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SHORT COMMUNICATION

Immune response in the larvae of the black soldier fly *Hermetia illucens*

A Zdybicka-Barabas[1], P Bulak[2], C Polakowski[2], A Bieganowski[2], A Waśko[3], M Cytryńska[1]

[1]Department of Immunobiology, Institute of Biology and Biochemistry, Faculty of Biology and Biotechnology, Maria Curie-Skłodowska University, Akademicka 19 St., 20-033 Lublin, Poland
[2]Institute of Agrophysics, Polish Academy of Sciences, Doświadczalna 4 St., 20-290 Lublin, Poland
[3]Department of Biotechnology, Human Nutrition and Science of Food Commodities, University of Life Sciences in Lublin, Skromna 8 St., 20-704 Lublin, Poland

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Abstract

The black soldier fly *Hermetia illucens* is an ecological decomposer used for biodegradation of organic waste. Its larvae can develop on a wide range of decaying plant and animal matter, including manure and food scraps, *i.e*., habitats that are extremely rich in various microorganisms. Living in such conditions requires very well-functioning immune mechanisms. However, the immune response processes have not been examined so far in *H. illucens* larvae. In order to shed light on the immune system in the black soldier fly, in the present study we have examined *H. illucens* hemocytes and analyzed the effects of immune challenge of *H. illucens* larvae on the activity of the key components of insect humoral immune response, *i.e*., phenoloxidase, lysozyme, and antimicrobial peptides.

Key words: *Hermetia illucens*; innate immunity; hemocytes; antimicrobial peptides; lysozyme; phenoloxidase

Introduction

The black soldier fly *Hermetia illucens* is an ecological decomposer used for biodegradation of organic waste (Čičkova *et al*., 2015). Its larvae develop through six larval instars on a wide range of decaying plant and animal matter, including manure, food scrapes, municipal garbage, and rotting plant material (Sheppard *et al*., 1994, 2002; Diener *et al*., 2011). Living in an environment that is extremely rich in various microorganisms, including many pathogenic ones, requires a very well-functioning immune system.

Insect immunity relies on cellular and humoral innate mechanisms, which have been well characterized in *e.g., Drosophila melanogaster*, a dipteran model organism (Lemaitre and Hoffmann, 2007; Buchon *et al*., 2014; Kleino and Silverman, 2014; Lindsay and Wasserman, 2014; Cytryńska *et al*., 2016). The hemolymph cells, hemocytes, are involved in processes of the cellular immune response, *i.e.,* phagocytosis, nodulation, and encapsulation (Lavine and Strand, 2002; Dubovskiy *et al*., 2016). In addition to prohemocytes, three types of hemocytes, *i.e*., plasmatocytes, crystal cells, and lamellocytes were characterized in *D. melanogaster* (Ribeiro and Brehélin, 2006), whereas those in *Anopheles gambiae* and *Aedes aegypti* were classified as granulocytes and oenocytoids (Strand, 2008; Hillyer and Strand, 2014).

An important role in humoral immune response against pathogens is played by phenoloxidase (PO) as well as antimicrobial peptides and proteins. PO activity is a result of fast activation of the PO system by pathogen-associated molecular patterns (PAMPs), including components of microbial cell walls, *i.e.,* bacterial lipopolysaccharide (LPS), peptidoglycan (PGN), and fungal β-1,3-glucan (Cerenius *et al*., 2008; Bidla *et al*., 2009; Lu *et al*., 2014). PO activity leads to formation of quinones and other cytotoxic intermediates of melanin, and finally to melanin deposition at the site of injury and around invading pathogens. Melanin as well as cytotoxic intermediate products exhibit strong antimicrobial activity and prevent spreading of the pathogens in an insect organism (Suguraman, 2002; Lee and Miura, 2014). In addition to activation of the PO system, recognition of pathogens results in induction of synthesis of defense peptides able to kill the invaders. The inducible antimicrobial peptides are mainly synthesized in the insect fat body and released into hemolymph, where they are essential components of systemic immune response. Seven families of defense peptides with different biochemical and antimicrobial properties have been

