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Changes in the apolipophorin-III level in the hemolymph of *Galleria mellonella* larvae after bacterial infection

Zmiana poziomu apolipoforyny-III w hemolimfie gąsienic *Galleria mellonella* po zakażeniu bakteryjnym

SUMMARY

The level of apoLp-III in hemolymph of *Galleria mellonella* larvae infected with entomopathogenic strains of *Pseudomonas aeruginosa*, *Serratia marcescens*, *Bacillus thuringiensis* var. *kurstaki*, *Bacillus thuringiensis* var. *alesti* or non-pathogenic bacterium *Escherichia coli* were studied. It was found that the amount of 18 kDa protein decreased within infection with *P. aeruginosa* and *S. marcescens* in comparison to its content in the hemolymph of non-infected larvae. Whereas, the inconsiderable increase of the apoLp-III level was observed in the hemolymph of infected larvae as a response to injection with *B. thuringiensis*. There was no difference in the apoLp-III level between hemolymph collected from larvae infected with *E. coli* and control naïve insects. *In vitro* studies also demonstrated apoLp-III degradation by *P. aeruginosa* and *S. marcescens* culture supernatants.

STRESZCZENIE

Po zakażeniu gąsienic *Galleria mellonella* entomopatogennymi bakteriami (*Pseudomonas aeruginosa*, *Serratia marcescens*, *Bacillus thuringiensis* var. *kurstaki*, *Bacillus thuringiensis* var. *alesti)* lub bakterią saprofityczną *Escherichia coli* zmieniał się poziom apoLp-III w hemolimfie. Stwierdzono, że ilość białka o masie 18 kDa, odpowiadającego apoLp-III zmniejszała się wraz z czasem trwania zakażenia gąsienic bakterią *P. aeruginosa* oraz *S. marcescens*. Natomiast nieznaczny wzrost ilości apoLp-III obserwowano w hemolimfie owadów, którym podano przez iniekcję bakterie *B. thuringiensis*. Poziom apoLp-III w hemolimfie gąsienic zakażonych bakterią E. *coli* był porównywalny z poziomem tego białka u owadów kontrolnych. Wykazano również, że w warunkach *in vitro*, apoLp-III ulegała degradacji po inkubacji z płynem pohodowlanym bakterii *P. aeruginosa* lub *S. marcescens*.

Key words: Apolipophorin-III, *Galleria melonella*, *Pseudomonas aeruginosa*, *Bacillus thuringiensis*, *Serratia marcescens*.

INTRODUCTION

Insect hosts defend themselves against bacterial and parasite infections with cellular and humoral immune response. An important role in insect immune response against invading pathogens is played by apolipophorin III (apoLp-III), a major exchangeable lipid transport protein found in hemolymph. The apoLp-III protein has been identified as an immune-mediating molecule (Dettloff et al. 2001; Weers, Ryan 2006). It can bind to lipopolysaccharide, lipoteichoic acid, as well as to β-1,3-glucan (Dunphy, Halwani 1997; Whitten et al. 2004). It has been demonstrated that injection with the native (Wiesner et al. 1997) and the recombinant protein (Niere et al. 1999) into *G. mellonella* larvae leads to an increase in antimicrobial activity within the hemolymph. It was proved *in vivo* and *in vitro* that apoLp-III and lysozyme can act synergistically, exactly apoLp-III by binding to bacterial cell wall components, increases peptidoglycan sensitivity to lysozyme (Halwani, Dunphy 1999). ApoLp-III is also engaged in cellular immunity regulation. This protein stimulates and increases phagocytosis of *Saccharomyces cerevisiae* by plasmatocytes and foreign bodies encapsulation (Whitten et al. 2004; Wiesner et al. 1997).

Proteolytic enzymes play crucial roles in homeostasis maintenance in both eukaryotes and prokaryotes. It is well established that these enzymes are produced as virulence factors by a large number of pathogenic microorganisms (Miyoshi, Shinoda 2000; Travis et al. 1995). They promote development within the infected host and interfere with its immune system (Hung et al. 2005; Miyoshi et al. 2002; Vilcinskas, Götz 1999).

Pseudomonas aeruginosa is a common environmental Gram-negative bacillus, which acts as an opportunistic pathogen in diverse hosts, including mammals, insects, nematodes and plants. During infection bacteria secrete a wide range of virulence factors, among them extracellular proteases. This group of enzymes includes at least four proteases: alkaline protease (aeruginolysin), protease IV, two elastases, namely LasA (staphylolysin) and LasB (pseudolysin) (Caballero et al. 2001).

