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Phenoloxidase activity in hemolymph of *Galleria mellonella* larvae challenged with *Aspergillus oryzae*

Aktywność oksydazy fenolowej w hemolimfie gąsienic *Galleria mellonella* infekowanych *Aspergillus oryzae*

SUMMARY

Immunization of *Galleria mellonella* larvae with filamentous fungus *Aspergillus oryzae* led to increase of phenoloxidase (PO) activity in hemolymph 24–72 h after treatment. In acidic/methanolic extracts of hemolymph collected from fungi-challenged insects additional peptide bands of molecular mass below 6.5 kDa were detected. When PO activity was determined in the presence of the extracts considerable decrease in the studied activity was noticed. We postulated that immune challenge of *G. mellonella* larvae with *A. oryzae* induced synthesis and/or release from storage compartments of peptides with inhibitory activity towards components of proPO system.

STRESZCZENIE

Immunizacja gąsienic *Galleria mellonella* zarodnikami konidialnymi grzybów nitkowatych *Aspergillus oryzae* powodowała wzrost poziomu aktywności oksydazy fenolowej w hemolimfie 24–72 godziny po podaniu immunogenu. W ekstraktach metanolowych hemolimfy owadów infekowanych *A. oryzae* stwierdzono obecność dodatkowych peptydów o masie cząsteczkowej poniżej 6,5 kDa. Wykazano, że składniki ekstraktów hemolimfy powodowały obniżenie poziomu aktywności oksydazy fenolowej w hemolimfie *G. mellonella*. Uzyskane wyniki wskazują, że immunizacja zarodnikami *A. oryzae* indukuje pojawianie się w hemolimfie peptydów hamujących aktywność układu oksydazy fenolowej *G. mellonella*.

Key words: *Galleria mellonella*, *Aspergillus oryzae*, phenoloxidase, innate immunity

List of abbreviations: DOPA – 3,4-dihydroxyphenylalanine; ISPI – inducible serine protease inhibitor; PAMPs – pathogen associated molecular patterns; PO – phenoloxidase; proPO – prophenoloxidase; PRRs – pattern recognition receptors; PTU – N-phenylthiourea; SDS/PAGE – sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

INTRODUCTION

Phenoloxidase (PO) is a copper-containing enzyme catalyzing the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones. Quinones are then converted on the pathway leading to formation of the brown-black pigment, melanin. In the insects, quinones and melanin are involved in the defence reactions against pathogens invading the hemocel, such as nodule formation and encapsulation. Melanin is also engaged in wound healing and cuticule sclerotization. Phenoloxidase exists in insect hemolymph or hemocytes as a proenzyme called prophenoloxidase (proPO). Conversion of proPO into its active form, phenoloxidase, needs limited proteolysis carried out by a serine proteinase (proPO-activating enzyme or proPO-activating proteinase), a member of a Ca^{2+}-dependent serine proteinase cascade known as proPO activating system. Prophenoloxidase activation and PO activity have to be tightly regulated due to high cytotoxicity of quinones and other melanization intermediates not only for pathogen cells but also for the host cells. The system is triggered by the pathogen associated molecular patterns (PAMPs), e.g. bacterial or fungal cell wall components (lipopolysaccharide, peptidoglycan, β-1,3-glucan) recognized by appropriate pattern recognition receptors. In the lepidopteran insects, recognition of PAMPs lead to release of the proPO system components from hemocytes called oenocytoids into the hemolymph. It was documented that simultaneously serine proteinases inhibitors (serpins) involved in the regulation of the proteinase cascade are released (Cerenius et al. 2008; Kanost et al. 2004; Lu, Jiang 2007).

Pye (1974) demonstrated activation of the proPO system in the hemolymph of *Galleria mellonella* larvae challenged with bacterial lipopolysaccharide and prophenoloxidase was purified and characterized by Kopáček et al. (1995). More recently it has been demonstrated that two hemolymph proteins, namely apolipophorin III and Gm protein-24, are involved in activation of proPO cascade in *G. mellonella* (Park et al. 2005). The proenzyme was histochemicaly detected in *G. mellonella* eonocytoids and granulocytes using dihydroxyphenylalanine (DOPA) as a substrate (Schmit et al. 1977). Literature data indicated that PO levels are elevated in response to injection of fungal components such as blastospores and conidiospores (Gillespie et al. 2000; Gillespie, Khachatourians 1992). In *Lymatria dispar* challenged with *Entomophaga* spp. activation of proPO was observed (Bidochka, Hajek 1998). It was also reported that infection of *Spodoptera exigua* with *Beauveria bassiana* caused alterations in the level and distribution of PO activity (Hung, Boucias 1992). Interestingly, Slepneva et al. (2003) demonstrated a decrease in DOPA-semiquinone and DOPA-quinone production in hemolymph of *G. mellonella* larvae infected with *Metarhizium anisopliae* which was correlated with inhibition of phenoloxidase activity in hemolymph (Slepneva et al. 2003).

