Pergamon
0305-0491(94)00069-7

Comp. Biochem. Physiol. Vol. 109B, No. 2/3, pp. 333–338, 1994

0305-0491/94 $7.00 + 0.00

Lipid circulation in spiders. Transport of phospholipids, free acids and triacylglycerols as the major lipid classes by a high-density lipoprotein fraction isolated from plasma of *Polybetes pythagoricus*

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A high-density lipoprotein (HDL) fraction was isolated from the hemolymphatic plasma of the spider *Polybetes pythagoricus* by density-gradient ultracentrifugation. Hydrated density (1.13 g/ml), electrophoretic mobility (SDS—PAGE) of apoproteins and lipid classes composition were determined. Lipids were identified by HP–TLC and auxiliary techniques; they were quantified by TLC–FID. The protein moiety is composed of two main apoproteins (250 and 76 kDa, respectively) and several polypeptides of low molecular weight. It resembles the apoliphorins of insects and some other arachnids. The lipid composition differs from most lipophorins. Phospholipids amount to more than 60% of total lipids, while diacylglycerols (2.4%) are supplanted by triacylglycerols (16.5%) as the main circulating energetic lipids.

Key words: Lipids; Transport; Spiders; Hemolymph; Lipoproteins; Lipophorin; Phospholipids; Triacylglycerols; Fatty acids; Arachnida.

Comp. Biochem. Physiol. 109B, 333–338, 1994.

Introduction

Lipid circulation systems in invertebrates have been studied only in those belonging to phyla Arthropoda and Mollusca. The mechanisms of lipid circulation are well-known in many arthropods; they are transported by means of high- (HDL) or very high-density lipoproteins (VHDL) in insects (Beenakkers *et al.*, 1985) or only by HDLs in crustaceans (Lee, 1989). Plasma lipid circulation in molluscs is comparatively less known, although it has been studied in our laboratory, firstly in bivalves (Pollero, 1987), then in cephalopods (Heras and Pollero, 1990), and recently in gastropods (Pollero *et al.*, 1992).

Regarding arachnids, there is little available information dealing with plasma lipoproteins. Lipoprotein fractions of high density have been detected in the hemolymph of four species belonging to the orders Araneida, Scorpionida, Solpugida and Acarina; according to their apoprotein components, in the first three ones they show characteristics similar to those of insect lipophorins (Haunerland and Bowers, 1989). The lipid composition of these lipoproteins was determined in only one

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Received 31 January 1994; accepted 9 May 1994.

species, *Eurypelma californicum* which, due to its high content of diacylglycerols and phospholipids, also showed similarity to that of insect lipophorins (Haunerland and Bowers, 1987).

In the present work, a high-density lipoprotein fraction was isolated from hemolymphatic plasma of the spider *Polybetes pythagoricus*. Although it presents some characteristics similar to the already-mentioned lipophorins, its lipid composition is markedly different.

Materials and Methods

Hemolymph collection and preparative ultracentrifugal fractionation

Wild specimens of *P. pythagoricus* were caught from barks of eucalyptus trees and bled on the same day. After severing of their legs, the spiders were placed in a tube and centrifuged at low speed to obtain hemolymph. The lipoprotein under study was isolated by density gradient ultracentrifugation. Aliquots of clear blue plasma were overlayered on 3 ml NaBr solution (density 1.21 g/ml) and centrifuged at 178,000 *g* at 10°C for 22 hr in a Beckman L8 70 M centrifuge with a SW 60 Ti rotor. As we assumed that the density of spider plasma was 1.006 g/ml, a saline solution of this density was run simultaneously as blank. The total volume of the tubes was fractionated from top to bottom into 0.3 ml fractions. A yellow band in the high-density zone of the gradient was separated as a whole fraction. The presence of proteins in each fraction was monitored by light absorption at 280 nm.

