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COMPARATIVE AND ONTOGENIC BIOCHEMISTRY

Changes of the Antioxidant Status and System of Generation of Free Radicals in Hemolymph of *Galleria mellonella* Larvae at Microsporidiosis

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Abstract—Changes of superoxide dismutase (SOD) and glutathione-S-transferase (GST) activities as well as of the content of SH-containing compounds were revealed in hemolymph of the native and the *Vairimorpha ephestiae* microsporidian-infected greater wax moth *Galleria mellonella* larvae. The SOD and GST activities in hemolymph of infected insects decreased at the stage of merogony, whereas during massive sporulation the enzymatic antioxidant activity in host tissues was higher than in control. By the ESR spectroscopy method, using the 1-hydroxy-3-carboxy-pyrrolidinespin-trap, generation of free radicals in hemolymph of infected insects was shown to decrease only at the stage of sporogony. The phenoloxidase activity in lymph was lower at acute microsporidiosis than in native larvae. The hemolymph concentration of thiol-containing proteins in infected insects did not differ from that in control. We suggest that decrease of generation of free radicals in hemolymph of the greater wax moth larvae at the stage of sporogony is due to a suppression of the prophenoloxidase system and an elevation of the antioxidant activity.

INTRODUCTION

Microsporidia are obligate intracellular parasites of animals of various taxonomic groups including representatives of the insect class [1]. In particular, microsporidia *Vairimorpha ephestiae* penetrate into fatty body of greater wax moth larvae in which development of the parasite occurs. On entering hemocoel, microsporidian spores are phagocytosed by hemocytes [2]. It cannot be ruled out that the parasite spores in the host hemocyte are disrupted under effect of high-reactive oxygen intermediates. The oxygen intermediates are possible to affect essentially the parasite development directly in cells of the damaged tissue. Such intermediates can be the superoxide anion radical, peroxide hydrogen, hydroxyl radical, nitrogen oxide, singlet oxygen, etc. [3]. Our previous studies using EPR-spectroscopy with a 1-hydroxy-3-carboxy-pirrolidin spin trap have shown formation of superoxide anion and other activated oxygen metabolites (AOM), first of all quinones/semiquinones—derivatives of dihydroxyphenylalanine, in hemolymph of intact larvae of *G. mellonella* and *Dendrolimus superans sibiricus* [4]. Semiquinones formed at melanogenesis play an essential role in such immune reactions in insects as incapsulation and granule formation [5–6]. Semiquinones are able to interact with molecular oxygen to produce superoxide anion and then hydroxyl radical. Thus, it has been found that

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prophenoloxidase activity in hemolymph of conenose bugs *Rhodnius prolixus* infected with trypanosome correlates with superoxide-anion formation [7]. It is quite reasonable that the organism has systems participating in protection of cells from possible injury by AOM.

A regulatory enzyme for superoxide is superoxide dismutase (SOD, EC 1.15.1.1) present in all aerobic organisms [3, 8]. The superoxide radical production and the SOD activity have been revealed in hemolymph and various tissues of lepidopterous insects [9–11]. We suggest that like in phagocytosis in mammals, in the insect hemopymph, superoxide is formed prior to phagocytosis and encapsulation, the SOD protecting insect cells from a damage by superoxide anion. A possible participation of high-reactive oxygen intermediates in respiratory processes and in melanization has been revealed in studies on generation of oxygen metabolites in hemolymph and hemocytes of various insects [6, 12, 13].

Glutathione-S-transferase (GST, EC 2.5.1.18) is a specialized regulatory enzyme for organic hydroperoxides produced during oxidative stress [3, 14]. Thiol-containing compounds are also essential components of maintaining the oxidation–reduction homeostasis in cells and tissues demonstrating a protective effect [15]. Studies of GST in insects mainly deal only with the physico-chemical properties of the isolated enzymes and their participation in detoxication of xenobiotics [14, 16–18]. Such non-enzymatic antioxidants as SH-containing compounds were studied predominantly in vertebrate animals [3, 15].