In the present study we investigated alterations of PO activity in hemolymph of *G. mellonella* larvae challenged with spores of filamentous fungus *Aspergillus oryzae*.

MATERIAL AND METHODS

Biological species

Larvae of the greater wax moth *Galleria mellonella* (Lepidoptera: *Pyralidae*) were reared on honey bee nest debris at 28°C in the dark. Last instar (seventh) larvae were used throughout the study.

Filamentous fungus *Aspergillus oryzae* CBS 133.52 was grown at 28°C on PDA slides (Potato dextrose agar) until sporulation and then stored at 4°C. Gram-negative bacterium *Escherichia coli* D31 was grown at 37°C in liquid LB medium (Luria Bertani Broth) till late logarithmic phase.

Insect immune challenge, hemolymph collection and preparation of hemolymph extracts

Immune challenge was performed by piercing of the larval abdomen with a needle dipped in a pellet of *A. oryzae* spores or *E. coli* cells. The insects were kept at 28°C in the dark and the hemolymph (20 ml per larvae) was collected 24, 48, 72 and 96 h after immunization with *A. oryzae* and 24 h after challenge with *E. coli*. Prior to hemolymph collection, the insects were chilled for 15 min at 4°C. The hemolymph samples were obtained by puncturing of larval abdomen with a sterile needle. 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 20000×*g* for 15 min at 4°C. Pooled supernatants were used immediately or stored at -20°C until used.

Proteins of molecular mass below 30 kDa and peptides were partially purified from the hemocyte-free hemolymph by acidic/methanolic extraction method as described previously (Cytryńska et al. 2007). Briefly, the hemolymph was diluted ten times with the extraction solution consisting of methanol/acetic acid, glacial/water (90:1:9) and mixed thoroughly. Precipitated proteins were pelleted by centrifugation at 20000×*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 appropriate volume of water (usually the water volume was 2/3 of the initial hemocyte--free hemolymph volume).

Phenoloxidase activity assay

Activity of phenoloxidase was determined in hemolymph of non-challenged as well as *A. oryzae*- and *E. coli*-challenged *G. mellonella* larvae according to the method described previously (Park et al. 2005). Two µl of acidic/methanolic extract of hemolymph (1 µg of total protein) in TBS (50 mM Tris-HCl pH 6.8, 1 mM NaCl) was combined with 2 µl of hemolymph five times diluted in TBS and 16 µl of TBS containing 5 mM $CaCl_2$ in wells of 96-well plate (final sample volume 20 µl). After 20 min of incubation at the room temperature 180 µl of 2 mM dopamine in 50 mM sodium phosphate pH 6.5 was added to the mixture. Phenoloxidase activity was determined by measuring absorbance at 490 nm over 160 min with 20-min interval using a microtiter plate reader (BioRad). The experiment was performed three times with four replicates.

Other techniques

Protein concentration was performed by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

Proteins and peptides of acidic/methanolic extracts of hemolymph were resolved in 16.5% polyacrylamide gels by Tris-tricine SDS/PAGE as described previously (Schägger, von Jagow, 1987).

RESULTS AND DISCUSSION

Changes of phenoloxidase activity in *G. mellonella* hemolymph after challenge with *A. oryzae*

We investigated the level of phenoloxidase activity in hemolymph of *G. mellonella* larvae immunized with spores of *A. oryzae*. As it is presented in Figure 1, the activity of PO was detected in hemolymph of the naïve (control) as

well as challenged larvae. However, the activity level in hemolymph of immunized insects was much higher in comparison to the control. The activity measured in hemolymph collected 24 and 72 h after challenge was calculated as 190–221% and 190–200% of the control, respectively. Interestingly, the level of PO activity measured in hemolymph 48 h after immunization was only 40–50% higher than in the control one, suggesting appearing at this time in the hemolymph of some inhibitors of proPO system activation and/or PO activity (Fig. 1).

