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MAIZE ROOT PEROXIDASES: RELATIONSHIP WITH POLYPHENOL OXIDASES

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Key Word Index—*Zea mays*; Gramineae; maize; aromatic amines; catalase; inhibitors; isoperoxidases; phenols; polyphenol oxidases; root.

Abstract—Maize root peroxidases (POD) may also have polyphenol oxidase (PPO) activity as shown by using 3-amino-9-ethylcarbazole or DOPA as hydrogen donor to detect isoenzymes after disc gel electrophoresis. Copper chelators inhibited POD activity, and since PODs are haemoproteins, it can be concluded that copper chelators are not entirely specific for Cu enzymes. This raises the question whether PPO are only Cu enzymes. In POD preparations contaminated by catalase, POD activity could be over-estimated; this could be due to the auto-oxidation of the hydrogen donor or to stimulation of PPO activity by oxygen, as demonstrated with DOPA, dopamine and gallic acid. No correlation was found between the chemical nature of the substrate and the type of peroxidatic or oxidatic oxidation.

INTRODUCTION

Peroxidases (donor: hydrogen peroxide oxidoreductase, EC 1.11.1.7) are characterized by their ability to oxidize many different hydrogen donors in the presence of hydrogen peroxide. This ability may change according to the origin of the plant extract. These enzymes which have been shown to include a large number of isoenzymes [1, 2] were also able to oxidize some phenolic hydrogen donors in the absence of hydrogen peroxide. A relationship between peroxidases (POD) and polyphenol oxidases (PPO) has also been demonstrated [3, 4] although this has been disputed [5]. The aim of the present work was to analyse the specificity of maize root isoperoxidases and their relationship with polyphenol oxidases.

RESULTS AND DISCUSSION

Two substrates were tested: 3-amino-9-ethylcarbazole (AC) for the peroxidases [6] and DL-3,4-dihydroxyphenyl alanine (DOPA) for polyphenol oxidases [7]. When using polyacrylamide disc gel electrophoresis, twelve isoperoxidases were found (Fig. 1A), the gels being stained with AC. With DOPA as hydrogen donor only nine coloured bands were obtained (Fig. 1B), with the same electrophoretic mobility as isoperoxidases stained with AC. The similarity of the electrophoretic mobility was also obtained by cationic disc gel electrophoresis [8]. This was also found for the five isoperoxidases present in purified horseradish peroxidases (HRP, Sigma type II [8]) and for three of the four covalently wall bound isoperoxidases from maize root extracts (Fig. 2). This confirms previous data about cell walls of *Riella helicophylla* [9] which indicate that oxidation in walls could occur without hydrogen peroxide. The absence of stain for isoperoxidases 1, 5 and 7 with DOPA and the different relative activity for other isoperoxidases using AC, could be attributed to different affinities of the isoenzyme for DOPA as compared with other hydrogen donors [4].

In the presence of hydrogen peroxide and DOPA, a significant band appeared (Fig. 1C). This corresponds to the auto-oxidation of DOPA and to the activation of PPO by the oxygen produced by catalase localized in this zone in the gel (Fig. 1D), and is not the result of peroxidatic or oxidatic activity of catalase. The evidence was obtained: (a) using 3-amino-1,2,4-triazole (AT), a potent inhibitor of catalase [10] without effect on POD

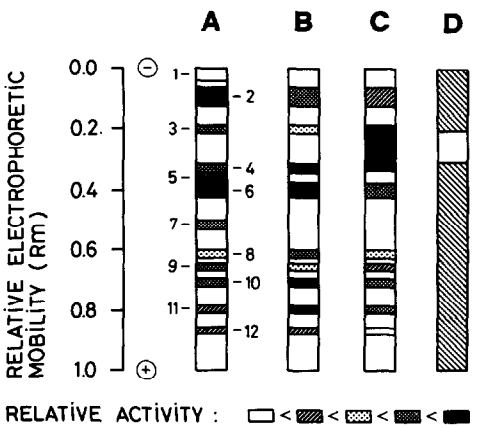

Fig. 1. Diagram showing relative activities for cytoplasmic isoperoxidases stained with 3-amino-9-ethylcarbazole and hydrogen peroxide (A); 3,4-dihydroxyphenylalanine without (B) and with (C) hydrogen peroxide. For catalase staining (D), soluble starch was incorporated during preparation of the gels. Isoenzymes were separated by anionic disc electrophoresis. The relative activity scale does not apply to gel D.

