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

Aldana LAINO, C. Fernando GARCIA and Mónica CUNNINGHAM

Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), CCT- La Plata CONICET-UNLP, 60 y 120 (1900) La Plata, Argentina.

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ABSTRACT: VHDL fraction contains hemocyanin as its major apoprotein and transports most of the circulating lipids in the spider *Polybetes 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; Soulages 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 *Polybetes 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

*Address correspondence to: Mónica Cunningham
cunninghammoni@gmail.com
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FIGURE 1. An adult female specimen of *Polybetes pythagoricus* (Araneae, Sparassidae).

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 % lípids (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

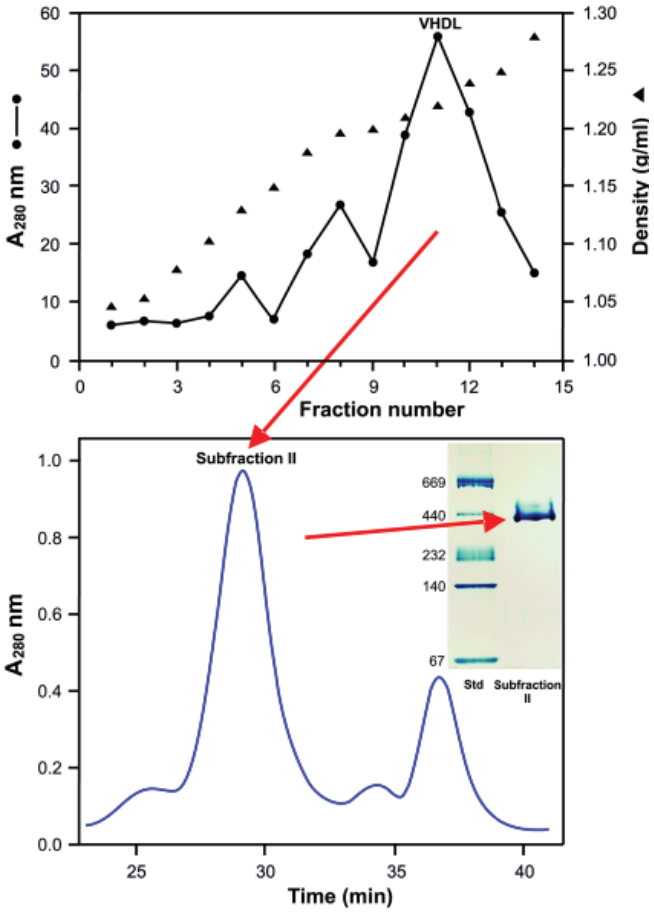


FIGURE 2. Protein distribution (absorbance at 280 nm) and density distribution in hemolymphatic fractions of *P. pythagoricus*. Hemolymph was centrifuged in a NaBr gradient and fractioned **(A)**. Elution profile from FPLC of the protein fractions isolated from the VHDL fraction obtained by density gradient ultracentrifugation **(B)**. Inset: Native-PAGE profile of the subfraction II isolated from FPLC (taken from Laino *et al.*, 2015).

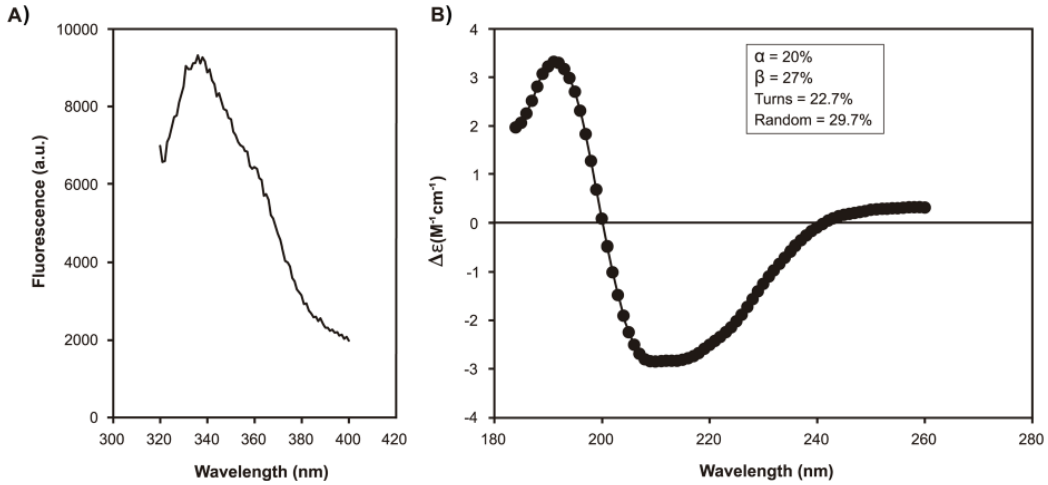


FIGURE 3. Intrinsic fluorescence emission spectrum (A) and Far-UV circular dichroism spectrum of subfraction II of VHDL fraction from *P. pythagoricus* (B).