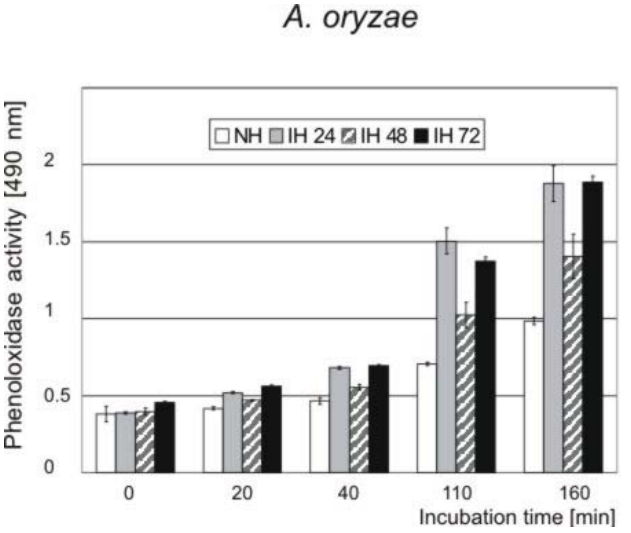

Fig. 1. Changes of phenoloxidase activity level in hemolymph of *G. mellonella* larvae after immunization with *A. oryzae.* Activity of phenoloxidase was measured in non-immune (NH) and immune hemolymph collected 24 h (IH24) 48 h (IH48) and 72 h (IH72) after challenge. The activity was determined using DOPA as a substrate and melanin formation was monitored at 490 nm. The diagrams represent the mean of three independent experiments ±SD

Changes in hemolymph peptidome after challenge of *G. mellonella* larvae with *A. oryzae*

Taking above presented results into consideration, in the following experiments we investigated alterations in hemolymph peptidome of *G. mellonella* larvae challenged with *A. oryzae*. As revealed by Tris-tricine SDS/PAGE, in extracts of immune hemolymph additional peptide bands appeared (Fig. 2). In the hemolymph collected 24, 48 and 72 h after treatment, one, two and three peptide bands corresponding to molecular mass below 6.5 kDa were detected, respectively, which were not noticed in the extracts of non-immune hemolymph. Moreover, in the hemolymph collected 72 and 96 h after immunization, another peptide band with molecular mass of 6.5 kDa was also noticed (Fig. 2). For comparison, in Figure 2 protein/peptide band pattern of extracts prepared from hemolymph of *E. coli*-challenged larvae is presented. The molecular mass of additional peptides

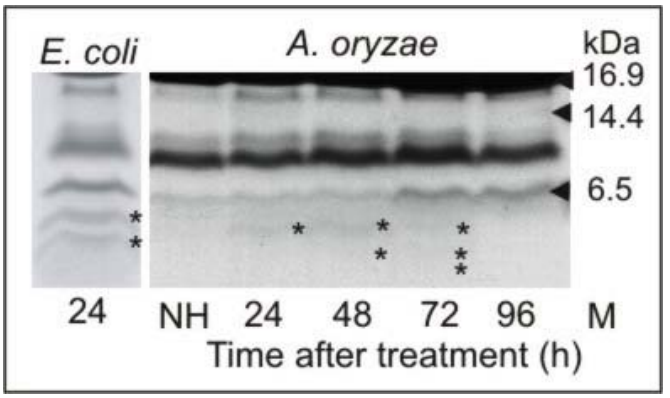

Fig. 2. Tricine SDS/PAGE of polypeptides obtained from *G. mellonella* hemolymph by acidic/methanolic extraction. The extracts were prepared from non-immune (NH) and immune hemolymph collected after *A. oryzae* or *E. coli* injection at the time indicated and resolved in 16.5% tricine SDS gels. Each line on the gel contained 20 µg of proteins. Additional peptide bands appearing in immune hemolymph are marked by the asterisks. The electrophoretogram is representative for three independent experiments. M – molecular weight standards

detected in hemolymph of immunized insects indicated an induction of defence peptide synthesis and/or release after challenge of larvae with *A. oryzae*. Among them could be cationic and anionic antimicrobial peptides as well as peptides with serine proteinase inhibitory activity. Induction of such peptides synthesis after immune challenge of *G. mellonella* has been described and the peptides have been characterized (Brown et al. 2009; Cytryńska et al. 2007; Fröbius et al. 2000). For example, three inducible serine protease inhibitors (ISPI-1,2,3) were purified from the hemolymph of zymosan-challenged *G. mellonella* larvae. The molecular mass of the inhibitors was determined to be 9.2 kDa (ISPI-1), 6.3 kDa (ISPI-2) and 8.2 kDa (ISPI-3). They were active against various serine proteinases, e.g. trypsin and toxic proteases Pr1 and Pr2 of *Metharizium anisopliae* (Fröbius et al. 2000).

