10.2478/v10067-011-0024-2

ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN – POLONIA

VOL. LXVI, 1 SECTIO C 2011

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Analysis of *Galleria mellonella* hemolymph proteins profile after metalloproteinase immune challenge

Analiza profilu białek hemolimfy *Galleria mellonella* immunizowanych metaloproteazami

SUMMARY

A comparison of the 2D gels of the metalloproteinase-immunized and control insects showed that elastase B and thermolysin were responsible for changes in the pattern of hemolymph proteins/peptides of *Galleria mellonella* larvae. It was found that the amount of apolp-III and lysozyme increased in response to injection of elastase B and thermolysin. Approximately 11 newly synthesized proteins/peptides were detected after metalloproteinase injection. We also observed that certain peptides were up-regulated depending on the metalloproteinases used. Some proteins/peptides which were present in the metalloproteinase-challenged larvae were absent in the hemolymph from the non-treated control larvae. They may be degradation products of hemolymph proteins treated with metalloproteinases.

STRESZCZENIE

Metodą elektroforezy dwukierunkowej wykazano zmiany w profilu białek i peptydów hemolimfy gąsienic *Galleria mellonella* immunizowanych elastazą B lub termolizyną. Stwierdzono zwiększoną ilość białka apoLp-III oraz lizozymu po iniekcji metaloproteinaz. W hemolimfie zaobserwowano 11 nowo zsyntetyzowanych białek/peptydów. Wykazano również, że niektóre białka/peptydy ulegały zwiększonej syntezie w zależności od zastosowanej proteazy. Niektóre białka/peptydy obecne w hemolimfie gąsienic immunizowanych metaloproteinazą nie występowały w hemolimfie gąsienic kontrolnych. Mogą to być produkty degradacji proteolitycznej białek hemolimfy.

K e y w o r d s: elastase B, *Pseudomonas aeruginosa*, immune response, *Galleria mellonella.*
S ł o w a k l u c z o w e: elastaza B, *Pseudomonas aeruginosa*, reakcja immunologiczna, *Galleria mellonella.*

INTRODUCTION

Metalloproteinases associated with human pathogenic bacteria and fungi have been playing a predominant role as virulence factors during pathogenesis (19, 23). They promote development within the infected host and interfere with its immune system (16, 17, 22, 24, 29). The therapeutic inhibition of metalloproteinases has become a novel strategy in the development of second-generation antibiotics (27, 28).

A thermolysin-like metalloproteinase released by the entomopathogenic fungus *Metarhizium anisopliae* and *Beauveria bassiana* inhibited phagocytic activity, attachment and spreading of plasmatocytes isolated from larvae of *G. mellonella* (13). *Bacillus thuringiensis* produces a metalloproteinase which degrades cecropin of the infected host (11). Recently, studies performed in our laboratory indicated that *P. aeruginosa* elastase B degraded inducible antimicrobial peptides in the hemolymph of *G. mellonella* (5). In addition, it was shown that elastase B stimulates the innate immune response in the greater wax moth (6).

The recognition system for a microbial pattern in *G. mellonella* is capable of sensing both microbial cell wall components, such as bacterial LPS, 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 which are absent in the host, e.g., microbial cell wall components, whereas the so-called danger model explains the activation of immune response by alarm signals from injured cells, such as those exposed to pathogens, toxins or mechanical damage (1, 21).

It was recently reported that the presence and activity of microbial metalloproteinases within the body of *G. mellonella* results in the formation of small peptides with molecular masses below 3 kDa, which are potent elicitors of innate immune responses (14). Peptidic fragments derived from hemolymph proteins or collagen IV serve as danger signals triggering a set of signalling pathways that lead to the induced expression of immune-related genes (2, 3, 4, 14).

In this study, we examined the changes in the pattern of hemolymph proteins/peptides in the metalloproteinase-challenged *G. mellonella* larvae.

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^{o} C in the dark. Last instar larvae (250–300 mg in mass) were selected for this study.

Pseudomonas aeruginosa strain ATCC 27853, the pyocyanin-producing strain, an isolate of moderate virulence to 7th instar larvae of *G. mellonella* (LD_{50}=17 cells) were used in this study. The bacterial cells were grown in nutrient broth for 24 h at 37^{o} C and pelleted by centrifugation at 20,000 g for 10 min at 4^{o} C.