Lipid analysis

Total lipids of the yellow band were extracted with chloroform/methanol according to the Bligh and Dyer method (1959). Lipids were separated on Merck high-performance thin-layer chromatography (HP–TLC) plates. Neutral lipids were resolved by developing the plates in hexane–diethyl ether–acetic acid (80:20:1.5, v/v). Polar lipids were resolved by the development of plates in chloroform–methanol–acetic acid–water (65:25:4:4, v/v). Hydrocarbons were separated from other neutral lipids by development in hexane–benzene (70:30, v/v). Reference lipids used as standards on HP–TLC plates were 1–2 and 1–3 diacylglycerols, cholesterol, tristearin, stearic acid, cholesteryl oleate, a mixture of hydrocarbons, choline and ethanolamine phosphoglycerides. For detection of separated constituents, the plates were exposed to iodine vapors or sprayed with sulfochromic acid solution and heated in an oven at 180°C. Acetic–sulfuric acid (1:1, v/v) and ethanolic ninhydrin were used as selective spray reagents to facilitate the lipid identification.

The quantitative determination of the lipid classes was performed by TLC coupled to a flame ionization detection (FID) in an Iatroscan apparatus model TH 10, after their separation on chromarods type S-III, using a triple development: hexane–benzene (70:30, v/v), benzene–chloroform–formic acid (70:25:2, v/v) and chloroform–methanol–water (70:25:3, v/v). Lipid classes were quantified by comparison with known amounts of standards run under the same conditions and using monoacylglycerol as internal standard (Ackman, 1990). Total lipids were calculated by the summation of individual lipid weights. The signals from the FID were registered on a Hewlett–Packard model HP-3396 A integrator.

Apoprotein analysis

The fraction isolated from the density gradient (yellow band) was extensively dialyzed against 10 mM Tris–HCl (pH 6.8) and analyzed by electrophoresis under dissociating conditions. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were carried out in both continuous 8% slab gels overlayered with 4% stacking gels and 4–20% gels, in a mini-slab electrophoresis unit (8 × 10 cm). The resolving gel buffer was 0.375 M Tris–HCl and the stacking gel buffer was 0.125 M Tris–HCl. The electrode buffer contained Tris–glycine 0.025 M Tris, 0.192 M glycine (pH 8.3). Proteins were visualized by staining with Coomassie Brilliant Blue. Molecular weight standards (HMW, Pharmacia, Uppsala, Sweden, and Marker kit, Sigma, St Louis, MO) were run in parallel lines.

Results

When total plasma of *P. pythagoricus* was centrifuged in a density gradient, two well-defined colored bands appeared: a yellow one, with clearly marked borders in the middle of the tube (Fig. 1), and another one, which was blue and wide, at the tube bottom. Absorbance measurements at 280 nm in each fraction along the total gradient showed a protein profile with two maxima: a small peak coincident with the yellow fraction, and a large protein peak coincident with the blue fraction. Plasma fractions between bands showed a low protein concentration. Absorption scanning at 200–600 nm recorded for each fraction showed the characteristic spectrum of hemocyanin only in those fractions corresponding to the blue band (results not shown). Since the yellow band had a density of 1.13 g/ml, it could be considered a high-density lipoprotein (HDL) fraction.

Aliquots of HDL plasma fraction, isolated by preparative ultracentrifugation, were utilized for the lipid composition analysis. After total lipid separation by HP–TLC, the following classes of lipids were identified: phosphatides of choline (PC) and ethanolamine (PE), diacylglycerols (DG), free fatty acids (FFA), triacylglycerols (TG) and hydrocarbons (HC). Three phospholipids with a lower R_f than PC, containing free amino groups (visualized with ninhydrin), were partially identified. Figure 2 shows a typical chromatogram of the HDL fraction from *P. pythagoricus* compared to that of an insect lipophorin (HDL) isolated from *Triatoma infestans*. Differences were observed in the relative contents of TG, DG, PC and PE among the HDLs from both sources.

The quantitative lipid composition, determined by TLC–FID, is shown in Table 1. Phospholipids with PC predominance represented more than half of the total lipids;

