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## COMPARATIVE AND ONTOGENIC BIOCHEMISTRY

# Generation of Reactive Oxygen Species and Activity of Antioxidants in Hemolymph of the Moth Larvae *Galleria mellonella* (L.) (Lepidoptera: Piralidae) at Development of the Process of Encapsulation

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**Abstract**—Activities of enzymatic antioxidants—superoxide dismutase, glutathione-S-transferase, and catalase—as well as generation of reactive oxygen species (ROS) in lymph of the honeycomb moth *Galleria mellonella* L. were studied at development of the process of encapsulation of nylon implants. It has been established that as soon as 15 min after piercing of cuticle with the implant the capsule is formed on its surface. Active melanization of the capsule has been shown to last for 4 h. A statistically significant increase of the ROS generation in lymph and a decrease of the enzymatic antioxidant activities in the insect hemocytes have been revealed after the implant incorporation. The authors suggest that the key role in maintenance of the oxidation–reduction balance in hemolymph at development of the encapsulation process is played by non-oxidative antioxidants.

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*Key words*: insect immunity, cellular immune reactions, reactive oxygen species, hemocytes, superoxide dismutase.

## INTRODUCTION

Invertebrates, specifically insects, have a developed system of innate immunity aimed at eliminating the invading pathogens and parasites. At penetration of parasite and injury of cuticle and epidermal cells, some substances attracting parasites are released into the hemocoele. Hemocytes mediating phagocytosis provide elimination of small objects not exceeding the size of the hemocytes themselves as well as encapsulation of the larger objects, which consists in their isolation and inactivation. The process of encapsulation is known to begin with the first minutes after penetration of the foreign object and occurs actively for 24 h [1, 2]. Hemocytes have been established to play the key role in the process of encapsulation by attaching to the surface of the particle and by participating in formation of the capsule [2]. Simultaneously, by the cascade principle, phenoloxides triggering melanogenes are activated, which results in formation of an insoluble polymer—melanin. It has been found that the process of melanization in insects is accompanied by formation of the potentially toxic reactive oxygen species (ROS) including semiquinone radicals [4], hydrogen peroxide

[5, 6], and superoxide anion [7, 8]. It has been shown that an increase of ROS generation occurs at development of myxoses in flies Drosophila [7, 9]. It has also been established the increase of the ROS generation to take place at development of mycoses and microsporidioses in larvae of *Galleria mellonella* [10, 11]. There has been shown the key role of ROS in encapsulation and elimination of Plasmodium in the mosquito Anopheles [12, 13]. The studies were carried out which indicate participation of hemocytes in the ROS production in insects [8, 14, 15]. It is suggested that the ROS formed during encapsulation can participate in elimination of parasite at the expense of the high reactional capability [16]. It is to be noted that the cytotoxic ROS action can affect both entomopathogens and the host cells and tissues. The cytotoxic ROS effect can lead to a non-controlled increase of lipid peroxidation; besides, ROS can damage DNA and protein molecules [17].

The body of insects, like of many other animals, contains a complex of antioxidant and detoxifying enzymes whose action is directed to the ROS elimination [18]. In animals, including insects, several most important components of the antioxidant system are identified; they are subdivided to enzymatic antioxidants (superoxide dismutase (SOD), catalase, glutathione-S-transferase (GT), peroxidase) and non-enzymatic antioxidants (phenol-containing compounds, α-tocopherol, ascorbic acid, high- and low-molecular thiols) [18, 19]. Enzymatic antioxidants are characterized by a high specificity of action directed against certain ROS forms. SOD catalyzes reaction of dismutation of superoxide anion-radical ($O^{2*-}$) to hydrogen peroxide ($H_2O_2$), catalase degrades $H_2O_2$, GT protects cells from xenobiotics and products of lipid peroxidation by their reduction, with participation of reduced glutathione [17, 18, 20].

