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Superoxide and hydrogen peroxide formation during enzymatic oxidation of DOPA by phenoloxidase

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Abstract

Generation of superoxide anion and hydrogen peroxide during enzymatic oxidation of 3-(3,4-dihydroxyphenyl)-DL-alanine (DOPA) has been studied. The ability of DOPA to react with $O_2^{\cdot-}$ has been revealed. EPR spectrum of DOPA-semiquinone formed upon oxidation of DOPA by $O_2^{\cdot-}$ was observed using spin stabilization technique of *ortho*-semiquinones by Zn^{2+} ions. Simultaneously, the oxidation of DOPA by $O_2^{\cdot-}$ was found to produce hydrogen peroxide (H_2O_2). The analysis of H_2O_2 formation upon oxidation of DOPA by $O_2^{\cdot-}$ using 1-hydroxy-3-carboxy-pyrrolidine (CP-H), and SOD as competitive reagents for superoxide provides consistent values of the rate constant for the reaction between DOPA and $O_2^{\cdot-}$ being equal to $(3.4 \pm 0.6) \times 10^5\,M^{-1}\,s^{-1}$.

The formation of H_2O_2 during enzymatic oxidation of DOPA by phenoloxidase (PO) has been shown. The H_2O_2 production was found to be SOD-sensitive. The inhibition of H_2O_2 production by SOD was about 25% indicating that H_2O_2 is produced both from superoxide anion and via two-electron reduction of oxygen at the enzyme. The attempts to detect superoxide production during enzymatic oxidation of DOPA using a number of spin traps failed apparently due to high value of the rate constant for DOPA interaction with $O_2^{\cdot-}$.

Keywords: *DOPA, phenoloxidase, superoxide, spin trapping, DOPA-semiquinone, hydrogen peroxide*

Introduction

Catechols are widely distributed in nature. It has been reported for a number of these compounds that their auto- and enzymatic oxidation leads to the formation of highly reactive *ortho*-semiquinone radicals, which can react with dissolved oxygen to give superoxide anion [1,2]. Among other catechols DOPA is involved in a number of important biological functions, demonstrating significant neurologic activity and being a key intermediate in the biosynthesis of melanin. Melanin is known as a photoprotective pigment in the skin and the main component of the pigment capsule formed around the parasite during the immune response of insects. The encapsulation is a fundamental defense mechanism of insects. The key enzyme of melanization is phenoloxidase (PO), which catalyzes the first stage of this process, namely the oxidation of DOPA. Earlier, we have shown the generation of DOPA-semiquinone in insect haemolymph during melanization [3]. Some authors [4,5] proposed that DOPA-semiquinone can reduce molecular oxygen to produce superoxide radical. Several papers reported the production of $O_2^{\cdot-}$ in insect haemolymph, therefore, identifying superoxide anion as cytotoxic molecule in the insect immune defense [6–9]. However, the methods applied for superoxide

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detection in those studies were nonspecific, e.g. measurement of superoxide mediated reduction of nitroblue tetrazolium (NBT) and its derivates. It should be noted that data obtained using the NBT-reduction method for superoxide radical detection appear to be conflicting [10].

Our previous attempts to detect superoxide production in insect haemolymph using EPR spin trapping method were unsuccessful [11]. Consistent with these results Kalyanaraman et al. also did not observe the reaction of DOPA-semiquinone with molecular oxygen in their study [12].

In the present work, we demonstrated the formation of superoxide anion and hydrogen peroxide during enzymatic oxidation of DOPA by PO.

Materials and methods

Chemicals

Xanthine (X), xanthine oxidase (XO) from buttermilk, 3-(3,4-dihydroxyphenyl)-DL-alanine (DOPA), phenoloxidase (PO) from mushroom, superoxide dismutase (SOD) from *Escherichia coli*, diethylenetriaminepentaacetic acid (DTPA), potassium superoxide (KO_2), 3-carboxy-proxyl (CP) were purchased from Sigma (St. Louis, MO, USA). 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO) was obtained from Oxis International (Portland, OR, USA). Triarylmethyl free radical, TAM OX063, was a gift from Nycomed Innovations Co. (Malmö, Sweden). 1-Hydroxy-3-carboxy-pyrrolidine (CP-H) was synthesized and kindly provided by Dr Kirilyuk from Novosibirsk Institute of Organic Chemistry.

