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RESEARCH REPORT

The role of *Pseudomonas aeruginosa* alkaline protease in activation of the antimicrobial activity in *Galleria mellonella* larvae

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Abstract

The role of *Pseudomonas aeruginosa* metalloprotease - alkaline protease in activation of the antimicrobial activity in *Galleria mellonella* larvae was investigated. The results of our *in vivo* study showed that injection of alkaline protease at a sublethal dose enhanced the antimicrobial activity in the hemolymph of *G. mellonella* larvae as a result of induction of defense peptides synthesis**.** We observed that the antibacterial activity against *E. coli* appeared in the hemolymph 4 h after the injection of both metalloprotease or heat-killed *P. aeruginosa*, reached the maximum level 24 h post injection, and next decreased slightly. Antifungal activity against *A. niger* was detected in the hemolymph 15 h and 24 h after the challenge in the case of the alkaline protease and *P. aeruginosa* cell treatment*,* respectively. We also noted that the antimicrobial activity level induced by the presence of the metalloprotease in the hemolymph was higher than the activity measured after the injection of the insects with *P. aeruginosa*. The results of our *in vitro* studies indicated that inducible antimicrobial peptides present in the hemolymph of protease- or *P*. *aeruginosa*-challenged larvae were digested by alkaline protease.

Key Words: antimicrobial activity; *Galleria mellonella*; alkaline protease; *Pseudomonas aeruginosa*

Introduction

The gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic pathogen which can cause severe and lethal infections in patients with a variety of diseases. The bacteria produce several extracellular proteolytic enzymes that have been implicated as virulence factors. Among them, there are elastase (pseudolysin) (Kessler *et al.,* 1998), LasA protease (staphylolysin) (Kessler *et al*., 1993), protease IV, serine protease (Engel *et al*., 1998), and alkaline protease (Morihara, 1963; Maeda and Morihara, 1995; Caballero *et al*., 2001). The alkaline protease (EC 3.4.24.40) also named aeruginolysin (APR) is a member of the serralysin family (Morihara *et al*., 1973) and, hence, belongs to the metzincin superfamily of metalloendopeptidases (Rawlings and Barrett, 1995). APR is homologous to the 50-kDa metalloproteinases secreted by *Serratia marcescens* and *Erwinia chrysanthemi* (Maeda and Morihara, 1995; Stöcker *et al*., 1995). This protease is implicated in hydrolysis of many biologically important proteins, including the α_1-proteinase inhibitor (Morihara *et al*., 1979), cytokines (Parmely *et al*., 1990), complement factors (Hong and Ghebrehiwet, 1992), laminin (Heck *et al*., 1986), matrix metalloproteinases (MMPs) (Twining *et al*., 1993), human γ-interferon, and tumor necrosis factor-α (Horvat and Parmely, 1988; Parmely *et al*., 1990).

Insects have developed a very effective immune system, the functioning of which relies on humoral and cellular innate mechanisms. Cellular reactions, *i.e.,* phagocytosis, nodulation, and encapsulation, are mediated by hemocytes, whereas antimicrobial peptides and proteins (AMPs) are important components of humoral immune response. Antimicrobial peptides are mainly produced in the fat body or hemocytes and are then released into the hemolymph. An impressive arsenal of defense peptides differing in biochemical and antimicrobial properties has been reported in hemolymph of immune-challenged *G. mellonella* larvae (Brown *et al*., 2008, 2009; Cytryńska *et al*., 2007). In addition, differences in the peptide sets and the kinetics of appearance in the hemolymph were detected after insect infection with various bacteria and fungi (Mak *et al*., 2010).

The recognition system for a microbial pattern in *G. mellonella* is able to sense both microbial cell

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wall components and endogenous immune stimulatory peptides generated by microbial metalloproteinases. The infectious non-self model postulates that the immune system is set into alarm by recognition of microbial pattern molecules. The danger model explains the activation of immune response caused by alarm signals from injured cells, such as those exposed to pathogens, toxins, or mechanical damage (Matzinger, 2002; Altincicek *et al*., 2009).

It has been shown that innate immune systems of mammals and insects share a high degree of structural and functional homology. Many studies have demonstrated that the greater wax moth *G. mellonella* may be used as an alternative model host for investigating virulence factors of human pathogenic bacteria, including *P. aeruginosa* (Madziara-Borusiewicz and Lysenko, 1971; Dunphy *et al*., 1986; Jarrell and Kropinski, 1982). A positive correlation in the virulence of *P. aeruginosa* mutants in mice and *G. mellonella* caterpillars was demonstrated (Jander *et al*., 2000). A study which was to find out the role of the type III secretion system in *P. aeruginosa* pathogenesis has revealed a high level of correlation between the results obtained in the *G*. *mellonella* model and cytopathology assays conducted with a mammalian tissue culture system (Miyata *et al*., 2003).

The recent studies performed in our laboratory indicated that *P. aeruginosa* elastase B degraded inducible antimicrobial peptides in the hemolymph of *G. mellonella* (Andrejko *et al*., 2009, Andrejko and Mizerska-Dudka, 2012), while elastase B injected at a sublethal dose was responsible for eliciting the humoral immune response in *G. mellonella,* and this protease seems to be a more potent elicitor than thermolysin (Andrejko and Mizerska-Dudka, 2011). The diverse effects of two *P. aeruginosa* clinical isolates on the parameters of *G. mellonella* immune response indicated that this model of insect could be useful for analysis of the virulence factors of different *P. aeruginosa* strains (Andrejko *et al*., 2013b). In an *in vivo* study, proteolytic degradation of peptides and proteins in *G. mellonella* body after infection by an entomopathogenic *P. aeruginosa* strain was demonstrated, confirming the important role of bacterial proteinases in its pathogenicity (Andrejko *et al*., 2014). Larval infection with *P. aeruginosa* strains differing in the profile of proteolytic enzymes induces synthesis of immune peptides, likewise injection of the saprophytic bacteria *E. coli* (Andrejko *et al*., 2009; Andrejko *et al*., 2014).

