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Note

Detection of peroxidase isoenzymes in polyacrylamide gels by means of hydroxycinnamic acid esters as substrates

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Peroxidase (E.C. 1.11.1.7) is an enzyme containing haeme-iron widely distributed in plants and animals, and catalyses the oxidative polymerization of many organic compounds activated by OH or NH_2 groups.

A large number of peroxidase isoenzymes are commonly found in plants, as can be readily demonstrated by polyacrylamide gel electrophoresis (PAGE). Peroxidases are responsible for lignification in healthy plants[1–5] and also for lignification due to infection[6,7]. Some of the peroxidase isoenzymes participate in the oxidation of indolyl-3-acetic acid (IAA)[8–12].

Liu and Gibson[13,14] introduced eugenol, the principal constituent of clove oil, as a physiological substrate for the detection and fluorimetric determination of peroxidase isoenzymes. The advantages of eugenol are that it is not a health hazard like benzidine or *o*-dianisidine, which are toxic, the former being a carcinogen[15,16], and its chemical structure is very similar to those of substrates of peroxidase occurring in nature, for example those found as precursors of lignification. In the course of our investigation of biochemical reactions leading to lignification in plants, we tested a number of hydroxycinnamic acids, their esters and the corresponding cinnamyl alcohols for their usefulness as substrates for the sensitive detection of peroxidase isoenzymes in PAGE.

EXPERIMENTAL

Hydroxycinnamic acids and their corresponding esters were prepared according to Pearl and Beyer[17]. Hydroxycinnamyl alcohols were obtained by reduction of the cinnamic acid esters with lithium aluminium hydride according to the method of Freudenberg and Hübner[18]. Eugenol and horseradish peroxidase were purchased from Sigma.

An apparatus for vertical gel electrophoresis was used as described earlier[19]. The compositions of the gels and buffers were the same as in our work on the extraction of peroxidase isoenzymes from woody tissues[5]. A volume of 5 – 50 μl of the peroxidase extract, depending on its activity, was used for an electrophoretic run. After electrophoresis, the gels were washed in running water for 1 h before being incubated with the different substrates.

For staining of the zones of enzymic activities with hydroxycinnamic acid esters and cinnamyl alcohols, the gels were immersed in 200 ml of a 0.1 *M* acetate buffer (pH

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= 5,0) saturated with substrate and to which 3 ml of 3% hydrogen peroxide was added. The gels were incubated overnight. For better dissolution of the substrate it was found useful to dissolve it in a few drops of acetone before pouring it into the buffer. Zones of peroxidase activity appeared from one to several hours as white opaque zones caused by oxidative polymerization of the phenolic substrates. The time after which the first bands of precipitated polymer could be detected depended on the activity of the enzymes and the kind of substrate.

To compare the specific reactivities of several hydroxycinnamic acid esters and alcohols with peroxidase isoenzymes from wood, an equimolar solution (0.5 m*M*) of ferulic acid ethyl and methyl esters, isoferulic acid methyl ester, *p*-coumaric acid methyl ester, coniferyl alcohol, *p*-coumaryl alcohol and eugenol was prepared. All these gels were incubated for exactly 12 h.

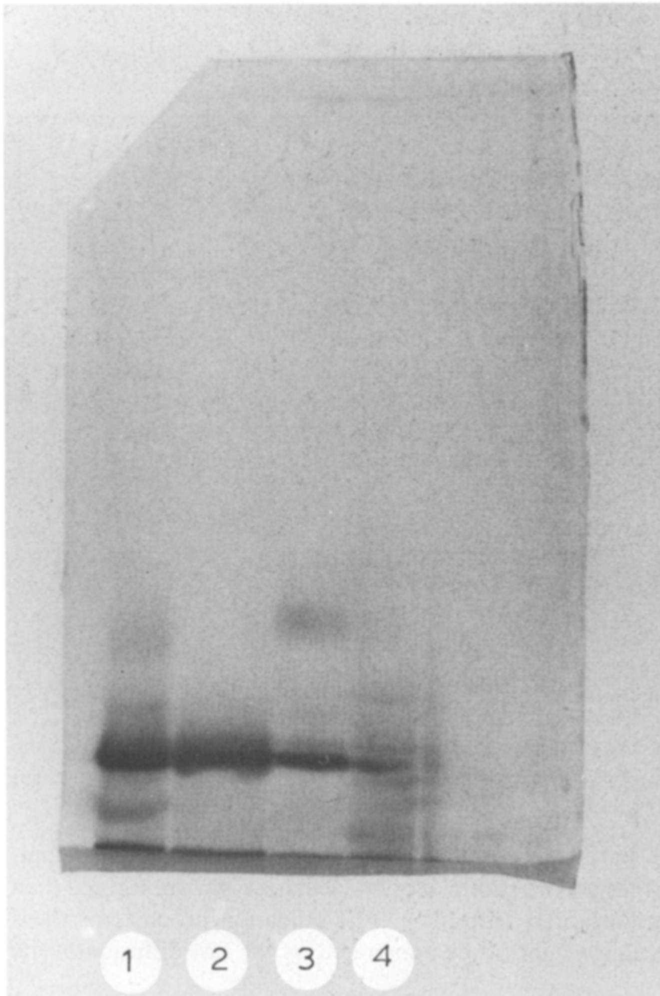

Fig. 1. Peroxidase isoenzymes from different trees stained with benzidine and hydrogen peroxide[20]. 1 = *Aesculus hippocastanum*; 2 = *Acer platanoides*; 3 = *Quercus sp.*; 4 = *Larix europaea*.