All experiments were carried out in appropriate buffer solutions, PBS (50 mM, pH 7.4, with 50 μM DTPA) or Tris–HCl (50 mM, pH 7.4), prepared using deionized water.

Sources of superoxide radicals

Superoxide radicals were generated by X/XO system. Xanthine was dissolved in 1 mM NaOH. The concentration of X in stock solution was 0.5 mM. The reaction was initiated by adding an aliquot of XO in buffer to the samples containing 0.05 mM X.

Saturated solution of KO_2 in dry DMSO (about 0.2 mM) was used as an alternative source of superoxide radicals [13]. Commercially available DMSO was purified by distillation from solid NaOH and stored over Molecular Sieves (4 A). Potassium superoxide solution was prepared by adding excess of KO_2 to DMSO and used within an hour.

Detection of superoxide radicals by spin traps

DEPMPO (10 mM) [14,15], TAM (10 μM) [16] and CP-H (1 mM) [17] spin traps have been used to detect formation of superoxide radicals. EPR spectrometer settings for DEPMPO spin trap experiments were as following: modulation amplitude 2 G, microwave power 20 mW. Samples were placed into 200 μl quartz flat cell for EPR measurements.

TAM is a paramagnetic spin trap, which specifically reacts with superoxide radicals resulting in the loss of EPR signal [16]. Production of superoxide radical was measured by monitoring time-dependence of EPR signal amplitude in 100 μl capillaries. EPR conditions were the following: modulation amplitude 0.02 G, microwave power 1 mW.

CP-H is sterically hindered hydroxylamine, which is oxidized by high oxidizing species with formation of the stable nitroxyl radical, CP [17]. CP-H was dissolved in oxygen-free (argon-bubbled) PBS. Concentration of CP-H in stock solution was 10 mM. Before the experiments, stock solution was kept frozen. The formation of CP radical in the samples has been studied by monitoring the amplitude of the low field component of EPR spectrum. The concentration of CP was determined using calibration dependence of EPR signal amplitude on concentration of pure CP. For EPR measurements samples were placed into 100 μl glass capillaries. EPR spectrometer settings were the following: modulation amplitude 1 G, microwave power 20 mW.

To inhibit the interaction of superoxide radical with spin trap, SOD (200 U/ml) was used as a competitive reagent. EPR measurements were performed using an ER 200D-SRC X-band EPR spectrometer (Bruker).

Determination of DOPA-semiquinone production

To detect the DOPA-semiquinone production the spin stabilization technique by Zn^{2+} was applied [18,19]. *Ortho*-semiquinone radicals form chelate complexes with divalent diamagnetic metal ions. The steady state concentrations of such complexes are much higher than those of the radical itself allowing their detection by EPR spectroscopy at room temperature. Samples were prepared in Tris–HCl buffer solution. The concentration of $ZnSO_4$ in samples was 0.5 M. The addition of $ZnSO_4$ to samples shifted the pH value of mixture to 5.8 and inhibited XO activity by about 40%. XO activity was stable under these conditions at least for 1 h. For EPR measurements, samples were placed into 200 μl quartz flat cell. EPR spectrometer settings were as following: modulation amplitude 0.16 G, microwave power 2 mW.

Determination of hydrogen peroxide production

The H_2O_2 was determined using ferrous oxidation-xylenol orange (FOX) assay [20]. The presence of H_2O_2 in solution causes the oxidation of Fe^{2+} to Fe^{3+}. Xylenol orange binds ferric ions forming a colored complex with absorption at 560 nm.

FOX system contains: 312.5 μM $FeSO_4$, 125 μM xylenol orange and 125 mM sucrose dissolved in NaCl–HCl buffer solution (0.3 M, pH 1.4). Mixtures of experimental samples (200 μl) with FOX system (800 μl) were incubated for 20 min and used for spectrophotometric assay. Experimental samples containing 200 U/ml of catalase were used as a control. The H_2O_2 concentration was determined by monitoring the absorption at 560 nm using calibration curve obtained with pure H_2O_2. The accuracy of the method was ± 0.8 μM of H_2O_2. Optical measurements were performed using UV-2401 (PC) CE spectrophotometer (Shimadzu).

UV-photolysis of DOPA solution

Photolysis experiments were carried out using 250 W mercury lamp. To remove infrared and visible spectral range the light was filtered through 10 cm of distilled water and ultraviolet filter (transmission at 250–400 nm).

