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Different forms of apolipophorin III in *Galleria mellonella* larvae challenged with bacteria and fungi

Agnieszka Zdybicka-Barabas*, Aneta Sowa-Jasiłek, Sylwia Stączek, Teresa Jakubowicz, Małgorzata Cytryńska

Department of Immunobiology, Institute of Biology and Biochemistry, Faculty of Biology and Biotechnology, Maria Curie-Skłodowska University, Akademicka St. 19, 20-033 Lublin, Poland

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ABSTRACT

Apolipophorin III (apoLp-III), a lipid-binding protein and an insect homolog of human apolipoprotein E, plays an important role in lipid transport and immune response in insects. In the present study, we have demonstrated a correlation in time between changes in the apoLp-III abundance occurring in the hemolymph, hemocytes, and fat body after immunization of *Galleria mellonella* larvae with Gram-negative bacteria *Escherichia coli*, Gram-positive bacteria *Micrococcus luteus*, yeast *Candida albicans*, and a filamentous fungus *Fusarium oxysporum*. Using two-dimensional electrophoresis (IEF/SDS-PAGE) and immunoblotting with anti-apoLp-III antibodies, the profile of apoLp-III forms in *G. mellonella* larvae challenged with the bacteria and fungi has been analyzed. Besides the major apoLp-III protein (p*I* = 6.5), one and three additional apoLp-III forms differing in the p*I* value have been detected, respectively, in the hemolymph, hemocytes, and fat body of non-immunized insects. Also, evidence has been provided that particular apoLp-III-derived polypeptides appear after the immune challenge and are present mainly in the hemolymph and hemocytes. The time of their appearance and persistence in the hemolymph was dependent on the pathogen used. At least two of the apoLp-III forms detected in hemolymph bound to the microbial cell surface. The increasing number of hemolymph apoLp-III polypeptides and differences in their profiles observed in time after the challenge with different immunogens confirmed the important role of apoLp-III in discriminating between pathogens by the insect defense system and in antibacterial as well as antifungal immune response.

Introduction

Insect apolipophorin III (apoLp-III), a homologue of human apolipoprotein E (apoE), is an abundant hemolymph protein playing an important role in lipid transport and immune response. The protein has been characterized so far in different insect species, e.g. *Bombyx mori*, *Galleria mellonella*, *Hyphantria cunea*, *Manduca sexta* (Lepidoptera), and *Locusta migratoria* (Orthoptera). The apoLp-III molecule is composed of five antiparallel amphipathic α-helices forming a bundle in which they are arranged in an up-and-down topology. The hydrophobic regions of the helices are directed into the interior, whereas the hydrophilic regions are presented on the surface of the bundle, preventing precipitation of apoLp-III in the aqueous environment of hemolymph. ApoLp-III is a lipid-binding protein. Upon lipid interaction, the apoLp-III molecule undergoes conformational changes leading to opening of the bundle of the helices [7,45]. The ability of lipid binding is an important feature of apoLp-III in lipid transport as well as in insect immunity. The role of apoLp-III in insect immune response has been studied especially in *Galleria mellonella* [50]. The protein is involved in pathogen recognition by interacting with bacterial lipopolysaccharides (LPS), lipoteichoic acids (LTA), fungal β-1,3-glucan, and binding to bacterial and fungal cell walls. It can be regarded as a signaling molecule, because upon infection/immunization the protein, after interacting with lipids, is included in lipophorin particles taken up by a particular type of hemocytes [14,15,18,31,34,36,47]. Antibacterial activity of apoLp-III and its involvement in antifungal activity of hemolymph has been demonstrated [35,51–53,55]. In addition, apoLp-III enhances the enzymatic (muramidase) activity of lysozyme [16,54]. Intrahemocoelic injection of apoLp-III increased antimicrobial activity of *G. mellonella* larval hemolymph [32,48]. Moreover, apoLp-III injection increased expression of genes encoding lysozyme and cecropin-like peptides in *H. cunea* [24].

* Corresponding author. Tel.: +48 81 37 50 50; fax: +48 81 37 50 50.
E-mail address: barabas@poczta.umcs.lublin.pl (A. Zdybicka-Barabas).

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Changes in the apoLp-III abundance in the hemolymph of *G. mellonella* larvae challenged with different bacteria (*Escherichia coli*, *Micrococcus luteus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) have been demonstrated [2,51]. Moreover, presence of apoLp-III isoforms in the hemolymph of *G. mellonella* and *L. migratoria* has been reported [10,42,44,51]. In the present study, we demonstrate a correlation in time between changes in the apoLp-III abundance occurring in the hemolymph, hemocytes, and fat body after immunization of *G. mellonella* larvae with bacteria and fungi. In addition, using two-dimensional electrophoresis (IEF/SDS-PAGE) and immunoblotting with anti-apoLp-III antibodies, we analyze the profile of apoLp-III forms in the fat body, hemocytes, and hemolymph of *G. mellonella* larvae challenged with bacteria and fungi. Also, we provide evidence that some of the apoLp-III-derived polypeptides appear in the hemolymph and hemocytes after the immune challenge.