In this study, we tested the level of antimicrobial activity in the hemolymph of *G. mellonella* larvae after the treatment with alkaline protease of *P. aeruginosa* clinical isolate. As we had shown earlier, this strain produced elastase B and alkaline protease when it grew in the LB medium and the minimal M9 medium, respectively. PCR analysis confirmed the presence of the *aprA* gene coding for alkaline protease in the genome of the *P. aeruginosa* clinical strain used in this study (Andrejko *et al*., 2013a). Furthermore, protease profile analyses of the larval homogenates revealed that this isolate produces alkaline protease during infection (Andrejko *et al*., 2014). We examined the effects of injection with alkaline protease on antimicrobial activity in *G. mellonella* larvae hemolymph. The kinetics and level of antimicrobial activity is compared to that obtained after injection of heat-killed *P. aeruginosa* cells. We also studied whether alkaline protease was able to degrade inducible antimicrobial peptides of *G. mellonella in vitro*.

Materials and Methods

Microorganisms

Pseudomonas aeruginosa ATCC 27853, an isolate with moderate virulence to the 7th instar larvae of *G. mellonella* (LD_{50} = 17 cells), and clinical strain PA 02/18 (received from the Department of Microbiology and Epidemiology, Military Institute of Hygiene and Epidemiology in Warsaw, Poland) were used in this study. The bacterial cells were grown overnight at 37 ^{o}C in Luria - Bertani broth (LB broth, Sigma) or M9 minimal medium supplemented with monosodium glutamate (0.13 M), glycerol (0.1 M), and $CaCl_2$ (0.01 M). *Escherichia coli* K12, strain D31, which is LPS-defective and streptomycin- and ampicillin-resistant (CGSC 5165) (Boman *et al*., 1974), was grown in Luria-Bertani broth (LB broth, Sigma) for 24 h at 37 ^{o}C and pelleted by centrifugation at 8,000*g* for 10 min at 4 ^{o}C. The filamentous fungus *Aspergillus niger* 71 was grown on PDA slides (5 % potato extract, 0.5 % dextrose, 1.7 % agar) at 28 ^{o}C until conidial spores emerged; it was then stored at 4 ^{o}C.

Insect immune challenge, hemolymph collection, preparation of hemolymph methanolic extracts

The larvae of *G. mellonella* were reared in darkness on honey bee nest debris at 28 ^{o}C and 70 - 80 % humidity. For immune challenge, the larvae were injected with 0.8 µg/larvae of alkaline protease (chromatographic fraction) or heat-killed (98 ^{o}C; 30 min) cells of *P. aeruginosa* ATCC 27853. The larvae were pricked with a thin needle plunged in the bacterial pellet from the 24 h culture of bacteria (approx. $5x10^4$ bacteria/larvae). After the treatment, the larvae were kept at 30 ^{o}C in the dark on sterile Petri dishes and the hemolymph was collected after the time indicated in the text. Hemolymph samples were obtained by puncturing larval abdomens 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 ^{o}C. Pooled supernatants were stored at -20 ^{o}C until used. The acidic/methanolic extracts of the hemolymph containing antimicrobial peptides and proteins below 30 kDa were prepared from cell-free hemolymph as described elsewhere (Andrejko *et al*., 2005; Cytryńska *et al*., 2007).

Protease purification

Alkaline protease was purified from the culture supernatant of the *P. aeruginosa* clinical strain PA 02/18. The bacterial cells were cultivated under aerobic conditions at 37 ^{o}C for 20 h, in M9 minimal

medium. Then, the bacterial culture was centrifuged at 8,000*g* for 15 min at 4 ^{o}C to pellet the cells. The resulting clear supernatant was used as the starting material. Proteins secreted into the growth medium were precipitated from the supernatant with ammonium sulfate (90 % of saturation). The precipitate was collected by centrifugation (8,000*g* for 15 min, 4 ^{o}C), dissolved in 50 mM Tris-HCl buffer (pH 8.0), and dialyzed overnight against the same buffer. The dialyzed solution was fractionated using ion-exchange chromatography on DEAE-cellulose column (DE 52, Whatman). The column was equilibrated with 50 mM Tris-HCl buffer, pH 8. Proteins bound to the column were eluted with a linear gradient of 0 - 0.7 M NaCl in the above buffer. The fractions were assayed for proteolytic activity and analyzed by zymography and immunoblotting as described below. The lyophilized samples were stored at -20 ^{o}C.

Proteolytic activity assay

The alkaline protease activity was measured using a modified method described by Howe and Iglewski (1984). 0.7 ml of the buffer (20 mM Tris-HCl, 1 mM $CaCl_2$, pH 8) and 0.3 ml of the enzyme-containing fraction were added to 10 mg of the Hide powder azure substrate (Sigma). The reaction mixtures (1 ml) were incubated at 37 ^{o}C for 1 h with constant rotation. The undissolved substrate was removed by centrifugation at 4,000*g* for 5 min. The absorbance of the reaction mixtures were then determined at 595 nm.

Zymography analysis

Gelatin zymography was conducted following the procedures described by Caballero and coworkers (2001). The samples containing alkaline protease (0.5 - 1.0 µg protein) were electrophoresed under non-reducing (30 mM Tris-HCl pH 6.8, 1 % SDS, 5 % glycerol and 0.04 % bromophenol blue) conditions using 10 % SDS - PAGE with 0.1 % gelatin. The gels were then soaked twice in 2.5 % Triton X-100 for 15 min and incubated at 37 ^{o}C for 24 h in gelatin gel substrate buffer (50 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, 1 µM $ZnCl_2$, 150 mM NaCl). The gels were stained for 60 min in 0.2 % amido black and then destained in 10 % acetic acid.

Immunoblotting

After resolution by SDS-PAGE, the proteins were electrotransferred onto PVDF membranes (90 min at 350 mA). The membranes were blocked with 5 % non-fat milk in TBS (10 mM Tris-HCl pH 7.5, 0.9 % NaCl). For detection of bacterial alkaline protease, the membranes were probed with rabbit antibodies against *P. aeruginosa* alkaline protease (1:1000) (kindly provided by Dr. R. Voulhoux, Laboratoire d'Ingénierie des Systèmes Macromoléculaires, CNRS UMR7255, Institut de Microbiologie de la Méditerranée, France). Goat anti-rabbit alkaline phosphatase-conjugated antibodies were used (1: 30,000) (Sigma) as second antibodies and visualization of the immunoreactive bands was performed with 5-bromo-4-chloro-indolyl-phosphate (BCIP) and *p*-nitro blue tetrazolium chloride (NBT) (Blake *et al*., 1984).