The stock solution of DOPA (1 mM) was stored on ice to prevent the H_2O_2 formation from auto oxidation of DOPA. During the experiment the concentration of H_2O_2 in DOPA stock solution did not exceed 1 μM. Mixtures of 0.1 mM DOPA in PBS and various concentration of SOD were placed in quartz cuvette with 2 mm optical pathway. Samples were irradiated for 1 h. The conditions of photolysis were chosen so that SOD activity did not change significantly. After the photolysis, the H_2O_2 concentration in the samples was measured by FOX assay.

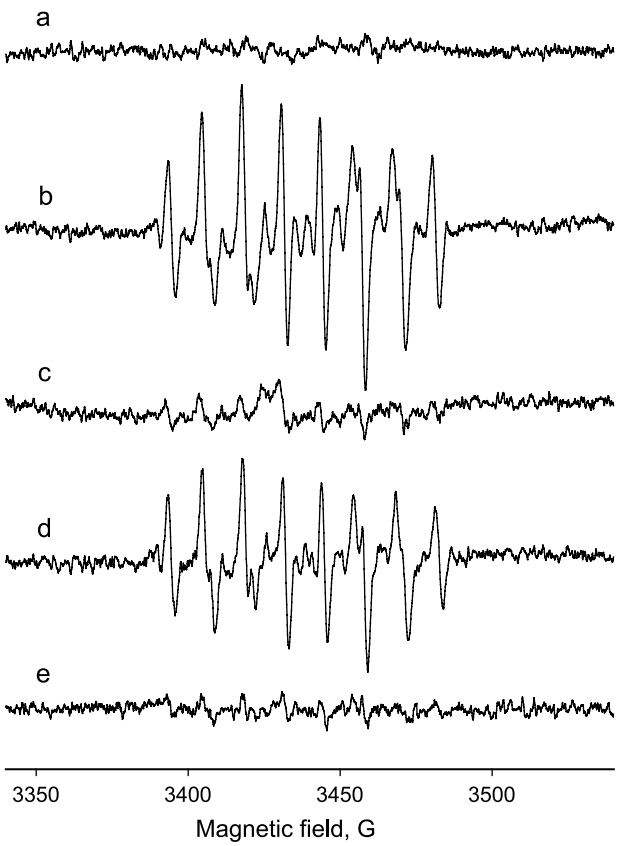

Figure 1. EPR spectra obtained in the presence of 10 mM DEPMPO in PBS buffer upon: (a) oxidation of DOPA (1 mM) by PO (20 U/ml); (b) oxidation of xanthine (0.05 mM) by XO (0.05 U/ml); (c) the same as (b) but in the presence of 10 μM DOPA; (d) addition of 5 μl KO_2 solution; (e) addition of 10 μM DOPA followed by addition of 5 μl KO_2.

Results and discussion

Detection of superoxide production during enzymatic oxidation of DOPA using spin traps

In this work, we have used several spin traps with different underlying chemical reactions with superoxide radical. Nitrone based spin trap DEPMPO is widely used to detect the superoxide anion due to the formation of comparatively stable DEPMPO-OOH adduct. The rate constant of superoxide trapping was recently reevaluated to be equal to 0.53 $M^{-1}s^{-1}$ [15]. Figure 1 demonstrates typical EPR spectra of DEPMPO-OOH adduct measured in X/XO system (1b) and after bolus addition of KO_2 (1d). However, EPR signal of spin adduct was not observed in DOPA/PO system (Figure 1a) at various DOPA concentrations (1–10 mM) and PO activities (5–100 U/ml). We have found that the presence of small amount of DOPA (10 μM) led to the significant decrease of the EPR signal both in X/XO system and upon addition of KO_2 (cf. Figure 1b and 1c, 1d and 1e). These results suggest that DOPA reacts with superoxide radical competing with the spin trap. Note that an ability of hydroquinones to react with superoxide has been previously reported [21].

