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Influence of the fungal infection on the production of reactive oxygen metabolites and the antioxidant state of haemolymph of *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae

Влияние грибной инфекции на продукцию реактивных кислородсодержащих метаболитов и антиоксидантный статус гемолимфы личинок большой вощиной огневки *Galleria mellonella* L. (Lepidoptera: Pyralidae)

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KEYWORDS: haemolymph; reactive oxygen metabolites, phenoloxidase, superoxide dismutase, glutathione-S-transpherase, SH-compounds, insect immunity.

КЛЮЧЕВЫЕ СЛОВА: гемолимфа, реактивные кислородные метаболиты, фенолоксидаза, супероксиддисмутаза, глутатион-S-трансфераза, SH-компоненты, иммунитет насекомых.

ABSTRACT. Effect of fungal infection (*Metarhizium anisoplia*) on the immune system of *Galleria mellonella* larvae was studied. It was shown by spin trapping method that inhibition of the production of the reactive metabolites of derivates of dihydroxyphenil-DL-alanine in haemolymph occur under the action infection. This inhibition correlated with decrease of phenoloxidase activity in haemolymph of *G. mellonella* at 24 hr of fungal infection. The activity of superoxide dismutase was decreased. At the same time the activity of glutathione-S-transpherase was also increased. The level of SH-compounds in haemolymph of *G. mellonella* was not changed by the mycosis. It appears that the reactive metabolites may be involved in cytotoxic reactions depressing the growth of parasites in insects and the antioxidants (enzymatic and nonenzymatic) may take part in elimination of toxic products of pathogens and also in neutralization of reactive oxygen species.

РЕЗЮМЕ. Изучено влияние энтомопатогенных грибов *Metarhizium anisoplia* на иммунную систему личинок *Galleria mellonella.* С использованием метода спиновых ловушек показано, что под действием инфекции происходит ингибирование продукции активированных кислородных метаболитов — производных дигидрооксифенил-DL-аланина в гемолимфе зараженных личинок. Ингибирование коррелировало с уменьшением фенолоксидазной активности в гемолимфе *G. mellonella* через 24 часа после инфицирования. Активность супероксидисмутазы уменьшалась в течение микоза, тогда как активность глутатион-S-трансферазы увеличилась. Уровень SH-компонентов не изменялся под действием микоза. Активированные кислородные метаболиты могут быть вовлечены в цитотоксические реакции, подавляющие рост паразита в насекомых, а антиоксиданты (ферментативные и неферментативные) могут принимать участие в элиминации токсических продуктов патогенов и также в нейтрализации активированных кислородных метаболитов.

Introduction

Insects have immediate non-inducible defense to entrap and neutralize parasites and pathogens. These processes are mainly carried out by the humoral and cellular immune systems [Ratcliffe et al., 1985; Carton, Nappi, 1997; Gillespie et al., 1997]. The cellular com-

ponents of the immune system of insects include the blood cells (haemocytes) which are involved in the phagocytosis, nodulation and encapsulation [Ratcliffe, 1993]. The humoral responses comprise various induced broad-spectrum antimicrobial peptides and constitutive proteins [Boman et al., 1991; Otvos, 2000] and agglutinins [Kubo et al., 1996; Drif, Brehelin, 1994]. An important role in the immune responses belongs to the prophenoloxidase cascade [Ashida, Brey, 1997; Johansson, Söderhäll, 1995]. The key enzyme of this cascade is phenoloxidase, which catalyzes the first stages of the melanization process. During this process the quinoid intermediates are generated. They can be involved in cytotoxic reactions in the insect [Nappi, Vass, 1993; Nappi, Ottaviani, 2000] and (or) can cause the production of the reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, hydroxyl radical [Nappi et al., 1995; Nappi, Vass, 1998; Arakawa, 1994; Leem et al, 1996]. In our previous work we have demonstrated by EPR method using a spin trap the generation of reactive quinoid intermediates and ROS in haemolymph and hemocytes of great wax moth *Galleria mellonella* and siberian moth *Dendrolimus superans sibiricus* caterpillar [Slepneva et al., 1999]. Therefore, insect organisms need the mechanism to regulate the production of oxygen radicals. Such defense mechanism against oxygen radicals including enzymatic and nonenzymatic components exists in all aerobic organisms [Fridovich, 1978]. A few reports show the presence of defense systems against ROS in insects, but these studies are related to the enzymatic antioxidants [Arakawa, 1994; Ahmad et al., 1991; Tower,1996; Sohal et al., 1993]. In present paper we report the influence of entomopathogenic fungi *Metarhizium anisoplia* on the production of the reactive metabolites and the antioxidant state of haemolymph of great wax moth *G. mellonella* larvae.

