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An Apolipophorin III Protein from the Hemolymph of Desert Locust, *Schistocerca gregaria*

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Abstract Apolipophorin III (apoLp-III) from insects and apolipoprotein A-I from humans, are major component of the lipoprotein and share various properties. ApoLp-III is an abundant hemolymph protein. Besides its crucial role in lipid transport, apoLp-III is able to associate with fungal and bacterial membranes and stimulate cellular immune responses. ApoLp-III was isolated and purified from the hemolymph of desert locust *Schistocerca gregaria* by ion-exchange and reversed-phase chromatography. The purity and the molecular weight of apoLp-III were determined at ~19,000 Da by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. According to similarities in the amino terminal sequence, molar mass and retention on reversed-phase analytical HPLC column, this protein is a *Schistocerca gregaria* homologue of *Locusta migratoria* apoLp-III.

Keywords Apolipophorin III · *Locusta migratoria* · *Schistocerca gregaria* · Hemolymph

Abbreviations

kDa	Kilodalton
TFA	Trifluoroacetic acid
PMSF	Phenylmethylsulfonyl fluoride
TPCK	*N*-Tosyl-phenylalanyl chloromethyl ketone
DTT	Dithiothreitol
SGCI	*Schistocerca gregaria* chymotrypsin inhibitor

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MUTMAC	4-Methylumbelliferyl *p*-(NNN-trimethylammonium)cinnamate
MUB	4-Methylubelliferone
AMC	7-Amino-4-methyl-coumarin

Introduction

Investigation of the molecular basis of insect physiology is a rapidly growing field of science, where research is driven not only by scientific interest but also by the hope of finding biologically active compounds and new ways in pest control. Locusts are widely studied mainly because of the agriculture point of view as they can cause extensive and serious damage to crops in many parts of the world. In the last two decades, most of the focus was on the natural pest control agents like phenols, terpenoids, essential oils, alkaloids, polypeptides, xavonoids, and other substances [1, 8, 10, 34].The species most commonly reared in the laboratory are: the lesser migratory grasshopper, *Melanoplus sangumipes*; the desert locust, *Schistocerca gregaria*; and the African migratory locust, *Locusta migratoria*. In a search for protease(s) and protease inhibitor(s) in the hemolymph of the desert locust, *S. gregaria*, we found and isolated two inhibitor peptides [19] and a prominent protein component called apolipophorin III (apoLp-III). Here, we describe the rearing of the desert locust *S. gregaria* in a lab as well as isolation, purification and partial characterization of apoLp-III protein. On the basis of its amino terminal sequence and several molecular properties, apoLp-III from *S. gregaria* is a homologue of a closely related locust species, *L. migratoria*.

ApoLp-III is a small lipid-binding protein [15]. The protein moiety consists of three apolipoproteins: apoLp-I (Mr, ~250,000), apoLp-II (Mr, ~78,000), and apoLp-III′ (Mr, 17,000) [16, 25, 28]. Whereas apolipophorins I and II are integral constituents of lipophorin [26], apoLp-III appears mostly free in the hemolymph, with only a small fraction associated with lipophorin [16]. Lipophorin is the major lipoprotein in the hemolymph of most insects [16] and serves as a reusable shuttle to transport various lipids including diacylglycerol, hydrocarbons, cholesterol, and carotenoids between tissues [4, 7, 13, 14]. ApoLp-III has high functional and structural similarity to the apoE422K [23, 37, 42] which has been successfully adapted for biotechnological applications [2, 9]. Importantly, and unlike other apolipoproteins, apoLp-III remains monomeric in the absence of lipid, even at high concentrations [37].

The apoLp-III molecules of different insects are similar in their amino acid sequence only if they are from closely related species, the homology ranges from 4% to 30% between distantly related and 60% to 70% between closely related species [24, 40], but they all posses the capacity to associate reversibly with lipid surfaces. ApoLp-III also shares a large number of properties with human exchangeable apolipoproteins [18], such as apolipoprotein A-I. In addition, analysis of X-ray crystal structures reveals that apoLp-III is very similar to the 22 kDa region of the N-terminal domain of human Apo-E [35], apoLp-III is known as a stabilizer of lipophorin particles when they are converted from a high density to a low-density form upon diacylglycerol loading [20, 36], a biological function which is in concert with the molecular properties of purified natural and recombinant apoLp-III molecules observed in vitro [21, 39]. This appears to be a temporary function, restricted to periods of elevated low-density lipophorin levels during intensive muscle activity, such as flight. Especially, in the African migratory locust, *L. migratoria*, and the tobacco hornworm, *Manduca sexta*, which use lipids as the major fuel for flight, the peptide adipokinetic hormone is released during flight and causes lipid stored in the fat body to