There are few studies on generation and regulation of SOD and GST in insects at pathogenesis. Thus, an increased level of superoxide generation in *Drosophila melanogaster* was recorded during encapsulation of the parasitoid *Leptopilina boulardi* [6]. It has been established experimentally that the superoxide production in hemolymph of the bug *Rhodnius prolixus* is elevated during encapsulation with the parasite *Trypanosoma rangeli* [7]. Activation of antioxidant systems at microsporidiosis has been studied only in vertebrate animals; the microsporidian spores were shown to be able to inhibit the respiratory burst of phagocytes [19]. There are no papers reported study of the antioxidant system and the AOM generation in insects infected with microsporidia. In the present work we carried out a comparative study of SOD and GST activities, free radical generation and measurement of concentrations of SH-compounds in larvae of *G. mellonella* in norm and at different stages of microsporidiosis produced by *V. ephestiae*.

MATERIALS AND METHODS

Used in this work were reduced glutathione (G-SH), 1-chloro-2,4-dinitrobenzene, Tetrazolium Nitroblue, xanthine oxidase, superoxide dismutase (SOD) from bovine erythrocytes (1.15.1.1), 3(3,4-dihydroxyphenyl)-DL-alanine (DOPA), phenylthiourea (PTU), bovine serum albumin (BSA), 3-carboxyproxyl obtained from Sigma (USA), bis(2,2,5,5-tetramethyl-3-imidazoline-1-oxyl-4-il) disulfide from Institute of Organic Chemistry, Siberian Branch of the Russian Academy of Sciences, Novosibirsk. 1-Hydroxy-3-carboxy-pirrolidin was a gift from I. Kirilyuk (Institute of Organic Chemistry, Siberian Branch of the Russian Academy of Sciences, Novosibirsk).

Experiments were carried out on larvae of the greater wax moth *G. mellonella*. Laboratory culture of the insects was kept in darkness at 28°C on a nutritive medium [20]. Microsporidian spores of *V. ephestiae* (Microsporidia: Burenellidae) were kindly provided by Yu.Ya. Sokolova (Russian Research Institute of Plant Protection, St. Petersburg). A peroral infection of *G. mellonella* was performed by maintaining the 3rd age larvae on a nutritive medium containing *V. ephestiae* spores (10^6 spores/g medium). Development of microsporidian infection was controlled in the hemolymph and in tissues, using a phase-contrast microscopy.

Antioxidant enzyme activities were determined at different stages of microsporidiosis: in the beginning of microsporidian vegetative reproduction, i.e., at the phase of meront formation (the 3rd day after infection), at the period of transition to sporogony (the 6th day after infection), and at the period of massive sporogony (the 13th day after infection).

Preparation of samples for determination of enzyme activities. Hemolymph from 5–7 larvae was collected in a tube on ice-bath by cutting pseudopodium with a glass capillary. The hemolymph was centrifuged for 5 min at 500 *g* and 4°C; hemocytes were removed and supernatant was used for deter-

minations of the enzyme activities. Homogenates of the fat body and intestine were prepared in 50 mM phosphate buffer (PB, pH 7.4) containing 150 mM NaCl at 4–6°C. The removed organs were ground in a glass homogenizer in PB in the ratio of 1 g per 2 ml of the buffer (5 larvae per sample). The organ homogenates were centrifuged for 15 min at 10 000 *g* at 4°C. The obtained supernatant was used for assay of the enzyme activities.

Phenoloxidase (PO) activity was determined spectrophotometrically (at 490 nm) in a reaction medium containing 1 mg DOPA, 1 ml PB, and 30 ml of the cell-free hemolymph. Incubation was performed in darkness for 5 min at 22°C [21]. The specific enzyme activity was expressed in units of changes of optical density (A_{490}) of the incubation mixture in the course of the reaction and calculated for 1 min per 1 mg protein.