Immune challenge

For immune challenge, the larvae were injected with 0.1 µg/larvae of elastase B (enzyme fraction in 10 mM ammonium acetate buffer) or 0.5 µg/larvae of thermolysin (Sigma) dissolved

in Ringer solution (172 mM KCl, 68 mM NaCl, 5 mM NaHCO3, pH 6.1). After the treatment, the larvae were kept at 30° C in the dark on sterile Petri dishes and the hemolymph was 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 melanisation. 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 (26). 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.

Purification of elastase B

Elastase B was isolated from *P. aeruginosa* growth medium. The cells were cultivated under aerobic conditions at 38° C for 20 h. Then the bacterial culture was centrifuged at 10,000 g for 60 min to pellet the cells. Then the supernatant was filtered through a 0.30-µm-pore-size filter (Millipore) to remove any remaining bacteria. The resulting clear supernatant was used as the starting material. Proteins secreted into the growth medium were precipitated from the filtrate with ammonium sulfate (90% of saturation). The precipitate was collected by centrifugation (20,000 g, 30 min, 4° C), dissolved in 10 mM ammonium acetate buffer (pH 6.6) and dialysed overnight against the same buffer. The dialysed solution containing 20 mg of protein was fractionated using ion-exchange chromatography. As the first step, anion-exchange chromatography on DEAE-cellulose column (DE 52, Whatman) was used. Proteins which did not bind to this resin were immediately fractionated by cation-exchange chromatography on phosphocellulose column (P-11, Whatman). Both columns were equilibrated with 10 mM ammonium acetate buffer. Proteins bound to the P-11 column were eluted with linear pH 6.6–9.0 gradient of the above buffer. The active fractions were pooled, concentrated on polyethylene glycol 20000 (PEG 20,000, dialysed overnight against 10 mM ammonium acetate buffer (pH 6.6) and stored in the same buffer containing 40% glycerol at -20° C.

Enzyme assay

The elastolytic activity was determined using a modification of the method by Caballero et al. (9). Elastin congo red 5 mg, (ICN Biomedicals Inc.) in 375 µl of 10 mM Tris-HCl (pH 8.0) with or without 6 mM EDTA was mixed with 5 µl enzyme solution (0.4 µg of protein) in 120 µl of the same buffer. The reaction mixture (500 µl) was incubated at 37° C for 24 h and centrifuged at 10,000 g for 15 min and the absorbance was recorded at 490 nm using a Smart-Spec TM 3000 (Bio-Rad) spectrophotometer.

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 (25). Protein bands were visualized by Coomassie Brilliant Blue R-250 staining.

Two-dimensional gel electrophoresis of hemolymph extract proteins

Two-dimensional gel electrophoresis was performed with 150 µg of hemocyte-free hemolymph extract protein using the Protean IEF focusing system (Bio-Rad) according to the manufacturer's recommendations. The sample was suspended in rehydratation buffer (8.8 mol/L urea; 2% W/V, CHAPS; 70 mmol/L DTT; 0.2 % W/V, Bio-Lytes) and loaded on 70 mm IPG strips (Bio-Rad). After separation of proteins in the first dimension, strips were equilibrated twice for 15 min in equilibration buffer (6 mol/L urea; 20% V/V, glycerol; 2% W/V, SDS; 375 mmol/L Tris-HCl, pH 8.8). The first step was done in equilibration buffer with 130 mmol/L DTT, the second equilibration buffer contained 135 mmol/L iodoacetamide. Then Tris-Tricine-SDS–PAGE was performed in 16.5% acrylamide gel under the conditions described above.

The concentration of proteins

The concentration of proteins was estimated by the Bradford method using bovine serum albumin (BSA) as a standard (7).

RESULTS AND DISCUSSION

The invertebrate immune response can be activated by danger signals from injured cells such as those exposed to mechanical damage, pathogens or toxins (21, 2). The special role in this model is played by microbial metalloproteinases which represent potent activators of innate immunity in insect (12). It is known that injection of bacterial thermolysin at a sublethal concentration into *G. mellonella* larvae mediates immune response (14). In our experiments, we used elastase B (pseudolysin, LasB), a thermolysin-like neutral zinc-metalloprotease which is an important virulence factor of the opportunistic human pathogen *P. aeruginosa* (Fig. 1).