RESULTS

Figs 1–4 show the staining results for peroxidase activity with different substrates (benzidine, *o*-dianisidine, eugenol and ferulic acid ethyl ester, respectively).

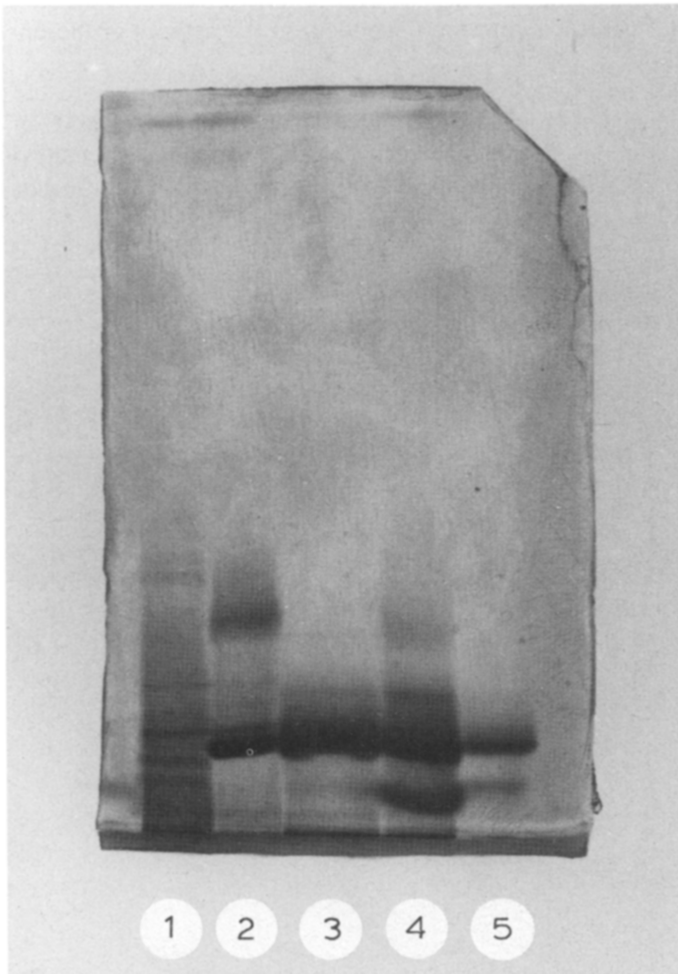

Fig. 2. Peroxidase isoenzymes from different trees stained with *o*-dianisidine and hydrogen peroxide[21]. 1 = *Larix europaea*; 2 = *Quercus sp.*; 3 = *Acer platanoides;* 4 = *Aesculus hippocastanum* (50 μl); 5 = *Aesculus hippocastanum* (10 μl).

The most and sharpest bands were obtained with ferulic acid ethyl ester stain. One reason for this might be that polymers of higher molecular weight are formed than during the oxidation of benzidine or *o*-dianisidine. The oxidation products of these substrates are of lower molecular weight, as demonstrated by the fast diffusion of the dyes formed in the gel.

Differences can be seen in the numbers of bands visible and also in the intensities of the zones. Particularly large differences exist between the eugenol and the ferulic