The bacterium *Bacillus thuringiensis* is a pathogen of many insect species and is actively used in biocontrol. After the peroral inoculation of *G. mellonella* with *Bt* increase the phagocytic activity and enhanced encapsulation rates has been observed (Dubovsky et al. 2008). It was shown that entomopathogenic strain *B. thuringiensis* produces an exoprotease which selectively destroys cecropins and attacins, two antibacterial peptides found in immune hemolymph from *Hyalophora cecropia* (Dalhammar, Steiner 1984).

Degradation of cecropins and attacins was also revealed *in vitro* with two proteases from an insect pathogenic strain of *Serratia marcescens* (Flyg, Xanthopoulos 1983). *S. marcescens* is a well known insect pathogen which can produce several hydrolytic enzymes (e.g. proteases and chitinase), some of which have been shown to be toxic to an insect (Kaŝka 1976; Ourth, Smalley 1980; Poinar et al. 1979).

In this paper we describe the effects of infection with entomopathogenic strains *P. aeruginosa, S. marcescens and B. thuringiensis* on apolipophorin III level in the hemolymph of *G. mellonella* larvae. We also studied whether culture supernatants of entomopathogenic bacteria are able to degrade apoLp-III protein *in vitro*.

MATERIAL AND METHODS

Biological species

The larvae of greater wax moth *G. mellonella* (Lepidoptera*: Pyralidae*) were reared on a natural diet-honeybee nest debris at 30°C in the dark. Last instar larvae (250–300 mg in mass) were selected for this study.

For immunization (*in vitro* test) and for infection (*in vivo* test), *Escherichia coli* K12, strain D31, LPS defective, streptomycin and ampicilin resistant (CGSC 5165) (Boman et al. 1974) was used.

For *in vivo* experiments, *G. mellonella* larvae were infected with *P. aeruginosa* strain ATCC 27853, *B. thuringiensis* var. *alesti, B. thuringiensis* var. *kurstaki* or *S. marcescens*. The bacterial cells were grown in nutrient broth for 24 h at 37°C and pelleted by centrifugation at 20 000 g for 10 min at 4°C.

Infection of insects

The larvae were injected with non-toxic doses of viable cells of bacteria suspended in sterile water: *P. aeruginosa* (20–30 cells/larvae); *B. thuringiensis* (130–150 cells/larvae); *S. marcescens* (80–100 cells/larvae) or *E. coli* (10^5 cfu). After the infection the larvae were kept at 28°C in the dark on sterile Petri plates and samples of the hemolymph were collected after the time indicated in the text.

Hemolymph collection

Prior to hemolymph collection, the insects were chilled for 15 min at 4°C. Hemolymph samples were obtained by puncturing larval abdomen with a sterile needle. The out-flowing hemolymph was immediately transferred into sterile and chilled Eppendorf tubes containing a few crystals of phenylthiourea (PTU) to prevent melanization. The hemocyte-free hemolymph was obtained by centrifugation at 200 g for 5 min and subsequently at 20 000 g for 10 min at 4°C. Pooled supernatants were stored at -20°C until used.

Preparation of acidic/methanol hemolymph extracts

Low molecular mass proteins and peptides were isolated from hemocyte-free hemolymph by the acidic/methanol extraction method adapted from Schoofs et al. (Schoofs et al. 1990). The hemolymph was diluted 10 times with the extraction solution consisting of methanol:glacial acetic acid:water (90:1:9) and mixed thoroughly. Precipitated proteins were pelleted by centrifugation at 20 000 g for 30 min at 4°C. The obtained supernatant was collected, vacuum dried and the pellet was stored at -20°C until needed. Before use, it was dissolved in an appropriate volume of sterile distilled water. The acidic/methanol hemolymph extract obtained as described above contained proteins and peptides of M_r below 30 kDa.

***In vitro* experiments**

For *in vitro* experiments samples containing acidic/methanol hemolymph extract (20 µg of protein) and culture supernatants of bacteria were incubated at 37°C for 60 min. The reaction was stopped by sample buffer addition and samples were stored at – 20°C.

Electrophoresis methods

Polyacrylamide gel electrophoresis of protein samples was performed by tricine SDS-PAGE (16.5% T, 3% C) according to Schägger and von Jagow (Schägger, von Jagow 1987).