associate with lipophorin for transport to flight muscles. Several publications, however, suggest that apoLp-III can have functions unrelated to lipid transport. Upregulation of apoLp-III production in the intersegmental muscle and some neuron populations of *M. sexta* have been reported during their programmed death [31–33]. It has also been reported that the apoLp-III of *Galleria mellonella* larvae has hemagglutinating properties [17] and it stimulates the antibacterial activity of hemolymph, indicating also a participation of apoLp-III molecules in the immune response of insects [24, 41].

The present study describes the purification and characterization of *S. gregaria* apoLp-III. Evidence is also presented to indicate that apoLp-III of *S. gregaria* is similar to the N terminus of *L. migratoria*.

Materials and Methods

Instruments

The FPLC apparatus including a Superose 12 gel filtration column was from Pharmacia (Uppsala, Sweden). Reversed-phase chromatography was performed using an ABI HPLC system and an Aquapore OD-300 (220×4.6 mm, 7 μm) column (Perkin-Elmer, USA). A pulsed-liquid-phase protein sequencer 471A was from ABI, Perkin-Elmer. Bruker Biflex MALDI-TOF mass spectrometer was from Bruker-Franzen Analytik, Bremen (Germany). The photometric measurements were performed on a Shimadzu UV 2101PC (Shimadzu, Japan) or a Hewlett Packard 8452 A (USA) diode-array spectrophotometer.

Chemicals

All chemicals used were of analytical or sequencing grade as appropriate. HPLC-grade acetonitrile was obtained from Romil (UK). *N*,*N*-Diisopropylethylamine, trifluoroacetic acid, and all chemicals for the sequencing were obtained from ABI Perkin-Elmer (USA). 4-Vinyl pyridine, 1,2-ethanedithiol, and phenol were from Aldrich (Hungary); guanidine-HCl was from Serva (Germany); β-mercaptoethanol, α-CCA, angiotensin II, ACTH (18–39), MUTMAC, and MUB DTT were from Sigma (Hungary). Bovine chymotrypsin was obtained from Worthington, Ohio, USA.

Rearing and Sampling from Hemolymph

Desert locusts were collected at the Tharparker Desert of Pakistan. The insects were kept in special cages approximately 2×3×3 ft, fitted with light bulbs in order to maintain the temperature between 35 and 40 °C that was necessary for their growth and reproduction. They were fed cabbage leaves sprinkled with glucose and water was supplied once a day in the form of soaked cotton wool pads. The hemolymph was collected from fifth stage larvae via a syringe needle inserted into the abdomen. (Approximately 50 μl of hemolymph can be obtained from one insect.) Usually, about 100 locust of both sexes were used to collect 5 ml of hemolymph and stored at −80 °C.

Purification of the ApoLp-III Homologous Protein

All the purification procedures were carried out at 0–4 °C. As the first step of purification, 1.0 mL of the crude hemolymph was diluted twofold with 1 mM HCl and centrifuged

(15 min, 10,000 rpm) to remove precipitates. The supernatant was fractionated by FPLC, on a Mono Q HR10/10 anion-exchange column (Amersham Pharmacia Biotech) in several runs. Prior to loading the samples, the column was equilibrated with a Tris–HCl buffer, pH 8.0. The elution was with a 0.0–0.5 M linear NaCl gradient (0.017 M/min), and the flow rate was 3.0 ml/min. The protein content of the effluent was monitored at 280 nm. The third peak of fractions were pooled (collected fractions are shown in black bars), dialyzed, and concentrated by lyophilization. The dry material was dissolved in ~300 μL of solvent A (0.1% TFA in water) and subjected to reversed-phase HPLC on an Aquapore OD-300 column (4.6×220 mm, 7 μm, ABI HPLC system, ABI Perkin-Elmer). The column was eluted with a 0–100%, linear gradient of solvent B (80% (*v*/*v*) aqueous acetonitrile, containing 0.1% TFA, 1.67%/min) at a flow rate of 1.0 ml/min. The effluent was monitored at 220 nm for protein content. 1.0 mL fractions were collected and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for the apoLp-III purification. The fractions corresponding to the apoLp-III were pooled (Black bars) and stored in ~76% (*v*/*v*) aqueous acetonitrile, 0.1% TFA solution on 0 °C.

