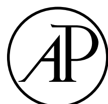
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Influence of fungal infection on the DOPA–semiquinone and DOPA–quinone production in haemolymph of *Galleria mellonella* larvae

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Abstract

The formation of reactive oxygen metabolites in haemolymph of *Galleria mellonella* larvae was studied by ESR spectroscopy. The inhibition of the production of the reactive oxygen metabolites of DOPA in haemolymph under the action of fungal infection was shown using spin trap 1-hydroxy-3-carboxy-pyrrolidine. This inhibition correlated with decrease of phenoloxidase activity in haemolymph of infected insects. Simultaneously, the decrease of production of DOPA–semiquinone was detected using method of spin stabilization by diamagnetic metal ions. Moreover, it was shown that the formation of DOPA–quinone was slowed down in haemolymph of infected insects. Our results suggest that the DOPA-derived quinones/semiquinones may be involved in immune response of insects as part of its defense mechanism.
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Keywords: ESR; Haemolymph; ROS; Phenoloxidase; DOPA–semiquinone; DOPA–quinone

The immune system of invertebrates comprises a variety of mechanisms and elements of defense against all types of pathogens or parasites. Melanotic encapsulation and the production of cytotoxic reactive intermediates are the important components of this system. The main role in the melanotic encapsulation belongs to the prophenoloxidase cascade [1,2]. The key enzyme of this cascade is phenoloxidase (PO), which catalyzes the first stage of the melanization process converting tyrosine to diphenols and corresponding quinones. During this process the potentially cytotoxic quinoid intermediates are generated. They can be involved in cytotoxic reactions in the insects [3,4] and/or can cause the production of the reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, and hydroxyl radical [5–8]. In our previous work we have studied by ESR method using the spin traps the generation of ROS and other reactive intermediates in haemolymph and hemocytes of great wax moth *Galleria mellonella* and siberian moth *Dendrolimus superans sibiricus* caterpillar. Our results suggest quinoid nature (DOPA-derived metabolites) of determined reactive intermediates [9]. At present time the role of such intermediates in immune response of insects is insufficiently studied.

In present work we have shown the generation of semiquinone radicals and quinones of DOPA and studied the influence of fungal infection on the production of these species in haemolymph of great wax moth *G. mellonella* larvae. It is known that semiquinones are important intermediates in various biological process [10]. They are also postulated as cytotoxic species in quinone-containing antitumor drugs [11,12]. Although very little is known about the role of semiquinones in cytotoxity of melanotic capsule, their high reactivity allows us to suggest significant role of semiquinones in this process.

o-Semiquinone radicals such as DOPA–semiquinone are very short-lived. However, these species form comparatively stable complex with diamagnetic divalent

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metal ions allowing its study under static conditions [13–15]. In this study DOPA–semiquinone radicals have been detected as their metal complexes with Mg^{2+} in haemolymph of insects.

Materials and methods

Chemicals. 1-Hydroxy-3-carboxy-pyrrolidine (CP-H) was synthesized and provided by Dr. I. Kirilyuk from Novosibirsk Institute of Organic Chemistry. 3(3,4-Dihydroxyphenyl)-DL-alanine (DOPA), phenylthiourea (PTU), superoxide dismutase from bovine erythrocyte (SOD), phenoloxidase from mushroom (PO), deferoxamine mesylate (Df), diethylenetriaminepentaacetic acid (DTPA), and 3-carboxyproxyl (CP) were all purchased from Sigma (USA).

Insects, fungi, and infected method. *Galleria mellonella* larvae were reared and maintained at 30 °C ± 1 °C in the dark [16]. The isolate *Metarhizium anisoplia* (1M-97) was obtained from collection of insect pathology laboratory (Institue of Animal Systematics and Ecology, Novosibirsk). The isolate 1M-97 was isolated from a field-collected Colorado potato beetle *Leptinotarsa decemlineata* cadaver, reisolated from larvae great wax moth (after a treatment of larvae *G. mellonella*) and subcultured not more than seven times on Sabouraud's glucose agar before use in this study. This isolate has high virulence for larvae *G. mellonella* (not published). The mortality of larvae from mycoses was registered on 72 h. Conidia were routinely mass produced on Sabouraud's glucose agar with 0.1% yeast extract. The conidia were suspended in 10 ml of sterile water containing 0.01% Tween 20. The number of conidia per milliliter was determined by using a haemocytometer. The larvae of *G. mellonella* were treated by immersion into the suspension of *M. anisopliae* conidia (2.0×10^6 conidia/ml) and returned to the diet cup, according to [17]. We have examined the haemolymph of larvae at 24 h after infection. At this time a conidia and hyphae have not been found in haemolymph while the melanotic spots on cuticle were registered.