Corresponding author:
Agnieszka Zdybicka-Barabas
Department of Immunobiology
Faculty of Biology and Biotechnology
Maria Curie-Skłodowska University
Akademicka 19 St. 20-033 Lublin, Poland
E-mail: barabas@poczta.umcs.lublin.pl

described in *D. melanogaster, i.e.,* attacins, cecropins, insect defensin, diptericins, drosocin, drosomycins, and metchnikowin (Uvel and Engström, 2007). In other insect species, a number of various defense peptides have been characterized to date, including anionic antimicrobial peptides (Cytryńska *et al*., 2007a; Scocchi *et al*., 2011; Mylonakis *et al*., 2016). Beside the defense peptides, hemolymph lysozymes play an important role in antimicrobial immune response in insects largely due to enzymatic muramidase activity (Hultmark, 1996). Lysozymes are usually constitutive components of insect hemolymph; however, they can act synergistically with defense peptides, and pathogen recognition may also result in a dramatic increase in their level and activity in hemolymph, which contributes considerably to effective antimicrobial defense (Yu *et al*., 2002; Chapelle *et al*., 2009; Zhang *et al.*, 2009; Zdybicka-Barabas *et al.*, 2012, 2013; Sowa-Jasiłek *et al*., 2014; Beckert *et al*., 2015).

The habitat of *H. illucens* larvae, which is extremely rich in various microorganisms, implies that the immune system of this insect species functions very efficiently and effectively. Recently, Park *et al.* (2015) have reported on purification and characterization of a *H. illucens* defensin-like peptide with activity against Gram-positive bacteria. However, although *H. illucens* larvae are used in composting piles, the immune response processes in these insects have not been examined so far and there are no data currently available in this area. In order to shed light on the immune system in the black soldier fly, in the present study we have examined *H. illucens* hemocytes and analyzed the effects of immune challenge of *H. illucens* larvae on activity of the key components of insect humoral immune response, *i.e*., phenoloxidase, lysozyme, and antimicrobial peptides.

Materials and Methods

Insect culture conditions

The larvae of *Hermetia illucens* (Diptera: Striatomyidae) were reared in laboratory conditions at 29 ^{o}C and substrate humidity of 50 - 80 % in the Institute of Agrophysics of the Polish Academy of Sciences in Lublin, Poland. The larvae were fed with feed consisting of protein 25 %, fat and oil 5 %, crude fiber 5.8 %, ash 5.7 %, lysine 1.25 %, calcium 1 %, phosphorus 0.97 %, methionine 0.4 %, and sodium 0.05 %. Last instar larvae were used in the experiments.

Microorganisms

Gram-negative bacterium *Escherichia coli* D31 and Gram-positive bacterium *Micrococcus luteus* ATCC 10240 were grown in LB (Lysogeny Broth) at 37 °C and 28 °C, respectively, until the logarithmic growth phase. Filamentous fungus *Aspergillus niger* was grown on solid PDA medium (5 % potato extract, 0.5 % dextrose, 1.7 % agar) at 28 ^{o}C until conidial spores were obtained and then stored at 4 ^{o}C. A conidial suspension for the antifungal activity assay (see below) was prepared as described in our previous paper (Mak *et al*., 2010).

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

An immune challenge with live Gram-negative bacterium *E. coli* or Gram-positive bacterium *M. luteus* was performed with a piercing method, essentially as described previously for *Galleria mellonella* larvae (Mak *et al*., 2010). Before immunization, the larvae were washed with sterile water and the puncture sites were disinfected with 70 % ethanol. The larvae were punctured with a sterile needle (control) or with a needle dipped in a pellet of live bacteria (40 larvae per group). Next, the larvae were stored in Petri dishes provided with food or in sterile conditions, and the hemolymph was collected 6 h, 24 h and 48 h after the treatment as well as from the unchallenged (naive) larvae. The hemolymph (5 µl per larva) was combined to obtain pooled samples. Hemocyte-free hemolymph was obtained by centrifugation at 200x*g* for 5 min and subsequently at 20,000×*g* for 15 min at 4 °C (Mak *et al*., 2010).

An acidic/methanolic extraction method was used for partial purification of antimicrobial peptides. The hemolymph was diluted ten times with the extraction solution (methanol/acetic acid, glacial/water; 90:1:9), mixed thoroughly, and centrifuged (20,000×*g*, 30 min 4 ^{o}C) in order to pellet precipitated proteins. The supernatant containing mainly proteins of M_r below 30kDa and peptides was collected and vacuum dried, and the pellet was stored at -20 ^{o}C until needed (Cytryńska *et al*., 2007a; Mak *et al*., 2010).

