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INHIBITION OF ENZYMATIC INDOLE-3-ACETIC ACID OXIDATION BY PHENOLS

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Key Word Index—Peroxidase; IAA oxidation; inhibition; free-radical chain reaction; substituted phenols; H_2O_2.

Abstract—The influence of natural and unnatural phenolic inhibitors on peroxidase-catalysed oxidation of indole-3-acetic acid (IAA) was investigated using chemiluminescent and spectrophotometric methods. The threshold effect under inhibition was observed for all studied phenolic compounds. The IAA oxidation stopped only if the concentration of inhibitor was more than some threshold value. The spontaneous reinitiation of the stopped reaction was also investigated. The system of chemical reactions, required for the description of threshold effect under inhibition and spontaneous reinitiation of stopped reaction, was proposed. Moreover, the effect of H_2O_2 on the inhibited IAA oxidation was studied. It was found that addition of H_2O_2 caused a reinitiation of stopped reaction. Threshold effect was observed for the reinitiation. The reinitiation took place only when the concentration of H_2O_2 was more than a threshold value which depended on the inhibitor concentration. The total quantity of H_2O_2 required for the reaction reinitiation on the addition of H_2O_2 a little at a time was more than that on addition by a single large portion. The results obtained are discussed in terms of free-radical chain reaction of enzymatic IAA oxidation.

INTRODUCTION

The aerobic oxidation of indole-3-acetic acid (IAA) catalysed by peroxidase can occur in the absence of added H_2O_2 [1]. Peroxidase-catalysed IAA oxidation is inhibited, in particular, by natural phenolic inhibitors of this reaction (caffeic, chlorogenic, gallic acids and the others). The mechanism of the phenol-inhibiting action on the enzymatic IAA oxidation was investigated early by a number of authors. Phenolic inhibitors were found to result in the lag phase of the reaction [2–6]. First, it was suggested that inhibitor reduces the free radical of IAA to IAA molecule with the formation of the inhibitor dimer [7]. However, this hypothesis required the participation of a cofactor in the reaction, while the lag can take place without a cofactor. Afterwards the opinion about a competitive mechanism of inhibition predominated [2–6, 8]. According to this hypothesis the inhibitor is the competing substrate; the end of the lag period coincides with the finishing of inhibitor oxidation.

In our previous paper we described the threshold effect under inhibition of peroxidase-catalysed IAA oxidation by caffeic acid: the IAA oxidation stops only if the caffeic acid concentration was more than some value [9]. In order to explain this phenomenon we supposed that caffeic acid is not a competitive inhibitor and acts as a trap of free radicals, participating in the chain reaction of IAA oxidation. The present paper describes the action of a number of natural and unnatural phenolic inhibitors on peroxidase-catalysed aerobic IAA oxidation. Moreover, the effect of H_2O_2 on the inhibited IAA oxidation was studied.

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RESULTS AND DISCUSSION

Inhibition of IAA oxidation

Studying the influence of natural and unnatural phenolic inhibitors on the peroxidase-catalysed aerobic oxidation of IAA, we observed a threshold effect. Only temporary decreases of the chemiluminescence intensity and rate of IAA oxidation were observed when the concentration of inhibitor was less than a threshold value. If the inhibitor concentration was more than this threshold value, then the chemiluminescence ended and the reaction stopped. The threshold inhibitor concentration depends on the chemical nature of the inhibitor (Table 1). The value of threshold inhibitor concentration did not depend on the period of the storage of the IAA solution. The effect of enzyme and substrate concentrations and the moment of inhibitor addition was similar to that described in our previous paper for caffeic acid as inhibitor [9].

