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INVESTIGATION OF HYDROGEN PEROXIDE FORMATION IN PLANTS

K. STICH and R. EBERMANN

University of Agriculture, 1180 Vienna, Gregor Mendelstr. 33, Austria

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Key Word Index—*Ailanthus glandulosa*; Simarubaceae; hydrogen peroxide; peroxidase isoenzymes; lignification; flavin coenzymes; Mn^{2+}; PAGE.

Abstract—A convenient and simple electrophoretic procedure was used to study the NAD(P)H-dependent generation of the hydrogen peroxide needed for the polymerization of coniferyl alcohol by peroxidases from the wood of *Ailanthus glandulosa*. The results showed that an NAD(P)H-dependent generation of hydrogen peroxide could be brought about by either: a FMN or riboflavin-dependent system; or a Mn^{2+}-dependent system. The most active system was the one incorporating Mn^{2+}, followed closely by that incorporating riboflavin. In nature it appears that the method of hydrogen peroxide formation is determined by the amounts of cofactors present in the lignifying tissue. Because no quantitative data are available in the literature, further studies of the concentrations of these cofactors in the plant cell-wall are needed.

INTRODUCTION

Lignin polymers are widely distributed in the plant kingdom. Their structures were elucidated during the fifties mainly by Freudenberg and others. Although not all of the structural features are known completely, it is generally accepted that lignin is a polymerization product of hydroxycinnamyl alcohols such as coniferyl, sinapyl and *p*-coumaryl alcohol. It is believed that polymerization is a radical reaction initiated by peroxidase (EC 1.11.1.7) and hydrogen peroxide [1, 2]. The origin of the rather large amount of hydrogen peroxide needed for polymerization remained unknown for many years [3]. In the past 10 years, however, two hypotheses about the biosynthesis of hydrogen peroxide in plant cells have been put forward. The first involves a biosynthetic route in which oxygen is reduced to hydrogen peroxide in the presence of NAD(P)H by a cell-wall bound peroxidase. The reaction is accelerated by Mn^{2+} and monophenolic substances like coniferyl alcohol [4–11]. In the second hypothesis, the reduction of atmospheric oxygen by NAD(P)H is mediated by flavin coenzymes [12]. In the latter case it is assumed that NADH is reducing the flavin coenzymes which can combine with oxygen to yield flavin peroxides. Hwang postulates that the flavin peroxides are able to replace hydrogen peroxide as a substrate of peroxidase in the lignification process [12].

This paper presents a series of model experiments, with peroxidase isoenzymes from extracts of *Ailanthus* wood, designed to determine which hypothesis is correct.

RESULTS

Based on the peroxidase detection method reported recently [13], a new technique for studying lignification activities was developed. With this technique, lignin formation on the polyacrylamide gel was visualized directly as white dim zones at the site of the lignifying enzymes.

NADH oxidation by peroxidase isoenzymes in the presence of Mn^{2+}

Figure 1 shows the polymerization of coniferyl alcohol by peroxidase enzymes in the presence of alcohol dehydrogenase, NAD^+, ethanol and different concentrations of Mn^{2+}. Strong zones of precipitated polymerization products were produced. Under the same experimental conditions, but without Mn^{2+}, very weak precipitation zones were visible after 48 hr of incubation. No precipitation of polymers was observed when the enzymes in identical wood extracts were inactivated by heat.

Alcohol dehydrogenase could be replaced by malate dehydrogenase. Similar results were obtained when the NADH-producing system was replaced by an NADPH-producing one (Fig. 2).

All of the polymerization reactions were inhibited when Tiron or catalase was present in the incubation mixture.

NADH oxidation by peroxidase isoenzymes in the presence of flavin coenzymes

Preliminary experiments showed that in the presence of both light and flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD), polymerization of phenolic monomers by peroxidase occurred in the absence of any NAD(P)H-producing system. No polymerization of coniferyl alcohol occurred in the dark. Therefore all of the following experiments were carried out in darkness.

Figure 3 shows the results obtained with NAD^+, ethyl alcohol and alcohol dehydrogenase as the NADH-yielding system in the presence of FMN in the dark. In contrast to the results of the experiments described in the previous section, the reaction was Mn^{2+}-independent and

Abbreviations: ADH, alcohol dehydrogenase (EC 1.1.1.1); MDH, malic dehydrogenase (EC 1.1.1.37); GDH, glucose-6-phosphate dehydrogenase (EC 1.1.1.49); Tiron, 4,5-dihydroxy-1,3-benzene disulphonic acid.