Antimicrobial activity assays

Antibacterial and antifungal activity was tested by the well diffusion assay against viable *E. coli* D31 (LB agar plates) and *A. niger* 71 (PDA plates), respectively, as described previously (Cytryńska *at al*., 2001; Mak *et al*., 2010). Wells on Petri plates were filled with 4 µl of the samples of the hemolymph or hemolymph extract. The diameters of the *E. coli* D31 and *A. niger* growth inhibition zones were measured after 24 h incubation at 37 ^{o}C and 28 ^{o}C, respectively. The level of antimicrobial activity was calculated as described by Hultmark and coworkers (1982). For evaluation of antibacterial and antifungal activity, commercial cecropin B (Sigma) and amphotericin B (Sigma), respectively, were used as standards.

Mortality assay

For *in vivo* experiments, the last instar (7th) non-feeding, wandering *G. mellonella* larvae were injected with alkaline protease at doses in a range of 0.8 - 2.0 µg protein/larvae. Sterile water was used for the control injection. After the challenge, insects were kept on sterile Petri dishes at room temperature in the darkness. The mortality of injected larvae was monitored 72 h after injection of the enzyme. Larval survival was calculated as a percentage (%) of live larvae (*i.e*., moving and exhibiting no change in the body colour) relative to the total number of larvae in the group. The experiments were performed in groups of 12 larvae and repeated three times.

In vitro assay

To evaluate the effect of *P. aeruginosa* alkaline protease on antimicrobial activity, the immune hemolymph (hemolymph collected from *P. aeruginosa*- or protease-challenged larvae) or the acidic/methanolic extracts were incubated at 37 ^{o}C for 10 min in the presence of alkaline protease (0.03 - 0.17 µg of total protein). After incubation, 4 µl of each sample were used to determine the antimicrobial activity by the well diffusion assay (see above). For SDS-PAGE electrophoresis, appropriate volumes of sample buffer were added to the samples and they were stored at -20 ^{o}C until needed.

SDS gel overlay method (bioautography)

Detection of antibacterial activity after SDS-PAGE and subsequent renaturation of polypeptides in the gels was performed as described elsewhere (Cytryńska *et al*., 2001). After resolution of the proteins, the gels were washed for 30 min in 2.5 % Triton X-100 (Bio Rad) for SDS removal. The gels were then washed in 50 mM Tris-HCl pH 7.5, and subsequently in LB broth, for 30 min in each step. To localize the peptide bands with antimicrobial activity, the gels were overlaid with nutrient agar containing viable *E. coli* D31 and EWL (2.5 mg/mL) and incubated for 6 - 12 h at 37 ^{o}C. Zones of bacterial growth inhibition were subsequently observed.

Other methods

Protein concentration was calculated using the Bradford method with bovine serum albumin (BSA)

as a standard (Bradford, 1976). Polyacrylamide gel electrophoresis of protein samples was performed by 10 % or 13.8 % glycine SDS-PAGE under reducing or non-reducing conditions according to Laemmli (Laemmli, 1970) or by Tris-tricine SDS-PAGE (16.5 % T, 3 % C) as described by Schägger and von Jagow (1987).

Results

Alkaline protease was purified from the culture supernatant of *P. aeruginosa* by ion-exchange chromatography on DEAE-cellulose. The activity assay showed that the chromatographic fraction was able to hydrolyze hide powder azure, a substrate for *P. aeruginosa* alkaline protease (data not shown). When the fraction obtained was analyzed by the zymography method using gelatin SDS-PAGE, a clear protease band with an apparent molecular mass 52 kDa was observed (Fig. 1A). It corresponded to the molecular mass of *P. aeruginosa* alkaline protease (Caballero *et al*., 2001; Andrejko *et al*., 2013a). The 52-kDa single protein band was recognized by specific anti-*P. aeruginosa* alkaline protease antibodies, confirming the presence of this protease in the chromatographic fractions (Fig. 1B).

In order to estimate the sublethal dose, we tested the toxicity of *P. aeruginosa* alkaline protease to the 7th instar larvae of *G. mellonella.* Four doses of alkaline protease (0.8, 1.2, 1.6 and 2.0 µg/larvae) were used. The insects were injected through the last proleg and mortality rates were determined over a 72 h period. The larvae treated with alkaline protease at the doses of 1.2, 1.6, and 2 µg/larvae exhibited ca. 30 %, 50 %, and 80 % mortality respectively, while the treatment with 0.8 µg/larvae of alkaline protease resulted in a 100 % survival rate (Fig. 2A). Dead larvae exhibited high melanization as well as loss of turgor and body integrity (Fig. 2B). On the basis of these results, the dose showing no toxic effects on the larvae was chosen for immune challenge of the insects, *i.e.,* 0.8 µg/larvae. For comparison, heat-killed cells of *P. aeruginosa* were used.

The kinetics of *in vivo* changes in the antimicrobial activity level in the hemolymph of *G. mellonella* larvae in response to the treatment with the metalloprotease and *P. aeruginosa* cells was investigated. The insects were injected with alkaline protease (0.8 µg of protein) or heat-killed *P. aeruginosa* cells. The hemolymph samples were collected 4, 8, 15, 24, and 48 h after injection and antibacterial and antifungal activity levels were measured (Figs 3, 4). Additionally, an acidic/methanolic extracts of hemolymph containing antimicrobial peptides and proteins below 30 kDa were also used as a source of immune peptides.

The results presented in Figures 3A and B showed that after metalloprotease injection, the appearance of antibacterial activity had similar kinetics to that obtained after the treatment with heat-killed *P. aeruginosa*. The well diffusion assay against *E. coli* demonstrated that antibacterial activity appeared in the hemolymph 4 h after injection of both the metalloprotease and bacteria. Then, the activity gradually increased, reaching the highest level 24 h after injection, and was equivalent to the activity of ca. 3.93 µM and 3.24 µM of cecropin B, respectively. Next, the antibacterial activity slightly decreased. In the control samples containing the hemolymph or hemolymph extract of untreated insects, no anti-*E. coli* activity was observed. Moreover, the level of antimicrobial activity in the protease-challenged larvae was slightly higher in comparison with that measured in the insect hemolymph treated with heat-killed *P. aeruginosa*. For example, the activity level after injection of the alkaline protease was approximately 1.2- fold greater in comparison to that detected in *P. aeruginosa* injected larvae, both 15 h and 24 h post challenge. The results suggest that, like the microbial elicitor, the alkaline protease strongly induced antimicrobial activity in *G. mellonella* hemolymph.