It is known that several enzymatic antioxidants, by inhibiting ROS generation, can also inhibit the process of melanogenesis. It has been shown that catalase and SOD can decrease intensity of oxidation of 3,4-dihydroxyphenylalanine (DOPA) and dopamine by phenoloxidase [21]. However, it is also established that SOD is necessary for production of tyrosyl-radical and the cytotoxic ROS participating in elimination of parasite [22].

Thus, on other hand, antioxidants participate in control of melanogenesis and can block it, but on the other hand, they act as a most important attribute of ROS activity directed against parasites. Unfortunately, most works describing role of antioxidants are performed *in vitro* and with use of exogenous enzymes and allow only modeling the processes occurring in the insect body [5, 21]. The role of ROS and antioxidants in the process of encapsulation of a foreign object remains unclear. Also poorly studied is significance of antioxidants in hemocytes playing the key role in melanization and encapsulation of foreign objects.

In this connection, the goal of our work was to study the antioxidative system components participating in control of ROS generation at development of the process of encapsulation of nylon implants.

## MATERIALS AND METHODS

*Reagents.* Used in the work were reduced glutathione, 1-chloro-2,4-dinitrobenzene (DNB), xanthine oxidase, Tetrazolium nitroblue (TNB), ethylenediamine tetraacetic acid (EDTA), dihydroxyphenylalanine (DOPA), and HEPES (Sigma, USA).

*Insects.* Laboratory population of *C. mellonella* was maintained at 28°C in an artificial nutrition medium that contained bee honey (15%), wax (15%), glycerol (15%), fat-free dry milk (15%), and corn and wheat flour (40%). The studies were performed on larvae of the 5–6th age.

*Implants.* Used in the experiments were implants, 2 mm in length and 0.5 mm in diameter, made of nylon thread. The implants were introduced into the larva hemocoele of the coneycomb moth of the 6th age through puncture in the 4th ventral cuticle segment. After different time periods—15, 30, 60 min, 4 and 24 h—the implants were removed and used for analysis of the degree of darkening and the thickness of their formed melanotic capsule. At the same time periods, the insect hemolymph samples were collected for study of the COD, catalase, and GT activities in hemocytes and of generation of ROS and hydrogen peroxide in lymph.

*Measurement of intensity of encapsulation.* The encapsulation intensity was estimated by the degree of darkening and the thickness of the melan-

otic capsule formed on the surface of nylon implant. To evaluate the degree of the capsule darkening, the removed implants were photographed from three different sides. The encapsulation intensity was estimated by the degree of the capsule darkening with use of the software Image J [23, 24].

To estimate thickness of the formed capsule, the removed implants were dehydrated in a series of acetone gradient and then placed to a mixture of Epon—Araldite resins for 24 h at 60°C. From the obtained blocks, semithin sections were prepared with aid of an LCB 3 ultratome. Thickness of melanotic capsules on the sections was estimated with aid of an Axioscop 40 light microscope (Carl Zeiss, Germany).

*Preparation of hemocyte and lymph samples.* To obtain samples of hemocytes and lymph, hemolymph was collected in plastic test tubes with chilled (4°C) anticoagulant containing (mmol/l): 62 NaCl, 100 glucose, 10 EDTA, 30 sodium citrate, 26 citric acid, pH 4.6. Then lymph was centrifuged for 5 min at 500 *g*. Supernatant was used as lymph samples. To obtain hemocyte sample, the obtained pellet was resuspended and washed out in anticoagulant 3 times for 5 min at 500 *g* and once in HEPES-buffer, pH 7.2, containing (mM): 10 HEPES, 140 NaCl, 5 KCl, 6 glucose.

*Determination of COD, catalase, and GT activities.* Activity of COD was determined spectrophotometrically at 560 nm by inhibition of rate of reduction of Tetrazolium nitroblue by superoxide-anion formed in the process of oxidation of xanthine by xanthine oxidase [25]. To 500 μl of reaction mixture containing 5 mg/ml BSA, 70 μM TNB, and 125 μM xanthine in phosphate buffer (pB), 80 μl of sample were added, after which the reaction was initiated by addition of 20 μl of xanthine oxidase solution (5.87 un. act./ml) and incubated at 28°C in darkness for 20 min. The specific enzyme activity was expressed in units of change of optical density of incubation solution as compared with the control one at 560 nm in the course of reaction for 1 min and per 1 mg protein.

