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Note

Electrophoretic detection of peroxidase isoenzymes in polyacrylamide gels using hydrogen peroxide generated by rose bengal–glutathione in the presence of light

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Peroxidase (E.C. 1.11.1.7) is an important enzyme occurring mainly in plants. Usually a large number of isoenzymes can be detected in plants. Peroxidase influences many metabolic processes, such as phenol polymerization[1–4], H_2O_2 detoxification[5], indolyl-3-acetic acid oxidation[6,7] and ethylene production[8,9].

Peroxidase is usually detected by adding the substrate H_2O_2 to the incubation solution, which has to contain convenient reaction partners such as benzidine, *o*-dianisidine, ethyl ferulate or eugenol. In the course of studies on oxidative polymerization in plants, we found that the dye rose bengal[10], known mainly as a singlet oxygen producer, is also able to produce the H_2O_2 necessary for peroxidase detection. H_2O_2 formation is increased in the presence of light and an electron donor such as glutathione.

EXPERIMENTAL

Chemicals

Rose bengal, *o*-dianisidine, *p*-coumaric acid, glutathione and catalase were purchased from Sigma (Munich, F.R.G.). All the other chemicals were of analytical-reagent grade (Merck, Darmstadt, F.R.G.).

Plant material and preparation of extracts

Wood splinters from chestnut (*Aesculus hippocastanum*) and juice pressed from potatoes (*Solanum tuberosum*) were used. The extraction of peroxidase isoenzymes from chestnut has been described earlier[4].

Polyacrylamide gel electrophoresis

Electrophoresis was performed as reported previously[11]. A volume of 100 μl of the crude plant extract was used for an electrophoretic run. After electrophoresis the gels were washed in running water for 1 h and subsequently for 20 min in distilled water.

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TABLE I

COMPOSITION OF THE INCUBATION MIXTURES

Component	*Fig. 1*	*Fig. 2*	*Fig. 3*	*Fig. 4*
Rose bengal (m*M*)	0.07	0.07	–	0.07
Glutathione (m*M*)	0.5	–	–	0.5
p-Coumaric acid (m*M*)	0.1	0.1	0.1	0.1
o-Dianisidine (m*M*)	0.2	0.2	0.2	0.2
Catalase (U per 200 ml)	–	–	–	200
3% H_2O_2 (ml)	–	–	3	–
pH	7.6	7.6	6.0	7.6

Incubation solution

The substances listed in Table I were dissolved in 200 ml of phosphate buffer.

The gels were incubated for peroxidase activity in the oxygen-saturated solutions for 6 h. During the incubation period, solutions were irradiated with light from a 100-W bulb, except the control solution (Fig. 3). After incubation the excess of dye can be easily removed by washing the gel with water. For comparison, the detection of peroxidase isoenzymes was carried out in the usual way according to ref. 12.

RESULTS

Figs. 1–3 show the staining results for isoenzymes with peroxidase activity. In Figs. 1 and 2 different methods of production of H_2O_2 with rose bengal (with and

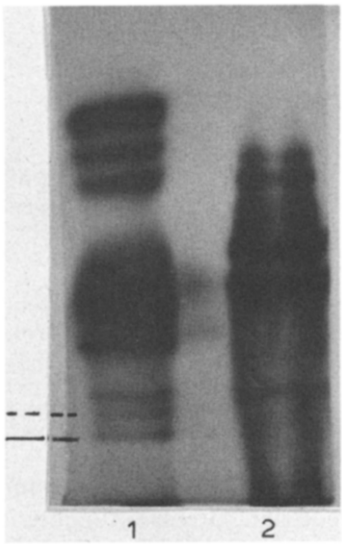

Fig. 1. Peroxidase isoenzymes from ***Aesculus hippocastanum*** **(1) and** ***Solanum tuberosum*** **(2) stained with** *o*-dianisidine. The H_2O_2 was produced by the system rose bengal–glutathione in the presence of light. The isoenzyme marked with a dotted line could be made visible only by incubation with rose bengal. The other, marked with a full line, could be detected by incubation with DOPA also.

