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DETECTION OF PEROXIDASE CATALYSED PHENOL POLYMERIZATION INDUCED BY ENZYMATICALLY REDUCED PARAQUAT

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Key Word Index—Paraquat; hydrogen peroxide; peroxidase; phenol polymerization; lignification.

Abstract—Phenol polymerization via hydrogen peroxide and plant peroxidases is investigated. Hydrogen peroxide is generated via enzymatically reduced paraquat with ferredoxin reductase, NADPH and oxygen. In order to study the paraquat dependent generation of hydrogen peroxide an electrophoretic procedure is applied using wood peroxidase isoenzymes separated on polyacrylamide gels, and phenolic substrates as sensitive hydrogen peroxide detectors. To quantify this process gels are scanned with a laser densitometer. The theory of hydrogen peroxide formation by paraquat is further supported by photometrical detection of the complex II of horseradish peroxidase and the fluorometric measurement of oxidized homovanillic acid. The effects of superoxide dismutase and catalase are investigated; hydrogen peroxide formation is enhanced by the former and inhibited by the latter.

INTRODUCTION

Paraquat (1,1′-dimethyl-4,4′-bipyridilium dichloride, methyl viologen), the active ingredient of many broad spectrum herbicides, is universally toxic in eucaryotes as well as in procaryotes [1–3]. The toxic effect of paraquat is believed to be due to the reductive formation of the monocation radical, which can be achieved in manifold ways [1–6]. The reduction of dioxygen by the paraquat radical leads to cytotoxic intermediates such as the superoxide radical and hydrogen peroxide [7–12].

An important way to remove hydrogen peroxide in plants is the peroxidase catalysed reduction to water at the expense of oxidative phenol polymerization. Though polymerised phenols are necessary for the fortification of cell walls they are toxic to cells at high concentrations acting as denaturating and complexing agents on proteins [13].

In this paper we describe for the first time that paraquat dependent phenol polymerization may contribute to the toxic action of this herbicide towards plants. To elucidate the reaction mechanism of this unphysiological *in vitro* 'lignification', enzymatically reduced paraquat monocation radical, wood peroxidases and horseradish peroxidase were applied to this model. To separate wood peroxidase isoenzymes and to detect phenol polymerization a gel electrophoretic method [14] was used. Quantitative investigations were done by gel scanning and by fluorometric measurements of paraquat dependent production of phenol polymerizates.

RESULTS

The detection of the enzymatical activity of peroxidase isoenzymes separated by non-denaturating polyacrylamide gel electrophoresis (PAGE) can easily be performed by incubating the gel strips in a solution containing the substrates of peroxidase, hydrogen peroxide and a phenolic compound [15–18]. Ferulic acid ethyl ester (FAEE) was used as the phenolic substrate and hydrogen peroxide (0.01 mM) was added in this experiment. FAEE, a derivative of cinnamic acid represents a chemical compound which is closely related to the natural occurring precursors of lignification [19]. The products of the phenolic polymerization due to the peroxidase isoenzyme activity are outlined in Fig. 1, lane 1. Submerging the gel in a solution containing FAEE, a NADPH-generating system [12], paraquat and ferredoxin reductase yielded the same isoenzyme pattern on the gel (Fig. 1, lane 2). This result strongly suggested that hydrogen peroxide was being generated by this system. The generation of hydrogen peroxide is dependent on the amount of reduction equivalents in the solution. Paraquat as the catalyst for hydrogen peroxide generation will not be exhausted. Addition of superoxide dismutase (SOD) to the incubation mixture led to an enhanced reaction rate (Fig. 1, lane 3). Addition of catalase inhibited all reactions (Fig. 1, lane 4). With *o*-dianisidine instead of FAEE the same electrophoretic results were obtained (data not shown). Experiments in the absence of either ferredoxin reductase, NADPH or paraquat were done, but there were no polymerization products seen in any case (data not shown).

Quantitative measurements of phenol polymerizates on the gel were done by scanning the gels with a densitometer and computer-assisted analysis. The staining intensities were converted to electrophoretic peaks and polymerizate concentration was calculated from the integration of peak areas. The amount of polymerized FAEE by means of paraquat (for concentrations see Fig. 1, lane 2) within six hr was equivalent to a hydrogen peroxide addition of 4 μM. With SOD added hydrogen peroxide generation was equivalent to 7 μM within the same time.