Materials and methods

Microorganisms

E. coli D31 and *M. luteus* ATCC 10240 were grown in LB broth at 37 °C and 28 °C, respectively. *Candida albicans* (wild-type; kindly gifted by Prof. A. Kędzia, Department of Oral Microbiology, Medical University of Gdańsk, Poland) and *Fusarium oxysporum* ET3 were grown in YPD medium (1% yeast extract, 2% peptone, 2% dextrose) and on solid PDA medium (5% potato extract, 0.5% dextrose, 1.7% agar) at 37 °C (*C. albicans*) and 28 °C (*F. oxysporum*), respectively [28].

Insects, immune challenge, collection of hemolymph, hemocytes, and fat bodies

The last instar larvae of the greater wax moth *G. mellonella* (Lepidoptera: Pyralidae) were reared on honeybee nest debris at 30 °C in the dark. Immune challenge with live pathogens was performed using the piercing method as described previously [28]. The hemolymph was collected from eight larvae per group at 0.25 h, 1 h, and 24 h after the treatment and combined to obtain pooled samples (20 μl/larva; $n = 8$). Hemocyte-free hemolymph was obtained by centrifugation at 200 × *g* for 5 min and subsequently at 20,000 × *g* for 15 min at 4 °C [11].

The hemocytes were isolated from the hemolymph ($n = 10$) using ice-cold insect physiological saline (0.1 M Tris–HCl pH 6.9, 150 mM NaCl, 5 mM KCl) supplemented with 0.02 mg ml^{-1} of phenylthiourea (PTU), as described previously [13,43]. The fat bodies were dissected from three individuals per each group using ice-cold Ringer buffer (172 mM KCl, 68 mM NaCl, 5 mM $NaHCO_3$, pH 6.1, osmolarity 420 mOsm) as described in our earlier paper [12]. The hemocyte pellets and the fat bodies obtained were frozen in liquid nitrogen and stored at −80 °C. Collection of the hemolymph, hemocytes, and fat bodies was repeated three times in independent experiments.

Preparation of extracts from the hemocytes and fat bodies

The hemocyte and fat body extracts were prepared using ice-cold buffer (50 mM Tris–HCl pH 7.4, 10 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 6 mM β-mercaptoethanol), essentially as described previously [12,13]. The hemocyte pellets and the fat bodies were suspended in 50 μl and 300 μl of the buffer, respectively, and were homogenized using a pellet pestle motor (Sigma–Aldrich). The resulting hemocyte and fat body extracts were centrifuged, respectively, once and three times at 20,000 × *g* for 15 min at 4 °C, and the supernatants were collected. Subsequently, the fat body supernatants were additionally centrifuged at 105,000 × *g* for 1 h at 4 °C to remove the microsomal fraction. The supernatants obtained containing soluble proteins were used immediately for two-dimensional (2D) electrophoresis. For SDS-PAGE, an appropriate volume of Laemmli sample buffer [26] was added and the samples were stored at −20 °C.

Binding of apoLp-III to bacterial and fungal cells

The experiments were carried out using hemolymph methanolic extracts containing apoLp-III as a main component. These extracts were prepared from cell-free hemolymph collected 24 h after immunization of the larvae with *E. coli* using a method described in detail elsewhere [11].

Adsorption of apoLp-III on the microbial cell surface was performed according to our published papers [51,53]. Briefly, properly prepared cells of bacteria (*E. coli*, *M. luteus*) and fungi (*C. albicans*, *F. oxysporum*) were incubated without (control) and in the presence of the hemolymph methanolic extracts (5 μg of total protein) for 5 min at 30 °C. After centrifugation and thorough washing (removal of non-adsorbed proteins), the cell pellets were suspended in 0.5 M ammonium formate pH 6.4 for detachment of the adsorbed hemolymph proteins. The protein samples were then analyzed by 2D electrophoresis (IEF/SDS-PAGE).

Electrophoretic analysis of proteins

One-dimensional polyacrylamide gel electrophoresis of proteins (3 μg and 20 μg of total hemolymph and hemocyte or fat body protein, respectively) was performed in denaturing conditions by 13.8% glycine SDS-PAGE according to Laemmli [26]. Two-dimensional polyacrylamide gel electrophoresis IEF/SDS-PAGE was carried out using the Protean System (BioRad) according to the manufacturer's instructions. In the first dimension, 7-cm long ReadyStrip™ IPG Strips pH 3–10 (BioRad) were used. For the 2D analysis of the hemolymph and hemocyte proteins, 100 μg and 150 μg of total protein were separated for stained gels and immunoblotting, respectively. In order to confirm the profile of the apoLp-III forms in the fat body, which is extremely rich in various polypeptides, samples containing up to 500 μg of total fat body protein were analyzed. The proteins in the gels were visualized after IEF/SDS-PAGE by silver or Coomassie Brilliant Blue R-250 staining.

Immunoblotting

The protein samples subjected to electrophoresis were electroblotted onto Immobilon P membranes (Millipore) for 1.5 h at 350 mA. For apoLp-III identification, the membranes were probed with rabbit polyclonal antibodies (1:2500) to *G. mellonella* apoLp-III (Agrisera, Sweden; custom ordered) and alkaline phosphatase-conjugated goat anti-rabbit IgGs (1:30,000) [51].

Other methods

The protein concentration was estimated by the Bradford method using bovine serum albumin (BSA) as a standard [6].

The densitometric analysis of bands was performed using the Quantity One computer imaging system (BioRad). The densitometric data are presented as mean ± standard deviation (±SD) for three independent experiments. Student's *t*-test was used for evaluation of the statistical significance of the differences ($^{*}p < 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$).