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THE ROLE OF PEROXIDASE IN THE METABOLISM OF INDOLE-3-ACETIC ACID AND PHENOLS IN WHEAT

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Abstract—A crude peroxidase preparation from leaves of young wheat plants oxidizes IAA in the presence of Mn^{2+} and a phenolic cofactor in the absence of exogenous H_2O_2. When exogenous H_2O_2 is supplied the enzyme oxidizes ferulic and *p*-coumaric acids. Ferulic acid causes a lag period in the oxidation of IAA and is oxidized itself during the lag.

INTRODUCTION

It is well known that plants contain enzymes capable of oxidizing IAA *in vitro* [1, 2]. These enzymes also possess peroxidase activity [3, 4] with the possible exception of enzyme preparations from tobacco [5] and from pea roots [6]. Peroxidases are found in plants in the form of a number of isoenzymes [7–9]. The wide variety of functions of peroxidases is puzzling. For example, they have been reported to oxidize a number of phenolic compounds to their dimers and/or lignin [10–15] and *p*-methylmercapto-α-oxo-butyric acid to ethylene [16, 17]. Recently Ivanova and Peive [18] found that horseradish peroxidase (HRP) and other plant peroxidases also possess nitrate reductase activity.

Numerous reports indicate that certain monophenols and *m*-diphenols act under certain conditions *in vitro* as cofactors or activators of IAA oxidase and certain *o*- and *p*-diphenols and polyphenols as inhibitors [19–24]. Cofactors are supposed to transfer radicals from the CI enzyme complex to IAA [24] or to take part in generating the CIII active enzyme complex from enzyme and oxygen [25, 26]. However, the steric interactions of cofactors with the enzyme are not excluded [27]. Phenolic inhibitors may act by trapping free radicals [25, 28, 29] or by changing the conformation of the enzyme [24, 28, 30–33].

Most studies on the mechanism of action of peroxidase have been performed with HRP [26, 31]. Basu and Tuli [34] found 3-methyleneoxindole to be more active in stimulating the growth of wheat coleoptiles than IAA and we have therefore studied the mechanism of IAA oxidation by wheat peroxidase.

RESULTS

The crude peroxidase preparation (1 U = 83·3 μg) from leaves of young wheat plants contains 6 cathodic and 5 anodic bands with peroxidase activity on starch gel electrophoresis at pH 8·3. It oxidizes IAA only in the presence of Mn^{2+} ions and a phenolic cofactor. Using 4.10^{-4} M IAA in the reaction mixture, the optimal concentration of Mn^{2+} ions is 10^{-3} M. Two phenolic cofactors were tested, 2,4-dichlorophenol (DCP) and 4-hydroxy-*trans*-cinnamic acid (HCA, *p*-coumaric acid); the optimal concentrations are 10^{-4} M for DCP and 4.10^{-5} M for HCA. In the presence of DCP the enzyme has one pH optimum 5·5–5·9 and in the presence of HCA two pH optima, 4·2–4·4 and 5·4–5·6. Using DCP as a cofactor, some interactions were observed, which are not found in the presence of HCA. For example, Mn^{2+} stops the peroxidative degradation of DCP. HCA was therefore used as a cofactor in further experiments. IAA is oxidized in the presence of both cofactors giving spectrally identical products with λ_{max} at 247 and 254 nm, corresponding to the spectra of 3-methyleneoxindole [35–37]. The reaction follows Michaelis–Menten kinetics with K_m IAA = $2·3.10^{-3}$ M. High concentrations of IAA

(higher than 17 μM/1 U) inhibit the enzyme reaction. The reaction is further inhibited by cyanide (10^{-6}–10^{-3} M) and azide (10^{-3} M) ions. If the reaction proceeds under optimal conditions, exogenous H_2O_2 has no effect on its rate. At high concentrations (10^{-4} M H_2O_2) even slight inhibition was observed. Catalase (2·5–10 μg/3 ml reaction mixture) has no effect on the activity.

The crude enzyme oxidizes ferulic (FA) and *p*-coumaric acids in the presence of 10^{-4} M H_2O_2. Both reactions follow Michaelis–Menten kinetics with the inhibition by high concentrations of substrates. $K_m^{FA} = 4.10^{-3}$ M and $K_m^{HCA} = 3.10^{-3}$ M. Both reactions have two pH optima, 4·3–4·5 and 5·4–5·6. Oxidation of *p*-coumaric acid is inhibited by IAA in the concentration ratio IAA/HCA of about 10 and higher. Oxidation of FA is also inhibited by IAA, but only in the concentration ratio IAA/FA of about 100 and higher. The inhibition is not of a pure competitive type. Lineweaver–Burk plots are curvilinear. Manganese has no effect on the course of the oxidation of phenolic acids. FA is first oxidized to a pink compound with λ_{max} *ca* 500 nm. This compound quickly turns to a yellow one with the λ_{max} at 430 nm. The resulting reaction product was chromatographed by TLC on cellulose in isopropanol:ammonia:water (8:1:1). It has R_f 0·1 and shows bright green fluorescence in UV light. After spraying with diazotized *p*-nitroaniline, followed with 10% aq Na_2CO_3 [38] its color turns to violet.