Two-dimensional gel electrophoresis was employed to investigate the changes in the pattern of hemolymph proteins/peptides after injection of LasB or thermolysin at sublethal concentration. Acidic/methanolic extracts of cell-free hemolymph were used as a source of immune peptides/proteins (MATERIALS AND METHODS). Control hemolymph extract was obtained from the non-treated larvae. Antimicrobial peptides are mainly cationic peptides with molecular masses between 4 and 20 kDa. Therefore, in the first dimension we used IPG strips with pI values ranging from 3 to 11 and in the second dimension 16.5% Tris-Tricine-SDS-PAGE electrophoresis.

A comparison of the 2D gels of the metalloproteinase-immunized and control insects showed that the protein spot patterns changed greatly after injection. The most abundant protein in the hemolymph extracts was apoLp-III consisting of a few isoforms with a molecular mass of 18 kDa but a different isoelectric point

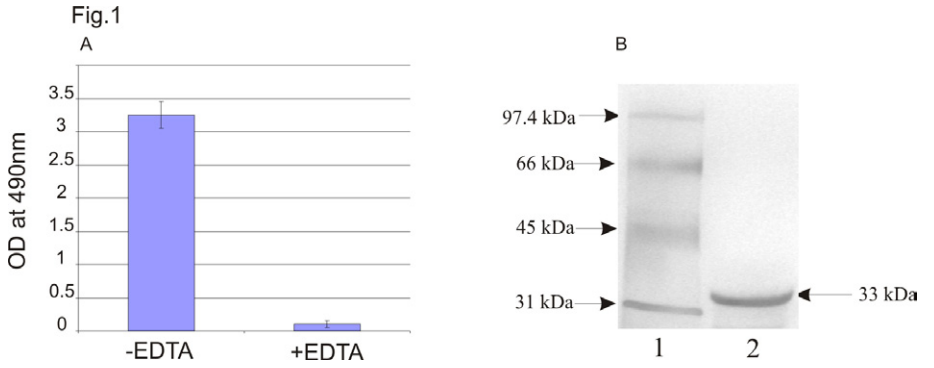

Fig. 1. Identification of elastase B in the culture supernatant of *P. aeruginosa*. A. Diagram represents proteolytic cleavage of elastin congo red by fraction obtained by ion-exchange chromatography on DEAE-cellulose and P-cellulose alone or in the presence of 6 mM EDTA. B. Proteins in the fraction were visualized by silver staining after glycine SDS-PAGE (lane 2). Low molecular mass standards are presented on lane 1. Each experiment was performed at least three times. Bars represent ±SD