The effect of proteins and peptides present in hemolymph extracts on the level of PO activity in *G. mellonella*

In the following experiments the effect of polypeptides present in the hemolymph extracts on phenoloxidase activity was studied. It was found that in the presence of the hemolymph extracts, the level of PO activity in hemolymph of non-immune as well as challenged insects decreased considerably (Fig. 3A–D). We noticed, that especially the extracts of the hemolymph collected 24 and 48 h after challenge with *A. oryzae* were considerably effective in inhibiting of PO activity. The effect was clearly evident after 110 and 160 min of incubation. In the

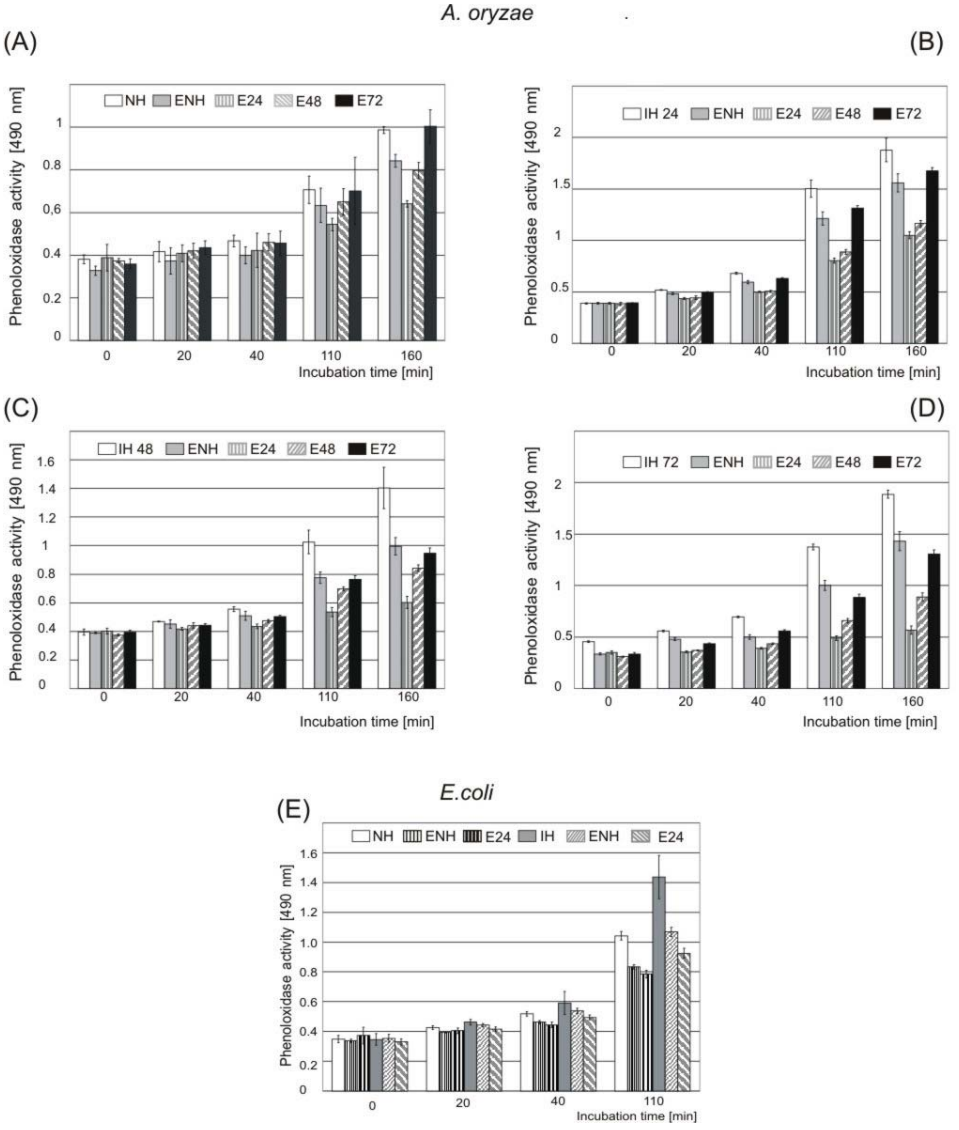

Fig. 3. The effect of hemolymph extracts of on the level of phenoloxidase activity in *G. mellonella*. PO activity was measured in non-immune (A) and immune hemolymph collected 24 h (B), 48 h (C) and 72 h (D) after challenge of larvae with *A. oryzae* or 24 h after challenge with *E. coli* (E). The activity was determined without and in the presence of extracts (1 µg of protein) prepared from non-immune hemolymph (ENH) and immune hemolymph obtained 24 h (E24), 48 h (E48) and 72 h (E72) after immunization. The activity was determined using DOPA as a substrate and melanin formation was monitored at 490 nm. The diagrams represent the mean of three independent experiments ±SD