Haemolymph collection. The haemolymph from larvae was collected into 1.5 ml Eppendorf tubes on ice [18]. The collected haemolymph was centrifuged for 5 min at 1000*g* at +4 °C temperature and a supernatant was used in experiments.

Determination of rates of ROS formation. CP-H spin trap [9,19] has been used for measuring the rates of reactive intermediate formation in haemolymph of insects. CP-H is nonspecifically oxidized by high oxidizing metabolites with formation of the stable nitroxyl radical, CP. The time-dependent accumulation of CP radical in the samples has been studied by monitoring the amplitude of low field component of ESR spectrum. The concentration of CP in the samples was calculated using calibration dependence of the ESR amplitude on concentration of CP obtained from Sigma. SOD was used as competitive reagent to clarify the contribution of superoxide radical into the kinetics of CP-H oxidation.

CP-H was dissolved in oxygen-free (argon-bubbled) PBS-D (PBS containing 50 μM Df, pH 7.4). Deferoxamine was used to decrease the self-oxidation of hydroxylamine catalyzed by traces of transition metal ions. The 10 mM stock solution of CP-H was stored frozen.

The samples were prepared in PBS-D. Haemolymph was diluted 40 times with PBS-D. The mixtures of haemolymph with CP-H (1 mM) were placed in 100 μl glass capillaries with an internal diameter of 1 mm for ESR measurements. Concentrations of DOPA and SOD in mixtures of haemolymph with spin trap were 0.2 mg/ml and 100 U/ml, respectively. We have found that DOPA does not contribute to the rate of CP-H oxidation at experimental concentrations used in the study.

ESR measurements were performed at room temperature using ER 200-D SRC X-band ESR spectrometer (Bruker). The ESR settings were the following: field center, 3474 G; field sweep, 50 G; time constant, 500 ms; microwave power, 20 mW; magnetic field modulation, 100 kHz; and modulation amplitude, 1 G.

Determination of PO activity. Phenoloxidase activity of the haemolymph was assayed spectrophotometrically according to [20] by mixing 40 μl haemolymph with 160 μl PBS and 200 μl DOPA (2 mg/ml in PBS). The mixture was incubated at 22 °C for 5 min in the dark in a cuvette placed in spectrophotometer and the change of OD per minute per milligram protein was measured.

Determination of DOPA–semiquinone production. The formation of DOPA–semiquinone in the samples was detected by monitoring the amplitude of low field component of ESR spectrum of DOPA–semiquinone complex with Mg^{2+}.

Samples were prepared in Tris–HCl-D (Tris–HCl containing 50 μM DTPA, pH 7.5). DTPA was used to decrease the rate of decay of the *o*-semiquinone radicals catalyzed by traces of transition metals. DOPA was dissolved in oxygen-free (argon-bubbled) Tris–HCl-D to prevent the reaction of autoxidation of DOPA. The concentration of DOPA in stock solution was 4 mg/ml. Haemolymph was diluted 200 times with Tris–HCl-D. The mixtures of haemolymph, $MgCl_2$ (0.5 mM), and DOPA (2 mg/ml) were used for ESR measurements.

ESR conditions were following: field center, 3480 G; field sweep, 20 G; time constant, 1 s; microwave power, 2 mW; magnetic field modulation, 100 kHz; and modulation amplitude, 1.6 G.

Determination of DOPA–quinone production. Production of DOPA–quinone was measured from ascorbic acid oxidation according to [21]. The observation of time dependence of absorption at 265 nm was used to follow kinetics of ascorbic acid oxidation.

DOPA was dissolved in oxygen-free (argon-bubbled) Tris–HCl-D. The concentration of DOPA in stock solution was 2.5 mM. Haemolymph was diluted 50 times with Tris–HCl-D. The mixtures of haemolymph, DOPA (1.25 mM), and ascorbic acid (1.2 mM), were used in this study. The measurements were carried out for 1 min to detect the initial linear fraction of the kinetics.

Optical measurements were performed using Hewlett Packard 8453 (Germany) spectrophotometer. Samples were placed into a quartz flat cell with an optical path length of 1 mm.

Statistics. All values are reported as means ± SE. Significance of differences between the indices was evaluated using Student's *t* test ($P < 0.05$).