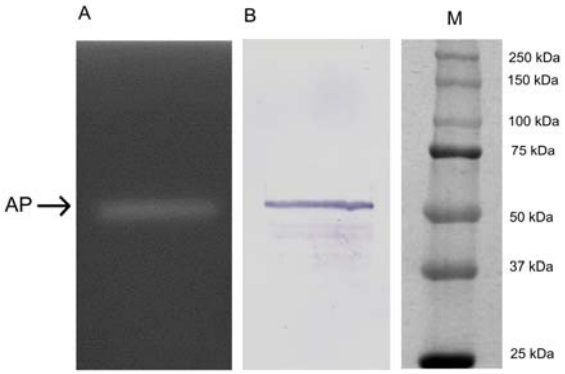


Fig. 1 Identification of alkaline protease in the fraction obtained by ion-exchange chromatography on DEAE-cellulose. (A) Zymography analysis. The samples (0.5-1 µg of protein) were electrophoresed under non-reducing conditions in 10% polyacrylamide gel containing 0.1 % gelatin. The gels were washed in Triton X-100, incubated at 37 ^{o}C for 24 h in a specific buffer, and stained in amido black as described in Materials and Methods. The zymogram shown is a typical representative of at least ten independent experiments. (B) Immunoblotting. The samples (ca. 5µg of protein) were resolved by SDS-PAGE, transferred onto PVDF membranes, and probed with anti-*P. aeruginosa* alkaline protease antibodies. A fragment of the membrane containing alkaline protease recognized by the antibodies is presented. M - molecular weight standards. AP – alkaline protease band is indicated by an arrow.

Antifungal activity toward *A. niger* was detected in the hemolymph collected 15 h after the challenge of *G. mellonella* larvae with the metalloprotease (Fig. 4A). The activity in this case persisted at a high level, corresponding to the activity of approx. 140 µM of amphotericin B, even 48 h after the treatment. In contrast, in the *P. aeruginosa*-challenged larvae, antifungal activity equivalent to the activity of 80 µM of amphotericin B appeared in the hemolymph 24 h after the treatment; 48 h after immunization, it decreased and corresponded to the activity of 48.5 µM of amphotericin B (Fig. 4B).