Polyacrylamide Gel Electrophoresis

The purity of the isolated protein was checked and the molecular weight was determined by electrophoresis in 15% polyacrylamide gel, in the presence of SDS. Before loading, the samples were denatured by 5 min boiling in a 1% SDS and 0.2% β-mercaptoethanol containing loading buffer. The gels were stained with Coomassie Brilliant Blue R-250.

Determination of Protein and Enzyme Concentrations

Protein concentration was determined according to Bradford [3]. The active enzyme concentration of bovine chymotrypsin was measured by active site titration with the fluorescent burst titrant, MUTMAC, using MUB for calibration [11].

Effect of DTT on the ApoLp-III

When the effect of reduction was investigated on apoLp-III, 20 nmole reducing agent DTT was added to 300 pmole protein samples in the assay buffer, in 30 μL final volume. After 90 min incubation at room temperature, apoLp-III sample with the reducing agent DTT was analyzed with analytical reversed-phase HPLC on Aquapore OD-300 column as described above. In the control experiment, the same procedure was used with the chymotrypsin.

N-terminal Sequence of ApoLp-III

Protein sample reduced (10 μg of peptide in 50 μl of 0.25 M Tris–HCl, 6 M guanidine-HCl, 1 mM EDTA, pH 8.5, and 1 μl of 10% β-mercaptoethanol, incubated at 37 °C for 2 h) and pyridylethylated (1 μl of 4-vinylpyridine at room temperature for 2 h) were purified by RP-HPLC and sequenced by automated Edman degradation, using a Beckman 890 M instrument (Beckman Instruments). The Beckman Quadrol program 05-22-85 was used and runs were 8–62 cycles. The amino acid sequences of apoLp-III along with that of the previously reported *L. migratoria* peptides are shown in Fig. 5.

Results

The elution profile of *S. gregaria* hemolymph on a Mono Q anion exchanger column is illustrated in Fig. 1. It demonstrates three major peaks, identified as peaks I, II, and III. Each fraction was tested for the presence of apoLp-III on SDS-PAGE using apoLp-III from *L. migratoria* as a reference. The results indicate that apoLp-III appears principally in peak III and in some fractions of peak II, but no apoLp-III is detected in the other fractions (data of SDS-PAGE are not shown).

The fractions which contained apoLp-III of peak III (black bars) were combined, dialyzed overnight against water, lyophilized, and further purified by reversed-phase high-performance liquid chromatography on Aquapore OD-300 analytical column. Samples were dissolved in 0.1% trifluoroacetic acid and eluted with a linear gradient of 0–100% acetonitrile in 0.1% trifluoroacetic acid (30 min, 1 ml/min). The elution profile is illustrated in Fig. 2 that demonstrates five distinct peaks that are designated as apoLp-III-A, apoLp-III-B, apoLp-III-C, apoLp-III-D, and apoLp-III-E, respectively. These five fractions were tested for apoLp-III on SDS-PAGE (data of SDS-PAGE are not shown). The results are shown in Figs. 3a and 4, those clearly reveal that apoLp-III-E peak is homogeneous, contains a single peak, and no other proteins. Fractions containing apoLp-III were pooled (black bars).

Rechromatography of 100 μL sample of the pooled fractions of apo-III-E loaded on the same column (Fig. 3a) or 1.0 μL sample on a reversed-phase capillary column (C-18, 5 μ, 150×0.5 mm, Perkin-Elmer—not shown) detected only one single component indicating a pure and homogenous protein. The analysis of heat-denatured, mercaptoethanol-reduced samples by SDS-PAGE with coomassie staining (Fig. 4) also confirmed the presence of only one protein in this fraction, without contamination. The estimated molecular mass of the protein was 19 kDa.

A 14-amino-acid portion of the amino terminal sequence was determined by Edman degradation, proved to be highly homologous to an apolipoprotein, apoLp-III, from the hemolymph of a closely related locust species, *L. migratoria* (Fig. 5). The sequence identity was 86% (12 out of 14 amino acids were the same) suggesting that the isolated protein was apoLp-III of *S. gregaria*. Although some other molecular properties of the isolated protein also support this conclusion (see "Discussion"), therefore, it is referred to as an "apoLp-III homologous" protein.