The enzyme is not capable of direct oxidation of IAA with exogenous H_2O_2. The presence of HCA as a cofactor in the reaction mixture is necessary. Ferulic acid is also able to function as a cofactor for peroxidative degradation of IAA, but a high concentration ratio IAA/FA of 100 and higher is needed. Also in this reaction manganese has no effect.

Ferulic acid introduces a lag period prior to the onset of IAA oxidation. Plotting the duration of this lag as a function of FA concentration yields a nonlinear function. Also plotting the duration of the lag as a function of FA or IAA concentration, with a constant ratio of IAA/FA (100), yields a nonlinear function. After the lag, IAA oxidation proceeds without any change. The duration of the lag depends also on the concentration of HCA. Increasing HCA concentration shortens the lag. Exogenous H_2O_2 (10^{-6} to 10^{-5} M) shortens or abolishes the lag, catalase (1–10 μg) has no effect on the duration of the lag. FA is itself oxidized during the lag. Products of this oxidation are spectrally and chromatographically identical with those of direct oxidation of FA with exogenous H_2O_2 and enzyme. Ferulic acid added to a reaction mixture in which IAA oxidation is already proceeding, induces a much shorter lag than when added before starting the reaction. For example: 2.10^{-6} M FA induces a lag about 10 min. When added 3 min after starting the reaction, it induces a lag of only 2 min.

In the absence of exogenous H_2O_2, wheat peroxidase itself generates it. The presence of Mn^{2+} ions, IAA and a phenolic cofactor is needed. Raising the Mn^{2+} ions concentration and/or the concentration ratio HCA/FA increases the rate of H_2O_2 formation. The rate of H_2O_2 formation is the rate-limiting step of the overall oxidation of substrates. Catalase (2–50 μg) does not affect the rate of H_2O_2 formation.

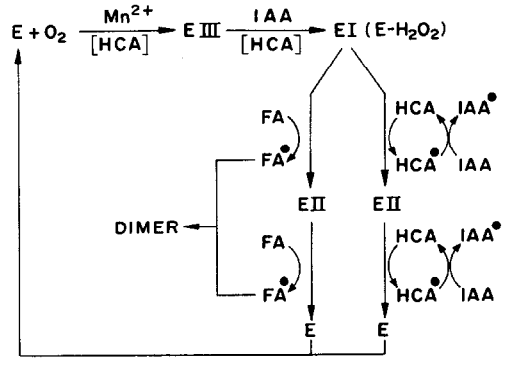
DISCUSSION

It is evident from the results that wheat peroxidase has two functions. It generates H_2O_2 (or peroxo-radical) in bound form (catalase does not affect it) and uses it to catalyze the oxidation of substrates. This is in agreement with the knowledge of the existence of intermediary enzyme complexes, described for HRP:complex HRP III, that reacts with IAA and forms H_2O_2 and complexes HRP I and HRP II, which take part in the peroxidative mechanism of substrate oxidation. HRP III complex is formed from ferroperoxidase and oxygen [26, 39–41].

Wheat enzyme is not capable of direct oxidation of IAA with exogenous hydrogen peroxide, and this is the main difference from HRP. This oxidation proceeds only in the presence of HCA or other phenolic cofactor. We suggest that direct transfer of an electron between IAA and the enzyme is not possible, probably due to the steric configuration. HCA may then act as a mediator of this transfer. The radicals IAA reacts further by the known mechanism [37, 42] to 3-methyleneoxindole as the main product of oxidation. If FA is added to the reaction mixture, the E I complex reacts preferentially with it, and radicals of FA are formed. These radicals dimerize and form a prod-

uct likely to be of guaiacylglycerol type [43]. Only after the concentration of FA decreases to a certain level, may interaction of the enzyme with HCA and IAA proceed.

Both phenolic acids and IAA probably interact with the enzyme and change its configuration. They may compete for the binding site, as Sano [35], Sirois and Miller [31] and others proposed. The concept of steric interactions is further supported by two facts (1) high concentrations of substrates inhibit the reaction and (2) the plot of the lag duration as a function of FA concentration, when FA/IAA is constant (= 100), is not linear. This is a further difference from HRP, where this plot is linear [26].