Materials and methods

Chemicals

1-Hydroxy-3-carboxy-pyrrolidine (CP-H) was synthesised and kindly provided by Dr. I. Kiriluyk from Novosibirsk Institute of Organic Chemistry. Biradical, bis (2,2,5,5-tetramethyl-3-imidazoline-1-oxyl-4-il) disulfide (RSSR) was synthesised as in [Khramtsov et al., 1989] and was kindly supplied by Dr. T. Berezina (Novosibirsk Institute of Organic Chemistry). 3,4-Dihydroxyphenil-DL-alanine (DOPA), phenylthiourea (PTU), deferoxamine mesylate (Df), superoxide dismutase from bovine erythrocyte (SOD), 3-carboxy-proxyl (CP) were all purchased from Sigma (USA).

Insects, fungi and infected method

G. mellonella larvae were reared and maintained at 30°C ±1°C in the dark [Tamarina, 1987]. The isolate *M. anisopliae* (1M-97) was obtained from collection of insect pathology laboratory (Institue of Animal Systematics and Ecology SB RAS, Novosibirsk). The isolate 1M–97 was isolated from a field-collected colorado potato beetls *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 isolated has high virulent for larvae *G. mellonella* (not published). The mortality of larvae from mycoses was registered on 72 hour. A 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 [Goettel, Inglis, 1997]. We have examined the haemolymph of larvae at 24 hour post infection. At this time a conidia and hyphae did not found in haemolymph, but the melanotic spots on cuticule were registered.

Haemolymph collection

The haemolymph was collected from a minimum of 10 larvae last instar into 1.5 ml Eppendorf tubes in ice. The collected haemolymph was centrifuged for 5 min at 1000 g at +4°C temperature, a supernatant was used in experiments.

Determination of rates of ROS formation

CP-H spin trap [Slepneva et al., 1999; Dikalov *et al.*, 1997] has been used for the measurement of the rate of ROS formation. CP-H is nonspecifically oxidized by ROS (superoxide, hydroxyl radicals, peroxynitrite and other high oxidizing metabolites) with formation of the stable nitroxyl radical, CP. The observation of time-dependent accumulation of CP radical by EPR was used to follow the kinetics of ROS formation in the samples by monitoring the amplitude of the low field component of the EPR spectrum. Experimental concentrations of CP in the samples were determined using calibration dependence of the EPR amplitude on concentration of CP obtained from Sigma. To clarify the contribution of superoxide radical into the kinetics of CP-H oxidation SOD was used as competitive reagent [Dikalov *et al.*, 1997].

All samples were prepared in PBS-D (PBS, containing 50 mM Df, pH 7.4). Haemolymph was diluted 40 times in PBS-D. The mixtures of haemolymph with CP-H (1 mM) were placed in 100 μl glass capillaries of an internal diameter of 1 mm for EPR 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 experimental concentration of DOPA used in the study does not contribute into the rate of CP-H oxidation.

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

Haemolymph enzyme assays

Superoxide dismutase (SOD) activity was determined based on the inhibition of reduction of nitroblue tetrazolium (NBT) by superoxide generated by the xanthine-xanthineoxidase system [McCord, Fridovich, 1969]. One unit of SOD is defined as the amount of enzyme which causes 50% of inhibition of reduction of NBT per minute per milligram protein.

Glutathione-S-transferase (GST) activity with glutathione (GSH) was measured using the substrate 1-chloro-2,4-dinitrobenzene as described for mammal [Habig, Jakoby, 1981]. GST activity was calculated as change of absorbance units at 340 nm per minute per milligram protein.

SOD and GST activities were assayed in presence of phenylthiourea (0.2 mg/ml).

Phenoloxidase (PO) activity of the haemolymph was assayed spectrophotometrically according to [Hung, Boucias, 1996] by addition of 40 μl haemolymph to160 μl of 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 into spectrophotometer and the change of absorbance at 490 nm was recorded. PO activity was calculated as change of OD per minute per milligram protein.

Protein determination

The method of Bradford [Bradford, 1976] was used to determine protein concentration with bovine serum albumin as the standard in the range of 50 – 500 μg /ml.