Spontaneous reinitiation

The reaction stopped by inhibitor was spontaneously reinitiated after a period of delay (Fig. 1). Spontaneous

Table 1. Minimal inhibitor concentration required for the cessation of IAA (1 mM) oxidation catalysed by peroxidase (0.1 μM)

Inhibitor	Threshold inhibitor concentration (μM)
Caffeic acid	0.24 – 0.26
Gallic acid	1.20 ÷ 1.32
Hydroquinone	0.52 – 0.64
Catechol	0.64 ÷ 0.80
3,4-Dimethylphenol	18.4 ÷ 20.0
4-Methoxyphenol	0.96 – 1.04
2-Aminophenol	0.96 ÷ 1.04
6-Chloro-4-nitro-2-aminophenol	1.20 ÷ 1.28

Inhibitor was added to the IAA solution before the peroxidase addition.

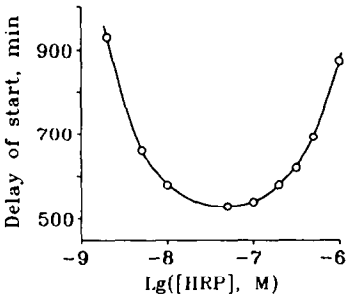


Fig. 2. The effect of the peroxidase concentration on the start delay (caused by the 6-chloro-4-nitro,2-aminophenol addition) of the IAA (1 mM) oxidation catalysed by peroxidase (0.1 μM). 6-Chloro-4-nitro,2-aminophenol was added to IAA before the addition of peroxidase. The digital logarithm of the peroxidase concentration (measured in mol l^{-1}) is shown on the horizontal axis.

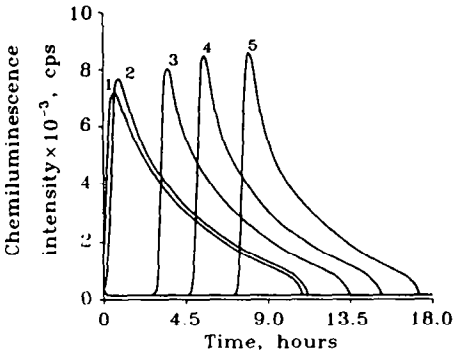


Fig. 1. The effect of 6-chloro-4-nitro,2-aminophenol on the chemiluminescence kinetics for the IAA (1 mM) oxidation catalysed by peroxidase (0.1 μM). 6-chloro-4-nitro,2-aminophenol was added to IAA before the addition of peroxidase. Concentrations of 6-chloro-4-nitro,2-aminophenol (μM): 0 (1), 1.15 (2), 1.32 (3), 1.48 (4), 1.64 (5).

reinitiation can be observed when inhibitor concentration only slightly exceeded the threshold concentration. Otherwise, the value of delay was more than the maximal duration of the experiment (36 hr). Logarithm of the delay was proportional to the inhibitor concentration. It was more evident at low pH values (when the rate of IAA oxidation is high). The delay did not depend on the incubation period of IAA-inhibitor mixture. Spontaneous reinitiation has been described earlier by the cited authors as a lag period [2–6]. However, none of these papers contained information about the influence of enzyme concentration on the lag period. Studying the effect of peroxidase concentration on the delay, we obtained an unexpected result—unmonotonous dependence (Fig. 2).

Mechanism of inhibition and reinitiation

In order to explain the obtained results we believe that phenolic inhibitors are the traps of free radicals R˙ participating in the chain reaction of IAA oxidation [9]. R˙ is a derivative of the substrate (S) and traces of R˙ are in the substrate solution [9].

