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THRESHOLD EFFECT DURING INHIBITION BY CAFFEIC ACID OF PEROXIDASE-CATALYSED OXIDATION OF INDOLYL-3-ACETIC ACID*

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Chemiluminescence has been used to study aerobic peroxidase-catalysed oxidation of indolyl-3-acetic acid (IAA). A threshold effect was found on inhibition of this reaction by its natural inhibitor — caffeic acid. At an inhibitor concentration below threshold, only a temporary fall in the intensity of chemiluminescence and the reaction rate was established; at an above-threshold concentration, chemiluminescence ceased, the reaction stopped and was not renewed throughout the observation period. It is shown that the reaction is not re-initiated by the addition of the enzyme; re-initiation of the arrested reaction was observed on exposure of the reaction mixture to visible light in the presence of a photosensitizer, Bengal rose; after initiation the reaction proceeded without light. It is assumed that in the experimental conditions employed enzymatic oxidation of IAA is a branched chain free-radical reaction and caffeic acid acts as a trap of the radicals involved in this reaction.

INTRODUCTION

Peroxidase-catalysed aerobic oxidation of a phytohormone — indolyl-3-acetic acid (IAA) — plays an important role in its metabolism [1]. Oxidation of IAA *in vivo* is regulated, in the main, through the action of the effectors of the reaction. A large number of natural activators and inhibitors of the oxidation of IAA on exposure to peroxidase are known [2]. Orthophenols with an OH group in the *para* position serve as activators (*p*-coumaric acid, ferrulic acid, umbelliferone). Among the inhibitors of the reaction are *ortho*-di- and trihydroxyphenols (caffeic, chlorogenic and gallic acids) and others. Detailed investigation of the mechanism of the inhibiting action of the phenols has shown that phenolic inhibitors cause delay in the working over of the substrate [3–6]. It was originally thought that the inhibitor reduces the free radical of IAA to the IAA molecule with the formation of the dimer of the inhibitor [7]:

$$2R - CH_2\,COO^{\bullet} + 2\,IOH \rightarrow 2\,R - CH_2 \cdot COOH + IO - OI,$$

where IOH is the inhibitor. Later, most often the assumption was advanced on the competitive mechanism of inhibition in which the inhibitors are in fact competing substrates, and the termination of the delay of the reaction is linked with termination of oxidation of the inhibitor

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[5, 6]. As confirmation of the competitive mechanism of inhibition, data are invoked on the influence of inhibitor on the spectral changes in the Soret region of peroxidase appearing on interaction of peroxidase and IAA [5, 6, 8]. However, it should be noted that these arguments are in themselves not sufficient to demonstrate the competitive mechanism of inhibition. In addition, the hypothesis presented is contradicted by a number of experimental findings. In particular, the Lineweaver–Burke graphs reliably show non-linear dependence [9]. Therefore, the mechanism of the action of phenolic inhibitors on the enzymatic oxidation of IAA calls for further detailed study.

MATERIALS AND METHODS

We used RZ 3.0 horseradish peroxidase, indolyl-3-acetic acid, salts as components of the phosphate buffer, caffeic acid and Bengal rose of Sigma (U.S.A.). Anfen — an original water-soluble free-radical trap — was synthesized and kindly made available by Volod'kin (Institute of Chemical Physics, Russian Academy of Sciences, Moscow). All the solutions were prepared using triple distilled de-ionized water.

The standard reaction mixture of volume 3 ml, unless otherwise stated, contained 1 mM IAA and 0.1 μM peroxidase in 0.067 M phosphate buffer, pH 7.4.