Results and discussion

Phenoloxidase plays the key role in the catalysis of the first stage of the melanization process and, therefore,

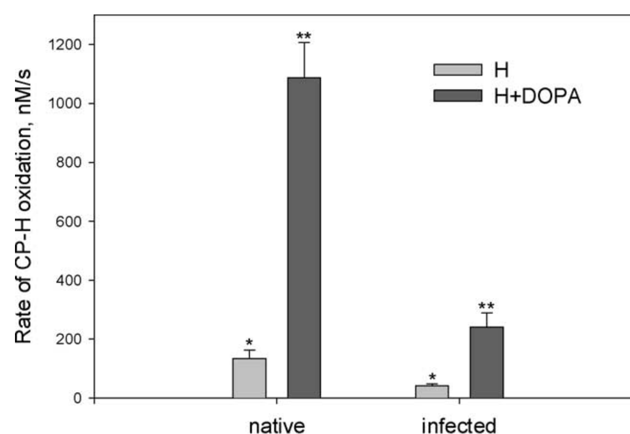

Fig. 1. ROS formation in native and *M. anisoplia* infected haemolymph (H) of *G. mellonella* measured as the rate of oxidation of hydroxylamine CP-H to nitroxyl radical CP. H+DOPA—haemolymph with DOPA. Data are means ± SE ($n = 12$; $*P < 0.05$; $**P < 0.05$).

in immune response of insects. Our previous studies [9] have demonstrated that PO significantly stimulates the production of ROS, which may be one of the cytotoxic agents responsible for the defense mechanisms against the pathogens.

In the present work we have studied the influence of fungal infection on the formation of ROS in haemolymph of *G. mellonella* using nonspecific spin trap CP-H. As seen in Fig. 1 the *M. anisoplia* infection significantly decreases (3.2 ± 1.2 times) the rates of ROS formation in cell-free haemolymph both in the presence and absence of DOPA. Addition of SOD to the mixture resulted in insignificant inhibition ($P > 0.05$) of DOPA-induced CP-H oxidation. These data indicate that quinoid precursors of melanin rather than superoxide radicals are responsible for the CP-H oxidation in haemolymph of insects. Thus the decrease of CP-H oxidation by fungal infection is likely related to reduction of PO activity and to corresponding decrease of the melanogenesis products. In agreement with this assumption PO activity in haemolymph was found to be significantly decreased by fungal infection (0.073 ± 0.02 U/mg protein/min) in comparison with control group (0.166 ± 0.04 U/mg protein/min) ($n = 5$; $P < 0.05$).

Earlier several groups [22,23] have observed ESR spectra of metal complexes of DOPA–semiquinone during photolysis of DOPA solution or its enzymatic oxidation using purified enzymes (such as PO and peroxidase). However, the detection of the ESR spectra of DOPA–semiquinone in any biological samples during melanization was not reported. We have shown by ESR spin stabilization method with Mg^{2+} that DOPA–semiquinone radicals are really produced in cell-free haemolymph of *G. mellonella*. The spectra obtained from oxidation of DOPA in haemolymph of *G. mellonella* (Fig. 2A) and with purified PO (Fig. 2B) are identical. The spectra were not registered in the presence of the specific inhibitor of PO, phenylthiourea, in haemolymph (Fig. 2C). These data indicate that DOPA–semiquinone radicals are formed during melanogenesis in haemolymph of insects and their production depends on PO activity.

It was shown that steady-state concentration of DOPA–semiquinone complex with Mg^{2+} depends on PO activity (Fig. 3). Therefore, it was of our interest to compare DOPA–semiquinone production in haemolymph of infected and intact insects. It was observed that steady-state concentration of DOPA–semiquinone complex was decreased as a result of mycosis practically to the level of DOPA autoxidation in buffer while it was about two times higher in the intact haemolymph (Fig. 4). In addition, it was shown that oxidation of ascorbic acid was also inhibited in about four times

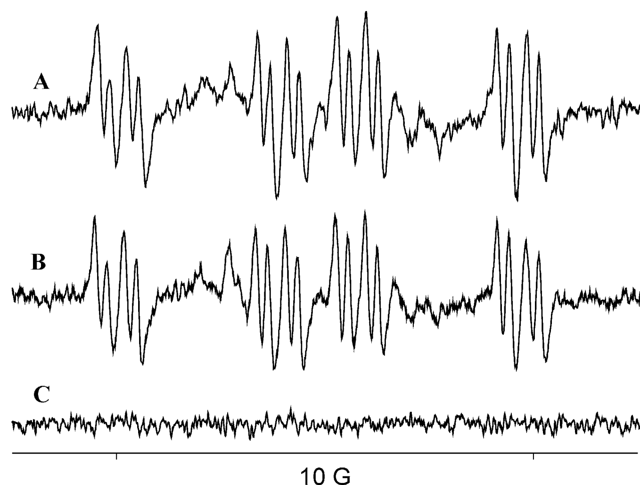

Fig. 2. ESR spectra of DOPA–semiquinone complexes with Mg^{2+} obtained: (A) from oxidation of DOPA in haemolymph; (B) from oxidation of DOPA with PO; and (C) in the presence of PTU in haemolymph. ESR conditions were: time constant, 1 s; microwave power, 2 mW; and modulation amplitude, 0.16 G.