Superoxide dismutase activity was determined spectrophotometrically at 560 nm from inhibition of reduction of Nitroblue Tetrazolium by superoxide anion formed in the course of oxidation of xanthine by xanthine oxidase [8]. Reaction mixture containing 5 mg/ml BSA, 70 mM Nitroblue Tetrazolium, and 0.125 mM xanthine in PB, was incubated in darkness for 20 min at 28°C. The reaction was initiated by adding 100 ml (5.87 units/ml) of xanthine oxidase solution in PB. The unit of the SOD activity was considered the enzyme amount required for the 50% inhibition of the Nitroblue Tetrazolium reduction for 1 min per 1 mg protein.

The glutathione-S-transferase activity was determined as related to 1-chloro-2,4-dinitrobenzene [22]. Incubation was performed for 3 min at 28°C in 100 mM (K^+, Na^+)-phosphate buffer (pH 6.5) containing 1 mM glutathione, 1 mM 1-chloro-2,4-dinitrobenzene, and the enzyme source. Concentration of 5-(2,4-dinitrophenyl)-glutathione formed in the course of reaction was measured spectrophotometrically at 340 nm. The specific enzyme activity was expressed as units of change of the optical density (A_{340}) of the incubation mixture in the course of reaction and calculated for 1 min per 1 mg protein.

Rate of the AOM formation was determined in a cell-free hemolymph by using a spin trap of 1-hydroxy-3-carboxy-pyrrolidine [4, 23] that is nonspecifically oxidized by various AOM (superoxide radical, hydroxyl radical, peroxynitrite and other highly reactive metabolites) to form stable nitrooxyl radical (SR). The rate of the AOM formation in the hemolymph samples was determined by the EPR method by monitoring the SR accumulation kinetics by an increase of low-field spectral component. The sample radical concentration was determined from dependence of the EPR spectral amplitude on the SR concentration. To evaluate contribution of the superoxide radical to kinetics of oxidation of 1-hydroxy-3-carboxy-pyrrolidine, SOD was used as a competitive reagent [23].

The specimens were prepared in PB-D (phosphate buffer, pH 7.4, containing 20 mM mesylate deferoxamine). For EPR measurements the hemolymph was diluted 40 times in PB-D, mixed with 1-hydroxy-3-carboxy-pyrrolidine (1 mM), and placed in glass capillaries, 100 ml in volume and 1 mm in internal diameter. The concentrations of DOPA and SOD in the mixtures of hemolymph and the spin trap of 1-hydroxy-3-carboxy-pyrrolidine were 0.2 mg/ml and 100 U/ml, respectively.

The EPR measurements were performed at room temperature using a ER 200D-SRC spectrophotometer of X-diapason (Bruker, Germany) under the following conditions: field of 3474 Hz, screening 50 Hz, extrahigh frequency power of 20 mW, high-frequency modulation of magnetic field of 100 kHz; amplitude modulation of 1 Hz.

Quantitative determination of SH-groups in low- and high-molecular compounds of hemolymph was performed by the EPR method, using a biradical reagent with disulfide bond, bis(2,2,5,6-tetramethyl-3-imidazoline-1-oxy-4-il) disulfide [24].

Protein concentration in the hemolymph and tissue homogenates was determined by Bradford's method [25]. To construct calibration curve, BSA was used.

The data obtained were treated statistically, with calculating the means and standard errors. The statistically significant difference was determined using Student's *t* criterion.

RESULTS AND DISCUSSION

The results of the present study have shown that microsporidia produce a suppressive action on phenoloxidase (PO) activity in the hemolymph of *G.*

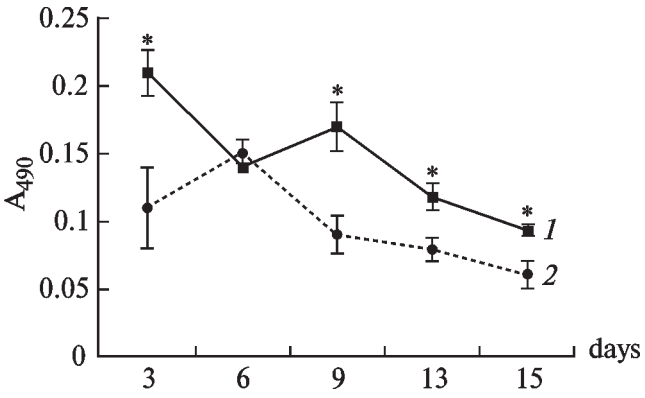

Fig. 1. Dynamics of phenoloxidase activity in the hemolymph of native (*1*) and microsporidian-infected (*2*) *G. mellonella* larvae ($n = 5$, asterisk designates statistically significant difference from control at the $p < 0.05$ level). *Abscissa*: time after infestation (days), *ordinate*: PO activity (A_{490}/mg/min).