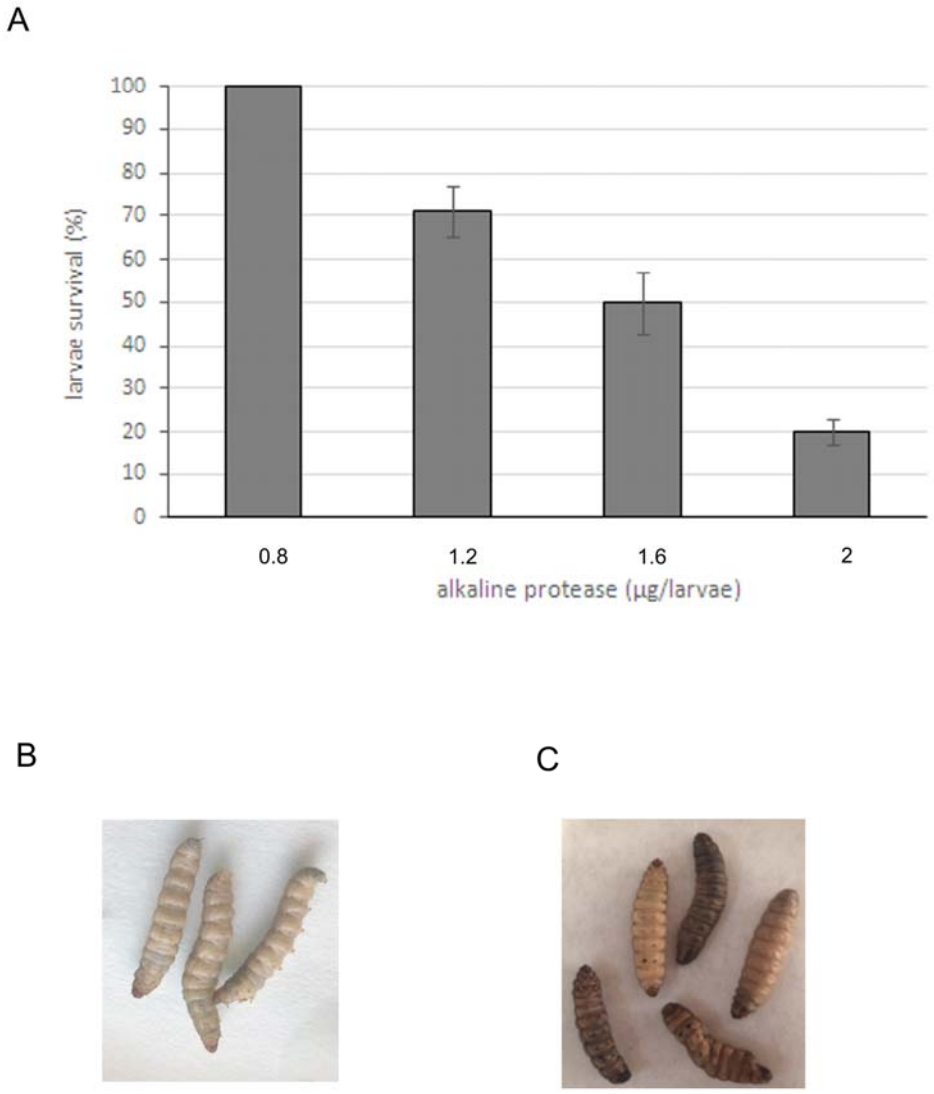


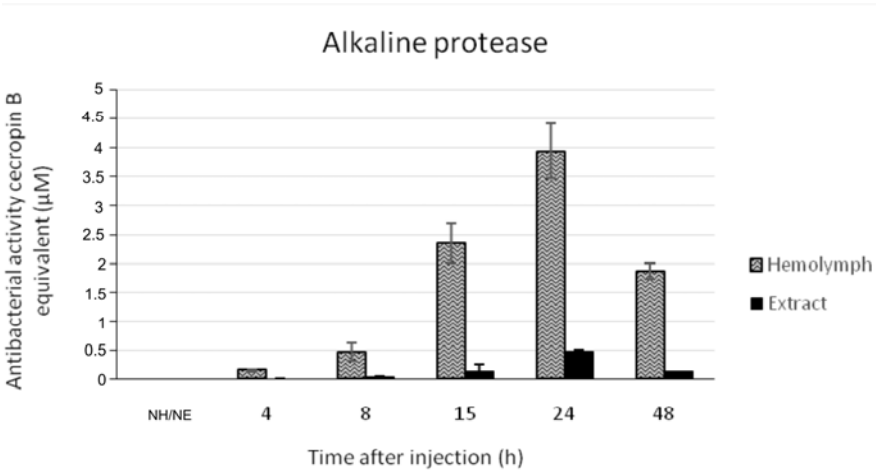
Fig. 2 (A) Mortality assay in *G. mellonella* larvae injected with alkaline protease. Twelve individuals were used per each group. All values represent the mean ±SD of three independent experiments. (B) larvae injected with alkaline protease (0.8 µg/larvae). (C) larvae after injection of alkaline protease (2 µg/larvae).

Additionally, the antibacterial activity of the hemolymph samples was tested by bioautography after resolution of polypeptides by SDS-PAGE and subsequent renaturation (Fig. 5A). To localize the polypeptide bands with antibacterial activity, the gels were overlaid with *E. coli* D31 (Cytryńska *et al*., 2001, 2007). The results obtained using the bioautography method revealed that the defense peptides with molecular mass corresponding to cecropin B were mainly responsible for the anti-*E. coli* activity in the studied hemolymph samples.

A striking difference between the level of antibacterial activity in the hemolymph and the hemolymph extract was observed. In contrast, no difference was detected in the case of antifungal activity (Fig. 4). Most probably, in addition to the antibacterial peptides present in the hemolymph extract, other polypeptide components of hemolymph, which are not recovered by methanolic extraction, are responsible for the antibacterial activity in hemolymph. On the other hand, the small difference in antifungal activity level between hemolymph and the hemolymph extract may implicate that mainly peptides and proteins with molecular mass below 30 kDa recovered during the extraction procedure to a high extent, are responsible for the antifungal activity.

The Tris-tricine SDS-PAGE analysis of *G. mellonella* larval proteins isolated from hemolymph of immune-challenged larvae showed that the same new peptide bands with molecular mass below 6.5 kDa appeared in the hemolymph samples in response to the injected microbial elicitor (*P. aeruginosa*) or alkaline protease (Fig. 5B). The presence of additional peptides in the hemolymph correlated also in time with the antimicrobial activity detected in the hemolymph of the immunized insects.

A



B

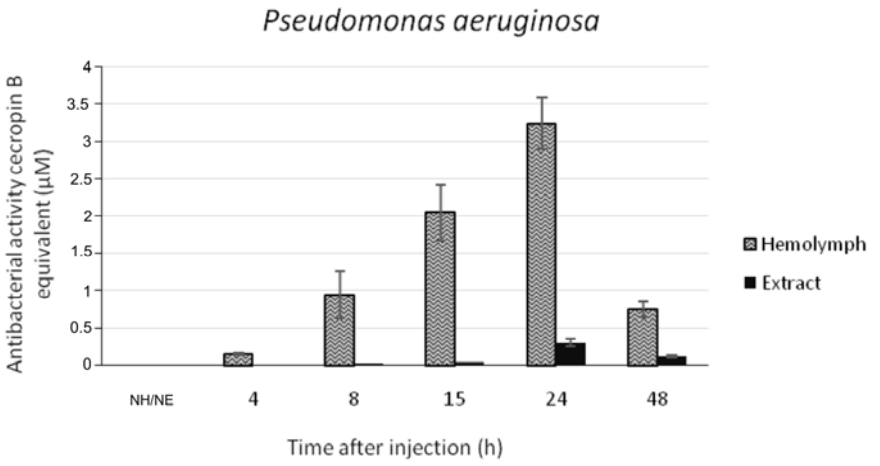


Fig. 3 Kinetics of the antibacterial activity in the hemolymph of alkaline protease- or heat-killed *P. aeruginosa*-challenged *G. mellonella* larvae. (A, B) The larvae were injected with the alkaline protease (0.8 µg protein) or heat-killed *P. aeruginosa* cells. The hemolymph samples were collected at specified time points after injection and methanolic extracts were prepared as described in section 2. The hemolymph/hemolymph extract samples were analyzed using the radial diffusion assay on solid agar plates containing live *E. coli.* The antibacterial activity was calculated as a cecropin B equivalent. The diagrams present the results ±SD of three independent experiments. NH = hemolymph from untreated larvae; NE = hemolymph extract from untreated larvae.

In order to test if AMPs, which are mainly responsible for antibacterial activity in the hemolymph of *G. mellonella*, are susceptible to *P. aeruginosa* alkaline protease, the activity in the hemolymph and the hemolymph extract from immune-challenged *G. mellonella* larvae was measured after *in vitro* incubation in the absence or presence of alkaline protease. For immunization, the larvae were injected with a chromatographic fraction containing alkaline protease (0.8 µg protein) and heat-killed *P. aeruginosa* cells. The well diffusion assay revealed that the antimicrobial activity was sensitive to the fraction containing alkaline protease. The results presented in Fig. 6A clearly showed that incubation of the hemolymph or the hemolymph extract in the presence of a fraction containing 0.17 µg protein for only 10 min completely abolished the anti-*E. coli* activity. In turn, the fraction added to the samples at a concentration of 0.03 µg protein significantly decreased antimicrobial activity, for example by ca. 69 % and 76 % in the hemolymph of *P. aeruginosa*-challenged and protease-challenged insects, respectively.