Antimicrobial activity assays

Well diffusion assay

The hemolymph antibacterial activity against *E. coli* D31 and *M. luteus* was detected by a growth inhibition zone assay on LB agar plates, essentially as reported (Mak *et al*., 2010). To improve the sensitivity of the method against Gram-negative bacteria, egg white lysozyme (EWL) at the final concentration of 2.5 mg/ml was added (Cytryńska *et al*., 2001). The hemolymph antifungal activity was detected using PDA agar plates containing *A. niger* conidia, as described previously (Mak *et al*., 2010). Lysozyme activity in the hemolymph was estimated using agarose plates containing freeze-dried *M. luteus* as reported (Jarosz, 1995). The activity of lysozyme was calculated from a standard curve made with egg white lysozyme (EWL, EC 3.2.1.17; Sigma-Aldrich).

Each well on the Petri dish was filled with 4µl of five times diluted hemolymph and the plates were incubated at 37 ^{o}C (*E. coli*) or 28 ^{o}C (*M. luteus*, *A. niger*). The diameters of bacterial and fungal growth inhibition zones were measured after 24-h and 48-h incubation, respectively. The level of anti-*E. coli* activity was calculated using the algorithm described previously (Hultmark *et al*., 1982) with cecropin B of *Hyalophora cecropia* (Sigma-Aldrich) as a standard.

Bioautography (SDS gel overlay method)

Detection of antibacterial activity in the hemolymph after SDS/PAGE and subsequent renaturation of polypeptides was performed as described previously (Cytryńska *et al*., 2001).

Briefly, after separation of proteins (300 µg of total protein per sample), the gels were washed in 2.5 % Triton X-100 (Bio Rad) for removal of SDS. Next, the polypeptides were renatured in 50mM Tris-HCl pH 7.5 and subsequently in LB. Finally, the gels were overlaid with nutrient agar containing *E. coli* D31 cells and 2.5 mg/ml EWL, and the zones of bacterial growth inhibition were observed after incubation for 6 - 12 h at 37 ^{o}C.

Phenoloxidase activity assay

The phenoloxidase activity in the hemolymph was determined on the basis of melanin formation according to the method described previously (Park *et al.*, 2005; Zdybicka-Barabas *et al.*, 2014). Briefly, 2 µl of non-diluted hemolymph was added to 18 µl of TBS (50 mM Tris-HCl pH 7.4, 150 mM NaCl) containing 5mM $CaCl_2$ in the wells of a 96-well plate and the mixture was incubated for 20 min at room temperature. Next, 180 µl of 2 mM dopamine in 50 mM sodium phosphate pH 6.5 was added and absorbance of the mixture was measured at 490 nm over 90 min at a 5-min interval using a microtiter plate reader (BioRad). The experiment was performed in triplicate on three independent occasions.

Other methods

Polyacrylamide gel electrophoresis of proteins was performed by 13.8 % glycine SDS/PAGE according to Laemmli (1970). Separation of proteins below 30 kDa and peptides was carried out by tricine SDS/PAGE (16.5 % T, 3 % C) (Schägger and von Jagow, 1987). The proteins and peptides were stained using Coomassie Blilliant Blue R-250 (0.25 %) or G-250 (0.025 %), respectively, after glycine SDS/PAGE and tricine SDS/PAGE. The protein concentration was estimated by the Bradford method using bovine serum albumin (BSA) as a standard (Bradford, 1976). For microscopic observations of hemocytes, the samples of freshly collected hemolymph (5µl) were placed onto microscopic slides, covered with coverslips, and the hemocytes were observed immediately using a contrast-phase microscope Olympus BH-2 (lens magnification 40×).