To study the effect of caffeic acid on the peroxidase-catalysed aerobic oxidation of IAA, we used the chemiluminescent method based on high sensitivity of the kinetics and chemiluminescence spectra appearing in the course of the reaction to the regime of its course [10]. We also used the spectrophotometric method. The formation of the oxidation products of IAA was observed from the difference in absorption at 242 and 296 nm (wavelengths for which the extinction coefficients of IAA are equal). To check on the chemiluminescent and spectrophotometric data, we used the traditional colorimetric method of determining the IAA concentration with the aid of the Salkowski reagent [11]. The differential optical density of the reaction mixture was determined with the Hitachi 557 spectrophotometer (Japan) using the two wavelength regime and a quartz cuvette with a length of the optical path 1 cm. The kinetics of chemiluminescence was measured with a chemiluminometer to our design. Six quartz cuvettes of diameter 30 mm with the reaction mixtures were placed on a turning drum, set in motion by a step-by-step motor. As radiation detector, we used a FEU-130 with calibrated spectral sensitivity working in the photon count regime. The region of spectral sensitivity of the FEU ranges from 200–600 nm. The FEU photocathode was placed beneath the cuvette, so that chemiluminescence was recorded through its bottom. The regime of the work of the chemiluminometer is detailed in [10]. For exposure of the reaction mixture to light we used the OVS-1 illuminator.

RESULTS

Study of the effect of caffeic acid on peroxidase-catalysed aerobic oxidation of IAA revealed a threshold effect. The presence of inhibitor in a below-threshold concentration caused a temporary fall in the intensity of chemiluminescence (Fig. 1) and the reaction rate (Fig. 2). At concentrations above threshold, chemiluminescence ceased and was not renewed throughout the period of continuous control (Fig. 1). The reaction of oxidation of IAA was

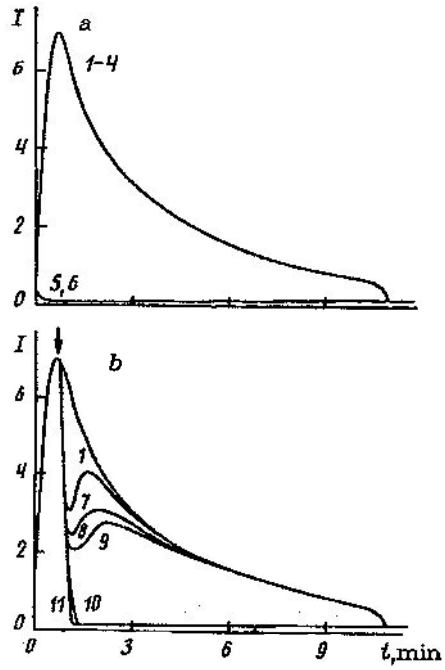

Fig. 1. Effect of caffeic acid (*1*, 0; *2*, 0.08; *3*, 0.16; *4*, 0.24; *5*, 0.33; *6*, 0.41; *7*, 1.3; *8*, 2.3; *9*, 3.2; *10*, 4.0; *11*, 4.9 μM) on the kinetics of the chemiluminescence of the system IAA (1 mM)–peroxidase (0.1 μM)–O_2: *a*, enzyme added to the mixture IAA–caffeic acid; *b*, caffeic acid added 45 min after initiation of the reaction by the enzyme (arrow indicates moment of adding caffeic acid). *I* is the intensity of chemiluminescence, 10^3 counts/s.

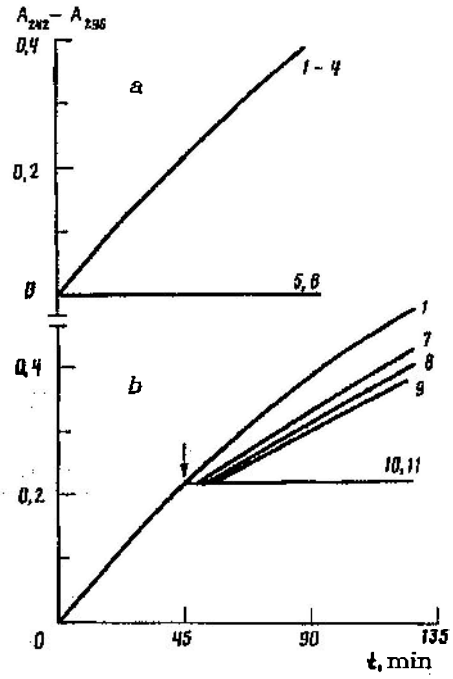

Fig. 2. Effect of caffeic acid on the kinetics of oxidation of IAA in the system IAA (1 mM)–peroxidase (0.1 μM)–O_2. For conditions, see Fig. 1.