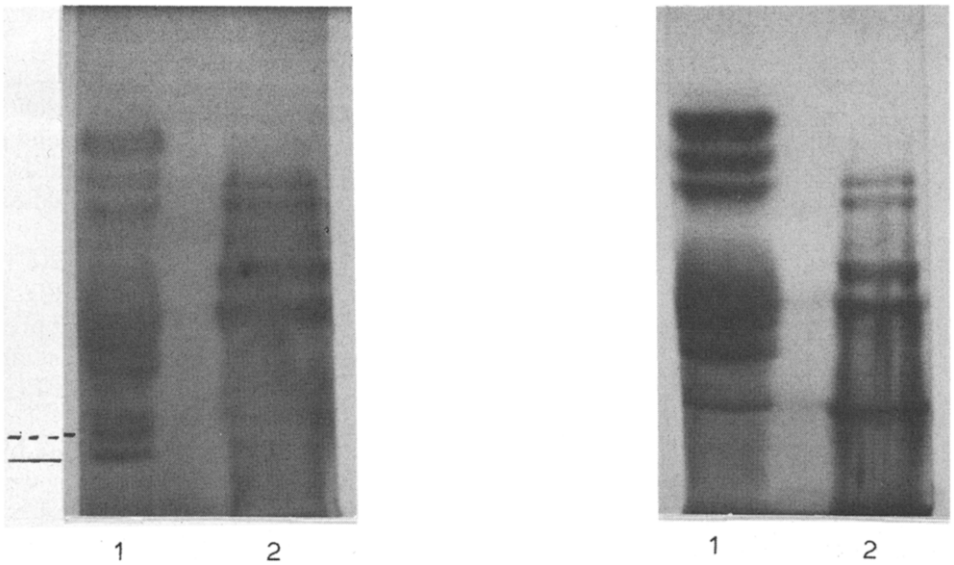

Fig. 2. **Peroxidase** isoenzymes from Aesculus **hippocastanum** (1) and *Solanum tuberosum* (2) stained with *o*-dianisidine. The H_2O_2 was produced by rose bengal in the presence of light. The isoenzyme marked with a dotted line could be made visible only by incubation with rose bengal. The other, marked with a full line, could be detected by incubation with DOPA also.

Fig. 3. Peroxidase isoenzymes from *Aesculus hippocastanum* (1) and *Solanum tuberosum* (2) stained with *o*-dianisidine and H_2O_2.

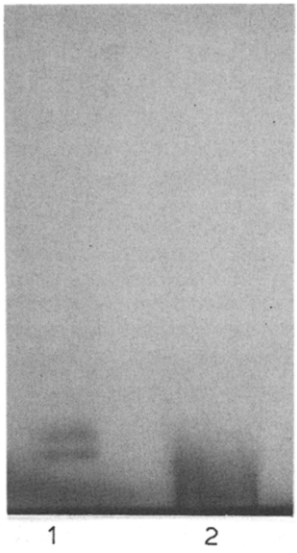

Fig. 4. Peroxidase isoenzymes **from** *Aèsculus hippocastanum* (1) and *Solanum tuberosum* (2) incubated with *o*-dianisidine, rose bengal, glutathione and catalase.

without addition of glutathione) were used. In Fig. 3 the development of the enzymatic activity in the ordinary way (addition of H_2O_2)[12] is shown for comparison.

By comparing Figs. 1–3, it can be seen that the patterns of isoenzymatic activities are identical over a wide range, although two additional electrophoretic zones appeared (marked in Figs. 1 and 2) that were not detectable with the other staining procedures (Fig. 3). Staining with rose bengal in the presence of light also furnishes the same electrophoretic zones as the direct addition of H_2O_2 to the incubation solution.

Evidence for the participation of H_2O_2 in the reactions described above is derived from the fact that the enzymatic activities were inhibited by catalase. Fig. 4 shows that all of the isoenzymes detectable by the ordinary peroxidase staining procedure were completely inhibited by the addition of 200 units of catalase per 200 ml.

In contrast, the two zones marked in Figs. 1 and 2 were not inhibited by catalase. H_2O_2 therefore cannot be considered as the substrate of these oxidases. The more slowly migrating of these bands could be identified as polyphenol oxidase by staining with DOPA[13] (not shown). The specificity of the other oxidase is not known.

Similar results were obtained when benzidine or ethyl ferulate was used instead of *o*-dianisidine.

The method described offers the possibility of the detection of peroxidase isoenzymes in polyacrylamide gels without the addition of H_2O_2.

REFERENCES

1 W. Nakamura, *J. Biochem.*, 62 (1967) 54.
2 Y. Nozu, *J. Biochem.*, 62 (1967) 519.
3 J. M. Harkin and J. R. Obst, *Science*, 180 (1973) 296.
4 R. Ebermann and K. Stich, *Phytochemistry*, 21 (1982) 2401.
5 Y. Nakano and K. Asada, *Plant Cell Physiol.*, 21 (1980) 1295.
6 M. C. Hoyle, *Plant Physiol.*, 60 (1977) 787.
7 O. P. Srivastava and R. B. van Huystee, *Phytochemistry*, 16 (1977) 1527.
8 R. L. Legge, *Phytochemistry*, 22 (1983) 2161.
9 H. S. Ku and S. F. Yang, *Plant Physiol.*, 45 (1970) 358.
10 V. S. Srinivasan, *J. Am. Chem. Soc.*, 100 (1978) 6513.
11 R. Ebermann and H. Bodenseher, *Experientia*, 24 (1968) 523.
12 *Worthington Enzyme Manual*, Worthington Biochemical Corp., Freehold, NY, 1972, pp. 43–45.
13 L. C. Van Loon, *Phytochemistry*, 10 (1971) 503.