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 $CaCl_2$ 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).

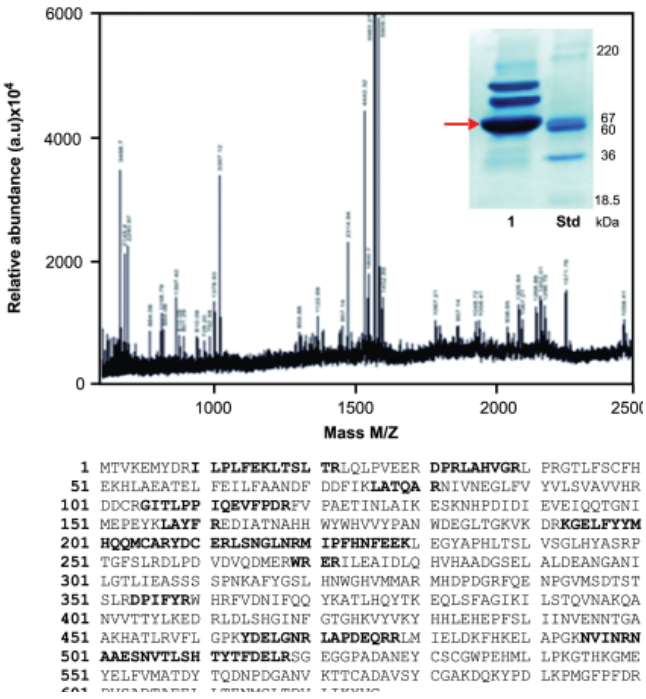
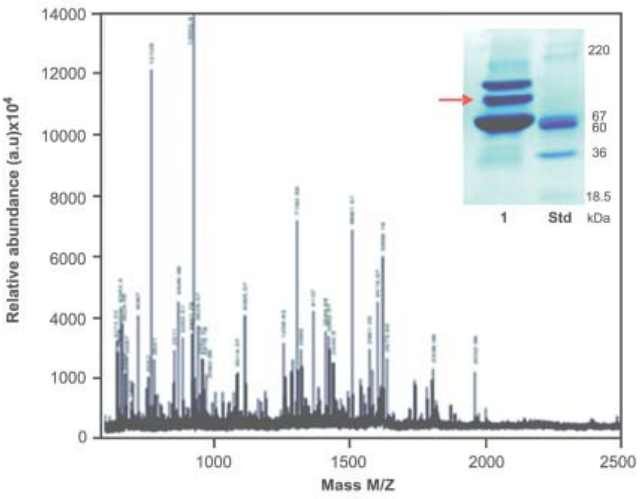


FIGURE 4. MALDI-TOF MS analysis of 67 kDa subunit of Amino acid sequence of subunit 3 in the spider *Cupiennius salei* (CAC44751). The matched peptides from *P. pythagoricus* are in bold letter. Inset: PAGE-SDS (4-23% acrylamide gradient slab). Lane 1: subfraction II. Lane 2: MW standards. Arrows indicate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



1 ISRKKETIGE EVMRLRQRLE QSNEMNGRLN RSLEELVKES EEK**TVTIEGH**
51 **EKELQR**LQEQ LASLRSEKES LEAVLFDTNT SLEEAEAKKD ALER**ENQDLL**
101 **IKQESHK**ALI ARLNKDLENA ERRAQDIK**IQ LTNAAANQEA EFLQKLSNLR**
151 TFSEENIKKL NDEKEQIR**QS LEKR**MQQSLQ ALESAKDAEI RQLREQFETL
201 QLHLDALNQQ HEEVILRAEN EKQQALLIAH RDKQAVIEKL EVTARELKNE
251 LENGDRLKRE MAARQEK**DRT TIGCLR**DEVT KMRTKAEEAR IR**AEEEMNRL**
301 **EVVAGSLR**EE KETLLKDAEE LKVQLRLSED RCDALNHQLQ DTHRKLKEAE
351 NSTDATRKDL TDTRRTLADS NIER**DKYANS NKE**LRDHVKR VEEQRRQQAR
401 TIEDALAKIS SLEETRNGLE QDKVRLQTIL KETETNVTKL NQELSNAQTD
451 IQKFQTDTTQ KDASEKELQA RLANEGEEKE RVLAELHQIK **KQMADLEGTL**
501 **CATR**QELGRA RCKANQDEHR FHQREQELCA RNEEGRGREK RLEDQKHNLE
551 VCLADATQQI QELKARLGGA EGRVRALDEQ LVQLECQKKE VENKLSSIGH
601 TLR**RIAGIQM DGSVSLPYRL** MSPSRR**YSPA RGGTGGR**SEG VCPNVLIDVD
651 PEMVRKGVRS LMQQIAHIER **ERDDFKVQLC TAR**K**QLQEGS DAQLRLETK**I
701 NKLQQHVRAV HEDKTNLEAR LAQKTSALQS TEETLRQKSD ELTALREKAT
751 QLEQSLTSTS EER**THLEERL DK**CR**QSGARL ESDKRHLQDE LARTEARATK**
801 LDLQR**VALEG DIQRLQMAMQ EK**DCTVRNQQ ERLENQQR**SL TQLEDRCVAL**
851 **KSTVDQLKE**R LQAAAITETE LRGEISGLQR HNADQSHTFA LGQDK**LKQLQ**
901 **K**TLSGSENER RVLAER**LDAA QHTINELR**RN QQASQDSTTR LQEQLAECEV
951 QKSTLESQLR LAKWNQETSE QLAATGGNLG GGSGGAGGNT SGDQELTR**QL**
1001 **ISSQR**ERNEL RNKVEALNEK IRTLERDQQR PAGKYDRSEK KSGEYDSNRI
1051 ESTTYSCGLD HAQLEQEGRE LRMKVRRLET LLAEKEAELA RSRAKLLESP
1101 SKASSLGGGS VGADVERYRS AQVQAERLLD AREQSHRQQV TRLENQIAML
1151 REQLAQEAKR RQQYILRSSR AGREMQQLRQ TIGDSLRNVA QDPLDTSLLE
1201 SEARRLDSAV SMSLPPTTSA TGRGRG