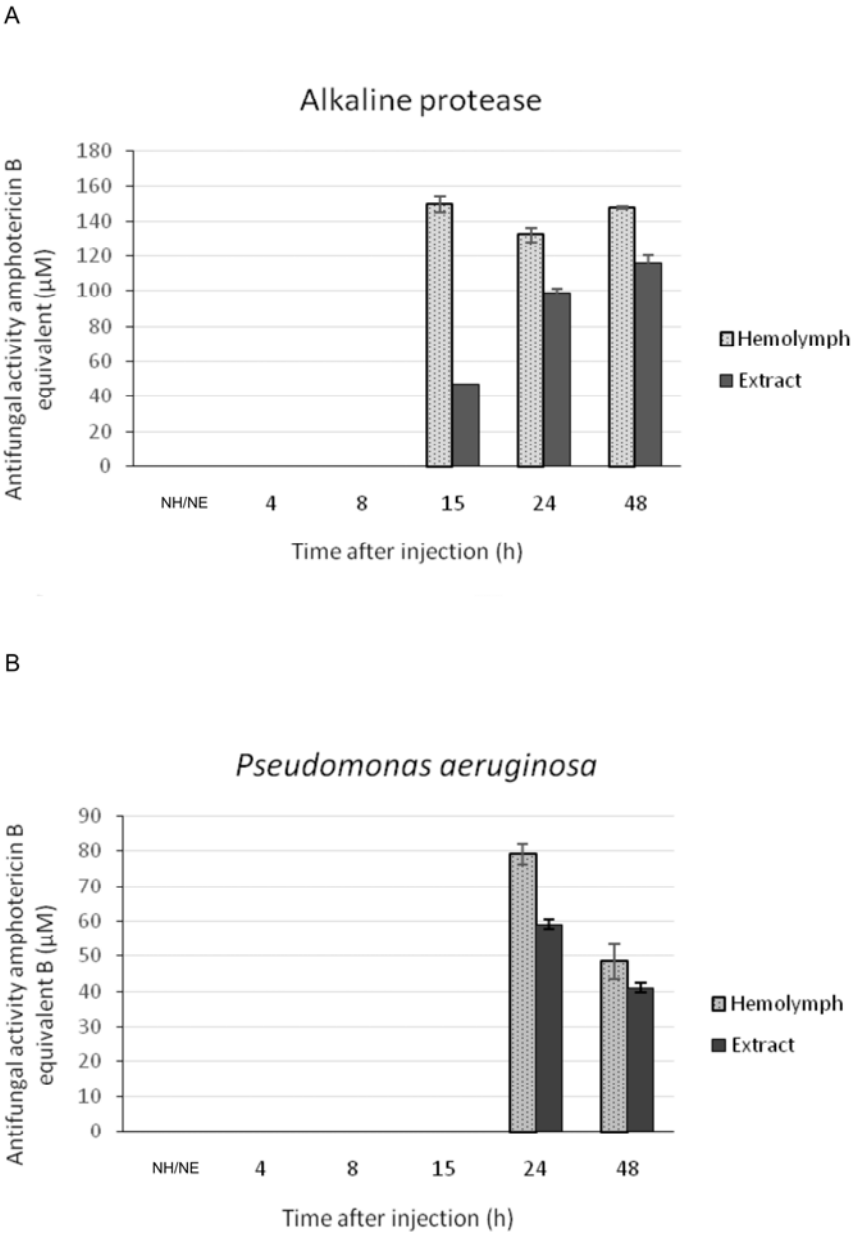


Fig. 4 Kinetics of the antifungal activity in the hemolymph of alkaline protease- or heat-killed *P. aeruginosa*-challenged *G. mellonella* larvae. (A, B) The larvae were injected with the alkaline protease (0.8 µg protein) or heat-killed *P. aeruginosa* cells. The hemolymph samples were collected at specified time points after injection and methanolic extracts were prepared as described in section 2. The hemolymph/hemolymph extract samples were analyzed using the radial diffusion assay on solid agar plates containing live *A. niger*. The antifungal activity as an amphotericin B equivalent. The diagrams present the results ±SD of three independent experiments. NH = hemolymph from untreated larvae; NE = hemolymph extract from untreated larvae.

The observation presented above was confirmed by testing the antibacterial activity by the bioautography method after electrophoretic separation of AMPs in polyacrylamide gels. The data presented in Fig. 6B showed *E. coli* growth inhibition zones in the hemolymph of the protease- and bacteria-challenged larvae (lane 2 and 5, respectively). Incubation of the same hemolymph samples in the presence of the proteolytic fraction, led to a significant decrease (lane 4 and 7) and complete abolition of the antibacterial activity (lane 3 and 6). The presented results indicate that *P. aeruginosa* alkaline protease, *i.e.,* the main proteinase of the clinical strain growing in minimal medium, may be responsible for degradation of antimicrobial peptides in *G. mellonella* hemolymph. It should be added that the other protein components of hemolymph involved in antibacterial activity (Fig. 6A) are highly sensitive to the alkaline protease activity, and this issue requires further investigations.