Results and Discussion

H. illucens *hemocytes*

The microscopic examination of the hemolymph revealed the presence of at least three types of morphologically different hemocytes, i.e. crystal cell-like, plasmatocyte-like, and granule-containing hemocytes (Fig. 1A). The oval shaped crystal cell-like hemocytes (approx. dimensions 18 µm × 15 µm) were non-adherent cells and contained evident crystal-like inclusions (approx. 12 µm in length and 2.5 µm in width), similar to those reported in *D. melanogaster* crystal cells (Ribeiro and Brehélin, 2006). Based on these characteristics, these cells may be involved in the melanization process. The two other types were adherent cells that formed filopodia-like projections, a feature that implicates their engagement in cellular immune response processes. The plasmatocyte-like cells were morphologically similar to some lepidopteran plasmatocytes (Cytryńska *et al.*, 2007b; Hori *et al.*, 2013; Wu *et al.*, 2016) and exhibited a tendency to aggregate (Fig. 1B). The granule-containing hemocytes resembled morphologically *Culex pipiens quinquefasciatus* granulocytes. Interestingly, in *C. pipiens quinquefasciatus*, a dipteran species, three hemocyte types, *i.e.,* oenocytoids, plasmatocytes, and granulocytes, were identified beside prohemocytes (Wang *et al.*, 2011). Although the *H. illucens* granule-like cells resembled *C. pipiens quinquefasciatus* granulocytes, the plasmatocyte-like cells were morphologically distinct from those in *C. pipiens quinquefasciatus*. In addition, hemocytes with undefined features were observed (Fig. 1B). Notably, nodule-like structures with a diameter approx. 25 - 35 µm were observed in the hemolymph of the bacteria-challenged *H. illucens* larvae (Fig. 1C), suggesting that nodulation may be one of the cellular immune response processes involved in fast elimination of invaders in *H. illucens* larvae.

Phenoloxidase activity in H. illucens *hemolymph*

The immune challenge of *H. illucens* larvae with the Gram-negative and Gram-positive bacteria led to a considerable increase in hemolymph PO activity. In comparison with the level of PO activity in the hemolymph of naive larvae (control), the enzyme activity increased 1.27-fold and 1.6-fold after the challenge with *E. coli* and *M. luteus*, respectively (Fig. 2A). Interestingly, the PO activity after sterile puncturing was by approx. 22 % lower than in the control hemolymph. If one takes this into consideration, the PO activity level after the challenge with *E. coli* and *M. luteus* was 1.6-fold and 2-fold higher, respectively (Fig. 2A). In addition to the changes in the hemolymph PO activity, effects of local PO activation that led to melanin deposition at the site of injury were observed on the surface of larval body (Fig. 2B). The results clearly indicate an important role of PO activity in *H. illucens* immune response against invading Gram-negative and Gram-positive bacteria.

Antimicrobial activity in H. illucens *hemolymph*

In addition to the evident changes in the PO activity level, the immune challenge of the *H. illucens* larvae induced antimicrobial activity in the hemolymph. The lysozyme and anti-Gram-positive bacterium *M. luteus* activity, both detected in the hemolymph of the naive larvae, increased considerably after the challenge and reached the highest level in the *E. coli*-challenged larvae (Tables 1, 2). In contrast, antibacterial activity measured against Gram-negative bacterium *E. coli* D31 was detected only in the hemolymph of the challenged larvae. It was induced by the bacterial challenge and by the sterile puncturing of the larvae. The level of anti-*E. coli* activity in the hemolymph of the sterile punctured as well as *M. luteus*- and *E. coli*-challenged larvae corresponded to the activity of 0.34 µM, 0.55 µM, and 1.2 µM of a cecropin B solution, respectively (Table 2). No antifungal activity against *A. niger* was detected in *H. illucens* hemolymph in our experimental conditions. On the other hand, the lysozyme activity in the hemolymph of naive larvae suggests constitutive synthesis of