Immunoblotting

The samples (20 µg protein) after tricine SDS-PAGE were electroblotted onto Immobilon membranes (Milipore) for 90 min at 350 mA. For apolipophorin-III identification, the membranes were probed with rabbit polyclonal antibodies (1: 2500) to *G. mellonella* apoLp-III. Antibodies were raised in rabbits by subcutaneous injection of purified recombinant apoLp-III with adjuvant (Agrisera, Sweden). The recombinant *G. mellonella* apoLp-III was a generous gift of Professor Paul M. M. Weers (Department of Chemistry and Biochemistry, California State University, Long Beach,

CA 90840, USA). Alkaline phosphatase-conjugated goat anti-rabbit IgGs (1: 30000) were used as the second antibodies and immunoreactive bands were visualized by incubation with *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

Other methods

The concentration of proteins was estimated by the Bradford method using bovine serum albumin (BSA) as a standard (Bradford 1976).

The densitometric analysis was performed using Quantity One computer imaging system (Bio-Rad, Hercules, CA).

RESULTS AND DISCUSSION

ApoLp-III is a very important element of insect immune responses. It has been shown that after bacterial infection, the apoLp-III protein levels in both larvae and pupal immune hemolymph increased in relation to hemolymph from non-infected *Heliothis virescens* (Hung, Ourth 2002). Similar results were reported for immune hemolymph of *Hyphantria cunea* (Kim et al. 2004). Recently was shown that immune-challenge with Gram-negative and Gram-positive bacteria led to an increase in the level of apoLp-III in *G. mellonella* hemolymph (Zdybicka-Barabas, Cytryńska 2011). Our previous report has shown that apoLp-III was degraded *in vitro* by extracellular serine protease IV produced by entomopathogenic strain of *P. aeruginosa* (Andrejko et al. 2005).

In this study we investigated the level of apoLp-III in the hemolymph *G. mellonella* larvae infected with entomopathogenic strains of *P. aeruginosa*, *S. marcescens*, *B. thuringiensis* var. *kurstaki*, *B. thuringiensis* var. *alesti* or non-pathogenic bacterium *E. coli*. As control was used the hemolymph of non-infected larvae. The insects were injected with sublethal doses, i.e. doses showing no toxic effects on larvae (MATERIAL AND METHODS). The level of apoLp-III was estimated in the extract samples of cell-free hemolymph collected in different periods of time after infection. To test apoLp-III protein levels the immunobloting technique with anti-apoLp-III antibodies was used.

When *G. mellonella* larvae were infected with the entomopathogenic strain *P. aeruginosa*, a remarkable increase in the amount of apoLp-III was observed after a short time postinjection (Fig. 1A). The densitometric analysis of immunoblots revealed that, in the hemolymph samples of larvae infected for 4 h, the apoLp-III content increased by 40% in comparison to control insects. This might indicate that in a short time after bacterial infection more apoLp-III is secreted into the hemolymph from fat body and hemocytes. After 18 h the level of protein presented in the tested sample significantly decreased. It may be caused by proteolytic degradation by extracellular *P. aeruginosa* proteases produced during infection. It is known from many studies that *P. aeruginosa* proteases are capable of degrading a variety of biologically active molecules associated with the human

humoral immune response (Caballero et al. 2008). We recently showed a decrease in the apoLp-III content *in vivo* after infection with *P. aeruginosa* (Andrejko et al. 2008).

From the data summarized in Figure 1B it appeared that in the hemolymph samples obtained from insects infected with *B. thuringiensis* var. *alesti*, the amount of apoLp-III slightly increased as early as 4 h after injection. Then, after 24 and 30 h of *B. thuringiensis* infection, the relative levels of apoLp-III increased approximately by 20% in relation to control (non-infected) organisms.

Likewise, apoLp-III level changed after injection of *G. mellonella* larvae with *B. thuringiensis* var. *kurstaki* (Fig. 1C). The intensity of apoLp-III bands observed on immunoblots 4, 18 and 30 h postinfection was somewhat greater than in the control larvae. The densitometric analysis revealed that the highest level of apoLp-III was observed in the hemolymph 24 h postinfection (by 16% in comparison to control). The obtained results indicate that *G. mellonella* apoLp-III appeared to be insensitive to the action of virulence factors of *B. thuringiensis*. However, literature data indicated that the *B. thuringiensis* metaloprotease has a vital role in virulence when the host is infected via the oral route (Fedhila et al. 2002).

In the following experiments, we tested the effect of *S. marcescens* infection on the level of apolp-III in the hemolymph of *G. mellonella* larvae. As revealed by immunoblots (Fig. 1D) the level of 18 kDa protein oscillated around the control larvae levels short time postinfection (4 h). However, starting from 18 h after the treatment, a significant decrease of apoLp-III content was observed. The obtained results seem to indicate that *G. mellonella* apoLp-III was degraded by proteases produced by entomopathogenic strain of *S. marcescens*. Studies performed earlier indicated that *in vitro* extracellular proteases produced by *S. marcescens* can elicit rapid and extensive damage to the rabbit cornea (Kreger, Griffin 1975). It was also shown that *S. marcescens* culture filtrates were cytotoxic to mammalian cells (Marty et al. 2002). The *S. marcescens*-derived protease serralysin is considered to play an important role in the pathogenesis of infection (Kida et al. 2007).