mellonella throughout the entire experiment, except for the 6th day after infection, which corresponds to the onset of sporogony (Fig. 1). This coincides with data of Sokolova and co-authors [30] who revealed a decrease of the PO activity in the hemolymph and hemocytes of the cricket infected with the *Nosema grylli* microsporidia. The decrease of the hemolymph PO activity could result from a parasite-induced inhibition of phenoloxidase directly or of prophenoloxidase cascade. The results obtained are in agreement with data of other authors who revealed that some entomoparasites, such as hymenopterous endoparasitoids [26], trypanosomes [27], entomopathogenic nematodes [28], entomopathogenic fungi [29], are able to suppress protective host reactions by inhibiting the hemolymph PO activity. The inhibitory effect of *V. ephestiae* on the PO activity is likely to be due to an adaptation of microsporidia to parasitism in the insect organism.

To evaluate the microsporidian effect on the rate of AOM production in hemolymph, we compared the rate of 1-hydroxy-3-carboxy-pyrrolidine oxidation by hemolymph of intact and infected *G. mellonella* larvae. In 3 days after infestation, at the phase of merogony, a statistically significant increase of the AOM production was found in hemolymph of infected insects (see table). It is to be noted that in 5 days after microsporidian infestation, i.e., in the beginning of sporogony, the AOM production in the hemolymph of infected insects decreased to the control level (see table). In 13 days after infestation, during the massive release of microsporidian spores into hemolymph and destruction of fat body cells, a statistically significant reduction of the rate of 1-hydroxy-3-carboxy-pyrrolidine oxidation occurred in the infected insect hemolymph as compared with control both with addition of DOPA and without it (see table). It is difficult to explain the marked rise of the 1-hydroxy-3-carboxy-pyrrolidine oxidation in the presence of DOPA in control at the 13th day of larval infestation by microsporidia. It cannot be ruled out that this is due to a total reduction of activities of several antioxidant enzymes in hemolymph at this stage of larval development as compared with the 3rd day after infestation.

Since 1-hydroxy-3-carboxy-pyrrolidine can be oxidized by several AOM species, we used SOD to elucidate contribution of the superoxide anion to 1-hydroxy-3-carboxy-pyrrolidine oxidation. The

Change in the rate of 1-hydroxy-3-carboxy-pyrrolidine oxidation (nM/s) in hemolymph of *G. mellonella* larvae after infestation with *V. ephestiae* microsporidian

Time after infestation (days)	Control larvae			Larvae infected with sporidia		
	hemolymph	hemolymph + DOPA	hemolymph + DOPA + SOD	hemolymph	hemolymph + DOPA	hemolymph + DOPA + SOD
3rd	84.6 ± 5	1015.4 ± 276.9	1000 ± 338.5	160 ± 30.8*	623 ± 184.6	1015 ± 284.6
6th	184 ± 6	1020.9 ± 298	950 ± 240	185 ± 15	880.7 ± 165.8	1005 ± 340
13th	227.3 ± 40.9	3181.8 ± 554.4	2727.3 ± 36.4	181.8 ± 42	772.7 ± 445.5**	636.4 ± 181.8**

Note: $n = 12$. Asterisks indicate statistically significant differences from control: one—$p < 0.05$, two—$p < 0.001$.

data obtained have demonstrated the absence of the SOD effect on the rate of AOM production activated by addition of DOPA into hemolymph (see table), which indicates a negligible contribution of the superoxide radical to the 1-hydroxy-3-carboxy-pyrrolidine oxidation by hemolymph. Nevertheless, this fact does not rule out a possibility of formation of superoxide.