The independence of threshold inhibitor concentration on the period of keeping of the IAA solution enables us to suppose that R˙ is in a quasi-equilibrium with the substrate:

$$S \rightleftharpoons R^{\cdot} \qquad (1).$$

As already mentioned, peroxidase-catalysed oxidation of IAA is an enzymatic free-radical chain reaction [10]. A crude scheme of the chain branched reaction is as shown:

$$S + R^{\cdot} \xrightarrow{HRP,\ O_2} 2R^{\cdot} \qquad (2),$$

where HRP is horseradish peroxidase. The increase of the chain reaction rate is limited by the square-law reaction of free radical R˙ destruction, for example, via dimerization:

$$R^{\cdot} + R^{\cdot} \rightarrow R - R \qquad (3).$$

As emphasized above we suppose that the phenolic inhibitors play the role of free radical R˙ trap:

$$R^{\cdot} + I \rightarrow P_1 + I^{\cdot} \qquad (4).$$

Here I and I˙ are inhibitor and free radical of inhibitor, respectively, P_1 is one of the products of this reaction. Free radical of inhibitor can, in turn, be reduced to the inhibitor molecule:

$$I^{\cdot} \rightarrow I \qquad (5),$$

where the donor of electron is not indicated. Besides that, the square-law reaction of 'unreversible' destruction of free radical I˙ takes place, for instance, via dimer formation:

$$I^{\cdot} + I^{\cdot} \rightarrow I - I \qquad (6).$$

It is supposed that I˙ is a less active radical than R˙ to the reaction (2); that is, I˙ cannot give rise to the development of the chain branched process.

The system of reactions (1)–(6) is the minimal one for the description of both threshold effect under inhibition and spontaneous reinitiation of inhibited reaction. According to this system the threshold effect under inhibition is described in the following way. Reactions (2)–(5) point to the existence of some critical concentration of the inhibitor, under which the free radical R' multiplication factor equals one. If the inhibitor concentration is more than the critical value, then the multiplication factor is less than one; if the inhibitor concentration is less than the critical value, then the multiplication factor is more than one. In the first case the reaction stops. In the second case the chain reaction develops as an avalanche, right up to the moment when the increase of the reaction rate is limited by the process (3). In this case the concentration of inhibitor relatively quickly decreases to zero by means of the processes (4) and (6). The critical concentration of inhibitor seems to correspond to the threshold inhibitor concentration which is determined experimentally.

Spontaneous reinitiation of the stopped reaction takes place apparently because, even after the reaction cessation, the concentration of free radical R' does not equal zero due to process (1). In this case slow 'unreversible' destruction of the inhibitor is realized by means of the processes (4) and (6). The moment comes when the concentration of the inhibitor becomes less than the critical value. Free radical R' multiplication factor becomes more than one and chain reaction develops as the avalanche.

Unmonotonous dependence of the delay on the peroxidase concentration (Fig. 2) points to the fact that the reaction (2) is not a single process with the enzyme and free radical R' participation. We suppose that when the enzyme concentration increases, some reaction of enzymatic destruction of R' (by Michaelis–Menten mechanism) becomes considerable:

$$R' + HRP \rightleftarrows R'HRP \longrightarrow P_2 + HRP \qquad (7),$$

where P_2 is the product of this reaction.

Effect of H_2O_2

Firstly the effect of H_2O_2 addition on the normally proceeding aerobic IAA oxidation was investigated. It was observed that H_2O_2 caused relatively weak alterations in the kinetics of IAA oxidation. Further H_2O_2 did not affect the absorption spectrum of peroxidase during the aerobic IAA oxidation. The addition of caffeic acid in overthreshold concentration to the normally proceeding IAA oxidation, in contrast with H_2O_2, resulted in significant changes in the absorption spectrum of peroxidase (Fig. 3).

Studying the effect of H_2O_2 on peroxidase-catalysed IAA oxidation, which was stopped by caffeic acid, we observed the threshold effect. If the concentration of added H_2O_2 was less than some threshold value, then only momentary increase of chemiluminescence intensity was observed (Fig. 4, curve 3). If the concentration of added H_2O_2 was more than the threshold value, then a significant increase of chemiluminescence intensity was

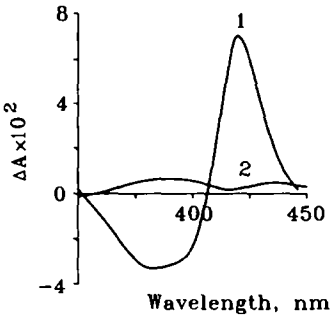


Fig. 3. The effect of caffeic acid on the difference absorption spectra between forms of peroxidase, forming in the IAA (1 mM)/peroxidase (0.1 μM) system, and ferriperoxidase. Spectra: 1, 3 min after the mixture of IAA and peroxidase; 2, 2 min after the addition of 0.15 mM of caffeic acid to the mixture IAA/peroxidase, incubated during 3 min.