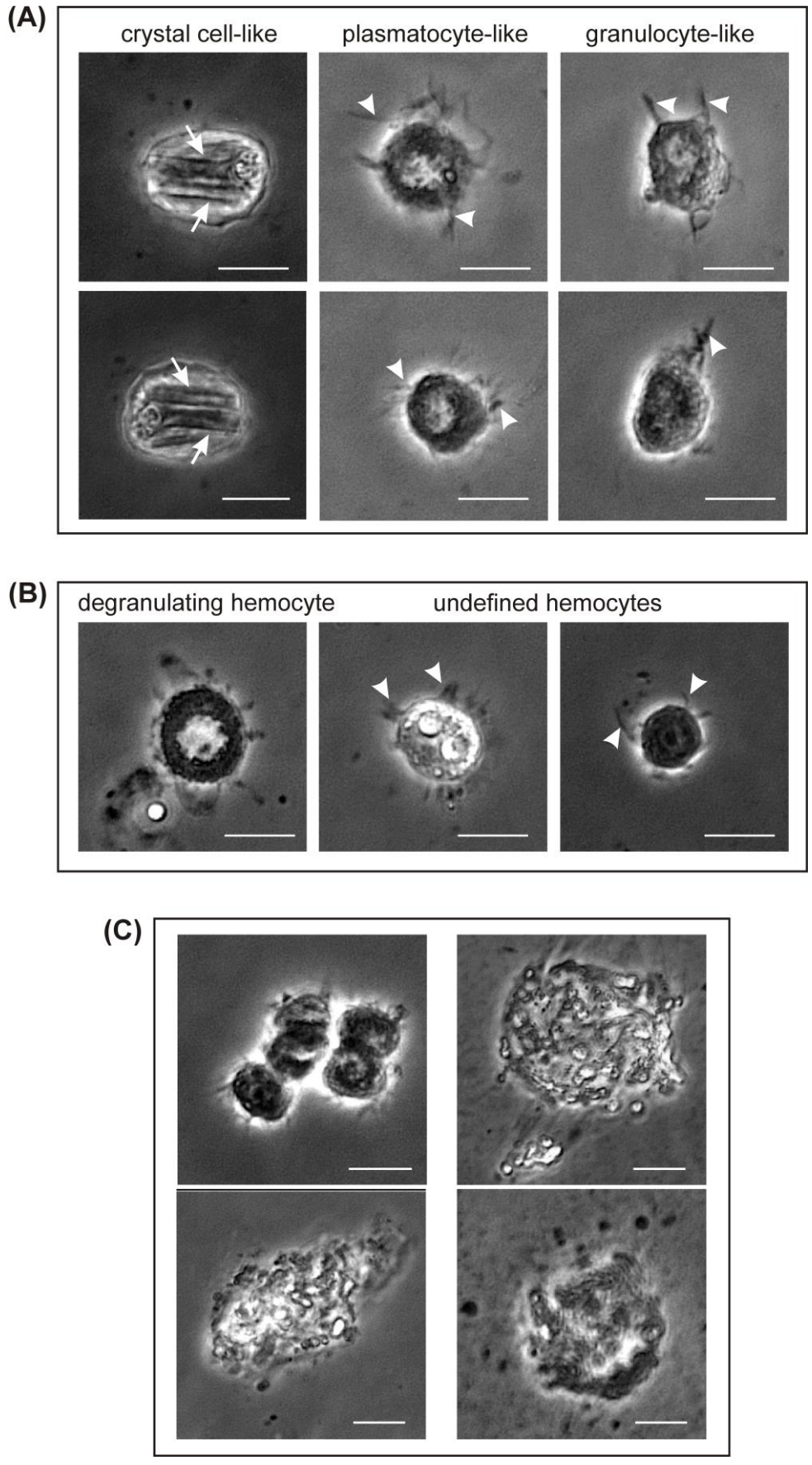

Fig. 1 *Hermetia illucens* hemocytes. The hemocytes (A, B) and cell aggregates or nodules (C) were observed in a contrast-phase microscope Olympus BH-2. The white arrows and arrowheads indicate, respectively, crystal-like intrusions and filopodia-like projections. Bar = 10 µm.