Activity of glutathione-S-transferase was determined spectrophotometrically at 340 nm by the rate of the rise of concentration of 5-2(2,4-dinitrophenyl)-glutathione, product of reaction of dinitrobenzene (DNB) and reduced glutathione, catalyzed by GT [26]. Incubation was performed at 22°C for 5 min in 500 μl of PB containing 1 mM glutathione, 1 mM DNB, and 20 μM of the experimental sample. The specific enzyme activity was expressed in units of change of optical density of the incubation mixture at 340 nm in the course of reaction for 1 min and per 1 mg protein.

Activity of catalase was determined spectrophotometrically at 240 nm by the rate of disintegration of $H_2O_2$ [27]. To 600 μl of reaction mixture (150 μl of 3% $H_2O_2$ in 400 μl of PB), 5 μl of the experimental sample were added and incubated for 10 min at 22°C. The specific enzyme activity was expressed in units of change of optical density of the incubation mixture at 240 nm in the course of reaction for 1 min and per 1 mg protein.

*Change of rate of ROS generation.* Study of rate of the ROS generation in lymph was performed by the method of electron paramagnet resonance (EPR) by using the sterically handicapped solution of hydroxylamine (SSH) that is oxidized by ROS with formation of stable nitroxyl radical, SR [28]. The SR solution (10 mM) was prepared before the experiment by dissolving sample in the argon-blown-off PB. The hemolymph sample was added by SR until a concentration of 1 mM. The mixture was placed into a glass capillary (50 μl). The SR formation rate was determined by an increase intensity of the low-pole component of EPR specter.

The adjustments of the EPR-spectrometer: amplitude of modulation 1 Gs, power of microwave irradiation 20 mW, the EPR-spectrometer ER 200D-SRC of X-diapason (Bruker, Germany).

*Determination of concentration of hydrogen peroxide.* Production of hydrogen peroxide in lymph by the FOX method (ferrous oxidation xylenol orange) was determined [29]. For this, hemolymph was diluted 10 times with phosphate buffer; added to experimental samples was 1 mM $NaN_3$ for inhibition of endogenous catalase, while to control samples—200 un. act./ml instead of $NaN_3$. Melanization was initiated by addition of 1 mM DOPA. Samples (200 μl) were incubated at 28°C for 30 min, then added to them were 800 μl of the FOX solution (312.5 μM $FeSO_4$, 125 μM xylene orange, and 125 mM sorbitol in 0.3 M HCl–NaCl buffer, pH 1.4), and incubated at room temperature for 20 min. To determine concentration of the formed hydroxide, intensity of optical absorption of mix-

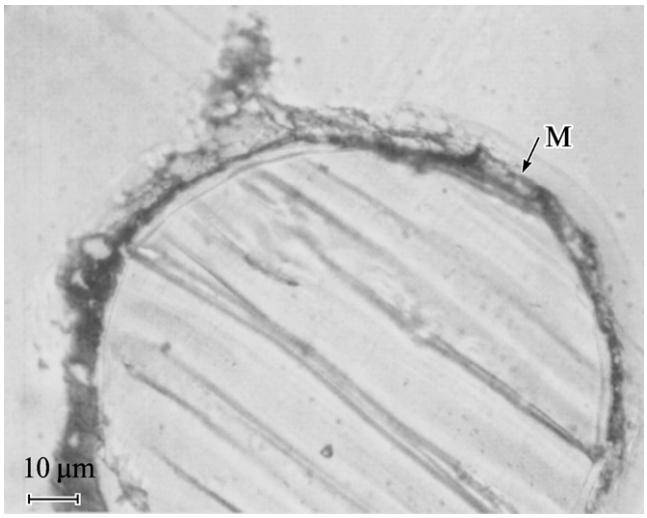


**Fig. 1.** Transverse section of the nylon implant incorporated into hemocoele of the larva *G. mellonella*. *Asterisk* indicates formation of melanin capsule (M) on its surface.