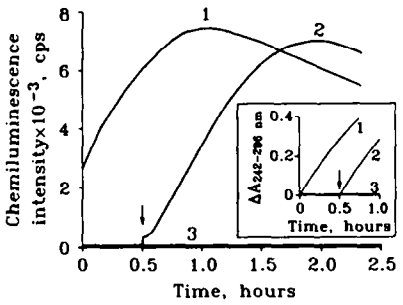


Fig. 4. The effect of H_2O_2 addition on the chemiluminescence from the system: IAA (1 mM)/caffeic acid (1 μM)/peroxidase (0.1 μM). Concentration of H_2O_2 (μM): 1, 0 (control experiment without caffeic acid); 2, 0.2; 3, 0.05. The arrow indicates the moment of H_2O_2 addition. Insert displays the spectrophotometric control of the IAA products formation in the conditions of this figure.

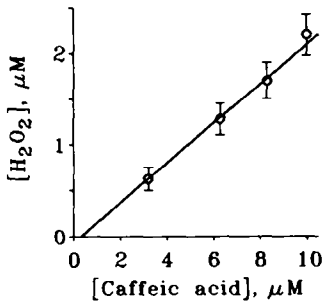


Fig. 5. The effect of caffeic acid concentration on the minimal H_2O_2 concentration required for the reinitiation of the reaction in the system: IAA (1 mM)/caffeic acid/peroxidase (0.1 μM).

detected and reaction reinitiation took place (Fig. 4, curve 2). The value of the threshold H_2O_2 concentration was proportional to the caffeic acid concentration (Fig. 5).

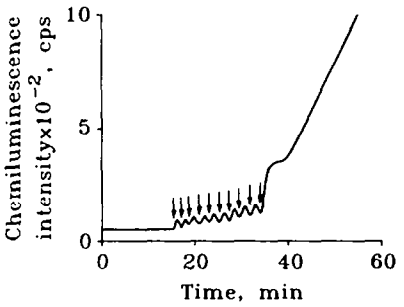
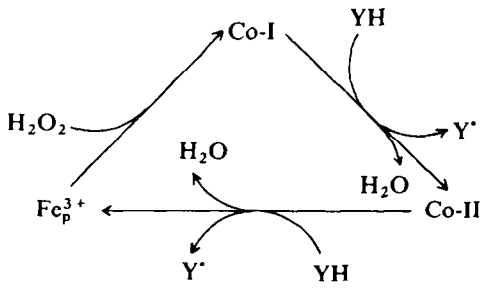


Fig. 6. The effect of the addition of H_2O_2 a little at a time on the chemiluminescence from the system: IAA (1 mM)/caffeic acid (1 μM)/peroxidase (0.1 μM). The arrows indicate the moments of addition of H_2O_2 (0.03 μM).

If H_2O_2 was added to the reaction mixture a little at a time, then the reaction reinitiation took place only when the total quantity of added H_2O_2 was more than some threshold value (Fig. 6). In this case the total quantity of H_2O_2 required for the reaction reinitiation was more than that added by a single large portion.

