and 121 kDa were excised, minced and de-stained using 100% acetonitrile, followed by four washes in 1 mL water. Gel pieces were incubated in 500 μL of 100 mM ammonium bicarbonate for 20 min, followed by 20-min incubation with 500 μL of 50% acetonitrile in 50 mM ammonium bicarbonate. Gel pieces were dried under vacuum, re-hydrated and digested with 50 ng/μL trypsin (sequencing grade, Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate at 4 °C overnight. Peptides were extracted and analyzed by MALDI-TOF MS in an ABI Voyager De Pro MALDI-MS using cyano-4-hydroxycinnamic acid as matrix and external standards for calibration. Peptide mass fingerprinting was carried out using MASCOT Matrix Sciences (London, UK) program for protein identification (http://www.matrixscience.com/).

N-terminal sequence

The subunit of 121 kDa was sequenced at Laboratorio Nacional de Investigaciones y Servicios en Péptidos y Proteínas [Federal Laboratory of Research and Services on Peptides and Proteins] (LANAIS-PRO, Universidad de Buenos Aires - Argentina - CONICET, www.uba.ar). Applied Biosystems 477 Protein/Peptide Sequencer was used, interfaced with a HPLC 120 for one-line phenylthiohydantoin amino acid analysis.

Trypsin treatment and SDS-PAGE

The purified subfraction II (25 μg) was incubated with trypsin (sequencing grade, Promega, Madison, WI, USA) in a protein/trypsin ratio of 1:0.015 (25μg/0.375μg) and 1:0.001(25μg/0.025μg) w/w in 50 mM ammonium bicarbonate pH 8 at 37 °C for 15 min. Proteolysis was stopped with ice. Control samples were incubated in the absence of trypsin under the same conditions. Samples were analysed under denaturing conditions (SDS-PAGE) in sample loading buffer according to Laemmli (1970) to prevent the reactivation of trypsin that occurs when using the sample buffer. The staining density was measured vertically for each track using image analysis (Image J, version 1.47). Also, subfraction II was analyzed with and without β-mercaptoethanol (4% final concentration) treatment by SDS-PAGE using a 4-23% polyacrylamide gel (Laemmli, 1970). Electrophoresis was finished after 90 min at room temperature, at a time in which the bromophenol blue band was at the lower end of the gel.

Fatty acid analysis

Fatty acid methyl esters (FAME) of total lipids were prepared from subfraction II with BF₃-MeOH, according to
Morrison and Smith (1992). The analysis was performed on 96.5% of lipids present in the subfraction II since these are hydrolysable lipids (12.7% tryacylglycerols, 13.7% free fatty acids, 4.8% diacylglycerols, 13.5% phosphatidylethanolamine, 40.2% phosphatidylcholine, 11.6% other phospholipids). The remaining fraction (3.5%) was cholesterol (Cunningham et al. 1996).

Gas-liquid chromatography (GLC-FID) was performed using an HP-6890 capillary GLC (Hewlett Packard, Palo Alto, CA) fitted with an Omegawax 250 fused silica column, 30 m x 0.25 mm, with 0.25 μm phase (Supelco, Bellefonte, CA). Peaks were identified by comparing the retention times with those from a mixture of standard methyl esters.

Results

Subfraction II of VHDL hemolymphatic fraction was isolated and purified as reported by Cunningham et al. (1996). Figure 2 shows the separation of the VHDL fraction by ultracentrifugation in density gradient and its purification through a molecular exclusion column using FPLC, and the purity of subfraction II was confirmed by electrophoresis, as previously reported (Cunningham et al. 1996). Native-PAGE showed a single protein of 420 kDa in agreement with previous reports (Cunningham et al. 1996; 1999).

The emission fluorescence spectrum obtained by excitation at 280 nm displayed a maximum at 336 nm (Fig. 3A), suggesting that tryptophan residues would be located in a hydrophobic environment (Lakowicz, 1999). The secondary structure of subfraction II was investigated by CD. From the far UV CD spectrum (Fig. 3B) and the spectral deconvolution with the program Varselec, it was estimated that the subfraction II contained 20% α-helix, 29% β-sheet, 22.7% turns and 29.7% unordered structure.