FIGURE 5. MALDI-TOF MS analysis of subunit of 105 kDa. Amino acids sequence of a protein in *Anopheles gambiae* (XP311738.5). 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 477ª 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 BF_3-MeOH, according to

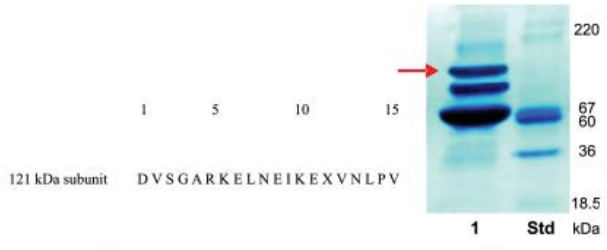


FIGURE 6. Sequence of N-terminal amino acids of subunit of 121 kDa. The X means that amino acids were not found to be licensed to that position. Inset: PAGE-SDS (4-23% acrylamide gradient slab). Lane 1: subfraction II. Lane 2: MW standards

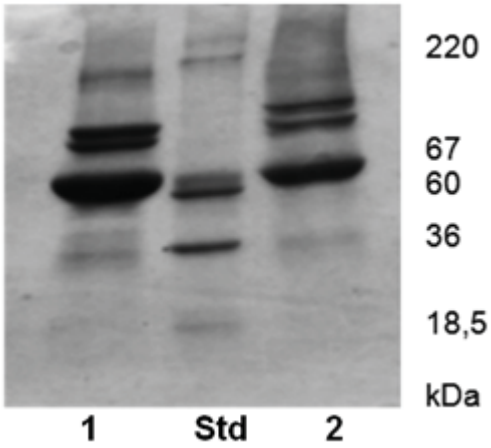


FIGURE 7. SDS-PAGE analysis (4-23% acrylamide gradient slab) of VHDL subfraction II from *P. pythagoricus* (25 µg). Lane 1: Subfraction II with β-mercaptoethanol (4% final concentration). Lane 2: Subfraction II without β-mercaptoethanol.

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).