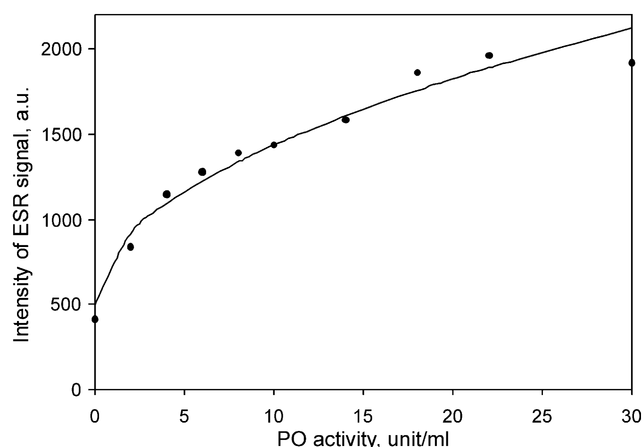

Fig. 3. Dependence of ESR signal intensity of DOPA–semiquinone complex with Mg^{2+} on PO activity.

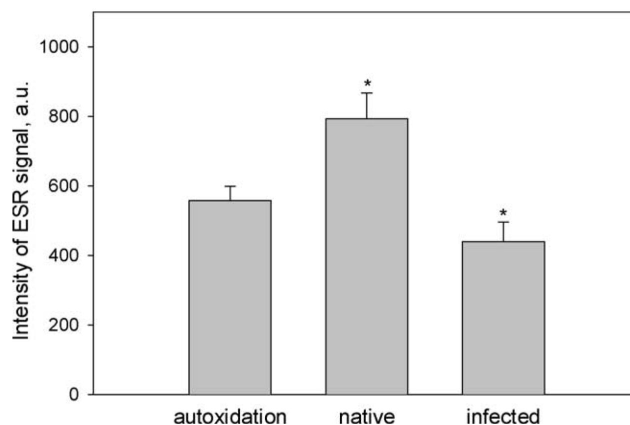

Fig. 4. Effect of fungal infection (*M. anisoplia*) on the DOPA–semiquinone production in haemolymph of *G. mellonella*. Data are means ± SE ($n = 5$; $^*P < 0.05$).

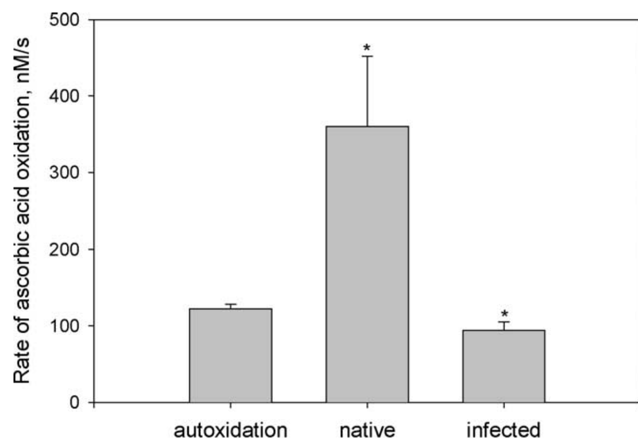

Fig. 5. Effect of fungal infection (*M. anisoplia*) on the DOPA–quinone production in haemolymph of *G. mellonella* measured as the rate of ascorbic acid oxidation. Data are means ± SE ($n = 5$; $^*P < 0.05$).

in infected haemolymph (Fig. 5) supporting decrease of the DOPA–quinone production. The decrease of DOPA-derived quinones/semiquinones production in the haemolymph of infected insects is apparently consequence of the reduction of PO activity as it was confirmed by our data obtained using spin trap CP-H (Fig. 1) and by direct measurements of PO activity.

In summary, our results demonstrate a decreased level of DOPA-derived quinones/semiquinones in haemolymph of infected insects that is probably responsible for these insects lacking vitality. It should be suggested that the level of production of such species in haemolymph of insects is very important for immune status of insects. The roles of DOPA–semiquinones/quinones produced during melanogenesis in insects are still far from understood. Taking into account high oxidizing ability of the quinoid intermediates our results allow one to consider them as cytotoxic molecules against internal parasites.

Acknowledgments

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