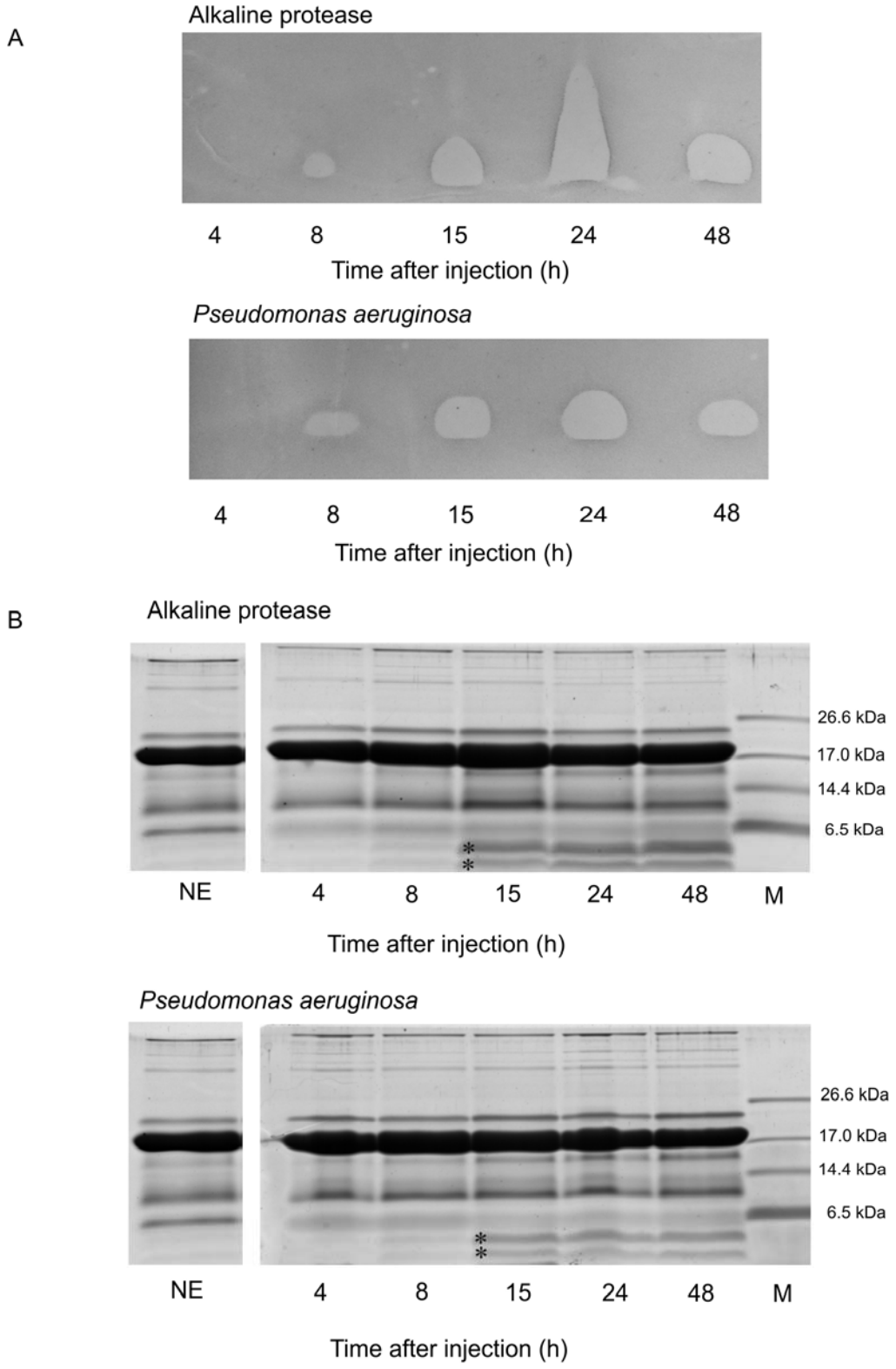


Fig. 5 (A) Bioautography of the hemolymph of *G. mellonella* larvae after injection of alkaline protease or heat-killed *P. aeruginosa* cells. The samples (120 µg of protein) collected at the indicated time points after injection were separated by glycine SDS-PAGE and after renaturation their antibacterial activity was detected by the gel overlay method as described in the Materials and Methods. (B) Analysis of peptide profiles in the hemolymph of alkaline protease- or *P. aeruginosa*-challenged *G. mellonella* larvae and control larvae. The methanolic extracts were prepared from the hemolymph collected at specified time points after injection. The proteins and peptides (20 µg of protein) were resolved by Tris-tricine SDS-PAGE and stained with Coomassie Brilliant Blue. The peptide bands appearing after the challenge are indicated by asterisks. NE = hemolymph extract from untreated larvae. M = molecular weight standards.