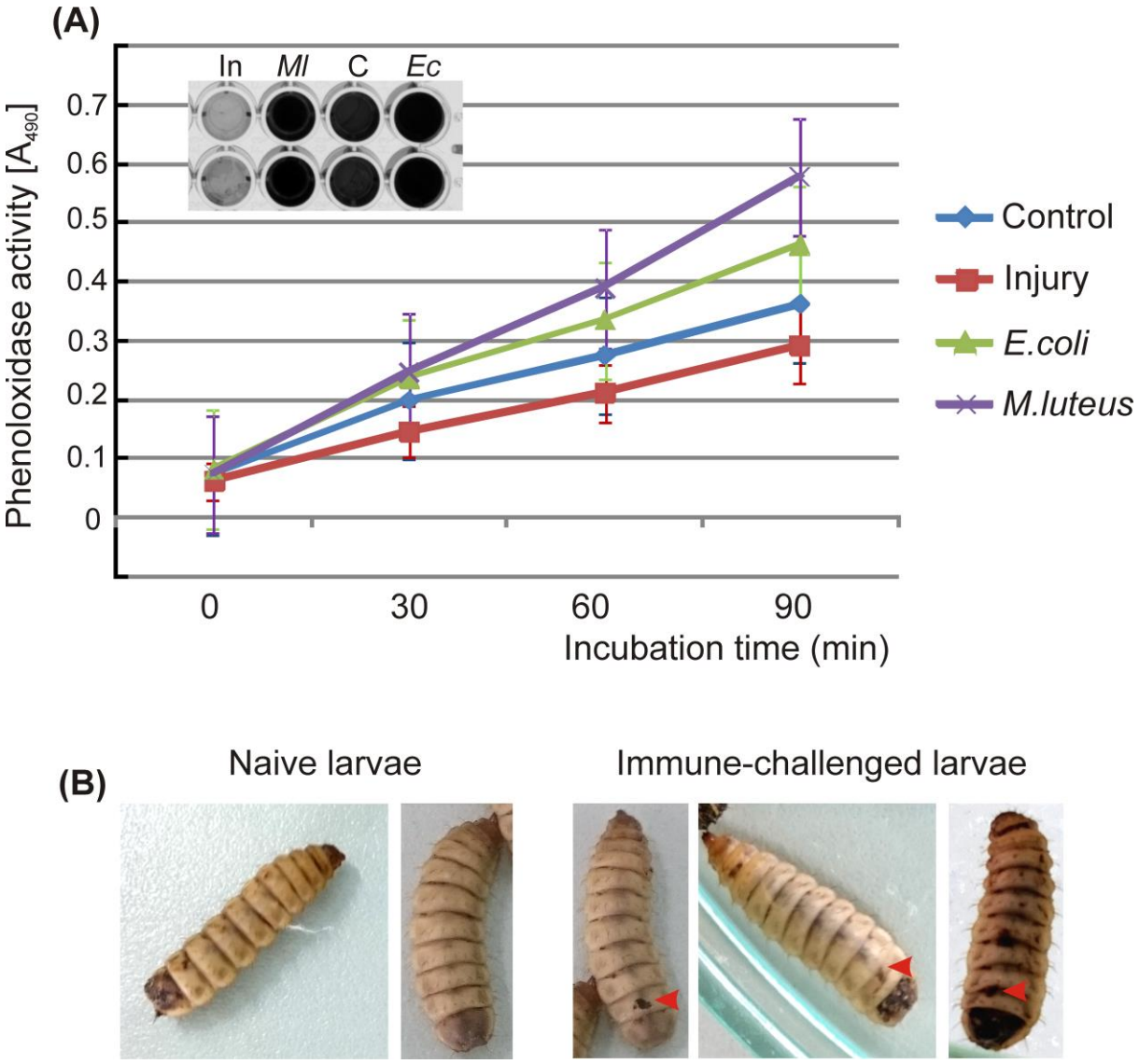

Fig. 2 Phenoloxidase activity in *H. illucens* larvae. (A) PO activity in the hemolymph. The larvae were immunized with sterile injury or bacteria-challenged, and the phenoloxidase activity was determined in the hemolymph collected 24 h after the treatment. The results are presented as ±SD from three independent experiments. The inset demonstrates melanin formed in the wells after 90 min incubation with DOPA as a substrate. C - naive larvae; In - sterile-injured larvae; *Ml* and *Ec* - *M. luteus*- and *E. coli*-challenged larvae, respectively. (B) Melanization localized at a site of injury is indicated by the red arrowheads.

this antibacterial factor in *H. illucens* larvae and its role in elimination of Gram-positive bacteria (*e.g., M. luteus*). It should be noted that a considerable increase in the lysozyme activity and induction of anti-*E. coli* activity was detected in the hemolymph of the larvae stored in the non-sterile conditions (*i.e.*, provided with food) after the sterile puncturing (Table 2). Such conditions reflect a possibility of invasion of pathogens present in food through mechanical injury in the natural habitat of *H. illucens* larvae. The results also indicate great adaptation of the *H. illucens* immune system to fight against pathogens.