To overcome the above competition between spin trap and DOPA we applied spin traps with higher trapping efficiency for superoxide. One of them is TAM that specifically reacts with superoxide with second order rate constant 3.1×10^3 $M^{-1}s^{-1}$ resulting in the loss of EPR signal intensity [16]. We used TAM to detect the formation of superoxide during oxidation of DOPA by PO. We have measured the kinetics of EPR signal amplitude of TAM in the DOPA/PO system in the absence and in the presence of SOD. As represented by Figure 2, SOD did not affect the kinetics of EPR signal amplitude of TAM indicating that the reaction of TAM with superoxide does not occur in this system. Note that the observed increasing of peak intensity of EPR signal of TAM is explained by the narrowing of its linewidth from 0.24 to 0.2 G due to depletion of dissolved oxygen during enzymatic oxidation of DOPA. At the same time, the integral intensity of the EPR spectrum of TAM did not change, supporting the lack of the reaction of TAM with superoxide.

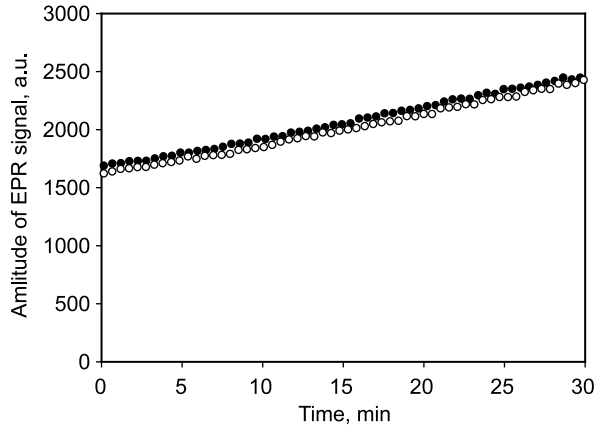

Figure 2. Time dependence of EPR signal amplitude of TAM (10 μM) in PBS buffer upon: (●) oxidation of DOPA (1 mM) by PO (20 U/ml); (○) the same as (●) but in the presence of SOD (200 U/ml).

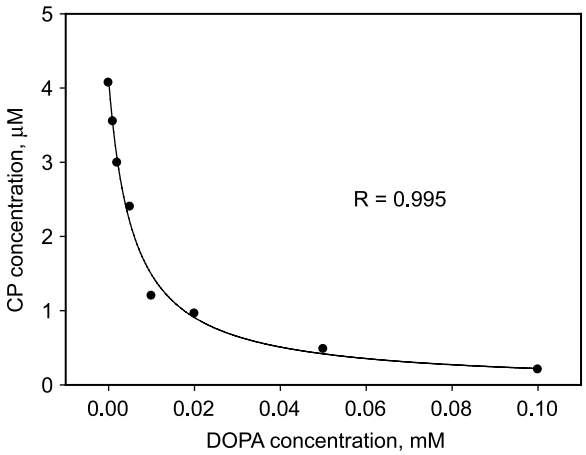

Figure 3. The influence of DOPA on CP-H oxidation by $O_2^{\cdot-}$. The samples were prepared as following: 5 μl of saturated solution of KO_2 in DMSO was added to the mixtures of CP-H (1 mM) and various concentrations of DOPA in PBS. The formation of CP was determined by monitoring of intensity of low field component of the EPR spectrum.

Spin traps based on sterically hindered hydroxylamines are also widely used as spin traps of superoxide with high trapping efficiency [17]. In particular, the cyclic hydroxylamine CP-H, is oxidized by superoxide radical to produce the stable nitroxyl radical, CP, with the rate constant $3.2 \times 10^3\,M^{-1}\,s^{-1}$ [17]. Similar to our data with DEPMPO and TAM, we did not observed superoxide-dependent CP-H oxidation during enzymatic oxidation of DOPA by PO [11,22].

Studies of the reaction of DOPA with superoxide

The data above allow to hypothesize that the reaction between DOPA and superoxide anion occurs. To test this hypothesis we studied the oxidation of CP-H by superoxide in the presence of DOPA. The solution of KO_2 in DMSO was used as a source of superoxide radical. The intensity of EPR signal of CP formed in mixture of CP-H with KO_2 has been measured. Figure 3 shows that an addition of DOPA to this mixture decreased the formation of CP supporting the existence of reaction between DOPA and $O_2^{\cdot-}$. Taking into account a competition between DOPA and CP-H for superoxide, the rate constant of the reaction between DOPA and $O_2^{\cdot-}$ was estimated as $5 \times 10^5\,M^{-1}\,s^{-1}$.