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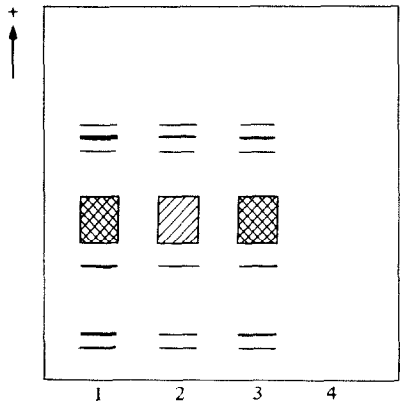


Fig. 1. Peroxidase isoenzymes from *Aesculus hippocastanum* separated on polyacrylamide gel electrophoresis stained with ferulic acid ethyl ester (FAEE), incubation time 6 hr, room temperature. Lane 1: incubation mixture A: 1 ml 0.1 M FAEE, 1 ml 1 mM hydrogen peroxide. Lane 2: incubation mixture B: 1 ml 0.1 M FAEE, 1 ml 0.1 M glucose-6-phosphate, 1 ml 50 mM NADP, 1 ml 0.1 M paraquat, 15 U glucose-6-phosphate dehydrogenase, 5 U ferredoxin reductase, up to 100 ml with 0.1 M phosphate buffer, pH 7.2. Lane 3: mixture B + 100 U SOD. Lane 4: mixture B + 300 U catalase.

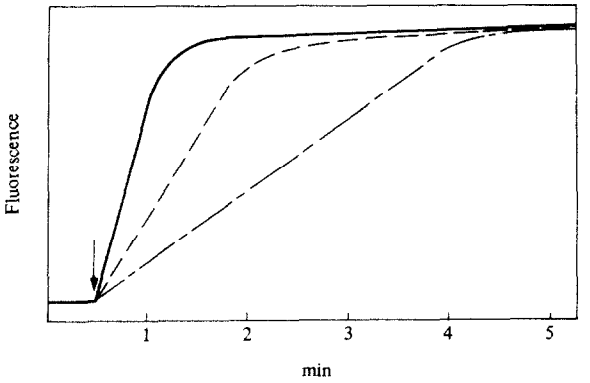


Fig. 2. Fluorometric measurement of the homovanillic acid dimer generated by peroxidase catalysed oxidation with hydrogen peroxide; ex = 315 nm, em = 425 nm, T = 20°; (——): mixture A: 100 μl 30 μM hydrogen peroxide, 100 μM 10 mM homovanillic acid, up to 2, 9 ml with 0.1 M phosphate buffer, pH 7.2; (—·—): mixture B: 100 μl 10 mM homovanillic acid, 100 μl 30 mM paraquat, 100 μl 0.5 mM NADPH, 10 μl ferredoxin reductase (1 U/ml), up to 2.9 ml with 0.1 M phosphate buffer, pH 7.2; (– – –): mixture B + 100 μl SOD (100 U/ml). The arrow indicates the addition of 100 μl horseradish peroxidase (40 U/ml) for each experiment.

Fluorometric investigation of the hydrogen peroxide generation by paraquat was accomplished using homovanillic acid as reducing agent which is converted to a highly fluorescent dimer by peroxidase catalysed oxidation with hydrogen peroxide [20]. The continuous increase of fluorescence when homovanillic acid was incubated with horseradish peroxidase, paraquat, NADPH and ferredoxin reductase is shown in Fig. 2. The amount of the fluorescent dimer formed was adequate to an addition of 3 μM hydrogen peroxide to homovanillic acid and horseradish peroxidase. The same reaction mixture with SOD added yielded a much faster increase of fluorescence, attributed to the enzymatically catalysed dismutation of the superoxide radical to hydrogen peroxide and oxygen. With boiled SOD the generation rate of fluorescence was not affected.