Mechanism of H_2O_2 action

The difference absorption spectrum of peroxidase has a peak at 420 nm during the normally proceeding aerobic peroxidase-catalysed IAA oxidation (see Fig. 3). This fact indicates that peroxidase is in the Co-III form [11], which cannot utilize H_2O_2 [11, 12]. That is why we observed only slight action of H_2O_2 upon the normally proceeding IAA oxidation (see above and refs [13, 14]). The peak at 420 nm disappears after the cessation of the reaction by caffeic acid (Fig. 1). This is likely to connect with a return of peroxidase to the initial ferric form (Fe_p^{3+}). Fe_p^{3+} can be easily oxidized by H_2O_2 [12]. In this case the addition of H_2O_2 initiates the ordinary peroxidase pathway of IAA (YH) oxidation (it should be noted that caffeic acid also can be a substrate of peroxidation). Such a process results in the formation of free radicals Y· according to the following scheme [15]:



The peroxidase pathway, as it is known, gives rise to the inactivation of enzyme [16, 17]. It is a possible reason for the slight decrease of the chemiluminescence intensity (Fig. 4, curve 2) in the case of the stopped reaction reinitiation in relation to that of the control sample (Fig. 4, curve 1).

An appearance of substrate free radical Y· causes the free radical chain reaction of oxidase IAA oxidation. The chain reaction develops as an avalanche (that is the reinitiation of the stopped reaction takes place) only if the free radical multiplication factor is more than one. In the presence of caffeic acid, this condition is arrived at when the concentration of free radicals is higher than some threshold value, which depends on the caffeic acid concentration [9]. So for avalanche-like development of chain reaction, the concentration of added H_2O_2 must also exceed some threshold value, which in turn, also depends on the caffeic acid concentration (Fig. 5). The tangent of the angle of slope for the line in Fig. 5 equals about 1/4. This value shows that one molecule of H_2O_2 is required for inactivation of four molecules of caffeic acid.

On addition of H_2O_2 a little at a time, each little portion is insufficient for the reinitiation of the stopped reaction. However, destruction of each H_2O_2 portion is accompanied by irreversible deactivation of some quantity of caffeic acid [9]. So the concentration of H_2O_2, which is required for the reaction reinitiation, decreased from portion to portion, and portion number 'n' gives rise to the reaction start. As already noted, the total quantity of H_2O_2 required for the reinitiation on addition a little at a time is more than that added by a single large portion. We suggest the following hypothesis which is able to explain this observation: in the first case, the reinitiation occurs due to the gradual decrease of caffeic acid concentration and in the second case, due to the extreme increase of free radical concentration.

EXPERIMENTAL

Horseradish peroxidase (RZ = 3.0), indole-3-acetic acid, caffeic acid, gallic acid and the components of phosphate buffer were obtained from Sigma. 3,4 Dimethylphenol, 2-aminophenol, 4-methoxyphenol, hydroquinone, catechol and H_2O_2 were obtained from Aldrich. 6-Chloro-4-nitro-2-aminophenol was obtained from Reachim (Russia). All solns were prepared using 3 × distilled deionized H_2O.

Except when otherwise stated standard reaction mixt. contained: 0.1 μM HRP and 1 mM IAA in 0.067 M phosphate buffer pH 7.4; the final vol. was 3 ml. When the effect of H_2O_2 on the inhibited IAA oxidation was studied the overthreshold concn of inhibitor (caffeic acid) was added to IAA soln before peroxidase addition.

We used a chemiluminescent method for the study of the peroxidase-catalysed IAA oxidation [9]. Intensity of chemiluminescence was measured with a specially designed chemiluminometer, slightly modified compared with that previously reported [18]. Six quartz cuvettes, each 3 cm diam., with reaction mixts were arranged on a rotating disc driven by a computer-controlled stepping motor. We also used the spectrophotometric method. Difference absorption spectra in Soret region of peroxidase were measured for the detection of alteration in the ferri-peroxidase concn. The formation of IAA oxidation products was observed by measurement of the differential A between 242 and 296 nm (wavelengths with

equal values of IAA absorbance) using the 'two wavelength mode'. All experiments were carried out at 22°.

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