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GENERAL BIOLOGY

Generation of Superoxide Radical and Hydrogen Peroxide in Insect Hemolymph in the Course of Immune Response

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Prophenoloxidase system, which is represented by a whole cascade of enzymatic reactions, one of the final products of which is melanin, plays a major role in insect immunity [1]. Reactive intermediates produced during melanogenesis may participate in cytotoxic reactions in the course of granule formation and incapsulation of a foreign agent in an insect organism. This group primarily involves *o*-semiquinones formed during enzymatic oxidation of *o*-hydroquinones (in particular, 3,4-dihydroxyphenylalanine, DOPA) [1, 2]. Such compounds can interact with oxygen to form superoxide anion ($O_2^{\bullet}$), which, after a number of transformations (dismutation and Fenton reaction), is eventually converted to other reactive particles, such as hydrogen peroxide and hydroxyl radical [1]. Earlier, we applied EPR to demonstrate the formation of DOPA *o*-semiquinones in the hemolymph of *Galleria mellonella* larvae during melanogenesis [3, 4]. Studies of the generation of superoxide and hydrogen peroxide in insect hemolymph are represented by a few papers. It should be noted that the formation of $O_2^{\bullet}$ has not yet been recorded directly. Some authors tested generation of $O_2^{\bullet}$ in insect hemolymph using the method based on reduction of nitro blue tetrazolium [1, 5, 6]. However, the nonspecificity of this method [7] does not allow the demonstration of $O_2^{\bullet}$ formation in insects' organism to be regarded as reliable data. Today, the most informative method of recording short-lived oxygen radicals in biological systems is the spin trap method in combination with EPR spectroscopy. The goal of this study was to investigate by this method the formation of $O_2^{\bullet}$ in the course of melanization and to determine a possible role of superoxide in immune response in insects.

We used three types of spin traps exhibiting different chemical properties with respect to the superoxide radical. The most widely used nitrone trap DEPMPO forms the paramagnetic adduct DEPMPO–OOH with a rate constant of 0.53 $M^{-1} s^{-1}$ [8]. Figure 1b shows a representative EPR spectrum of the DEPMPO–OOH adduct formed in the system of $O_2^{\bullet}$ generation (xanthine/xanthine oxidase)/ However, in the system of enzymatic oxidation of DOPA by phenoloxidase, which simulates the process of melanization, the EPR signal was absent (Fig. 1a). The concentration of DOPA and the activity of phenoloxidase varied in the ranges 1–10 mM and 5–100 U/ml, respectively. It was found that, in the presence of DOPA even at a low concentration (10 μM) in the $O_2^{\bullet}$ generation system, the EPR signal intensity considerably decreased (Figs. 1b, 1c). This finding led us to assume that DOPA interacts with $O_2^{\bullet}$ by competing with the trap. The ability of various hydroquinones to interact with superoxide was described in [9].

To rule out the competition between the trap and DOPA, a trap with a greater effectiveness of interaction with superoxide is required. Earlier, we recorded superoxide in the hemolymph of *Gall. mellonella* larvae using a trap derived from cyclic hydroxylamine, 1-hydroxy-3-carboxy-pyrrolidine (CP–H). Oxidation of this trap yields a stable nitroxyl radical, 3-carboxyproxyl (CP), with a rate constant of 3.2×10^3 $M^{-1} s^{-1}$ [10]. Using this trap, we recorded no superoxide generation in insect hemolymph during melanization. In this study, we used a trap based on the triarylmethyl C-centered radical, TAM, which reacts with superoxide in a specific manner with a rate constant of 3.1×10^3 $M^{-1} s^{-1}$, lacking its paramagnetic properties in the course of this reaction [11]. We used TAM for testing superoxide formation during DOPA oxidation by phenoloxidase. For this purpose, we recorded the kinetics of the EPR signal

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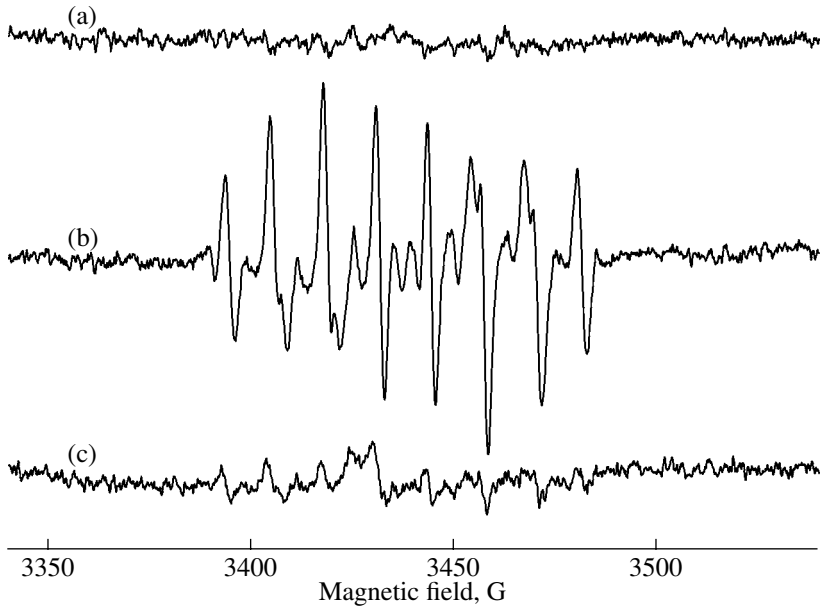