presence of the extract of hemolymph collected 24 h and 48 h after immunization, PO activity in the hemolymph of non-immune insects was decreased by 23.3-46.3% and 10–19.5%, respectively (Fig. 3A). Interestingly, the enzyme activity in immune hemolymph was inhibited to a much higher extent. The activity in hemolymph collected 24 h after challenge was decreased by 44.7–46.7% and 39.5–40% by extracts of hemolymph obtained 24 and 48 h after treatment, respectively (Fig. 3B). These extracts were even more effective in inhibiting of PO activity in hemolymph collected 48 and 72 h after immunization. The extract prepared from hemolymph obtained 24 h after challenge decreased PO activity by 50% and 64.3–71.1% in the hemolymph collected 48 and 72 h after immunization, respectively. The extract from the hemolymph obtained 48 h after challenge decreased the studied activity by 31.2–35.7% and 52.6–53.6% in the hemolymph collected 48 and 72 h after treatment (Fig. 3C, D). Interestingly, when hemolymph extracts of larvae immunized with *E. coli* were used, similar inhibitory effect was observed. PO activity in hemolymph collected 24 h after challenge with bacteria was inhibited by 37.1% in the presence of extract prepared from that hemolymph (Fig. 3E). The ability of the hemolymph extracts to decrease PO activity was well correlated with appearing of additional peptides in larval hemolymph after immune challenge, demonstrated in Figure 2. The inhibitory effect of components present in hemolymph extracts was also evident when melanin formation in the wells during incubation was observed (Fig. 4).

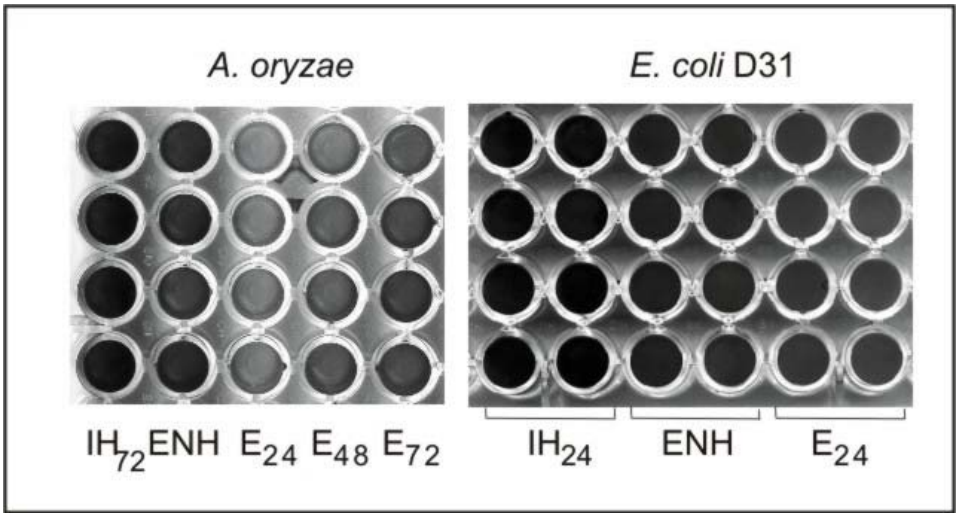

Fig. 4. Inhibition of melanin formation by extracts of non-immune and immune *G. mellonella* hemolymph. The picture presents fragments of the 96-well plate used for measurement of PO activity demonstrated as diagrams in Figure 3D (*A. oryzae*) and Figure 3E (*E. coli*)

Our results suggested that along with synthesis and release of inducible antimicrobial peptides/proteins, immunization of *G. mellonella* larvae with filamentous fungus *A. oryzae* or bacteria *E. coli* induced synthesis and/or release of inhibitors of proPO system and/or PO activity. Such inhibitors could control proPO system activation and allow avoiding toxic effects of quinones and other melanization intermediates on the host cells. Potential candidates for this function could be ISPI-1,2,3 exhibiting inhibitory activity against serine proteinases (Fröbius et al. 2000). It is also possible that immune challenge causes synthesis and release of peptides inhibiting PO activity. For example, from the hemolymph of housefly *Musca domestica*, phenoloxidase inhibitor (POI) containing 38 amino acids was isolated. One of the tyrosine residues present in the polypeptide chain is hydroxylated to DOPA, which is crucial for its competitive inhibitory activity (Daquinag et al. 1995). Homologous molecules were studied in *Anopheles gambiae* and *Manduca sexta* (Lu and Jiang 2007; Shi et al. 2006).