When *G. mellonella* larvae were infected with the non-pathogenic bacterium *E. coli* D31, which is a well-known immune response elicitor, there was no significant difference in the apoLp-III content in comparison to the level measured in non-infected insects (Fig. 1E).

The *in vivo* results prompted us to test whether apoLp-III was degraded by virulence factors presented in culture supernatants of entomopathogenic bacteria (*P. aeruginosa*, *S. marcescens*, *B. thuringiensis* var. *alesti* and *B. thuringiensis* var. *kurstaki*) and supernatant of non-pathogenic bacterium *E. coli* D31. As a source of apoLp-III, cell-free hemolymph extract from immune-challenged *G. mellonella* larvae were used. For immunization larvae were injected with *E. coli* D31 (MATERIAL AND METHODS). Samples containing immune hemolymph extracts (20 μg of protein) and culture supernatants were incubated at 37°C for

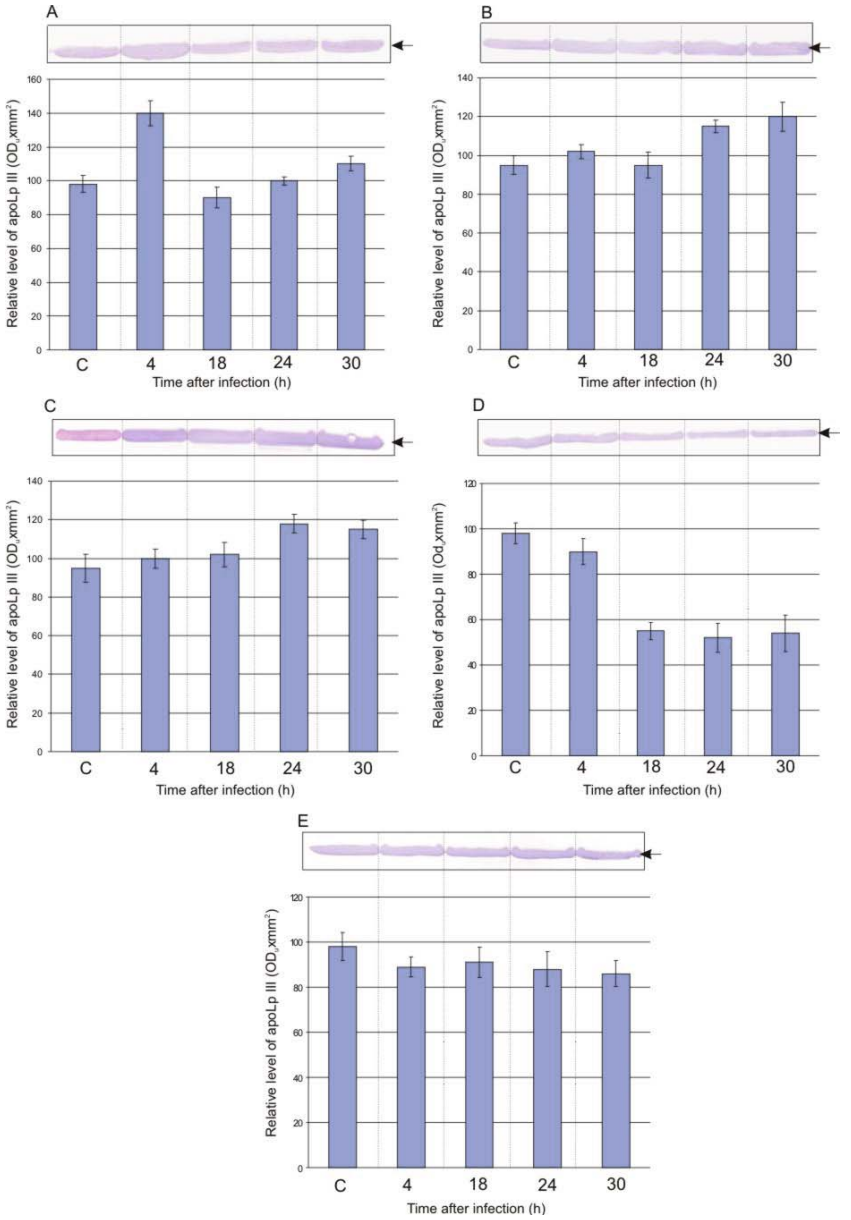

Fig. 1. ApoLp-III level in the hemolymph extracts obtained from *G. mellonella* larvae after bacterial infection. The immunoblotting analysis of hemolymph extract collected from infected larvae at the indicated time points. Samples (20 µg of protein) after tricine-SDS-PAGE were transferred onto Immobilon membranes and probed with anti-apoLp-III antibodies. The membranes of the representative experiments are shown. The diagram presents the results of densitometric analysis of bands containing apoLp-III. Bars = ±SD of three independent experiments. A – *P. aeruginosa*; B – *B. thuringiensis* var. *alesti*; C – *B. thuringiensis* var. *kurstaki*; D – *S. marcescens*; E – *E. coli*

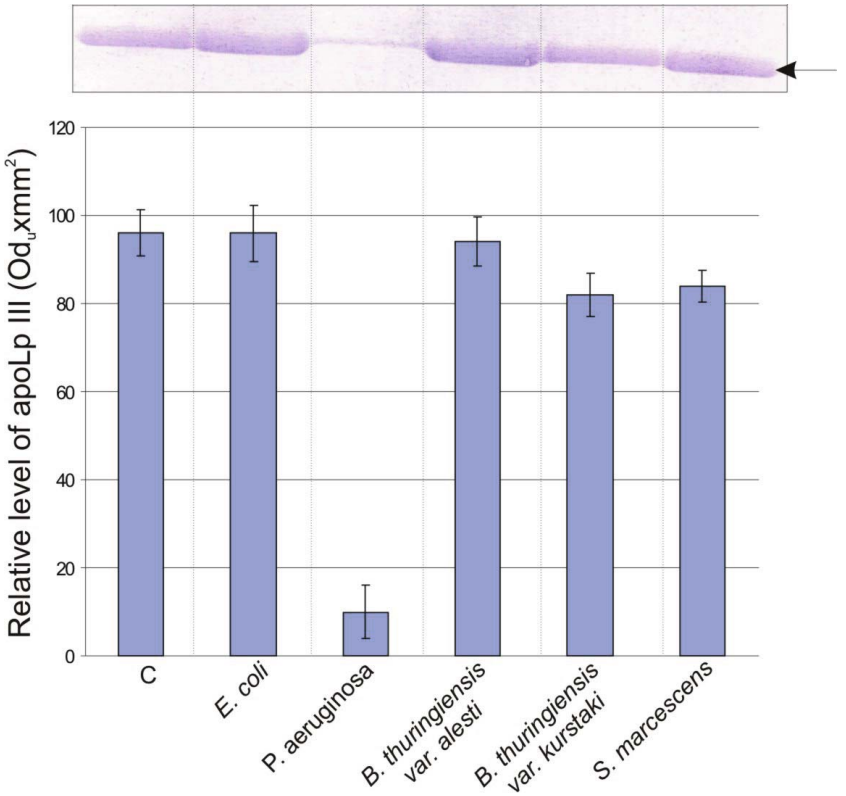