It is reasonably to propose the formation of DOPA-semiquinone and H_2O_2 upon the reaction of DOPA with $O_2^{\cdot-}$, namely:

$$QH_2 + O_2^{\cdot-} \rightarrow Q^{\cdot-} + H_2O_2 \qquad (1)$$

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

To confirm this assumption we have examined the formation of DOPA-semiquinone in this reaction. The spin stabilization technique of *ortho*-semiquinones by Zn^{2+} ions [18,19] was used to detect short-lived DOPA-semiquinone radical. The EPR spectrum of DOPA-semiquinone complex with Zn^{2+} was observed upon oxidation of DOPA by $O_2^{\cdot-}$ (see Figure 4, insert). The intensity of the observed EPR signal of DOPA-semiquinone depended on the rate of superoxide generation (Figure 4). Note that X/XO system generates both superoxide anion as well as H_2O_2. However, in an independent experiment we have shown that DOPA is not oxidized by H_2O_2 (data not shown). Thus, as we proposed, DOPA-semiquinone is formed due to reaction (1).

To study H_2O_2 formation upon oxidation of DOPA by superoxide we have used the UV-photolysis of DOPA solution. It is well known that the photolysis of hydroquinones, including DOPA, leads to the

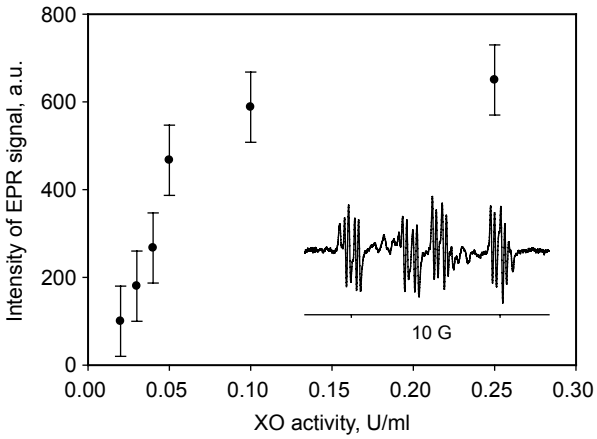

Figure 4. The dependence of EPR spectra intensity of DOPA-semiquinone complex with Zn^{2+} on the rate of $O_2^{\cdot-}$ production. The reaction mixtures were: DOPA (10 mM), $ZnSO_4$ (0.5 M), xanthine (0.05 U/ml) and XO. All samples were prepared in Tris–HCl buffer solution. EPR spectrometer settings were as following: modulation amplitude 1.6 G, microwave power 2 mW. *Insert:* the typical EPR spectrum of DOPA-semiquinone complex with Zn^{2+} (modulation amplitude 0.16 G).

production of superoxide anion according to the Equation (2) [23]:

$$QH_2 + O_2 \xrightarrow{h\nu} Q^{\cdot-} + O_2^{\cdot-} + 2H^+ \quad (2)$$

FOX assay (see "Materials and Methods" section) was used to measure the H_2O_2. The formation of H_2O_2 during photolysis of DOPA solution in the presence of various SOD concentrations has been studied. Figure 5 shows the decrease of H_2O_2 yield on SOD concentration. The observed decrease in the hydrogen peroxide production can be explained by competition of reactions (1) and (3), proceeding in the photolytic mixtures.

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (3)$$

In agreement with the stoichiometry of the reactions (1) and (3) the photolysis of DOPA solution in the absence of SOD and in the presence of its excess (200 U/ml) results in two-fold difference in H_2O_2 accumulation (about 18 and 9 μM, correspondingly, see Figure 5). These results confirm that the interaction of DOPA with $O_2^{\cdot-}$ leads to the production of H_2O_2 according to reaction (1). The dependence of H_2O_2 formation on SOD concentration, in the assumption of competition between DOPA and SOD for superoxide, is described by the following equation:

$$[H_2O_2] = \text{const}\frac{k_{DOPA}[DOPA] + 1/2k_{SOD}[SOD]}{k_{DOPA}[DOPA] + k_{SOD}[SOD]} \quad (4)$$

The result of the fitting equation (4) to the experimental data is shown in Figure 5 yielding $k_{DOPA} = (3.4 \pm 0.6) \times 10^5\,M^{-1}\,s^{-1}$. This value is in a good agreement with the value estimated using CP-H as competitive reagent ($5 \times 10^5\,M^{-1}\,s^{-1}$) and is reasonable close to the constants for other catechols (e.g. $5.4 \times 10^4\,M^{-1}\,s^{-1}$ for adrenaline [21]).