The two proteins apoLp-III and bovine chymotrypsin enzyme responded differently to DTT reduction. The retention of chymotrypsin on reversed-phase HPLC column increased (Fig. 3b) while that of the purified apoLp-III homologous protein did not change (Fig. 3a). This

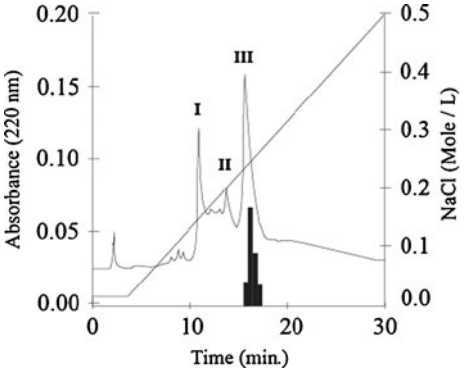

Fig. 1 Elution profiles of ion exchange FPLC. Ion-exchange FPLC of the crude hemolymph. The peak III fractions (*black bars*) were collected and subjected subsequently to reversed-phase HPLC

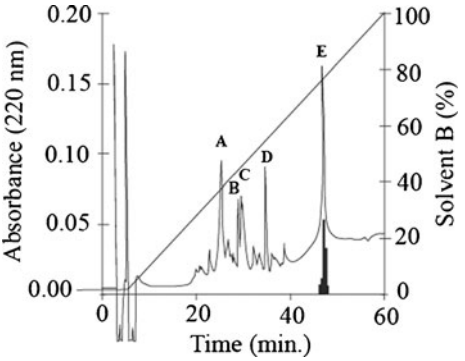

Fig. 2 Elution profiles of reversed-phase HPLC. Reversed-phase HPLC of the protein peak from the ion-exchange FPLC. The peak "E" fractions (*black bars*) were collected and subjected to reversed-phase capillary column

indicated that the molecular structure of the former altered on reduction, but in the case of the latter it remained the same or probably does not have the cysteine bridges.

Discussion

ApoLp-III is an abundant hemolymph protein normally found in an unbound state and can be isolated either from lipophorin or from crude hemolymph. In this paper, the rearing of

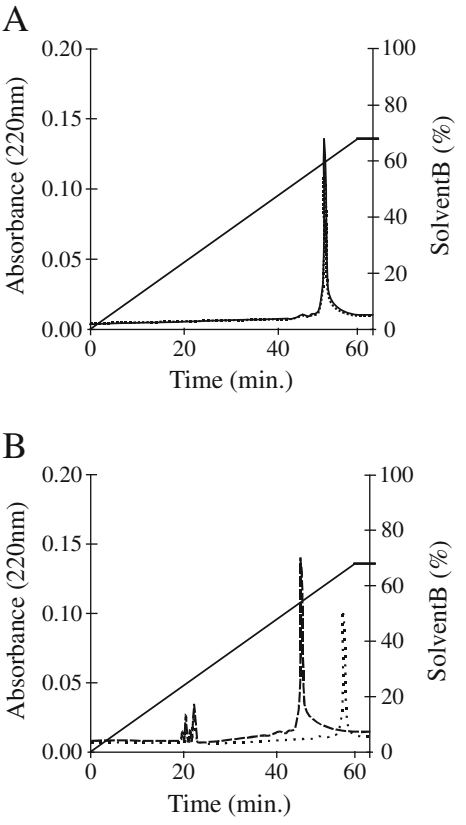

Fig. 3 **a** Checking the purity of the apoLp-III homologous protein and the effect of DTT reduction with analytical reversed-phase HPLC. HPLC profile of the apoLp-III homologous protein before reduction (*solid line*) and after reduction (*broken line*). For the details of DTT reduction see also "Materials and Methods." **b** Checking the effect of DTT reduction with analytical reversed-phase HPLC. HPLC profile of bovine chymotrypsin before reduction (*dashed line*) and after reduction (*broken line*). The conditions of chromatography were the same as in (**a**) and are described in "Materials and Methods." For the details of DTT reduction, see also "Materials and Methods"