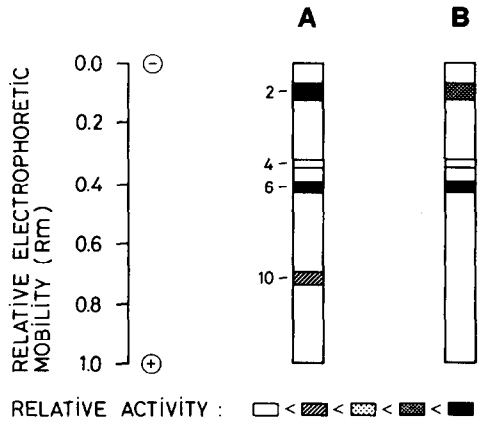

Fig. 2. Diagram showing relative activities of covalent wall-bound isoperoxidases stained with 3-amino-9-ethylcarbazole (A) and DOPA (B).

and PPO activity (Table 1) or on POD and PPO isoenzyme staining [8]; (b) after incorporation of AT at 0.01 mM in the staining medium containing hydrogen peroxide, catalase was not revealed and the highly coloured band did not appear in the gels stained with DOPA and hydrogen peroxide. This indicates that interference with catalases could occur in the presence of some hydrogen donors (DOPA and other phenolic substrates: Table 2) with hydrogen peroxide. Consequently, measurements of peroxidase activity with phenolic hydrogen donors have been done in the presence of AT to prevent such artefacts during the estimation of enzyme activity. This interference may cause many disparities reported about the nature of the oxidation (peroxidatic or oxidatic) of some phenolic substrates by plant extracts.

The lower activity of DOPA oxidase in the presence of hydrogen peroxide (Fig. 1C) is a consequence of the inhibitory effect of hydrogen peroxide at high concentration as demonstrated with other hydrogen donors such as syringaldazine [2]. As in other plant extracts [4, 11], maize root PPO activity is due to POD. This observation questions the copper nature of some PPO, since POD are clearly haemoproteins [4]. Only tyrosinases [12] and laccases [13] are clearly copper in nature. Maize root extracts are unable to oxidize tyrosine (Table 2) so they do not contain tyrosinase. No laccases were present in these extracts, because no coloured bands appeared in the gel in the absence of hydrogen peroxide with typical substrates for laccases as *p*-phenylenediamine, guaiacol and syringaldazine [14, 15]. In the absence of tyrosinase and laccase, the copper nature of PPO could be questioned in many other plants. The copper nature or the participation of copper in the PPO reaction was often only supported by the inhibitory action of copper chelators [16–22]. Such copper chelators (nos 5–8) were found not only to reduce PPO activity, but also the POD activity as metal (nos 1–3) or specific Fe (no 4) chelators (Table 1). Consequently (no 6) and other copper chelators were not specific for PPO, in contradiction to data previously published [23, 24]. Consequently, if the absence of tyrosinase or

Table 1. Relative variation (in % relative to the control) of the peroxidase (POD) and polyphenol oxidase (PPO) activities measured in the presence of different types of effectors

Effectors*	POD	PPO
(1) EDTA	+7	−36
(2) 2,3-Dimercaptopropanol (BAL)	−97	−74
(3) 4,5-Dihydroxy-1,3-benzenedisulphonic acid	−82	−17
(4) 2,2'-Dipyridyl	−17	+2
(5) Phenylthiocarbamide	−68	−6
(6) Sodium diethyldithiocarbamate	−42	−16
(7) Salicylaldoxime	−37	−8
(8) Thioglycolic acid	−76	−62
(9) 3-Amino-1,2,4-triazole	−2	0

*All effectors were used at 1 mM except no 6 (0.1 mM) and no 9 (10 mM).

Estimation of POD with guaiacol and hydrogen peroxide (pH 6; 30°), and of PPO with DOPA and without hydrogen peroxide (pH 7; 40°). In controls, activities corresponding to the relative absorbances changes (470 or 450 nm) were respectively 0.08 ± 0.006/min/mg fr. wt for POD and 0.0011 ± 0.0008/min/mg fr. wt for PPO.

Table 2. Substrates used with or without hydrogen peroxide to stain POD or PPO activities of maize root isoenzymes after electrophoretic separation by anionic disc gel electrophoresis: coloured bands appeared (+) or did not appear (−) in the gel after staining

Substrates	$+H_2O_2$	$-H_2O_2$
(10) *o*-Phenylenediamine	+	−
(11) *p*-Phenylenediamine	+	−
(12) Benzidine	+	−
(13) 3-Amino-9-ethylcarbazole	+	−
(14) 4'-Amino-2',5'-diethoxybenzanilide	+	−
(15) Protocatechuic acid	−	−
(16) Gentisic acid	−	−
(17) Gallic acid	+	+
(18) Vanillic acid	−	−
(19) Syringic acid	−	−
(20) Catechol	+	+
(21) Guaiacol	+	−
(22) *p*-Methoxyphenol	+	−
(23) Hydroquinone	+	+
(24) Pyrogallol	+	−
(25) Phloroglucinol	+	−
(26) Tyrosine	−	−
(27) *p*-Coumaric acid	−	−
(28) Ferulic acid	+	−
(29) Sinapic acid	+	−
(30) Caffeic acid	+	−
(31) Chlorogenic acid	+	−
(32) DOPA	+	+
(33) Dopamine	+	+
(34) Umbelliferone	−	−
(35) Eugenol	+	−
(36) Vanilline	+	−
(37) Syringaldehyde	+	−
(38) Syringaldazine	+	−

laccase was not really shown, the copper nature of some PPO could be doubtful.