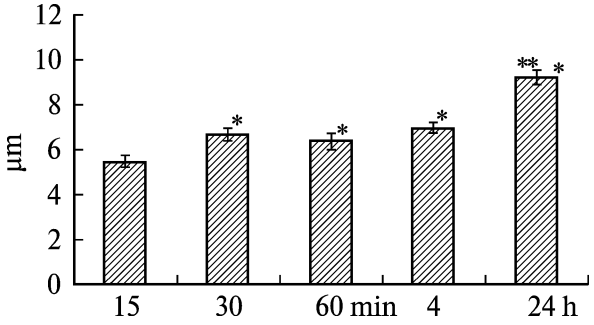


**Fig. 2.** Thickness of melanotic capsule (*vertical axis*, μm) on the implant surface at development of the encapsulation process in larvae *G. mellonella* ($n = 25$ for each variant). *Asterisks* indicate: one—statistical significance of differences ($p < 0.05$) between the indicated variants and the variant of 15 min; two—statistical significance of difference ($p < 0.001$) between the indicated variant and the remaining ones. *Horizontal axis*: time after the transplant incorporation. (the same for Figs. 3–5).

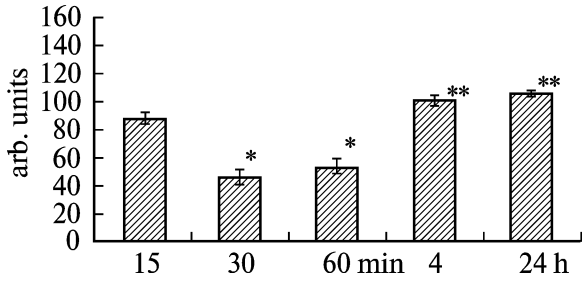


**Fig. 3.** Intensity of the implant darkening (*vertical axis*, arb. units) at development of the encapsulation process in larvae *G. mellonella* ($n = 15$ for each variant). *Asterisks* indicate: one—statistical significance of differences ($p < 0.01$) between the indicated variants and the variant of 15 min; two—statistical significance of difference ($p < 0.01$) between the indicated variant and the remaining ones.

tures at 560 nm was measured and the previously obtained calibration straight line for hydrogen peroxide was used. Optical measurements were performed on an UV-2401 ($p$C) CE spectrophotometer (Shimadzu, Japan).

*Determination of concentration of protein.* Concentration of protein in hemolymph and in tissue homogenates was determined by the method of Bradford [30]. To construct calibration curve, BSA was used.

*Statistical analysis.* The obtained results were treated statistically by calculating the mean and its error. To check normal distribution of data, the W-criterion of Shapiro–Wilk was used. The statistical significance of differences was determined by Student's criterion with aid of software STATISTICA 6.0. The data are presented as the mean ± standard error of the mean ($x \pm S_x$).

## RESULTS

*Intensity of encapsulation.* Analysis of thickness of the capsule envelope has shown that as soon as 15 min after incorporation of implant, capsule with the wall thickness of 5.4 ± 1.4 μm is formed (Figs. 1 and 2). After 30 min the wall thickness reached the value of 6.6 ± 2.01 μm and subsequently did not change statistically significantly ($p \geq 0.05$) for 4 h (Fig. 2). After 24 h the capsule wall thickness amounted to 9.2 ± 2.2 μm and exceeded statistically significantly ($p \leq 0.05$) all previous values.