Interestingly, when the immune-challenged larvae were incubated in the sterile conditions, the challenge with *E. coli* induced the lysozyme, anti-*M. luteus,* and anti-*E. coli* activity, whereas the challenge with sterile puncturing and *M. luteus* caused only an increase in the lysozyme and anti-*M. luteus* activity. In the hemolymph of the sterile punctured and *M. luteus*-challenged larvae, no anti-*E. coli* activity was detected (Table 1). This suggests discrimination between Gram-positive and Gram-negative bacteria by the *H. illucens* immune system, similarly to *D. melanogaster*, in which such a phenomenon is well documented (Goto

Table 1 Antimicrobial activity in the hemolymph of *H. illucens* larvae kept in sterile conditions

Antimicrobial activity	Time after challenge (h)	Experimental group			
		Control larvae	Sterile- injured larvae	*M. luteus*-challenged larvae	*E. coli*-challenged larvae
Lysozyme activity [µg/ml]	6	2.24±0.2	2.82±0.18	3.08±0.18	4.08±0.27
	24	2.63±0.27	3.98±0.7	12.59±0.8	20.42±0.3
	48	3.98±0.95	6.03±1.1	4.47±0.34	8.51±0.4
Anti-*M. luteus* activity (mm)	6	12.6±0.3	16.4±0.1	16.0±0.15	16.3±0.3
	24	10.4±0.25	17.0±0.2	18.6±0.25	20.4±0.15
	48	12.9±0.2	16.4±0.3	17.2±0.1	18.0±0.5
Anti-*E. coli* activity [µM]	6	nd	nd	nd	1.3±0.26
	24	nd	nd	nd	1.66±0.42
	48	nd	nd	nd	nd

H. illucens larvae were sterile-injured or immunized with *E. coli* or *M. luteus* and kept in sterile conditions for 48 h. The hemolymph was collected 6 h, 24 h, and 48 h after the challenge. Next, the lysozyme, anti-*M. luteus,* and anti-*E. coli* activity was determined by a well diffusion assay. The results are presented as ±SD from three independent experiments. nd = not detected

and Kurata, 2006; Silverman *et al*., 2009; Reumer *et al*., 2010).

The hemolymph antibacterial activity estimated against *E. coli* with the diffusion well assay was determined by the presence of inducible antimicrobial peptides with molecular mass corresponding to cecropin B, as revealed by bioautography (Fig. 3A) and tricine SDS/PAGE of the hemolymph methanolic extracts (Fig. 3B). Electrophoretic analysis of hemolymph proteins indicated that, despite induction of defense peptides, the bacterial challenge of *H. illucens* larvae led to appearance of an additional protein with molecular mass approx. 43 kDa. Interestingly, the *E. coli* challenge resulted in appearance of two other proteins with molecular masses approx. 58 kDa and 27 kDa (Fig. 3C). Determination of the identity of these proteins and their role in *H. illucens* immune response requires further study.

In conclusion, it is important to note that the immune challenge of *H. illucens* larvae with Gram-positive *M. luteus* induced only anti-Gram-positive bacterial activity, possibly resulting from increased lysozyme activity. In contrast, in the hemolymph of larvae immunized with Gram-negative *E. coli*, besides anti-Gram-positive bacterial activity also anti-Gram-negative bacterial activity was detected. This is consistent with the data reported recently (Park *et al*., 2015). These authors demonstrated that a defensin-like peptide purified from the hemolymph of *H. illucens* larvae challenged with Gram-positive *Staphylococcus aureus* was active against Gram-positive bacteria and not against Gram-negative ones (Park *et al*., 2015). Our results suggest that, depending on the bacteria used for the immune challenge (containing diaminopimelic-type PGN or lysine-type PGN in the cell wall), synthesis of different sets of antimicrobial peptides is induced in *H. illucens*, as in the case of *D. melanogaster* (Silverman *et al*., 2009; Lindsey and Wasserman, 2014; Kleino and Silverman, 2014) and *G. mellonella* (Mak *et al*., 2010) reported previously.