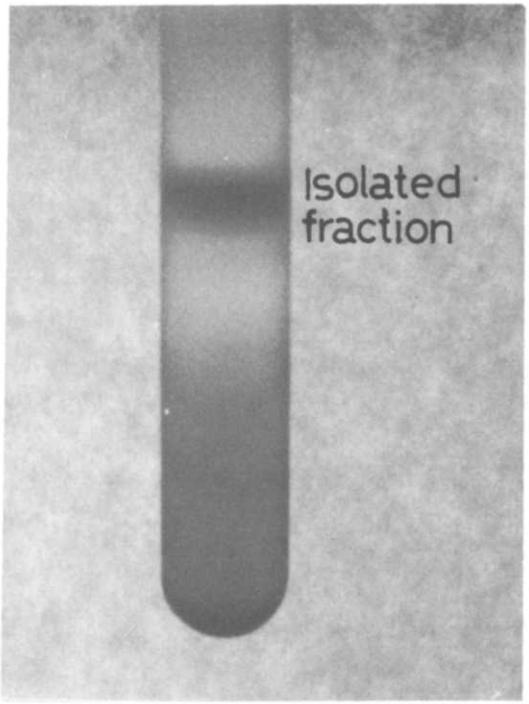

Fig. 1. Density gradient ultracentrifugation of *P. pythagoricus* hemolymphatic plasma on NaBr δ 1.21 g/ml.

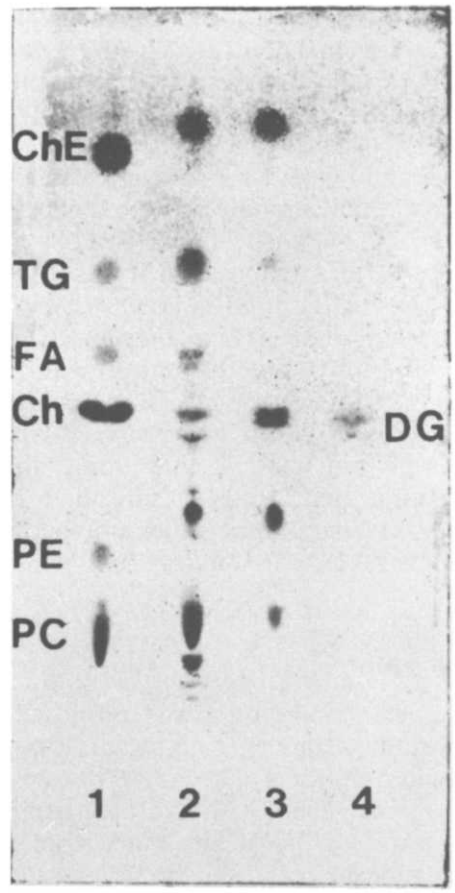

Fig. 2. Thin-layer chromatographic separation of neutral and polar lipids of the HDL from *P. pythagoricus* plasma. Separated constituents were detected by charring with sulfochromic acid solution. See solvent systems and conditions in the text. Lanes 1 and 4: standards; Lane 2: HDL from *P. pythagoricus*; Lane 3: lipophorin (HDL) from *Triatoma infestans*.

FFA and TG were also important lipid fractions, whereas DG and HC were found in small proportions. Similar proportions of phospholipids (66.0%), FFA (21.5%), TG (8.7%), DG (2.8%) and HC (1.0%) were also found in fresh whole plasma which was analyzed immediately after collection. Table 1 also includes the total protein value in the HDL fraction, which exceeds, in mass, that of the lipid moiety.

Other aliquots of *P. pythagoricus* HDL were used to study the constituent apoproteins. The electropherogram in Fig. 3, obtained in a continuous polyacrylamide gel under dissociating conditions, shows two main protein bands of 250 and 76 kDa, respectively, and some other polypeptides of minor molecular weight.

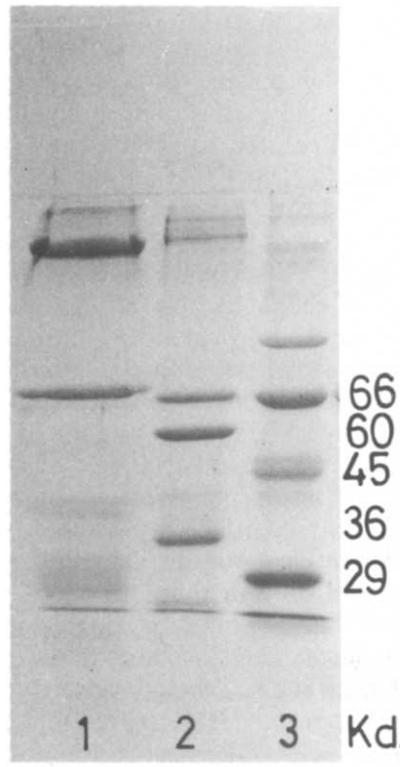

Fig. 3. SDS–polyacrylamide gel electrophoresis of apoproteins from the HDL of *P. pythagoricus*. Lane 1: sample; lanes 2 and 3: molecular weight standards.