A peptide mass fingerprinting of the 67, 105, 121 kDa subunits was obtained by trypsin cleavage and MALDI-TOF MS analysis (Figs. 4 and 5). The matching correlation was significant at p< 0.05 (test based in Mowse Score, ions score is -10*Log(P)) for 67 kDa and 105 kDa subunits. The search for matching peptides identified that 10 peptides present in the subunit of 67 kDa were identical to subunit 3 of the spider Cupiennius salei (Ctenidae) hemocyanin (21% sequence) (CAC44751) (Fig. 4). Concerning the 105 kDa subunit, 19 peptides were identical to a protein from the insect Anopheles gambiae (Culicidae) (20% sequence) (XP311738.5) (Fig. 5). As the 121 kDa subunit did not show sequence identity to any reported peptide, the N-terminal amino acid sequence was determined (Fig. 6).

Subfraction II was subjected to SDS-PAGE under oxidizing and reducing conditions to determine the nature of the interactions that hold together protein subunits. The molecular weight of the three protein bands was equal under both conditions, indicating the absence of intermolecular disulfide bridges (Fig. 7).
The est quantity of associated lipids was found in subfraction II. Fraction, using a molecular exclusion column, and the great-

Also, the susceptibility to trypsin of the three protein bands was assayed to estimate the relative exposure of these sub-

Discussion

Lipoproteins in spiders are only known in: E. californicum (Theraphosidae) (a nomen dubium that probably corre-

Works in other arthropods (insects and crustaceans) have shown 34% to 36% α-helices, 35% to 47% β-sheets, and 15% to 31% of random coil for different subspecies of HDLs of the insect Manduca sexta (Lepidoptera, Sphingidae) (Ryan et al. 1992); and 35% α-helices, 16% β-sheets, 20% turns for lipoproteins of the decapod crustacean Macrourbachium borellii (Palaeomonidae) (Garcia et al. 2006). A slightly higher content of β-sheet than that of α-helices components, 29% and 21.6% respectively; and 22.7% turns is reported here for P. pythagoricus.

Comparison with hemocyanins from different arachnids is hampered by the fact that P. pythagoricus hemocyanin is a component of a 420 kDa hemolymphatic protein. However, the maximum fluorescence emission of this large protein was 336 nm, i.e. similar to that reported for crustaceans lipoproteins (334 nm), and as discussed by Garcia et al. (2006) the fluorescence properties of proteins are a convenient tool for studying their binding to different hemolymphatic lipids. However, more studies are needed to further clarify these aspects.

Subfraction II from P. pythagoricus was also analyzed by SDS-PAGE under oxidizing and reducing conditions. The study showed no differences between the electrophoretic bands (67, 105 and 121 kDa) profiles obtained under both conditions, indicating the absence of intermolecular disulfide bridges in them. The higher susceptibility of greater molecular weight proteins to trypsinolysis suggests that these subunits are more exposed in the aqueous medium and, therefore, to trypsin. It is important to point out the only existing difference between subfraction II of hemolymphatic VHDL fraction and HDL-2 of P. pythagoricus is the small amount of 105 and 121 kDa proteins present in HDL-2 (Cunningham et al. 1996). This difference may explain the fact that these protein fractions are not involved in lipid dy-

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abundant 115 kDa hemolymphatic polypeptide in the spider *Brachypelma albopilosa* (Valerio 1980) (Theraphosidae), which they annotated as a possible apolipoprotein.

Characterization of fatty acids in hemolymphatic lipoproteins of arachnids seems restricted to two species, *E. californicum* and *G. rosea*. In both species the majority unsaturated fatty acids extracted from total lipids of very high density proteins were 18:1 and 18:2, which represent 52% in *E. californicum* and 43% in *G. rosea* (Stratakis et al. 1993; Laino et al. 2015). Whereas those analysed in the subfraction II of VHDL of *P. pythagoricus* constitute 29%. With respect to majority saturated fatty acids (16:0 y 18:0) constitute 39 and 37% in *E. californicum* and in *G. rosea* respectively, being represented in our model of study by a greater percentage (54%). In the present work it was observed a greater percentage of saturated fatty acids in *P. pythagoricus* compared to the other two species, this difference may be linked to numerous variables that may affect the composition of fatty acids, for instance the diet as described by Schartau and Leidescher (1983). New works on the characterization of lipids and fatty acids are necessary to arise some speculations on the subject.

Results herein reported extend our understanding of the structure of subfraction II, which contains hemocyanin and is responsible for a major part of lipid transport in *P. pythagoricus*.

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