The obtained value of the rate constant for the reaction of $O_2^{\cdot-}$ with DOPA is much higher than that for spin traps used for superoxide detection during enzymatic oxidation of DOPA. This fact can explain the absence of the superoxide-dependent adducts formation in spite of possible $O_2^{\cdot-}$ generation. Nevertheless probable superoxide generation during the enzymatic oxidation of DOPA should be accompanied by accumulation of hydrogen peroxide according to reaction (1). Figure 6 shows H_2O_2 formation during enzymatic oxidation of DOPA. At the same time we have observed the decrease of H_2O_2 yield in the presence of SOD. Note that this decrease was about 25% and did not reach 50% as in the photolysis experiment, indicating that only fraction of H_2O_2 was formed from superoxide. The rest of H_2O_2 can be formed directly via two-electron enzymatic process:

$$QH_2 + O_2 \xrightarrow{PO} Q + H_2O_2 \quad (5)$$

where Q is DOPA-quinone.

Taking into account the present data and our previous results [3], we suggest the following scheme of the reactions proceeding during initial stage of enzymatic oxidation of DOPA:

$$QH_2 + O_2 \xrightarrow{PO} Q + H_2O_2$$

$$QH_2 + Q \leftrightarrow 2Q^{\cdot-} + 2H^+$$

$$Q^{\cdot-} + O_2 \rightarrow Q + O_2^{\cdot-}$$

$$QH_2 + O_2^{\cdot-} \leftrightarrow Q^{\cdot-} + H_2O_2$$

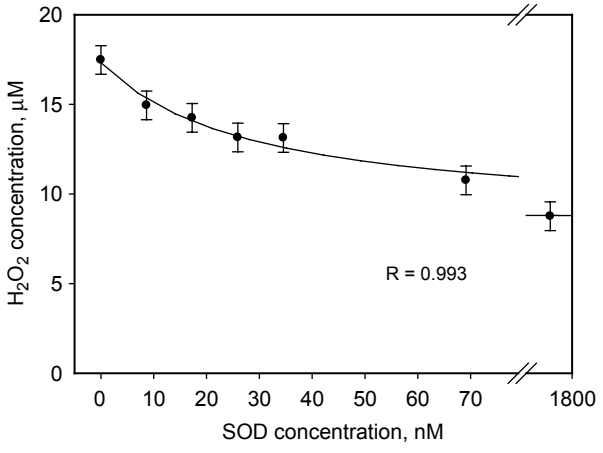

Figure 5. The formation of hydrogen peroxide during photolysis of DOPA solution in the presence of various concentration of SOD. The solution of DOPA (0.1 mM) and various concentrations of SOD in PBS was placed in quartz cuvette with 2 mm optical pathway and irradiated for 1 h. To quantify the hydrogen peroxide concentration in the samples FOX assay was used (see "Materials and Methods" section).

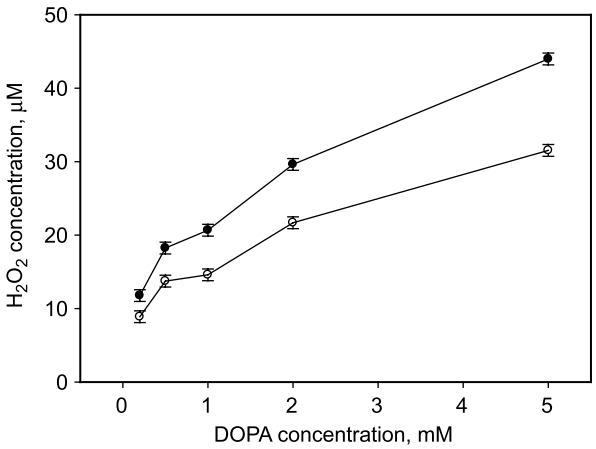

Figure 6. The formation of hydrogen peroxide in the reaction of enzymatic oxidation of DOPA in PBS buffer: (●) various concentrations of DOPA were oxidized by PO (5 U/ml) for 30 min; (○) the same as (●) but in the presence of 200 U/ml of SOD.

In summary, the present data demonstrate H_2O_2 and superoxide formation during initial stage of DOPA oxidation by PO. These reactive oxygen species as well as DOPA-semiquinone may play significant role in the mechanism of antiparasitic defense of insects as intermediates of melanization process.

Acknowledgements

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