Quantitative determination of SH- groups by biradical disulfide

Previously developed EPR method [Khramtsov et al., 1989; Nohl et al., 1995] has been used for quantitative determination of SH- groups in low- and high-molecular weight compounds in haemolymph. The reaction between biradical RSSR and thiols was initiated by mixing 180 μl of haemolymph (20 diluted times with PBS-D) and 20 μl of 2 mM biradical solution in acetonitrile. The mixtures were placed in 100 μl glass capillaries of an internal diameter of 1 mm for EPR measurements. The concentration of thiols in haemolymph was calculated using the linear calibration dependence of the amplitude of low-field component of EPR spectrum on thiol concentration at 3 minutes after mixing reagents for low-molecular weight thiols and at 30 minutes – for high-molecular weight thiols. These experimental conditions were obtained from independent experiments with precipitation of the haemolymph proteins by trichloroacetic acid.

Statistics

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

Results

As seen in Fig. 1 the *M. anisoplia* infection significantly decreased (3.2±1.2 times) the rates of CP formation both in cell-free haemolymph and DOPA-induced one versus control samples. Addition of SOD to the mixtures resulted in not significant inhibition ($P = 0.06$) of DOPA-induced CP-H oxidation.

The phenoloxidase activity in haemolymph was significantly decreased by fungal infection (0.073 ± 0.02 units/mg protein/min) in comparison with control group (0.166 ± 0.04 units/mg protein/min) ($P< 0.05$).

SOD activity was significant decreased by the fungal infection from 0.30 ± 0.02 units/mg protein/min in the haemolymph of native larvae to 0.23 ± 0.01 units/mg protein/min in the infected haemolymph ($P< 0.05$) (Fig. 2). However, the activity of GST was significantly increased in haemolymph of infected larvae and was 0.38 ± 0.14 and 0.12 ± 0.03 units/mg protein/min for native larvae ($P< 0.05$) (Fig. 3).

The concentrations of both low and high molecular weight SH-compounds were not significantly changed in haemolymph of *G. mellonella* larvae under the action of fungal infection and were 0.2–0.4 mM and about 1mM, respectively (Fig. 4).

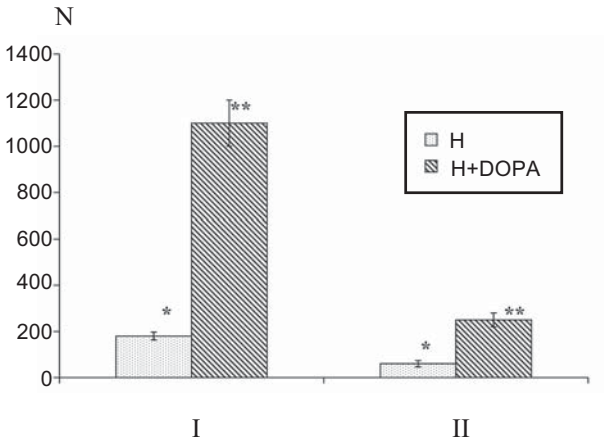

Fig. 1. ROS formation in native and *Metarhizium anisoplia* infected haemolymph (H) of *Galleria mellonella* measured as rate of oxidation of hydroxylamine CP-H to nitroxyl radical CP: N — Rate of CP-H oxidation (nM/s), mean ± S. E. (n = 12; *, $P< 0.05$; **, $P< 0.05$); I — native, II − fungal infected; H — haemolymph, H + DOPA — haemolymph with DOPA.

Рис. 1. Образование активированных кислородных метаболитов в гемолимфе *Galleria mellonella* интактных и зараженных *Metarhizium anisoplia,* измеренное как скорость окисления гидроксиламина CP-H в нитроксильный радикал CP: N — скорость окисления CP-H (нМ/сек), $\bar{x} \pm x$ (n = 12; *, $P< 0.05$; **, $P< 0.05$); I — интактные, II — зараженные; H — гемолимфа, H + DOPA — гемолимфа с DOPA.