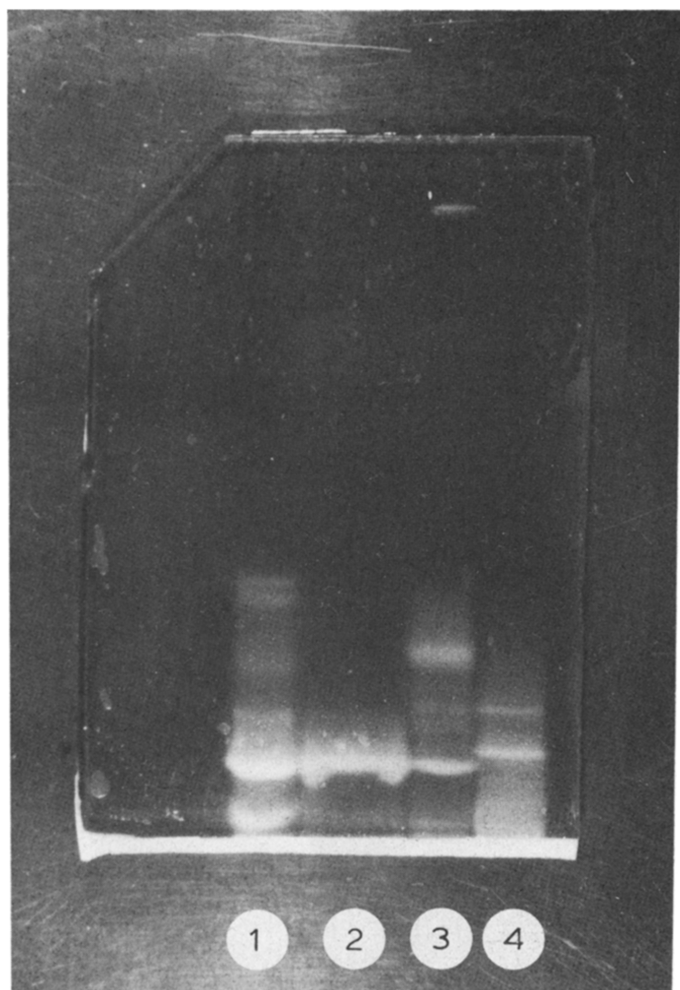

Fig. 3. Peroxidase isoenzymes from different trees stained with eugenol and hydrogen peroxide. 1 = *Aesculus hippocastanum*; 2 = *Acer platanoides;* 3 = *Quercus sp.;* 4 = *Larix europaea.*

acid ethyl ester stains, demonstrating the varying specifity of peroxidases towards different substrates, although some of the isoenzymes react with each of the substrates used. Liu and Gibson[13] reported similar results for the reactions of eugenol and benzidine with peroxidases.

A surprising fact is that isoferulic acid methyl ester yields oxidative polymerization products with peroxidases, similar to those obtained by oxidation of the compounds having a free hydroxyl group in the *para*-position. Free hydroxycinnamic acids such as ferulic acid did not furnish any higher polymers, as could be concluded from the fact that no precipitation zones could be detected on the gel.

As can be seen from Fig. 4, ferulic acid ethyl ester is the most active of the substrates investigated in terms of the number of the zones revealed, the intensity of the zones and the rate at which the precipitating oxidation products are produced, and

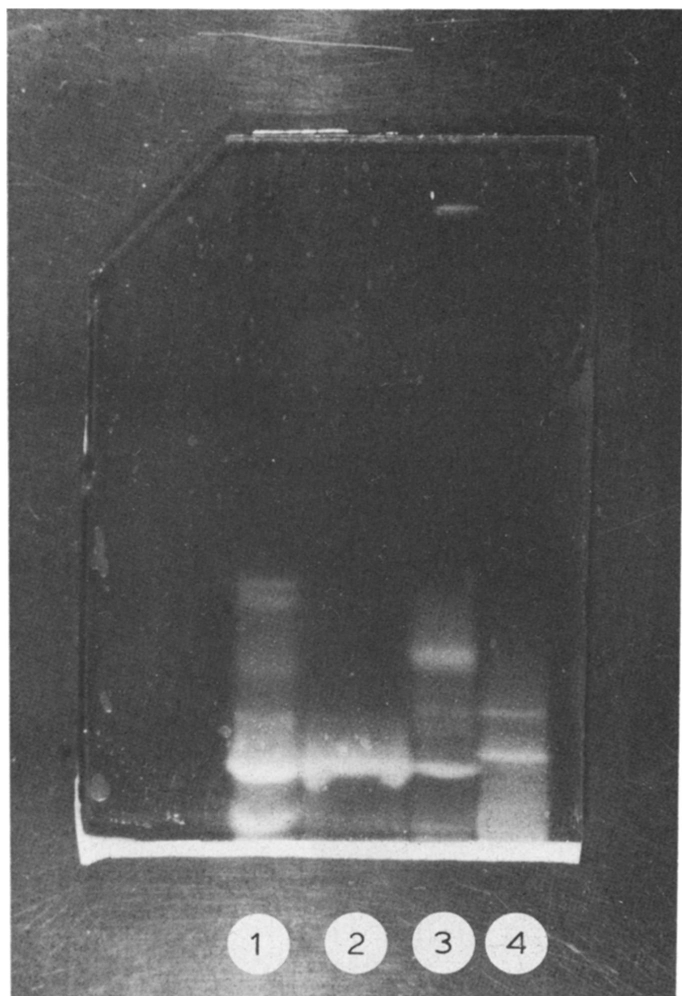

Fig. 4. Peroxidase isoenzymes from different trees stained with ferulic acid ethyl ester and hydrogen peroxide. 1 = *Aesculus hippocastanum;* 2 = *Acer platanoides;* 3 = *Quercus sp.;* 4 = *Larix europaea.*

it reacts the most sensitively with peroxidases. As a substrate for revealing zones of peroxidase activity it has the advantage of allowing highly sensitive detection of enzymic activity, producing non-diffusing, stable, white precipitation zones. It can easily be prepared by esterification of ferulic acid with ethanol and it is not toxic.

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