Scheme 1. The proposed mechanism of the oxidation of phenolic acids and IAA by wheat peroxidase.

Similar steric interactions also probably take place during H_2O_2 formation. Both HCA and FA probably act as cofactors for H_2O_2 formation, like the Mn^{2+} ions. Increasing HCA concentration in the presence of constant FA concentration stimulates H_2O_2 formation, indicating that HCA and FA compete for the binding site of the peroxidase. HCA appears to be a more effective cofactor. These conclusions are further supported by the fact, that FA added to the reaction mixture where IAA oxidation is already proceeding induces a much shorter lag than when added before starting the reaction. This result indicates that in the absence of FA, a high enough concentration of the E–H_2O_2 complex is formed to oxidize very quickly FA added later.

The proposed reaction mechanism of wheat peroxidase presented in the scheme is very similar to that of Gelinas [26] for HRP, with the exception that HCA functions as a necessary cofactor for the reaction of IAA with E I and E II. Mn^{2+} ions do not affect the oxidation of any substrate by exogenous H_2O_2. They are needed for the formation of E–H_2O_2 (E I) only. We do not envisage the formation of an inactive complex of FA with E III, as was suggested by Gelinas [26] for HRP. As may be seen from our results, FA forms a complex with wheat peroxidase, which is active in E–H_2O_2 formation, even if much less than is that of HCA. DCP, which was used as a cofactor for HRP by Gelinas [26] is a synthetic compound differing in some aspects of its action from naturally occurring HCA. Therefore HCA was used in our studies.

The functioning of HCA as a necessary cofactor for the reaction of E I and E II with IAA may be of physiological significance. HCA is a key-metabolite for the synthesis of a number of phenolic compounds and therefore it may be prevented by IAA from oxidation under certain conditions as already suggested by Siegel *et al.* [11]. Then the system peroxidase/phenols would be the site of action of IAA. Whether phenols or IAA are oxidized would depend on the concentration ratio of diphenols/monophenols/IAA. Recently also Sirju and Wilson [44] assigned to the system IAA/IAA oxidase/peroxidase a critical rôle in the regulation of lignification vs cell proliferation.

EXPERIMENTAL

Preparation of the enzyme. Wheat seed (cv Jubilar) was surface sterilized with 80% EtOH, soaked in H_2O, and cultivated on wet cheesecloth for 4 days in the dark and 8 days under continuous illumination (2500 lx). Cell sap was pressed out from the leaves on a pre-cooled household fruit press in the presence of 0·5 M KPi buffer pH 6 with 1% thiourea as an inhibitor of polyphenoloxidase [45]. It was established that thiourea does not influence the peroxidase activity. The homogenate was centrifuged 30 min at 20000 *g* and proteins of supernatant were precipitated with solid $(NH_4)_2SO_4$ to 90% saturation. The ppt was dissolved in 1% thiourea, and salt and phenols were removed on a Sephadex G 25 column. The elution was performed with H_2O. Temp throughout all procedures was 2–4°. The fractions containing proteins were freeze-dried and used as a crude preparation of wheat peroxidase.

Enzyme assays. The oxidation of IAA was followed by the *A* at 247 nm [5, 46]. The reaction mixture (3 ml) contained 10^{-3} M Mn^{2+}, 4.10^{-4} M IAA, 4.10^{-5} M HCA (or 10^{-4} M DCP) and 30 μg of enzyme, all in 0·05 M KPi buffer pH 5·5 (when DCP used pH 5·8). The reaction proceeded at 30 ± 0·5°. The 0·05 M acetate buffer was used for measurement at pH lower then 5·2.

The degradation of phenolic acids was also followed by *A* at 310 and 320 nm [26] for HCA and FA, respectively. Reaction mixture (3 ml) contained 10^{-4} M H_2O_2, 30 μg of enzyme and various concentrations of acids, all in 0·05 M KPi buffer pH 5·5. The reaction proceeded at 30 ± 0·5°.

Assay of H_2O_2 formation. The formation of H_2O_2 was followed as the degradation of FA. The reaction mixture (3 ml) contained 2.10^{-5} M FA, 6.10^{-3} M IAA, 10^{-3} M Mn^{2+}, 4.10^{-5} M HCA, when stated and 125 μg of enzyme, all in 0·05 M KPi buffer pH 5·5. The reaction was followed by A at 320 nm at 30 ± 0·5°.

Starch gel electrophoresis was performed according to the method of Siegel and Galston [7].

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Abbreviations: IAA—indole-3-acetic acid; FA—ferulic acid; HCA—4-hydroxycinnamic acid; HRP—horseradish peroxidase; DCP—2,4-dichlorophenol; C I–C III—complexes of horseradish peroxidase; E I–E III—complexes of wheat peroxidase.

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