Table 2 Antimicrobial activity in the hemolymph of *H. illucens* larvae kept in non-sterile conditions

Experimental group	Antimicrobial activity	
	Lysozyme activity [µg/ml]	Anti-*E. coli* activity [µM]
Control larvae	7.2±3.6	nd
Sterile-injured larvae	11.47±0.73	0.34±0.06
M. luteus-challenged larvae	26.12±5.84	0.55±0.09
E. coli-challenged larvae	35.48±10.83	1.2±0.48

H. illucens larvae were sterile-injured or immunized with *E. coli* or *M. luteus* and kept in non-sterile conditions for 24 h. Next, the hemolymph was collected, and the lysozyme and anti-*E. coli* activity was determined by a well diffusion assay. The results are presented as ±SD from three independent experiments. nd = not detected.

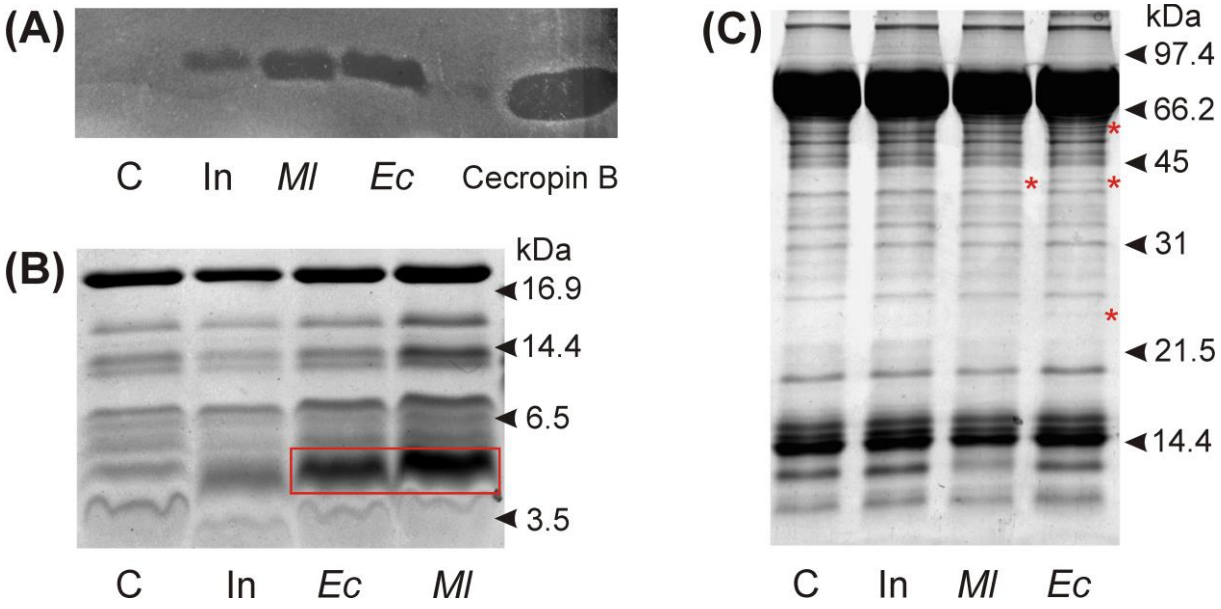

Fig. 3 Antimicrobial activity (A) and changes in peptide (B) and protein (C) patterns in the hemolymph of immunized *H. illucens* larvae. The hemolymph was collected from naive larvae (control - C) and from sterile-injured (In), *M. luteus* (*Ml*)-, or *E. coli* (*Ec*)-challenged larvae 24 h after the treatment. (A) Antimicrobial activity detected by bioautography after separation of the hemolymph polypeptides (300 µg of total protein) in 13.8 % polyacrylamide gel and subsequent renaturation. Cecropin B (1µg) was used as a control peptide. A gel fragment presenting clear zones (darker areas) of *E. coli* growth inhibition is shown. (B) Electrophoretic analysis of *H. illucens* hemolymph peptides. The peptides extracted from 5 µl of the hemolymph with the acidic/methanolic extraction method were diluted in 20 µl of sample buffer and separated by tricine SDS/PAGE. Additional peptide bands appearing in the larval hemolymph after the bacterial challenge are encircled by a red frame. (C) Electrophoretic analysis of *H. illucens* hemolymph proteins. The hemolymph samples (40 µg of total protein) were prepared in 20 µl of Laemmli sample buffer and separated by SDS/PAGE in 13.8 % polyacrylamide gels. Additional protein bands appearing in the larvae hemolymph after bacterial challenge are indicated by red asterisks.

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