Estimation of intensity of implant darkening showed a statistically significant increase of the melanization degree for the first 4 h after the implant incorporation. The process was running actively for the first 15 min of encapsulation (Fig. 3). The darkening intensity increased 2–3 times by the 60th min as compared with the encapsulation in-

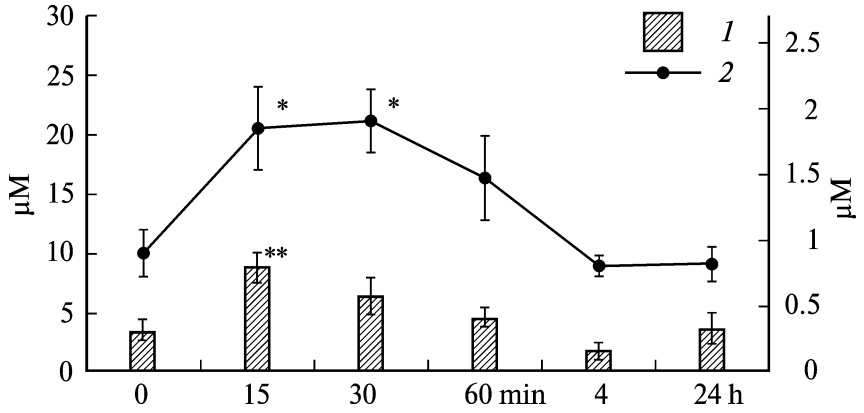


**Fig. 4.** Concentration of peroxide (*vertical axis*, *left*, μm, 1) and intensity of oxidation of SR-H (*vertical axis*, *right*, μm, 2) in hemolymph at development of the encapsulation process in larvae *G. mellonella* ($n = 15$ for each variant). *Asterisks* indicate: one—statistical significance of differences ($p < 0.01$) between the indicated variants and the variants of 0, 4, and 24 h; two—statistical significance of difference ($p < 0.01$) between the indicated variant and the variants 0, 60 min, 4 and 24 h.

tensity after 15 min ($p \le 0.05$; Fig. 3); after 4 h, a statistically significant ($p \le 0.05$) 2–3-fold increase as compared with the first hour is revealed. It is to be noted that after 4 and 24 h the capsule darkening intensity did not differ statistically significantly (Fig. 3).

*Generation of ROS at development of the process of encapsulation.* Study of ROS generation in the insect lymph by the SSH oxidation rate has revealed a twofold rise of the ROS generation 15 and 30 min after the implant incorporation ($p \le 0.05$; Fig. 4). At further development of the encapsulation process the ROS generation rate decreased to the values that did not differ statistically significantly from control (native insects). Analysis of hydrogen peroxide formation in lymph at development of encapsulation has shown similar tendencies. The lymph peroxide concentration increased statistically significantly ($p \le 0.05$) 15 min after the implant incorporation (Fig. 4). Subsequently, its concentration decreased until the level found in native insects (Fig. 4).

*Activities of SOD, catalase, and GT at development of process of encapsulation.* Evaluation of activities of antioxidants in hemocytes in the process of encapsulation has established a statistically significant decrease of the SOD activity 15, 30, 60 min, and 4 h after the implant incorporation (Fig. 5a). The intracellular catalase activity also decreased statistically significantly—2.2 times 15 min after the beginning of the encapsulation process (Fig. 5b). From the 30th min and to the 4th hour of the encapsulation process, the catalase activity was statistically significantly, 1.5 times lower than the control values (Fig. 5b). Activity of glutathione-S-transferase decreased sharply (3 times) by 15 min of the encapsulation development and remained decreased 2 times for 4 h (Fig. 5c). 24 hours after the implant incorporation, activities of SOD, catalase, and glutathione-S-transferase reached control values.

## DISCUSSION

The obtained results indicate the process of hemocyte adhesion and melanization to occur actively as soon as 15 min after incorporation of implant into hemocoele through the cuticle puncture (Figs. 2 and 3). These processes occur synchronously: adhesion of hemocytes is accompanied by initiation of melanogenesis, i.e., the melanin envelope is formed. This seems to be due to phenoloxidase activation of hemocytes at their spreading on the surface of foreign object. Several studies showed a rise of the phenoloxidase activity of hemocytes at their binding with antigen owing to activation of the prophenoloxidase system [2, 31]. It is to be noted that the capsule thickness on the implant surface 30, 60 min, and 4 h after its incorporation changed insignificantly (Fig. 2). During these time periods the