REFERENCES

Bidochka M. J., Hajek A. E. 1998. A nonpermissive entomophthoralean fungal infection increases activation of insect prophenoloxidase. J. Invertebr. Pathol. 72, 231–238.

Bradford M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brown S. E., Howard A., Kasprzak A. B., Gordon K. H., East P. D. 2009. A peptidomic study reveals the impressive antimicrobial peptide arsenal of the wax moth *Galleria mellonella*. Insect Biochem. Mol. Biol. 39, 792–800.

Cerenius L., Lee B. L., Söderhäll K. 2008. The proPO system: pros and cons for its role in invertebrate immunity. Trends Immunol. 29, 263–271.

Cytryńska M., Mak P., Zdybicka-Barabas A., Suder P., Jakubowicz T. 2007. Purification and characterization of eight peptides from *Galleria mellonella* immune hemolymph. Peptides 28, 533–546.

Daquinag A. C., Nakamura S., Takao T., Shimonishi Y., Tsukamoto T. 1995. Primary structure of a potent endogenous DOPA-containing inhibitor of phenol oxidase from *Musca domestica*. Proc. Natl. Acad. Sci. 92, 2964–2968.

Fröbius A. C., Kanost M. R., Götz P., Vilcinskas A. 2000. Isolation and characterization of novel inducible serine protease inhibitors from larval hemolymph of the greater wax moth *Galleria mellonella*. Eur. J. Biochem. 267, 2046–2053.

Gillespie J. P., Bailey A. M., Cobb B., Vilcinskas A. 2000. Fungi as elicitors of insect immune responses. Arch. Insect Biochem. Physiol. 44, 49–68.

Gillespie J. P., Khachatourians G. G. 1992. Characterisation of the *Melanoplus sanguinipes* hemolymph after infection with *Beauveria bassiana*. Comp. Biochem. Physiol. B 103, 455–463.

Hung S. H., Boucias D. G. 1992. Influence of *Beauveria bassiana* on the cellular defence response of the beet army warm, *Spodoptera exigua*. J. Invertebr. Pathol. 60, 152–158.

Kanost M. R., Jiang H., Yu X. Q. 2004. Innate immune responses of a lepidopteran insect, *Manduca sexta*. Immunol. Rev. 198, 97–105.

Kopáček P., Weise C., Götz P. 1995. The prophenoloxidase from the wax moth *Galleria mellonella*: purification and characterization of the proenzyme. Insect Biochem. Mol. Biol. 25, 1081–1091.

Lu Z., Jiang H. 2007. Regulation of phenoloxidase activity by high- and low-molecular-weight inhibitors from the larval hemolymph of *Manduca sexta*. Insect Biochem. Mol. Biol. 37, 478–485.

Park S. Y., Kim C. H., Jeong W. H., Lee J. H., Seo S. J., Han Y. S., Lee I. H. 2005. Effects of two hemolymph proteins on humoral defense reactions in the wax moth, *Galleria mellonella*. Dev. Comp. Immunol. 29, 43–51.

Pye A. E. 1974. Microbial activation of prophenoloxidase from immune insect larvae. Nature 251, 610–613.

Schägger H., von Jagow G. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem.166, 368–379.

Schmit A. R., Rowley A. F., Ratcliffe N. A. 1977. The role of *Galleria mellonella* hemocytes in melanin formation. J. Invertebr. Pathol. 29, 232–234.

Shi L., Li B., Paskewitz S. M. 2006. Cloning and characterization of a putative inhibitor of melanization from *Anopheles gambiae*. Insect Mol. Biol. 15, 313–320.

Slepneva I. A., Komarov D. A., Glupov V. V., Serebrov V. V., Khramtsov V. V. 2003. Influence of fungal infection on the DOPA-semiquinone and DOPA-quinone production in haemolymph of *Galleria mellonella* larvae. Biochem. Biophys. Res. Commun. 300, 188–191.