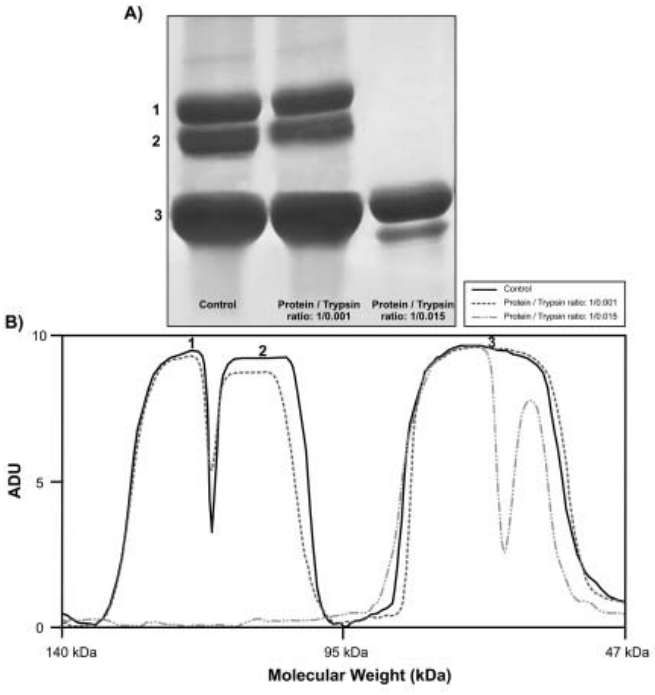


FIGURE 8. Effect of trypsin on the electrophoretic pattern of VHDL subfraction II from *P. pythagoricus* apolipoproteins. We incubated subfraction II samples with different ratio of trypsin for 15 min at 37°C. After incubation we added sample treatment buffer and placed the samples in a boiling water bath for 5 min. We then electrophoresed the samples on a 4-23% acrylamide gradient SDS slab gel (A). Densitometry profiles for the same samples. Solid line, subfraction II control; dotted line, subfraction II incubated with 0.025 µg of trypsin and dot/dash line, subfraction II incubated with 0.375 µg of trypsin (B).

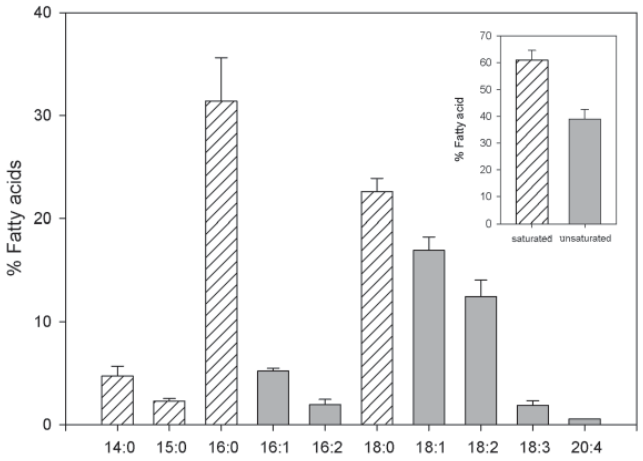


FIGURE 9. Fatty acid composition of VHDL subfraction II of *P. pythagoricus.* We expressed the data as weight percentage of the different fatty acid classes quantified by GLC. Values are mean ± SD of three independent analyses. Inset: % saturated and unsaturated fatty acids.

Also, the susceptibility to trypsin of the three protein bands was assayed to estimate the relative exposure of these subunits to the aqueous medium. SDS-PAGE and densitometry analysis of proteins after digestion showed extensive degradation of 105 and 121 kDa bands, but not of the 67 kDa band (Fig. 8).

GLC chromatography of subfraction II showed that 16:0 and 18:0 (31 and 22%) were the predominant saturated fatty acids present, whereas 14:0 and 15:0 were found in a lesser proportion (less than 6%). The major unsaturated acids were 18:1 and 18:2 (17% and 12%, respectively); as a whole, polyunsaturated acids represented less than 6%. When the proportion between saturated and unsaturated fatty acids was computed, it was found that the saturated ones represented 60 % of the total (Fig. 9).

Discussion

Lipoproteins in spiders are only known in: *E. californicum* (Theraphosidae) (a nomen dubium that probably corresponds to *Aphonopelma hentzi* (Girard 1852), following Nentwig, 2012), *L. mirabilis* (Theridiidae), *P. pythagoricus* (Sparassidae) (Cunningham *et al.* 2007) and G. rosea (Theraphosidae) (Laino *et al.* 2015). In three of them, hemocyanin is associated to hemolymphatic lipids, thus forming high density lipoproteins (HDL) in *L. mirabilis*, of high (HDL) and very high density (VHDL) in *P. pythagoricus* and very high density in G. rosea. In *P. pythagoricus*, three subfractions were purified from the VHDL hemolymphatic fraction, using a molecular exclusion column, and the greatest quantity of associated lipids was found in subfraction II.

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 *Macrobrachium borellii* (Palaemonidae) (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 dynamics in this species (Laino *et al.* 2009).

MALDI-TOF MS analysis of the 67 kDa subunit showed that it was identical to subunit 3 of hemocyanin in the spider *Cupiennius salei* (Ballweber *et al.* 2002) indicating the presence of hemocyanin in subfraction II, which agrees with a maximum absorption of copper in this subfraction (Cunningham and Pollero, 1996). On its part, MALDI-TOF MS analysis of 105 kDa subunit from *P. pythagoricus*, a sequence identity was found with a protein from the insect *Anopheles gambiae* (Culicidae). The fact that any other protein identity was not observed among arachnids may possibly be related to the scarce information available. Indeed, Trabalon *et al.* (2010) mention that only 9,499 entries are found in the NCBInr database for the whole order Araneae, which groups more than 42,000 species. This paucity of data may also explain that MALDI-TOF MS analysis of the 121 kDa subunit was not identical to any other peptide in the data base. However, Trabalon *et al.* (2010) found an also

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 speculation 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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