Fig. 1. EPR spectra obtained in the present of the spin trap DEPMPO (10 mM) during (a) oxidation of DOPA (1 mM) by phenoloxidase (20 U/ml), (b) oxidation of xanthine (0.05 mM) by xanthine oxidase (0.05 U/ml), and (c) oxidation of xanthine (0.05 mM) by xanthine oxidase (0.05 U/ml) in the presence of DOPA (10 μM).

amplitude in two samples: in the absence of superoxide dismutase (SOD) and in the presence of saturating concentration of SOD. It appeared that SOD had no effect on the kinetics, which suggests the absence of reaction between superoxide and TAM in the system specified. Generalizing the results of experiments with the used of spin traps, we assumed that the impossibility to record $O_2^{\cdot}$ with the use of spin traps during melanization may be the result of effective interaction of DOPA with $O_2^{\cdot}$. To test this hypothesis, we studied the oxidation of the spin trap CP–H by superoxide in the presence of DOPA. A solution of KO_2 in DMSO was used as a source of superoxide in these experiments. The intensity of the ERP signal of CP formed in the mixture of CP–H and KO_2 in dependence on DOPA concentration was determined. As seen from Fig. 2, DOPA present in the mixture suppressed the formation of CP, thereby confirming that the reaction between DOPA and superoxide indeed takes place. Taking into account the competition between DOPA and CP–H for superoxide, we estimated the rate constant of the reaction of DOPA with superoxide, which amounted to 5×10^5 M^{-1} s^{-1}. The value of this constant is greater by orders of magnitude than the rate constants of the spin traps that we used to study superoxide generation in the course of enzymatic oxidation of DOPA. Thus, this result can explain the fact that superoxide adducts of the traps used cannot form in this system, despite the possible generation of the superoxide radical.

It was natural to assume that the interaction of DOPA with superoxide yields DOPA-semiquinone and hydrogen peroxide:

$$QH_2 + O_2^{\cdot} \rightarrow Q^{\cdot} + H_2O_2, \quad (1)$$

where QH_2 is DOPA and $Q^{\cdot}$ is DOPA-semiquinone.

This assumption was corroborated experimentally [12]. The formation of short-lived DOPA-semiquinone was recorded by EPR spectroscopy using spin stabilization by Zn^{2+} ions. Hydrogen peroxide formation during UV photolysis of DOPA solution, when the superoxide radical is known to form [12], was demonstrated.

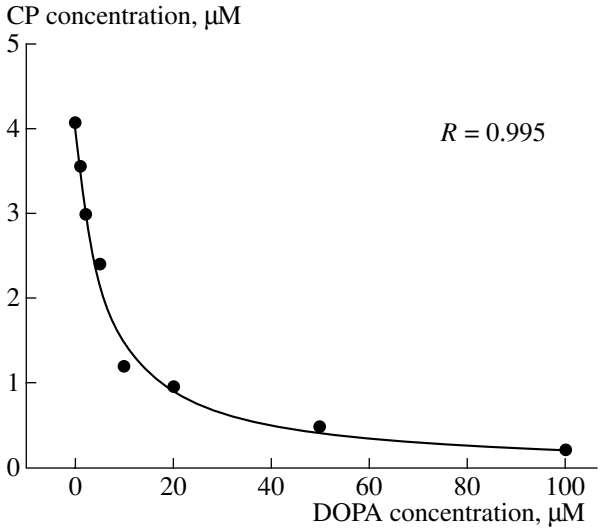

Fig. 2. Effect of DOPA on the oxidation of the spin trap CP-H (1 mM) by superoxide. The concentration of CP was determined by the intensity of the EPR spectrum.

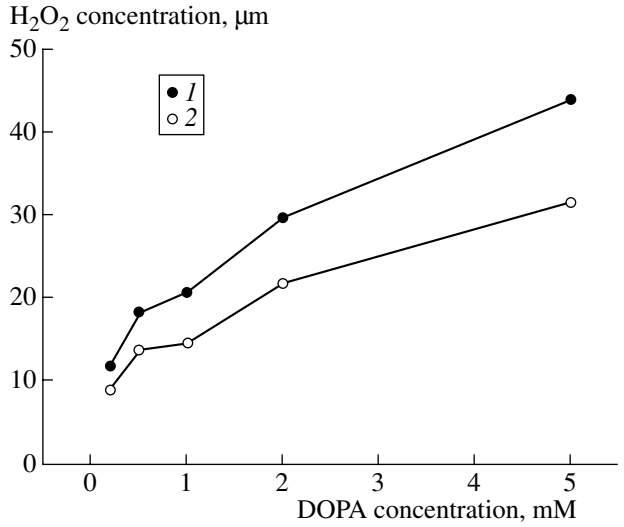

Fig. 3. Hydrogen peroxide generation in the reaction of enzymatic oxidation of DOPA. DOPA was incubated in phosphate buffer in the presence of phenoloxidase (5 U/ml) for 30 min (*1*) in the absence and (*2*) presence of SOD (200 U/ml).