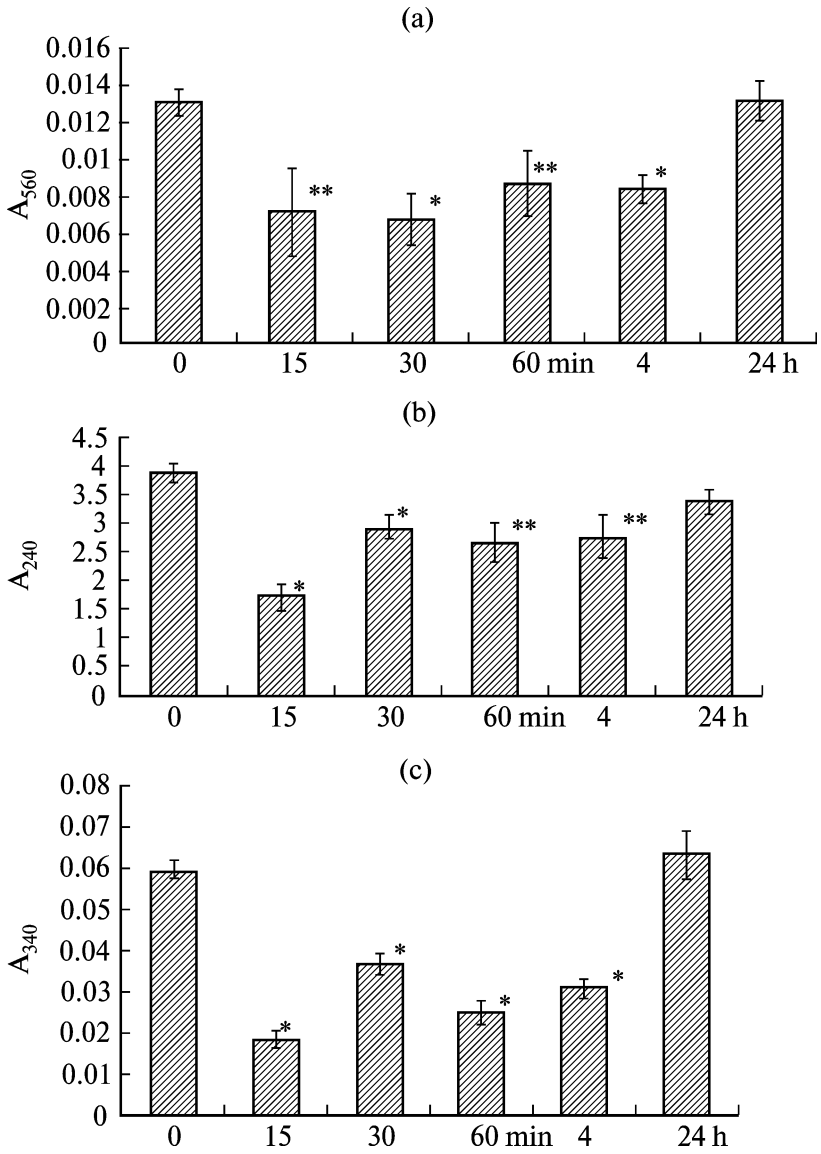


**Fig. 5.** Activities of SOD (a), catalase (b), and GT (c) (*vertical axis*, A/min/mg proteins) in hemocytes at development of the encapsulation process in larvae *G. mellonella* ($n = 20$ for each variant). (a) and (b), *asterisks* indicate: one—statistical significance of differences ($p < 0.001$) between the indicated variant and all remaining ones; two—statistical significance of difference ($p < 0.05$) between the indicated variant and the variants 0, 60 min, 4 and 24 h. (c), *asterisk* indicates statistical significance of differences ($p < 0.001$) between the indicated variant and the remaining ones.

capsule melanization process occurred sufficiently actively as compared with period of 15 min after the beginning of encapsulation, which is indicated by an increased implant melanization after 30, 60 min, and 4 h (Fig. 3). These results indicate that alongside with the fast process of hemocyte attachment and formation of the main mass of capsule by the 15th min, the process of its melanization continues for 4 h. The absence of changes in the capsule darkening intensity at the time period between 4 and 24 h can indicate completion of the initial stage of the intensive capsule melanization by the 4th hour. Analysis of the capsule thickness has shown its statistically significant (by 30%) increase by the 24th hour as compared with the 4-h point (Fig. 2). This increase might possibly occur owing to attachment of native hemocytes to the surface of the melanized capsule.