By photometric recording of the spectra of horseradish peroxidase during reaction with hydrogen peroxide, the transition of native peroxidase to the complex II was observed by a shift of the Soret band from 402 nm to 418 nm and new absorption maxima at 530 nm and 552 nm. The following addition of a phenolic substrate led to regeneration of free peroxidase with its typical spectrum [21]. The spectra of horseradish peroxidase incubated with paraquat, NADPH and ferredoxin reductase before and after the addition of homovanillic acid, which is in perfect accordance with the spectra of peroxidase complex II and native peroxidase described above, are shown in Fig. 3.

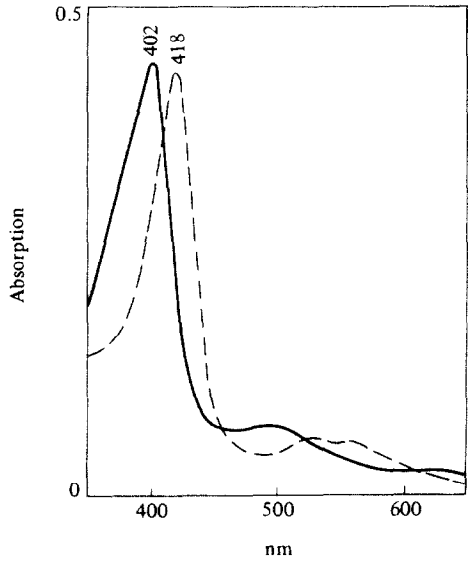


Fig. 3. Photometric measurement of the spectra of horseradish peroxidase during the reaction with NADPH/paraquat/ferredoxin reductase-generated hydrogen peroxide, at room temperature; (– –): 100 μl 10 mM paraquat, 100 μl 0.5 mM NADPH, 10 μl ferredoxin reductase (1 U/ml), 100 μl horseradish peroxidase (200 U/ml), up to 1.0 ml with 0.1 M phosphate buffer, pH 7.2, measured 10 min after incubation. (—): Addition of 100 μl 10 mM homovanillic acid, measured 1 min after addition.

DISCUSSION

This investigation presents evidence that paraquat toxicity in plants might be partially due to an increased rate of hydrogen peroxide induced phenol polymerization. Hydrogen peroxide is necessary for the peroxidase catalysed oxidative phenol polymerization (lignification, tannin production) which is essential to the formation of structure properties, defence against pathogens, wound

healing and others. Extensive phenol polymerization caused by excessive formation of hydrogen peroxide leads to irreversible protein damage, well documented by heartwood formation in trees [22].

To confirm the mechanism of hydrogen peroxide production by paraquat [23] the time dependency of the reaction was investigated fluorometrically. A continuous increase of fluorescence was observed when horseradish peroxidase was incubated with paraquat, NADPH, ferredoxin reductase and homovanillic acid. Peroxidase catalyses the oxidation of homovanillic acid to its highly fluorescent dimer at the expense of hydrogen peroxide. Superoxide dismutase accelerated the reaction indicating that superoxide radical has been generated faster than hydrogen peroxide.

Further evidence for the hydrogen peroxide formation is given by recording the spectra of horseradish peroxidase. The reaction of hydrogen peroxide with peroxidase led immediately to the typical absorption maxima of peroxidase complex II. Formation of complex II could also be demonstrated during the incubation of peroxidase with paraquat, NADPH and ferredoxin reductase. After addition of a phenolic substrate a rapid shift to the spectrum of free peroxidase was observed.

The polymerization of lignin precursor substances in polyacrylamide gels can serve as a simplified model for the *in vitro* demonstration of metabolic processes. In nature the primary cell wall represents the matrix where peroxidase catalysed lignification takes place [24]. In polyacrylamide gels the products of phenol polymerization are also covalently bound to a matrix, mimicing the naturally occurring conditions. One must emphasize that such a carrier system means a considerable approach towards the more lipophilic *in vivo* environment, than any kind of experiments done in aqueous buffer solutions. On the other hand it furnishes a sensitive and specific detection method for intermediately produced hydrogen peroxide.

Hydrogen peroxide produced by enzymatically reduced paraquat leads to a peroxidase isoenzyme pattern on the gel within some hours under the experimental conditions described above. The acceleration of the reaction rate by SOD is based on the much faster enzymatic production of hydrogen peroxide from superoxide radical than the non-enzymatic dismutation. However, the same isoenzyme pattern could be obtained when hydrogen peroxide is directly added. As expected these reactions were inhibited by catalase.