arrested, confirmed by spectrophotometric measurements (Fig. 2). In the completely inhibited reaction mixture (at an inhibitor concentration above threshold), the reaction was not renewed on adjusting the enzyme concentration to values exceeding that of inhibitor. Dilution of the completely inhibited reaction mixture with buffer in any proportions also did not take the reaction mixture out of the "resting state". The introduction into the completely inhibited reaction mixture of an extra amount of substrate initiated the reaction, and the kinetics of chemiluminescence in this case was the same as in the reaction mixture, not containing inhibitor at the same concentrations of enzyme and substrate.

The value of the threshold concentration of inhibitor rose with increase in the concentrations also of substrate when the inhibitor was added to the enzyme-initiated reaction. When the enzyme was added after preparing the IAA–caffeic acid mixture, the threshold concentration of caffeic acid did not depend on the enzyme concentration, but was directly proportional to the substrate concentration. A similar result was observed on adding substrate to the enzyme–inhibitor mixture. The value of the threshold concentration of inhibitor depended on the moment of its addition (Fig. 3).

An effect similar to that of caffeic acid was found on introducing into the IAA–peroxidase–O_2 system a substance unspecific for this reaction — anfen, known as a radical trap. The threshold level of inhibition for this substance was far higher than for caffeic acid at ~25 μM (on addition 1 min after the start of the reaction).

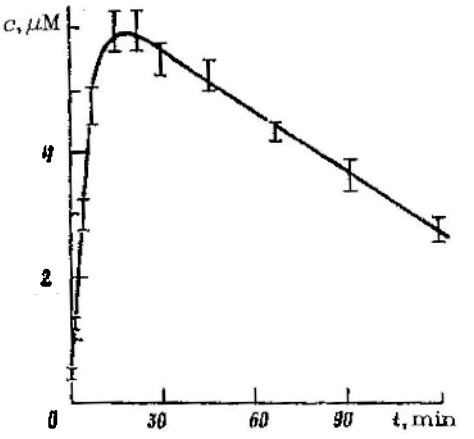

Fig. 3. Dependence of the threshold concentration of caffeic acid (c) on the moment of its addition to the system IAA (1 mM)–peroxidase (0.1 μM)–O_2.

In the completely inhibited reaction mixture, the reaction could be re-initiated by exposure to visible light in the presence of Bengal rose as photosensitizer. After initiation the reaction proceeded without light. The radiation dose necessary for initiation depended on the inhibitor concentration.

DISCUSSION

The impossibility of initiating the completely inhibited reaction by introducing an extra amount of enzyme clearly indicates that caffeic acid does not de-activate the enzyme. The independence of the threshold concentration of inhibitor on the peroxidase concentration (when the inhibitor is added to IAA before addition of the enzyme) also indicates the non-enzymatic pathway of inhibition. In addition, the threshold concentration of inhibitor is about three orders lower than the initial concentration of substrate in the reaction mixture, which rules out the possibility of withdrawing the substrate from the reaction through its binding to caffeic acid.