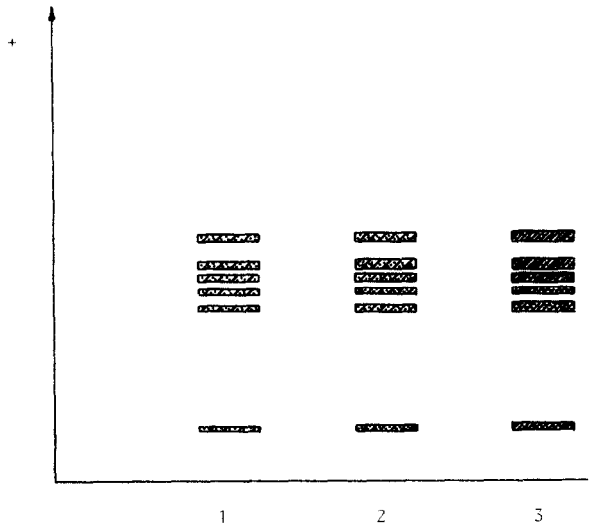


Fig. 1. Polymerization of coniferyl alcohol by peroxidase isoenzymes in the presence of ADH, NAD^+, ethanol and $MnCl_2$ (pH 7.4). (1) 0.1 mM $MnCl_2$; (2) 0.2 mM $MnCl_2$; (3) 1.2 mM $MnCl_2$.

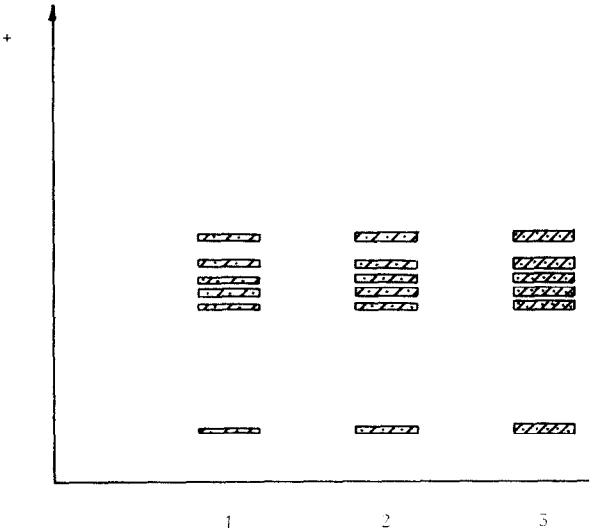


Fig. 3. Polymerization of coniferyl alcohol by peroxidase isoenzymes in the dark in the presence of ADH, NAD^+, ethanol and FMN (pH 7.4). (1) 0.1 mM FMN; (2) 0.2 mM FMN; (3) 1.2 mM FMN.

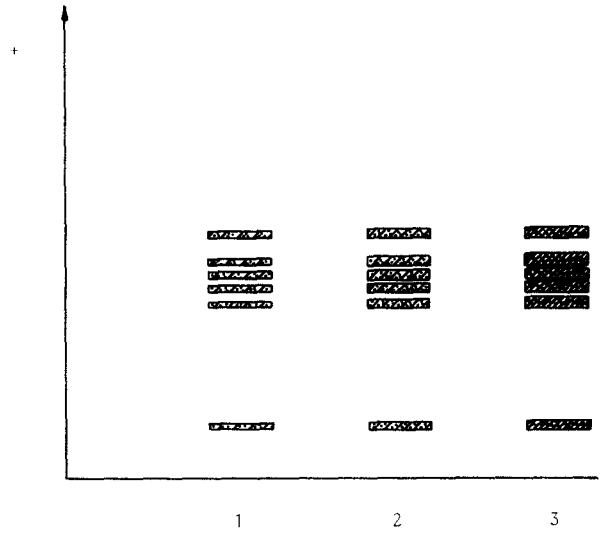


Fig. 2. Polymerization of coniferyl alcohol by peroxidase isoenzymes in the presence of GDH, $NADP^+$, G-6-P and $MnCl_2$ (pH 7.4). (1) 0.1 mM $MnCl_2$; (2) 0.2 mM $MnCl_2$; (3) 1.2 mM $MnCl_2$.

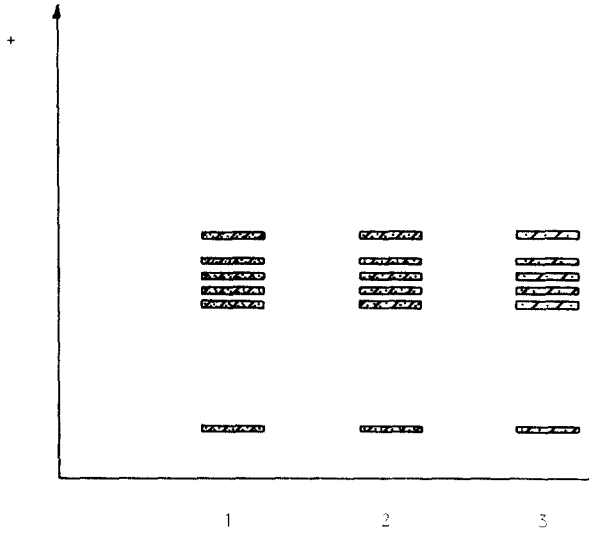


Fig. 4. Polymerization of coniferyl alcohol by peroxidase isoenzymes in the presence of ADH, NAD^+, ethanol (pH 7.4) and equimolecular amounts of the cofactors Mn^{2+}, riboflavin and FMN. (1) 0.1 mM $MnCl_2$; (2) 0.1 mM riboflavin; (3) 0.1 mM FMN.