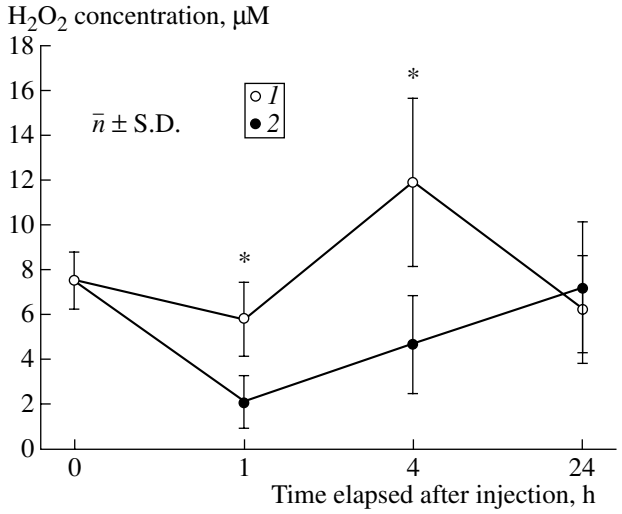

Fig. 4. Hydrogen peroxide generation in the hemolymph of (*1*) insects injected with *B. thuringiensis* and (*2*) control insects. The asterisk marks the values that statistically differed from the control at $p < 0.05$ ($n = 7–20$).

Therefore, if the superoxide radical forms in the course of melanization, it can be detected by accumulation of hydrogen peroxide according to reaction (1). Figure 3 shows the formation of hydrogen peroxide in the course of enzymatic oxidation of DOPA and demonstrates that the yield of H_2O_2 decreases in the presence of SOD. This indicates that H_2O_2 derived from the superoxide radical. According to the stoichiometry of reactions (1) and (2), the yield of hydrogen peroxide in the presence of SOD should decrease by a factor of 2:

$$2Q_2^{\cdot} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2. \quad (2)$$

However, as seen from Fig. 3, it decreased only by ~25%. This means that not all hydrogen peroxide forms from superoxide in the course of melanization. Part of it forms directly on the enzyme via two-electron reduction of molecular oxygen:

$$QH_2 + O_2 \xrightarrow{PO} Q + H_2O_2, \quad (3)$$

where *Q* is DOPA-quinone.

Thus, our results are indicative of formation of H_2O_2 and the superoxide radical at the initial stage of melanization—oxidation of DOPA by phenoloxidase. These reactive oxygen species may play a major role in the protective mechanism of insects in infection.

We studied the effect of phagocytosis on the generation of hydrogen peroxide in the hemolymph of *Gall. mellonella* larvae. For this purpose, 4 μl of a suspension of formalin-treated vegetative cells of *Bacillus thuringiensis* spp. *galleriae* strain 69-6 (one-day-old culture, 10^4 cells per larva) was injected into the hemocoel of the sixth instar larvae. The control insects were injected with 4 μl of a sterile 150 mM NaCl solution. Then, 1, 4, and 24 h after the injection, the hemolymph was isolated from the larvae by the standard procedure described in [6], and the generation of hydrogen peroxide in it was determined by the method of FOX [13]. The results of this experiment are shown in Fig. 4. Within 4 h after the injection of the bacterial suspension into the hemocoel of larvae, the level of hydrogen peroxide in insect hemolymph significantly increased compared to the control. One day after the injection, the level of hydrogen peroxide in the hemolymph of insects that were and were not injected with *B. thuringiensis* was similar. These results indicate that injecting insects with bacterial cells is accompanied by an increased production of reactive species (in particular, H_2O_2) in the first hours after injection. The production of reactive oxygen species is believed to be a component of the melanotic cascade and cytotoxic reactions, which was recorded earlier in studying cellular immunity processes in various insects [14]. Probably, H_2O_2 may be also involved in both melanogenesis and cytotoxic reactions in insects injected with *B. thuringiensis.* The addition of SOD to the hemolymph had no effect on the production of hydrogen peroxide in these experiments. It is known that *Gall. mellonella* hemolymph contains endogenous SOD [15]. Hence, it is not possible to make an unambiguous conclusion on the involvement of superoxide in the formation of hydrogen peroxide in this particular case.

Summarizing the results obtained in this study, it can be concluded that the superoxide radical forms in the course of melanization (enzymatic oxidation of DOPA). However, taking into account the discovered ability of DOPA to effectively interact with $O_2^{\cdot}$, the

potential role of this oxidative particle as a cytotoxic molecule in the immune response of insects at the stage of incapsulation is unfeasible. It is more likely that hydrogen peroxide, which yields the OH radical via the Fenton reaction, and the highly reactive melanization intermediates, *o*-quinones/semiquinones, may play this role.

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