The nature of the hydrogen donor relating to the mode of oxidation is uncertain. Many compounds were tested (Table 2) (after anionic disc gel electrophoresis) with or without hydrogen peroxide. They are aromatic amines (nos 10–14), benzoic acids (nos 15–19), phenols (nos 20–26), cinnamic acids (nos 27–31), catechol amines (nos 32–33), coumarins (no 34) and others (nos 35–38). Three kinds of reactions are observed. The first does not produce coloured bands in the presence or in the absence of hydrogen peroxide (nos 15, 16, 18, 19, 27, 34), but they can be oxidized without production of coloured bands, as previously observed [25] with *p*-coumaric acid (no 27). Such compounds are not of interest for this study using zymogram analyses. The second only produced coloured bands in the presence of hydrogen peroxide (nos 10–14, 21, 22, 24, 25, 28–31, 35–38); they are typical substrates for POD in maize roots. In the last type, coloured bands were obtained with or without hydrogen peroxide. Catechol (no 20) and hydroquinone (no 23) having an increasing colouration with increasing hydrogen peroxide concentration, are not able to distinguish POD and PPO activity. With gallic acid (no 17) and dopamine (no 33), the same results as with DOPA (typical PPO substrates) were obtained; they produced interference with catalase, as discussed before. Consequently, from the present data, no correlation between the chemical nature of the hydrogen donor and the type of oxidation (peroxidatic or oxidatic) can be found.

EXPERIMENTAL

Plant material. Maize roots (*Zea mays* L. cv. LG 5: Association Suisse des Sélectionneurs, Lausanne) were grown as previously described [26]. After 48 hr germination (dark; 19°), primary roots (15 ± 2 mm) were selected. Apical segments (10 mm) were prepared and immediately frozen in liquid N_2.

Enzyme extraction. Root segments were ground in liquid N_2, homogenized in a NaPi buffer (Sorensen 0.066 M, pH 6) and centrifuged (18 000 *g*, 20 min, 4°). The supernatant was the crude extract of cytoplasmic peroxidases. Covalent wall-bound peroxidases were extracted using cell-wall hydrolysing enzymes (cellulase BDH 0.4% and Driesalase Kyowa Hakko 0.1% in acetate buffer pH 4) as previously described [27].

Peroxidase assays. Activity was determined colorimetrically [27] using guaiacol as hydrogen donor in 3,3-dimethylglutaric buffer (pH 6).

DOPA oxidase assays. The colorimetric method was adapted from ref. [3]. Extracts were incubated at 40° with 1 ml DOPA (12.5 mM) in Sorensen buffer, pH 7. The *A* change at 450 nm, which increased linearly, was estimated after 20 min.

Inhibitors were dissolved in *N,N'*-dimethylformamide (DMF) and used at 1 mM in the medium except 0.1 mM for Na diethyldithiocarbamate (DIECA) and 10 mM for 3-amino-1,2,4-triazole (AT).

Isoenzyme separation. Anionic polyacrylamide disc gel electrophoresis [28] was used to separate isoenzymes in extracts made in 20% sucrose. The electrophoresis was carried out in Tris–Gly buffer at pH 8.5. Bromophenol blue 0.005% was used as tracking dye.

Isoenzyme staining. Aromatic amines and phenols were used as hydrogen donors.

Aromatic amines. o- and *p*-phenylenediamine were dissolved in DMF and used at 0.05% in a NaPi buffer (pH 6); H_2O_2 was at 0.03%. Other aromatic amines were also used: benzidine [29], 3-amino-9-ethylcarbazole [6], Fast Blue BB [30].

Phenolic. Gels were immersed in the dark in NaPi buffer (pH 6) containing the phenolic at 0.25% dissolved in DMF; DOPA was used at 0.5%, and H_2O_2 (0.03% final concn) was added. Guaiacol was prepared according to ref. [27], syringaldazine as in ref. [2]. For catalase investigation, soluble starch at 0.125% was incorporated in the gels during their preparation, and the activity resolved after refs [31, 32].

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