there was less phenolic polymer produced (cf. Figs. 1 and 3), although the activity of the enzymes and the other parameters were the same in both sets of experiments. When the concentrations of Mn^{2+} and FMN were reduced, similar results were obtained.

When riboflavin was used instead of FMN or FAD, the intensity of the precipitation zones was only slightly weaker than in the presence of an equimolar quantity of Mn^{2+}, but more polymer formation occurred than in an FMN-containing system (Fig. 4).

When NADH-yielding systems were replaced by NADPH-producing ones, such as a system with glucose-6-phosphate dehydrogenase, analogous results were obtained. The formation of polymers was inhibited by either Tiron or catalase.

DISCUSSION

By means of an electrophoretic method which involves the formation of lignin-like polymers, the activities of wood peroxidase isoenzymes in the presence of hydrogen peroxide produced by different cofactors have been investigated for the first time.

It has been assumed that NAD(P)H is necessary for hydrogen peroxide formation. Present conceptions differ in the kind of cofactors needed. In the first case, a cell-wall bound peroxidase in the presence of Mn^{2+} and NADH is believed to be responsible for hydrogen peroxide formation [4, 5, 7, 9] and our results with peroxidase isoenzymes

from *A. glandulosa* support the work of Gross and coworkers [5, 6] and Halliwell [7] as a possible route for hydrogen peroxide formation in plants.

In the second route proposed for hydrogen peroxide formation [12], Mn^{2+} is replaced by flavin coenzymes, which after reduction by NAD(P)H produced by dehydrogenase shuttles react with molecular oxygen to yield flavin peroxides. Hwang [12] postulates that these flavin peroxides serve as substrates for peroxidase during lignification. In his work, rather high concentrations of FMN were used (about 78 mM). It appears unlikely that such high FMN concentrations occur under physiological conditions. We used much smaller amounts of FMN (0.1, 0.2 and 1.2 mM), and formation of phenolic polymers was observed. However, in comparison with the experiments using equimolar concentrations of Mn^{2+} as cofactor, the amount of polymers produced was smaller.

In addition to the reaction mechanism presented by Hwang [12], several other mechanisms are possible (Scheme 1) [14]. All our experiments were carried out at pH 7.4. Under these conditions, the decay of flavin peroxide to the superoxide radical $O_2^{\cdot-}$ is the favoured reaction. This conclusion can be drawn from the fact that polymer formation is completely inhibited by the $O_2^{\cdot-}$ scavenger Tiron [15]. The superoxide radical can participate in lignification in two ways: first, it forms hydrogen peroxide and oxygen by self-dismutation; second, it combines with peroxidase to form compound III according to Halliwell [7].

$$2\,O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$$

Enhanced polymer formation was detected when FMN was replaced by an equimolar amount of riboflavin in the incubation mixture. The difference in reactivity must be ascribed to the different polarity of the molecule since the reaction mechanism with oxygen is the same in both cases.

As stated above, the formation of lignin-like polymers with flavin coenzymes was observed in the presence of light in the absence of an NAD(P)H-producing system. No formation occurred in the dark. It is known [16] that flavin coenzymes, especially FMN, are easily transformed to an excited state (Fl_{Ox}*) by light. These excited molecules are then able to react in a photoelectron transfer reaction with compounds like coniferyl alcohol to give resonance-stabilized radicals.

The flavin radical $HFl^{\cdot}$ is an intermediate in the reaction and is in equilibrium with the oxidized (Fl_{Ox}) and reduced ($Fl_{Red}H_2$) forms of flavin. Therefore, the reduced form of the flavin coenzyme is produced by the light reaction as well and reacts with oxygen to form flavin peroxide according to the reaction scheme outlined above. Coniferyl alcohol is converted to the phenoxy radical, which reacts with itself to give a polymer. As a result, a precipitate is formed in the solution.

The experimental evidence leads to the conclusion that at least two possible routes could be responsible for hydrogen peroxide formation. Whether there is one major route or all routes operate simultaneously seems to be determined by the amounts of cofactors present in the lignifying tissue. Since no quantitative data are available in the literature, a final answer to this question cannot be given until the concentrations of the cofactors in the cell wall have been measured.