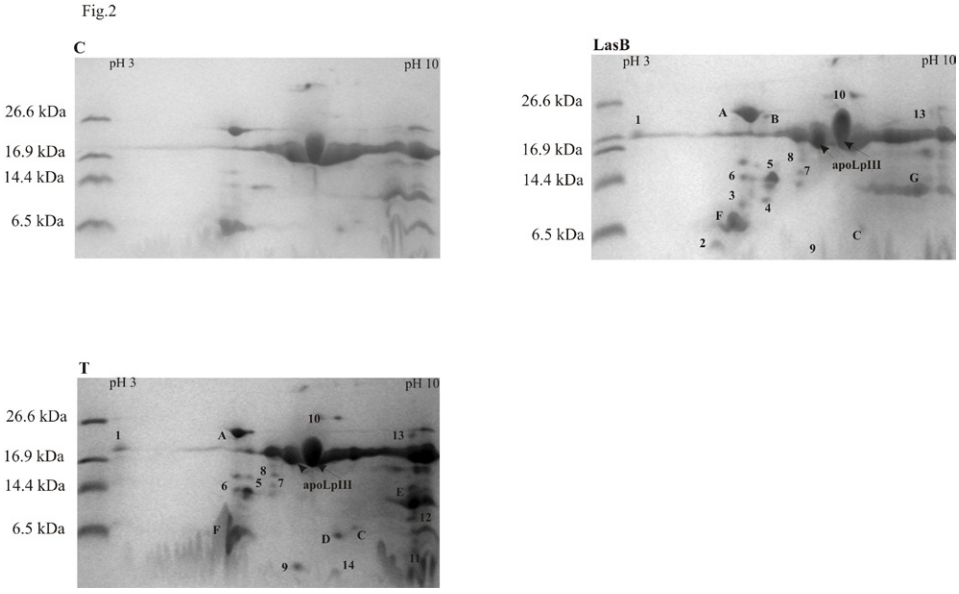

Fig. 2. Two-dimensional analysis of hemolymph extract proteins/peptides from (T) thermolysin-challenged (0.5 µg/larvae), (Las B) LasB-challenged (0.1 µg/larvae) or (C) untreated larvae. The samples (150 µg of protein) were separated according to their isoelectric points on immobilized 3–11 pH-gradients, then separated by 16.5% Tricine SDS/PAGE. The newly synthetised proteins/peptides are indicated by numbers (1–14). The differently regulated proteins/peptides are indicated by letters (A–G). Three independent experiments were carried out

(Fig. 2). It was found that the amount of 18 kDa protein increased in response to injection of elastase B or thermolysin (Fig. 2). These results are in line with our data reported previously (6). It was also 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 (30).

Approximately 11 newly synthesized proteins/peptides were detected after LasB (spots 1, 10–13) and thermolysin injection (spots 1, 5, 14) (Fig. 10).

Since most of the immune peptides appeared in the pH range of 4–11, the characteristic peptide with a molecular mass of 18 kDa and pI 3 drew our attention (spot 1 in Fig. 2) in the metalloprotease-immunized larvae. Attacins are a family of proteins, active against Gram-negative bacteria, with a molecular mass in the range 20–23 kDa (15). This peptide could therefore belong to this family.

Among the newly synthesized peptides, we observed a few peptides with a molecular mass below 6.5 kDa which could be induced antimicrobial peptides. According to the literature data, it can be conjectured that these peptides belong to the antimicrobial *G. mellonella* peptides (10). It is known that *G. mellonella* can release at least 18 antimicrobial peptides from 10 families to defend itself against invading microbes (8).

Spot 2 (4 kDa, pI 4) was observed after LasB challenge. This seems to be the Gm anionic peptide (with a molecular mass 4.8 kDa, pI 4.51) which exhibited antibacterial activity (*Micrococcus luteus*, *Listeria monocytogenes*) and was effective in inhibition of filamentous fungi growth (*Aspergillus niger*, *Trichoderma harzianum*) (10).

Upon thermolysin injection (Fig. 2) appearance of an immune peptide with a molecular mass of 4 kDa and pI 10 (spot 11) was observed. This may have been Gm proline-rich peptide 1 characterized by Cytryńska et al. (10), Lee et al. (18) and Mak et al. (20).

The induction of novel peptide with a molecular mass of 4 kDa and pI 6 (spot 9 in Fig. 10) was observed after injection of either LasB or thermolysin. There are at least three different peptides with a molecular mass 4 kDa and pI 6 isolated from immunized *G. mellonella* larvae, namely: defensin, a defensin-like, and a cecropin D-like peptide (10).

We also observed several differently regulated proteins in the hemolymph of the immunized larvae. It seems that certain peptides were up-regulated depending on the metalloproteinases used. Increased expression of 5 proteins/peptides was observed in the case of LasB injection (spots A, B, C, F ,G) as well as in the case of thermolysin in comparison with the control insects (A, C, D, E, F).

The molecular mass (14kDa) and its isoelectric point (9.3) of spot E in Figure 2 suggested that it was *Galleria* lysozyme. In this report, the level of lysozyme was greatly increased in the hemolymph thermolysin-challenged larvae.

Two-dimensional SDS-PAGE analysis of hemolymph samples from larvae injected with sublethal doses of bacterial metalloproteinases revealed changes in expression of a number of proteins associated with the immune response. It should be emphasized that some proteins/peptides which were present in the metalloproteinase-challenged larvae were absent in the hemolymph obtained from the non-treated control larvae. These results indicate that they may be degradation products of hemolymph proteins treated with metalloproteinases.

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