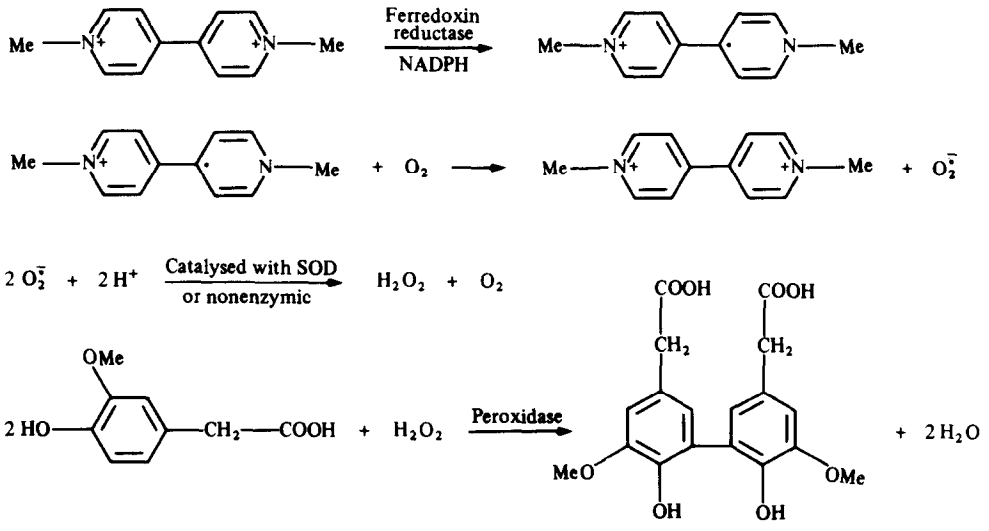
Our results presented in this paper strongly point to the nonphysiological catalytic action of paraquat leading to an increased polymerization of phenolic constituents. The mechanism of the hydrogen peroxide generation and subsequent phenol polymerization can be summarized according to Scheme 1.

EXPERIMENTAL

Chemicals. Horseradish peroxidase, glucose-6-phosphate-dehydrogenase, ferredoxin reductase, glucose-6-phosphate, $NADP^+$, NADPH and paraquat were purchased from Sigma, homovanillic acid from Serva. Ferulic acid Et ester was prepared according to ref. [25]. All other chemicals were of analytical grade.

Polyacrylamide gel electrophoresis. Wood samples from *Aesculus hippocastanum* were carefully milled and extracted overnight at 4° (1 g in 4 ml buffer) according to ref. [15]. The filtered soln (50 μl) was used for one electrophoretic run. PAGE was carried out as described earlier [14]. A water-cooled block gel apparatus and a sepn gel with 15% acrylamide, cross linked 1:75 with bismethyleneacrylamide, pH 8.6, was used. The electrophoresis buffer was a Tris–glycine system, pH 8.9. For a run of 4 hr a constant voltage of 300 V and a starting current of 100–120 mA were used. After sepn the gels were washed in running H_2O for 1 hr and incubated for 6 hr in a soln containing 0.1 mM ferulic acid Et ester, 1 mM glucose-6-phosphate, 15 U glucose-6-phosphate-dehydrogenase, 0.5 mM $NADP^+$, 0.1 mM paraquat and 1 U ferredoxin reductase. The same incubation mixt with either SOD (100 U) or catalase (300 U) added was also used. For comparison one gel was incubated with 0.01 mM H_2O_2. All assays were carried out in 0.1 M Pi buffer, pH 7.2, at room temp.

Densitometric measurements were carried out on a soft laser scanning densitometer equipped with a computer data processor.



Scheme 1.

Fluorometric measurements were carried out on a recording spectrofluorometer equipped with a thermoelectric cooler to maintain a constant temperature of 20°. The rate of change in the fluorescence of the homovanillic acid dimer was measured according to ref. [20].

Photometric measurements were carried out on a recording spectrophotometer. Absorption maxima of horseradish peroxidase and horseradish peroxidase complex II were compared with those given in ref. [21].

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