Study of ROS generation in the process of encapsulation in the insect lymph by the rate of oxidation of the SSH probe revealed an increase of the ROS generation rate 15 and 30 min after the implant incorporation (Fig. 4). An increase of the hydrogen peroxide production was also found 15 min after the beginning of the encapsulation process (Fig. 4). Earlier, the melanization process was established to be accompanied by release of ROS [14, 21, 32], but data in favor of their participation in the hemocyte-mediated encapsulation process were occasional [7, 13]. Our data have confirmed the capsule formation and its melanization to occur with participation of ROS. Besides, the revealed ROS release into lymph confirms the hypothesis of the key role of radicals in elimination of pathogens penetrating into the hemocoele [16].

It is to be noted that the capsule melanization process on implant occurs actively for the first hours (Fig. 3), with a decrease of the ROS concentration recorded by the first hour of the encapsulation process (Fig. 4). The ROS inactivation in the insect hemolymph seems to be owing to the antioxidant system. In our studies, we did not note a rise of the COD, catalase, and GT activities in hemocytes of the honeycomb moth larvae at development of the encapsulation process (Fig. 5). The generation of highly reactive ROS might probably be controlled by non-enzymatic antioxidants present in lymph. The non-enzymatic antioxidants are known to participate in the process of regulation of oxidation–reduction processes in lymph [13, 33]. Also known are extracellular forms of antioxidant enzymes that can provide inactivation of superoxide-anion, hydrogen peroxide, and organic peroxides [17].

Besides, we observed a decrease of activity of enzymatic antioxidants in hemocytes for the first 4 h after the implant incorporation (Fig. 5). These changes might probably be a consequence of disturbance of the oxidation–reduction balance in cells after activation of hematocytes due to development of the encapsulation process. This suggestion agrees with the fact of increase of ROS production in hematocytes at contact with antigen and development of reaction of phagocytosis [8, 15, 34]. These processes can be compared with the change of the balance of antioxidants—activated oxygen metabolites due to development of inflammatory reactions at contact with antigen [17]. Moreover, in some cases at inflammatory reaction there is fixed the granulocyte state close to apoptosis, which is one of the immunity mechanisms in vertebrates [35]. Thus, it cannot be ruled out that the revealed decrease of the antioxidant activity of enzymes in insect blood can indicate the state of the induced apoptosis and, as a consequence,—the elevated "readiness" of hemocytes to participation in defense reactions at the expense of destruction and release to lymph of various activators of immune reactions (calcium, chemokinins, activators of prophenoloxidase, etc.).

Thus, based on the presented results, it can be suggested that mechanisms of the encapsulation process in insects allow isolating rapidly the foreign object by formation of capsule. In the process of melanization, activated oxygen metabolites can be generated; they can participate in destruction of the invaded pathogen. Probably, in the insect body there exist the mechanisms that provide localization of the process of melanization and ROS generation within the limits of the penetrated foreign object. This, on one hand, in the site of invasion of parasite, allows using in the full measure the highly reactive compounds mediating activity of cell immunity, while, on the other hand, can prevent further increase of generation of these unspecific compounds in lymph, i.e., can prevent destruction of the own body tissue. It cannot be ruled out that these functions are performed by the hemolymph antioxidant system. As shown by our studies, it is hardly probable that the process of ROS inactivation in lymph is controlled by antioxidant enzymes of hemocytes. Probably the key role in maintenance of the oxidation–reduction balance in hemolymph during development of the encapsulation process is played by the lymph non-enzymatic antioxidants.

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