EXPERIMENTAL

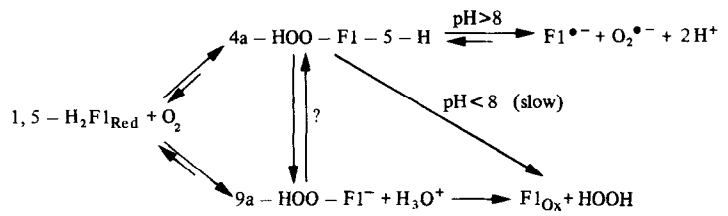
Chemicals. Alcohol dehydrogenase (from equine liver), glucose-6-phosphate dehydrogenase (type IX), malic dehydrogenase (from pigeon muscle), catalase (from bovine liver), glucose-6-phosphate, L(−)-malic acid, NAD^+, $NADP^+$, FMN, FAD, riboflavin and Tiron were purchased from Sigma Chemical Co. (München, West Germany). Coniferyl alcohol was prepared according to ref. [17]. All the other chemicals used were reagent grade (Merck).

Plant material and preparation of wood extracts. Trunk pieces from *A. glandulosa* Desf. (6 years old) were used. Extracts made from lignifying tissue and adjacent sapwood were prepared as described previously [2]. Preliminary experiments showed that the extraction procedure was also suitable for the extraction of cell-wall bound isoenzymes. The results of the extraction were comparable with those obtained by Mäder [18].

PAGE. Electrophoresis was performed as reported previously [19]. After electrophoresis, the gels were washed in running H_2O for 1 hr.

Incubation solutions. The substances of Tables 1 and 2 were dissolved in 200 ml 0.05 M Tris–HCl buffer, pH 7.4. 0.5 mM buffer was used for coniferyl alcohol. The gels containing the peroxidase isoenzymes were incubated in the freshly prepared solns overnight. Formed polymers were recognized as dim zones of precipitates.

$$ArOH + Fl_{Ox} \xrightarrow{h\nu} Fl_{Ox}^{*} + ArOH \rightarrow Fl^{\cdot+} + ArOH^{\cdot}; \rightarrow HFl^{\cdot} + ArO^{\cdot}$$
$$2\,HFl^{\cdot} \rightleftharpoons Fl_{Ox} + Fl_{Red}H_2$$



Scheme 1.

Table 1. Compositions of the incubation mixtures containing Mn^{2+} as cofactor

ADH (U/200 ml)	10	10	10	10	10	10	—	—	—	—
MDH (U/200 ml)	—	—	—	—	—	—	200	—	—	—
GDH (U/200 ml)	—	—	—	—	—	—	—	50	50	50
Ethanol (mM)	88	88	88	88	88	88	—	—	—	—
L(−)-malic acid (mM)	—	—	—	—	—	—	8	—	—	—
G-6-P (mM)	—	—	—	—	—	—	—	1.8	1.8	1.8
NAD^+ (mM)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	—	—	—
$NADP^+$ (mM)	—	—	—	—	—	—	—	0.4	0.4	0.4
$MnCl_2$ (mM)	—	0.1	0.2	1.2	0.2	0.2	0.2	0.1	0.2	1.2
Catalase (U/200 ml)	—	—	—	—	2000	—	—	—	—	—
Tiron (mM)	—	—	—	—	—	10	—	—	—	—
$MgCl_2$ (mM)	—	—	—	—	—	—		1.2	1.2	1.2

Table 2. Compositions of the incubation mixtures containing riboflavin or flavin coenzymes as cofactors

ADH (U/200 ml)	—*	10	10	10	10	10	10	10	—	—
MDH (U/200 ml)	—	—	—	—	—	—	—	—	200	—
GDH (U/200 ml)	—	—	—	—	—	—	—	—	—	50
Ethanol (mM)	—	88	88	88	88	88	88	88	—	—
L(−)-malic acid (mM)	—	—	—	—	—	—	—	—	8	—
G-6-P (mM)	—	—	—	—	—	—	—	—	—	1.8
NAD^+ (mM)	—	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	—
$NADP^+$ (mM)	—	—	—	—	—	—	—	—	—	0.4
FMN (mM)	0.2	0.1	0.2	1.2			0.2	0.2	0.2	0.2
FAD (mM)	—	—	—	—	0.1	—	—	—	—	—
Riboflavin (mM)	—	—	—	—	—	0.1	—	—	—	—
Catalase (U/200 ml)	—	—	—	—	—	—	2000	—	—	—
Tiron (mM)	—	—	—	—	—	—	—	10	—	—
$MgCl_2$ (mM)	—	—	—	—	—	—	—	—	—	1.2

*Blank.

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