Discussion

The term lipophorin was proposed by Chino and colleagues (Chino *et al.*, 1981) to designate the insect diacylglycerol-carrying lipoproteins which, with hydrated densities comparable to HDL in man, transport lipids from sites of absorption or storage to sites of utilization. Also characteristic of lipophorins is the presence of two apoproteins of 250–280 and 78–85 kDa, respectively (Chapman, 1983). On this basis, the extension of the term "lipophorin" to designate the two HDLs of tarantula, the only spider lipoproteins in which both lipid and protein moieties have been characterized in detail up to now (Haunerland and Bowers, 1987), seems appropriate since they contain diacylglycerols as the major lipids and both characteristic apoproteins. Although lipophorins have several unifying structural restrictions that govern the molecular organization (Soulages and Brenner, 1991), the lipid composition and the protein:lipid ratios differ among species and developmental stages. Therefore, DG predominance is not a strict condition of lipophorins, since they are of minor significance in *Periplaneta americana* and scanty in the beetle *Leptinotarsa decemlineata* (Katagiri and de Kort, 1991); the protein proportion is higher in the lipophorin of the wax-moth *Galleria mellonella* (Thomas, 1979) than in other insect species. Within this scope, the HDL isolated from *P. pythagoricus* in this work, could be also considered as a "lipophorin".

The two main apoproteins of *P. pythagoricus* HDL show an electrophoretic behavior similar to those of insect apolipophorins, as well as to arachnid apolipoproteins, with the exception of

Table 1. Composition of HDL isolated from the hemolymph plasma of *Polybetes pythagoricus*

Component	Percent w/w
Lipid classes	
Hydrocarbons	2.4 ± 0.7
Triacylglycerols	16.5 ± 3.7
Diacylglycerols	2.4 ± 0.3
Free fatty acids	17.4 ± 0.9
Phosphatidyl etanolamine	15.2 ± 0.6
Phosphatidyl choline	29.9 ± 4.1
Other phospholipids	16.2 ± 1.4
Total lipids (0.89 mg/ml hemolymph)	27.7%
Total proteins (2.32 mg/ml hemolymph)	72.3%
Hydrated density (g/ml)	1.13

The HDL was isolated by ultracentrifugation in density gradient. Lipids were identified after separation by HP-TLC and quantified by TLC–FID. Proteins were measured by colorimetry.
Results are the average of three determinations (45 animals) ±SD.

velvet mite HDL (Haunerland and Bowers, 1989). In some insects, a third apolipoprotein, (ApoLp III), with a low molecular weight (17 kDa) has been found in the adult lipophorins (Kawooya *et al.*, 1984; Chino and Yazawa, 1986) which, in association with the main apoproteins, increases the lipophorin lipid-carrying capacity. It is speculated that low molecular weight polypeptides accompanying the major apoproteins in *P. pythagoricus* HDL, could play a similar role. Nevertheless, since no protease inhibitors were used during the assays, we cannot discard the possibility that these small polypeptides have a proteolytic origin.

Despite the above considerations, this high-density fraction isolated from plasma of *P. pythagoricus* presents a quite different lipid composition to those of most lipophorins. Due to its high proportion of phospholipids, it is more closely related to the crustacean HDLs rather than to the lipophorins of insects in general and tarantula. On the other hand, the small amount of DG and the relatively high content of TG suggest a substitution of DG by TG as circulating energetic lipids and, consequently, that the DG are not a direct energy source in this spider. In the Colorado potato beetle lipophorin, the DG are also low but they are substituted by hydrocarbons as the main neutral lipids (Katagiri and de Kort, 1991). In contrast, the hydrocarbon content in *P. pythagoricus* HDL is low and the neutral lipids represent, all together, only the fourth part of total lipids. As a consequence, we can suppose that this lipoprotein particle has special structural characteristics. The fact that two genera of spiders within the same order, such as *Eurypelma* and *Polybetes* show lipid compositions markedly different in their HDLs, makes difficult any generalization regarding the lipid transport, the lipid requirements in arachnid tissues and the evolutive process of lipoproteins.