Fig. 2. The effect of bacterial culture supernatants on apoLp-III level *in vitro*. Samples containing immune hemolymph extract (20 µg of protein) and culture supernatant were incubated at 37°C for 60 min. The reactions were stopped by sample buffer addition. Then the samples were subjected to tricine SDS-PAGE and immunoblotting with anti-apoLp-III antibodies. The diagram presents the results of densitometric analysis of bands containing apoLp-III. Bars = ±SD of three independent experiments

60 min. The reactions were stopped by sample buffer addition. Then the samples were subjected to tricine SDS-PAGE and immunoblotting with anti-*G. mellonella* apoLp-III antibodies (MATERIAL AND METHODS).

It was found that apoLp-III was effectively digested by enzyme presented in *P. aeruginosa* supernatant (Fig. 2). The densitometric analysis of immunoblots revealed approximately 90% decrease of apoLp-III level in comparison to control samples. These data are in line with our previous studies (Andrejko et al. 2005). The amount of apoLp-III slightly decreased in hemolymph exposed to the action of *S. marcescens* and *B. thuringiensis* var. *kurstaki* culture supernatants (by 16 and 18%, respectively) in comparison to its content in the hemolymph of non-infected larvae.

As can be seen in Figure 2, in the hemolymph samples incubated with *E. coli* or *B. thuringiensis* var. *alesti* culture supernatants, no change in the apoLp-III protein level in comparison to control samples was observed.

The results of *in vitro* and *in vivo* experiments support the conclusion that extracelullar proteases produced by *P. aeruginosa* and *S. marcescens* can effectively degrade apoLp-III in the hemolymph of *G. mellonella* larvae.

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