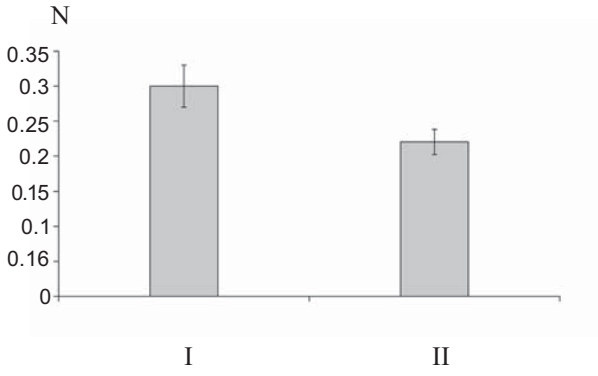

Fig. 2. Effect of fungal infection (*Metarhizium anisoplia*) on the SOD activity of *Galleria mellonella* haemolymph: N— SOD activity (Units/mg proteine/min), mean ± S. E. (n = 7; $P< 0.05$); I, II as on Fig. 1.

Рис. 2. Влияние грибной инфекции (*Metarhizium anisoplia*) на активность супероксиддисмутазы в гемолимфе *Galleria mellonella*: N — активность супероксиддисмутазы (Ед./мг белка/мин),(n = 7; $P< 0.05$); I, II как на рис. 1.

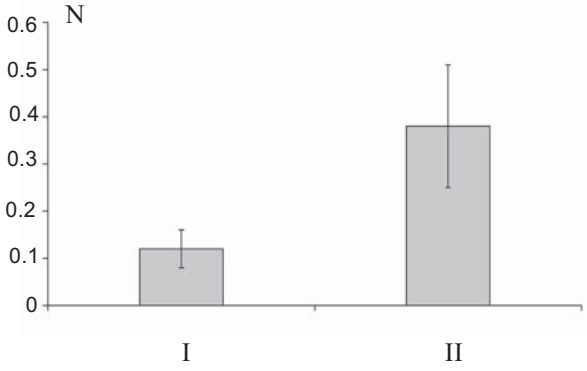

Fig. 3. Effect of fungal infection (*Metarhizium anisoplia*) on the GST activity of *Galleria mellonella* haemolymph: N — G-S-T activity (Units/mg proteine/min), mean ± S. E. (n = 6; P < 0.05); I, II as on Fig. 1.

Рис. 3. Влияние грибной инфекции (*Metarhizium anisoplia*) на активность глутатион-S-трансферазы в гемолимфе *Galleria mellonella*: N — активность глутатион-S-трансферазы (Ед./мг белка/мин), $\bar{x} \pm x$ (n = 6; P< 0.05); I, II как на рис. 1.

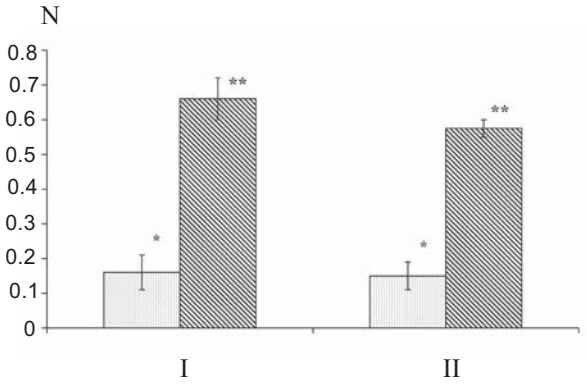

Fig. 4. Concentrations of thiols in native and *Metarhizium anisoplia* infected haemolymph of *Galleria mellonella* measured at 3 and 30 min after addition of biradical RSSR: N — SH-groups concentration (mM), mean ± S. E. (n = 8; *, P> 0.05; **, P> 0.05); I, II as on Fig. 1.

Рис. 4. Концентрация тиолов в гемолимфе *Galleria mellonella* интактных и зараженных *Metarhizium anisoplia,* измеренное после добавлений бирадикала RSSR через 3 и 30 мин.: N — концентрация SH-групп (mM), $\bar{x} \pm x$ (n = 8; *, P> 0.05; **, P> 0.05); I, II как на рис. 1.

Discussion

During the development of fungal infection the fungal protein inhibitors and secondary metabolites may be able to enter larvae of *G. mellonella* suppressing components of the immune system and change the balance in system antioxidants – produced ROS, that in turn can provoke the dysfunction in insect organism.

Our previous study have demonstrated that the phenoloxidase (PO) catalyzing the first stages of the melanogenesis, plays significant role in the production of ROS [Slepneva et al., 1999].

At 24 hr *Beauveria bassiana* causes a significant suppression of the ability of haemocytes to spread over substrate, possibly as a result of destabilization of the cytoskeleton of the insect haemocytes [Hung and Boucias, 1993; Mazet et al., 1994].