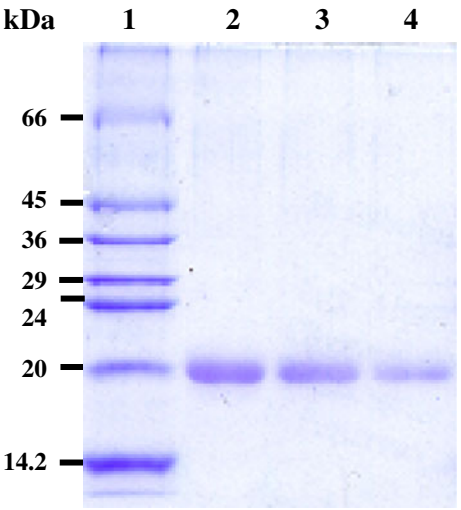

Fig. 4 SDS-PAGE of the purified apoLp-III homologous protein. Checking of purity and molecular mass determination: *lane 1*, molecular weight standard; *lanes 2–4* are 5, 10, and 15 μg loading, respectively, of heat-denatured and β-mercaptoethanol-reduced apoLp-III homologous protein

the locust, isolation of the hemolymph from the locusts, purification, and a brief characterization of a 19-kDa apoLp-III from the desert locust, *S. gregaria*, is described.

Fourteen-amino-acid segment, sequenced from the N terminus of the protein (Fig. 5), exhibits similarity with the N-terminal sequence of an insect lipid transport protein, apoLp-III, particularly with apoLp-III of the closely related locust species, *L. migratoria*.

The isolated protein was found to be homogenous and free of contaminating protein(s) by SDS-PAGE and by capillary and analytical reversed-phase HPLC (Figs. 3a and 4). Additionally, the *S. gregaria* apoLp-III migrated similarly as *L. migratoria* apoLp-III on HPLC and was resistant to reduction of disulfide bridges (Fig. 3a, b). It is based on a number of similarities with the apoLp-III of *L. migratoria*: The amino terminal segment of the *L. migratoria* protein is 86% homologous to the sequenced; 14-amino-acid amino terminal region of our protein and its molecular mass is also 19 kDa (Fig. 5). The retention of *L. migratoria* apoLp-III that was pure and homogenous (a generous gift of Dr. Van der Horst) on reversed-phase HPLC column, observed under slightly different conditions [21, 39] is similar to the retention of the protein that we purified (both elute late, at about 80% acetonitrile), and neither change upon reduction (see "Results" and [21]). The resistance of both the molecular structure (retention time) are consistent with a protein devoid of disulfide bridge(s) (cysteins) in its structure(Fig. 3a, b), which is a well known character of apoLp-III molecules [6, 12, 21, 22, 30, 37–40]. These results strongly support the conclusion that our protein is apoLp-III.

Mammalian blood contains a variety of lipoproteins that serve different lipid transport functions and perform these functions in different ways. In general, insects have a single lipoprotein species, which may serve several different functions and may even change its size, density, and apoprotein complement as it alters its function [4, 27]. This lipoprotein

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                                                1         10   14
                                                |         |    |
ApoLp-III homologous protein from S. gregaria   DA-GQVNIAETVQQL
ApoLp-III of L. migratoria                      DAAGHVNIAEAVQQL
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Fig. 5 The amino terminal sequence of the protein isolated from the hemolymph of *S. gregaria* aligned with the N-terminal sequence of the apoLp-III of *L. migratoria*

has been given the name lipophorin in recognition of its function as a lipid carrier. The use of diacylglycerol to fuel sustained flight is well documented in insects. Lipophorin is known to participate in the transport of diacylglycerol from fat body to flight muscles. Thus, apoLp-I11 may function to stabilize the lipid loaded form of lipophorin or as a recognition signal at muscle cells to identify it as a loaded carrier, or both. Therefore, lipophorin is considered to act as a reutilizable shuttle for lipid transport [4]. It is also well documented that apoLp-III in insects is involved in the molecular pattern recognition, synergistic action with antimicrobial peptides and lysozyme, activation of hemocytes during non-self recognition, and detoxification of bacterial cell wall components. Moreover, it has opsonizing activity and its hemagglutinating as well as anti-oxidant properties were also described [5, 17, 29, 37].

In conclusion, the present study describes an isolation and purification of apoLp-III and provides a molecular basis for the physiological observations carried out by others. This knowledge may facilitate the future studies of the function of *S. gregaria* apoLp-III.

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