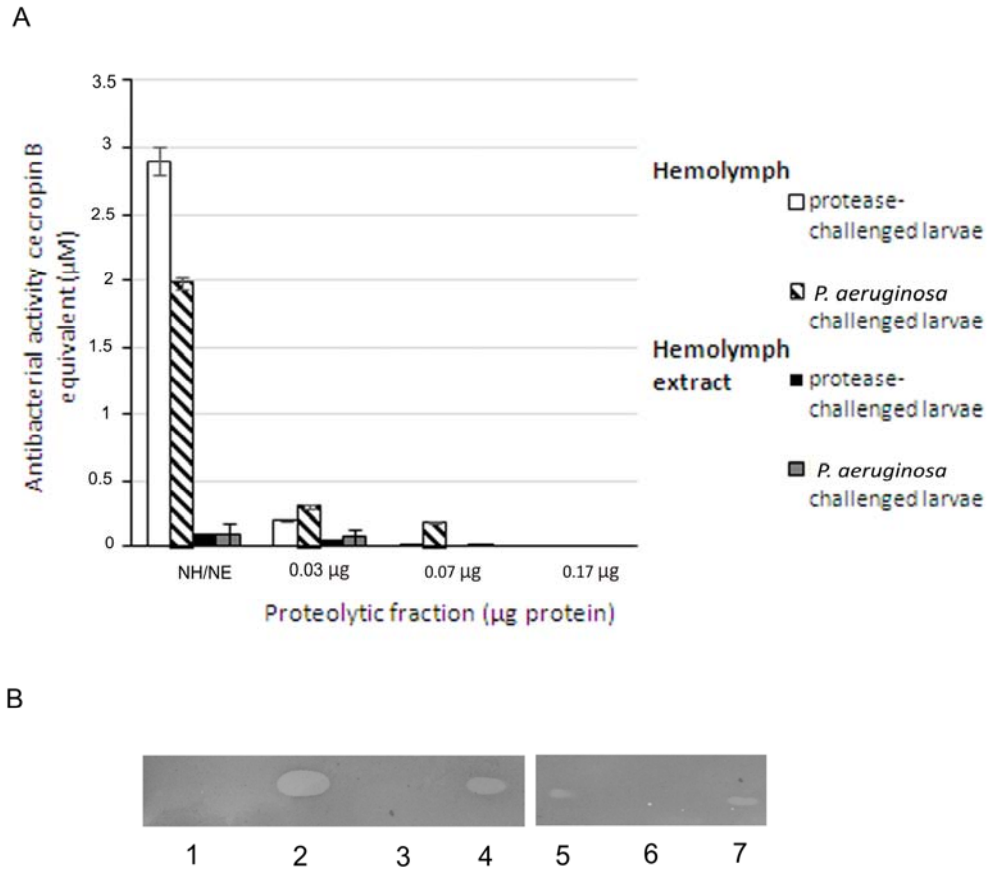


Fig. 6 The effect of *P. aeruginosa* alkaline protease on the antibacterial activity in *G. mellonella* hemolymph *in vitro*. (A) Immune hemolymph (120 μg of total protein) or hemolymph extracts (20 μg of total protein) were incubated without (control) or in the presence of the alkaline protease. Then, antibacterial activity was tested by the well diffusion assay and calculated as a cecropin B equivalent. The diagrams present the results ±SD of three independent experiments. (B) Bioautography. The samples of the hemolymph (120 μg of protein) of protease- or *P. aeruginosa*-challenged larvae were incubated with alkaline protease (0.03 and 0.17 μg) at 37 ^{o}C for 10 min. Then, the samples were separated by glycine SDS-PAGE and, after renaturation, their antibacterial activity was detected by the gel overlay method as described in the Materials and Methods. Hemolymph from untreated larvae (lane 1); hemolymph of protease-challenged larvae collected 24 h after the challenge (lane 2); hemolymph of protease-challenged larvae treated with the enzyme fraction (0.17 μg of protein) (lane 3); hemolymph of protease-challenged larvae treated with the enzyme fraction (0.03 μg of protein) (lane 4); hemolymph of *P. aeruginosa*-challenged larvae collected 24 h after the challenge (lane 5); hemolymph of *P. aeruginosa*-challenged larvae treated with the enzyme fraction (0.17 μg of protein) (lane 6); hemolymph of *P. aeruginosa*-challenged larvae treated with the enzyme fraction (0.03 μg of protein) (lane 7).

Discussion

It is known that induction of antimicrobial peptides and proteins in response to an immune challenge is one of the most important mechanisms of humoral defense in invertebrates (Hetru *et al*.,1998). In addition, it has been shown that hydrolysis of *G. mellonella* hemolymph proteins by collagenolytic enzymes, such as thermolysin, results in formation of small-sized protein fragments which elicit innate immune responses (Altancicek and Vilcinskas, 2006). It was demonstrated that the protein fragments and LPS induced expression of a similar spectrum of immune-related genes encoding antimicrobial peptides, such as gallerimycin, gloverin, IMPI and lysozyme (Altancicek *et al*., 2007). As demonstrated by us previously, *P. aeruginosa* elastase B was responsible for eliciting the humoral immune response in *G. mellonella* larvae (Andrejko and Mizerska-Dudka, 2011).

In the present study, we were interested to determine whether injection of other *P. aeruginosa* protease, namely alkaline protease mediates activation of *G. mellonella* innate immunity. The results of our *in vivo* studies showed that like the microbial elicitor (*P. aeruginosa*), the alkaline protease injected at a sublethal dose strongly induced antimicrobial activity in insect hemolymph. After metalloprotease injection, the appearance of antibacterial activity had similar kinetics to that obtained after the treatment with heat-killed *P. aeruginosa*. In contrast, the antifungal activity

appeared in hemolymph of the protease-challenged larvae much earlier and persisted longer at a high level than in the hemolymph of the bacteria-injected insects. This suggested that the metalloprotease treatment induced broader spectrum of antimicrobial peptides and proteins in comparison with the heat-killed bacteria.

The electrophoretic analysis of hemolymph peptides after protease treatment confirmed that the activation of innate immune response was correlated with subsequently synthesized antibacterial peptides. Two peptide bands with molecular mass below 6.5 kDa were observed in the hemolymph samples in response to the injected microbial elicitor or alkaline protease, but not in the hemolymph of the non-treated larvae.

It is known that the immune system of the Lepidoptera moth *G. mellonella* is able to distinguish between different classes of microorganisms and responds to the invading pathogen accordingly (Mak *et al*., 2010). It can be assumed that injection of microbial elicitors, i.e. Gram-negative bacteria *P. aeruginosa* or *E. coli*, to the hemocel leads to the synthesis of a similar set of peptides. However, further detailed research is required in the case of antimicrobial peptides appearing in the hemolymph of alkaline protease-challenged larvae.

It is commonly known that among the enzymes of human pathogenic bacteria and fungi, thermolysin-like metalloproteinases seem to play a predominant role during pathogenesis (Miyoshi and Shinoda, 2000; Altancicek *et al*., 2007). In our previous papers, we demonstrated that extracellular proteinases present in a *P. aeruginosa* culture supernatant degraded immune-relevant proteins and peptides in *G. mellonella* hemolymph *in vitro* (Andrejko *et al*., 2005; Andrejko *et al*., 2008, 2009; Andrejko and Mizerska-Dudka, 2012).

From this study, we concluded that *P. aeruginosa* alkaline protease may also be responsible for degradation/inactivation of inducible antimicrobial peptides in *G. mellonella* hemolymph. The results of series of experiments obtained with the well diffusion assay and with bioautography method for assessment of antimicrobial activity revealed inhibition of anti-*E. coli* activity in insect hemolymph incubated with alkaline protease.

As presented in this paper, the larvae treated with 0.8 µg of alkaline protease survived and antimicrobial activity was simultaneously detected in their hemolymph. It cannot be excluded that alkaline protease induces *G. mellonella* immune response in two ways: (i) it is recognized by the insect immune system as non-self and (ii) it can act as a danger signal and activate proteolytic cascades in the hemolymph. Similarly, the decrease in the antimicrobial activity observed 48 h after the challenge could be a result of: (i) silencing of the immune response by the insect immune system, or (ii) proteolytic activity of alkaline protease. The results of our study suggest that the first possibility is more probable, as the larvae injected with 0.8 µg of alkaline protease survived; however, the effects observed could be a result of both of these processes. Bacterial proteolytic enzymes seem to have a dual role in pathogenesis. In the initial phase of infection, they activate the insect immune system, thereby enhancing the antimicrobial activity in the hemolymph of infected insects. Next, as bacteremia progresses, proteolytic enzymes may degrade certain proteins/peptides e.g. defense peptides.

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