We assume that caffeic acid acts on a certain third hypothetical substance R. This substance is evidently present in trace amounts in the substrate solution, as confirmed by the possibility of re-initiating the completely arrested reaction by introducing into the reaction mixture an additional quantity of substrate solution. Moreover, the substance R is produced in the course of the reaction of oxidation of IAA as indicated by the influence on the inhibition threshold of the moment of adding the inhibitor (Fig. 3) and also of the concentrations of enzyme and substrate. The nature of the substance R is not known. However, the following indirect findings suggest that R is a free radical. In fact, re-initiation of the arrested reaction may be caused by exposure to light in the presence of sensitizer — Bengal rose — which on exposure to light gives a high yield of triplet excited molecules [12]. Being in essence biradicals, they are capable of inducing the effective formation of free radicals. Furthermore, it is known that peroxidase–oxidase reactions to which aerobic oxidation of IAA by peroxidase also belongs may follow a chain free-radical mechanism [13].

The formation of free radicals in the course of enzymatic oxidation of IAA was investigated by the authors of [14], who identified the skatole-radical and the superoxide

anion-radical. If the assumption on the free radical nature of R is true, then caffeic acid must evidently act as a trap for the free radicals R$^{\cdot}$ (taking into account the assumption on the free-radical nature of the hypothetical substance R, then instead of the designation R we shall use R$^{\cdot}$). The proposed hypothesis is confirmed by the identity of the action of caffeic acid and anfen on the enzymatic oxidation of IAA.

Thus, to explain the threshold effect we assume that the enzymatic oxidation of IAA is a branched chain free-radical reaction and that caffeic acid quenches the free radicals involved in the chain reaction through radical transfer:

$$R^{\cdot} + I \rightarrow R + I^{\cdot}, \quad (1)$$

The free radicals of the inhibitor I$^{\cdot}$ which form in this case evidently do not lead to the development of a free-radical chain and are rapidly destroyed as a result, for example, of the formation of dimers:

$$I^{\cdot} + I^{\cdot} \rightarrow I - I. \quad (2)$$

When the inhibitor concentration is above threshold, the concentration of R$^{\cdot}$ falls to a value at which the multiplication coefficient of the radicals becomes less than unity and the reaction stops. If the inhibitor concentration is below threshold, the multiplication coefficient of the radicals remains above unity, the chain reaction develops like an avalanche, the concentration of R$^{\cdot}$ rises and the inhibitor is rapidly destroyed in the processes (1) and (2). Then the chain reaction develops by the usual kinetics.

Thus, the threshold effect detected by us on inhibition by caffeic acid of peroxidase-catalysed oxidation of IAA and study of the patterns of threshold inhibition clearly show that the earlier conclusions on the competitive nature of inhibition [5, 6, 8] are mistaken.

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REFERENCES

1. K. Dörfling, in *Plant Hormones. A System Approach*, Mir, Moscow (1985).
2. V. V. Polevoi, in *Phytohormones*, Leningrad State University, Leningrad (1982).
3. W. A. Andreae, *Nature*, **170**, 83 (1952).
4. G. W. Schaeffer, J. G. Buta and F. Sharpe, *Physiol. Plantarum*, **20**, 342 (1967).
5. D. A. Gelinas, *Plant Physiol.*, **51**, 967 (1973).
6. T. T. Lee, *Plant Physiol.*, **59**, 372 (1977).
7. J. A. Sacher, *Amer. J. Bot.*, **50**, 116 (1963).
8. T. T. Lee, G. L. Rock and A. Stoessl, *Phytochemistry*, **17**, 1721 (1978).
9. N. F. Haard, *Z. Pflanzenphysiol.*, **89**, 87 (1978).
10. S. N. Krylov, V. V. Lazarev and L. B. Rubin, *Dokl. Akad. Nauk SSSR*, **310**, 1000 (1990).
11. J. A. Sacher, *Plant Physiol.*, **37**, 74 (1962).
12. D. C. Neckers, *J. Photochem. Photobiol. A: Chemistry*, **47**, 1 (1989).
13. V. R. Fed'kina, F. I. Ataullakhanov and T. V. Pronnikova, *Teoret. i eksp. khim.*, **24**, 172 (1988).
14. C. Mottley and R. P. Mason, *J. Biol. Chem.*, **261**, 16860 (1986).