In present paper we have demonstrated that activity of PO was decreased in plasma of *G. mellonella* larvae after 24 hr fungal infection (Fig. 5). That corresponds to sharp stage of mycosis during the treatment by high virulent strain of fungi. We used the fungal contamination which is more similar to a natural infection then injection of blastospores into haemocoel. The decrease of PO activity in haemolymph of *G. mellonella* correlated with the inhibition of the production of the reactive metabolites which we determined by EPR method using spin trap CP-H (Fig. 1). To clarify the contribution of superoxide radicals into the CP-H oxidation SOD was used as competitive reagent. Addition of SOD to the mixtures did not significant inhibit DOPA-induced CP-H oxidation. These data indicate that a semiquinoid metabolites, but not superoxide radicals, are mainly responsible for the oxidation of CP-H in haemolymph of *G. mellonella*.

The progressive inhibition of PO activity in haemolymph of larvae by mycosis possibly results from the depletion of requisite enzyme activators or the increasing of protease inhibitors amount that may decrease or fully suppress the activity of serine proteases. It is known that these proteases are involved in a stepwise process of activation of the proPO system [Johansson and Söderhäll, 1995]. Thus, presence of the several kinds of protease inhibitors were found in the haemolymph of the silkworm *Bombyx mori* [Eguchi, 1993], in cuticle of *Manduca sexta* [Sugumaran, Nellaiappan, 2000].

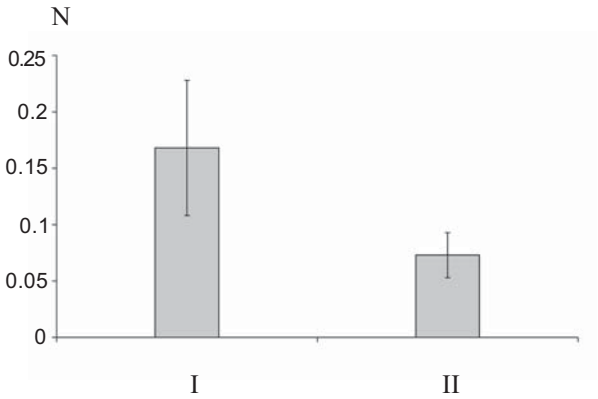

Fig. 5. Effect of fungal infection (*Metarhizium anisoplia*) on the PO activity of haemolymph of *Galleria mellonella*: N — PO activity (A_{490}/mg proteine/min), mean ± S. E. (n = 5; P< 0.05); I, II as on Fig. 1.

Рис. 5. Влияние грибной инфекции (*Metarhizium anisoplia*) на активность фенолоксидазы в гемолимфе *Galleria mellonella*: N — активность фенолоксидазы (A_{490}/мг белка/мин), $\bar{x} \pm x$ (n = 5; P< 0.05); I, II как на рис. 1.

It was found that rates of haemolymph melanization and PO activity were significantly dimished in several species of lepidopterans larvae during the parasitism by both ecto- and endoparasitoids [Stoltz, Cook, 1983; Beckage et al., 1990; Kitano et al., 1990; Richards, Edwards, 2000].

Fig. 2 shows that the activity of SOD was decreased by fungal infection possibly as result of the inhibition or destruction of the enzyme by fungal metabolites. At the same time the GST activity was increased (Fig. 3), that is possible because of need to eliminate toxic fungal metabolites. It is known that the increase of GST activity was registered under the insecticide treatment of insects [Hayaoka and Dauterman, 1982]. Probably GST takes part in elimination of insecticides as well as fungal metabolites.

As it was found (Fig. 4) the level of SH-compounds in haemolymph of *G.mellonella* was not changed by mycosis. SH-compounds of haemolymph may be involved in the processes of the elimination of fungal metabolites and the trapping of free radicals. Stability of the level of SH-compounds in haemolymph indicates the significant role of these compounds in the defense against different free radicals and parasite metabolites in insects.

The roles of produced oxygen metabolites and of the antioxidant system of insects in the defense mechanism against parasites are still unclear. However, it appears that the oxygen metabolites may be involved in cytotoxic reactions depressing the growth of parasites in insects. At the same time the antioxidants (enzymatic and nonenzymatic) may take part in elimination of toxic products of pathogens and destroyed tissues of organism itself and also in neutralization of ROS.

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