In 3 days after the insect infestation, i.e., at the early stages of the microsporidian development, a decrease of the SOD activity was revealed in hemolymph of the wax moth larvae (Fig. 2). In 6 days after infestation, no significant difference in the SOD activity was found between the infected and control insects. In 13 days after the infestation by microsporidian there was recorded a rise of the SOD activity in hemolymph of infected larvae (Fig. 2).

To establish the role of antioxidant enzymes in protection of cells from potential damage by organic hydroperoxides produced during oxidative stress, we measured the GST activity that plays the key role in elimination of hydroperoxides from cells [3]. The results obtained have shown that in 3 days after infestation the GST activity in hemolymph of *G. mellonella* larvae decreases 2.4 times as compared with control (Fig. 2). In 6 days after infestation, there was no statistically significant difference between the control and infected larvae. In 13 days after infestation, an elevation of the GST hemolymph activity was observed in infected larvae (Fig. 2). This might have been due to the GST detoxicational function [31], as at acute microsporidiosis a release of toxic molecules into hemolymph is possible as a result of disturbance of the total metabolism and destruction of host cells.

For the first 6 days after infestation, concentration of thiol-containing proteins in hemolymph did not change at microsporidiosis in comparison with control. In 13 days after infestation, a statistically non-significant increase of the content of SH-containing compounds was found in hemolymph of infected insects.

As a results of our studies, it has been revealed that activity of the antioxidant system and generation of free radicals in hemolymph of *G. mellonella* larvae change depending on the stage of microsporidian development in the insect organism. The initial stages of parasite development are accompanied by a rise of the AOM production and a simultaneous decrease of the antioxidant enzyme activity, which might be due to a peculiarity of interaction between the parasite and host at this stage of the microsporidian life cycle, when the antagonistic interactions between the microsporidia and host may be "veiled" [32]. The suppressive action of the parasite on the free radical production in hemolymph of the greater wax moth larvae at acute microsporidiosis is due, on one hand, to inhibition of the prophenoloxidase system as one of the free radical sources and, on the other hand, to an increase of activity of antioxidants at massive microsporidian sporogony in tissues of the host insect. Apart from the organism protection from AOM, the antioxidant enzymes might participate in detoxication and elimination of the toxic substances that accompany destructive cell changes at microsporidiosis.

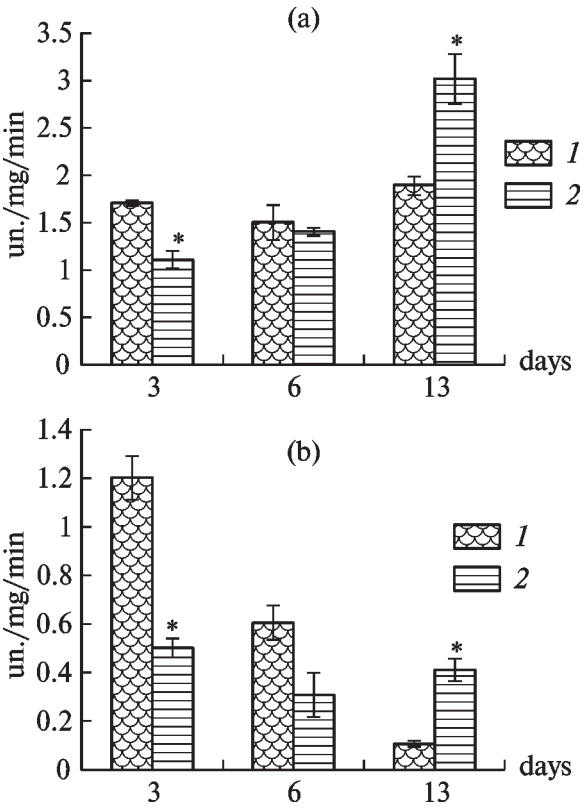

Fig. 2. Activities of SOD (a) and GST (b) in hemolymph of native (*1*) and microsporidian-infected (*2*) *G. mellonella* larvae in different days after infestation; asterisk designates statistically significant difference from control at the $p < 0.05$ level.

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