In conclusion, results drawn from the present study are coincident with those obtained by Haunerland and Bowers (1987, 1989) in relation to the similarity existing between *P. pythagoricus* Apo-HDL and apolipophorins of insects and almost all the arachnids. However, differences concerning quality and quantity of lipids, which are transported in this plasma fraction, are also shown. Similar apoprotein compositions and notoriously different lipid compositions lead us to think that the apoproteins do not necessarily determine the associated lipids in the lipoprotein particle. In view of such a composition of the *P. pythagoricus* HDL, it would be interesting to perform some physical studies on the structural organization of this spider lipoprotein.

Acknowledgements—This research was supported by grants from CONICET, Argentina and Efamol Research Institute, Canada. M.C. is Fellow of the CIC BA; R.P. and A.G. are Members of Carrera del Investigador Cientifico of the CIC BA and CONICET, respectively.

References

Ackman R. G. (1990) An overview of analyses by Chromarods-Iatroscan TLC-FID. *J. Planar Chromat.* **3**, 450–490.

Beenakkers A. M., Van der Horst D. J. and Van Marrewijk W. J. A. (1985) Insect lipids and lipoproteins, and their role in physiological processes. *Prog. Lipid Res.* **24**, 19–67.

Bligh E. G. and Dyer W. J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917.

Chapman M. J. (1983) A broad perspective on invertebrate and lower vertebrate apolipoproteins: crustaceans, insects, fish, amphibians, and reptiles. In *CRC Handbook of Electrophoresis* (Edited by Lewis L. A. and Naito H. K), Vol. IV, pp. 285–310. CRC Press, Boca Raton, FL.

Chino H. and Yasawa M. (1986) Apolipophorin III in locust: purification and characterization. *J. Lipid Res.* **27**, 377–385.

Chino H., Downer R. G. H., Wyatt G. R. and Gibert L. I. (1981) Lipophorins, a major class of lipoproteins of insect hemolymph. *Insect Biochem.* **11**, 491.

Haunerland N. H. and Bowers W. S. (1987) Lipoprotein from the tarantula, *Eurypelma californicum*. *Comp. Biochem. Physiol.* **86B**, 571–574.

Haunerland N. H. and Bowers W. S. (1989) Comparative studies on arthropod lipoproteins. *Comp. Biochem. Physiol.* **92B**, 137–141.

Heras H. and Pollero R. J. (1990) Occurrence of plasma lipoproteins in octopods. Partial characterization and interorgan transport of lipids. *J. exp. mar. Biol. Ecol.* **140**, 29–38.

Kawooya J. K., Keim P. S., Ryan R. O., Shapiro J. P., Samaraweera P. and Law J. H. (1984) Insect apolipophorin III: purification and properties. *J. biol. Chem.* **259**, 10733–10737.

Katagiri C. and de Kort S. (1991) Characterization of Colorado potato beetle lipophorin: a hydrocarbon-rich diacylglycerol-poor lipophorin. *Comp. Biochem. Physiol.* **100B**, 149–152.

Lee R. F. (1989) Lipoproteins from the hemolymph

and ovaries of marine invertebrates. In *Advances in Comparative and Environmental Physiology* (Edited by Gilles *et al.*), Vol. 7, pp. 187–207.

Pollero R. J. (1987) Transport of cholesterol in the hemolymph of the mollusc *Diplodon delodontus*. *Comp. Biochem. Physiol.* **88A,** 577–580.

Pollero R. J., González Baró M. R. and Garin C. (1992) Lipid transport in snails. Partial characterization of a high-density lipoprotein isolated from *Ampullaria canaliculata* plasma. *Comp. Biochem. Physiol.* **102B,** 717–720.

Soulages J. L. and Brenner R. R. (1991) Study on the composition–structure relationship of lipophorins. *J. Lipid Res.* **32,** 407–415.

Thomas K. K. (1979) Isolation and partial characterization of the haemolymph lipoproteins of the wax moth, *Galleria mellonella*. *Insect Biochem.* **9,** 211–219.