

Substrate Specificity of Peroxidase Isoenzymes for Hydrogen Donors

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Abstract. The investigation of the substrate specificity of the anionic peroxidase isoenzymes, isolated from the zone of differentiation of the primary roots of *Zea mays*, for some representatives of phenolic compounds and aromatic amines, as hydrogen donors, is reported. The investigation was carried out electrophoretically with peroxidase isoenzymes partially purified by a combination of gel filtration by Sephadex G-25 and Sephadex G-100. A difference in the substrate specificity of the individual isoenzymes is observed. It was established that the anionic peroxidase isoenzymes showed a similarity in total number and relative activity on staining with bivalent phenols and difference on staining with trivalent phenols, as hydrogen donors. A greater number of isoenzymes was stained with benzidine and *o*-dianisidine and a lesser number with *o*- and *p*-phenylendiamine. The substrate specificity of the peroxidase isoenzymes was compared for guaiacol and benzidine. The substrate specificity of peroxidase isoenzymes was discussed as regards their diverse role in the plant metabolism.

The multiple molecular forms of the enzymes, catalyzing one and the same chemical reaction, could differ significantly between themselves by a number of physico-chemical properties: molecular weight, pH and temperature optimum, substrate specificity. The investigations show that the multiple molecular forms of the peroxidase — the isoenzymes differ as regards their preferences to diverse hydrogen donors (FARKAS and STAHMANN 1966, MACKO and NOVACKÝ 1966, MARKLUND *et al.* 1974). It is assumed that these specific substrates and other characteristics of the isoenzymes of the peroxidase can explain the polyfunctionality and the flexibility in the behaviour of the enzyme. Our aim in the investigation is to compare the substrate specificity of the anionic peroxidase isoenzymes for several hydrogen donors, representatives of the phenolic compounds and the aromatic amine group, which are the most frequently used in investigations with peroxidase.

MATERIAL AND METHODS

We carried out the investigations with enzyme extract from the zone of differentiation of a primary root of 64-h seedlings of *Zea mays* cv. Kn-2L-611. The seeds were germinated on moist filter rolls in the dark, in a growth

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TABLE 1

 R_m values of the anionic isoenzymes of the peroxidase with phenol compounds as hydrogen sources

Iso-enzymes	Guaiacol 		Catechol 		Pyrogallol 		Phloroglucinol 			
	S_d	t	S_d	t	S_d	t	S_d	t	S_d	t
1	0.0797	0.0740	0.0097	$t_8 = 0.058$	0.0830	0.0091	$t_8 = 0.91$	0.0465		
2	0.1543	0.1705	0.0109	$t_6 = 1.47$	0.1698	0.0077	$t_9 = 2.00$	0.1636	0.0096	$t_7 = 0.96$
3	0.2435	0.2840	0.0051	$t_4^{**} = 7.90$	-	-	-	0.2445	0.0014	$t_4 = 0.71$
4	0.4217	0.4480	0.0240	$t_3 = 1.05$	0.4440	0.0109	$t_7 = 2.00$	-	-	-
5	-	-	-	-	0.6590	-	-	-	-	-
6	0.7100	0.7100	0.0158	$t_5 = 0.00$	-	-	-	0.6993	0.0114	$t_4 = 0.93$

** - significant at probability about 0.1

chamber at 25 °C. All procedures of the extraction and the partial enzyme purification through gel filtration by Sephadex G-25 and G-100, are described in another publication of ours, (DENCHEVA and KLISURSKA 1979 and DENCHEVA et al., in print).

The separation of peroxidase isoenzymes was accomplished by disc electrophoresis on polyacrylamide gel, by the method of Davis (*cf.* MAURER 1971). The electrophoresis was carried out for 1.30 h at 2 mA per tube. The staining of the isoenzymes was achieved by means of colour reactions with the following hydrogen donors, at suitable conditions: phenolic compounds — guaiacol, catechol, pyrogallol and phloroglucinol in a concentration 0.08 M and final concentration of H_2O_2 0.05%, pH 6.0 of the incubation medium; aromatic amines — benzidine, *o*-dianizidine, *o*-tolidine in a concentration of 0.004 M and final concentration of H_2O_2 0.003%, pH 5.0 of the incubation medium; *o*-phenyldiamine, *p*-phenyldiamine in a concentration of 0.5% and final concentration of H_2O_2 0.03%, pH 6.0 of the incubation medium. The conditions for resolution and staining of the isoenzymes, the amounts of protein applied and the substrate concentrations were chosen after preliminary assays with a view to staining the greatest possible number of isoenzymes, well differentiated in the gel. The stained isoenzyme patterns were recorded densitometrically. We judged of the differences and the similarities in the substrate specificity of the individual isoenzymes for the different hydrogen donors by their number and values of the relative electrophoretic mobility (R_m). The data for R_m were processed statistically by Student-Fisher method (*cf.* ROKITSKIĬ 1967).

RESULTS

Densitometric scans of the anionic isoenzymes of the peroxidase stained with phenolic compounds as hydrogen donors (Fig. 1) show the similarity in the total number and the relative activity of the individual isoenzymes with guaiacol (A) and catechol (B) and the difference in their activity with pyrogallol (C) and phloroglucinol (D). Five isoenzymes are stained with guaiacol and catechol. The similarity between them is confirmed by the statistical analysis of R_m data (Table 1), which show only isoenzyme-3 to differ. Four isoenzymes are stained with pyrogallol and phloroglucinol. Comparing the isoenzyme spectra stained with pyrogallol and phloroglucinol and guaiacol, we can see that isoenzyme-3 does not stain with pyrogallol and

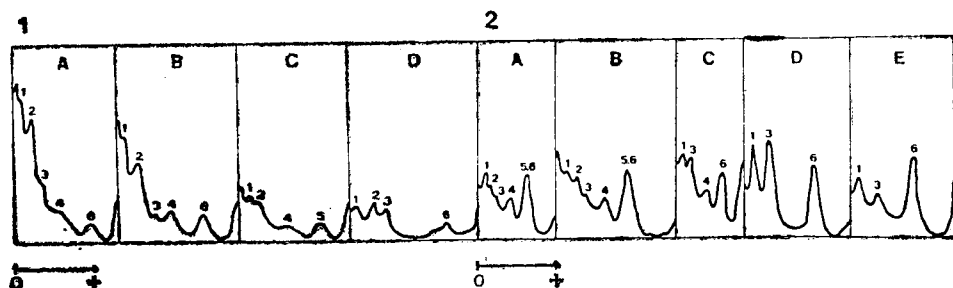


Fig. 1. Densitometric scans of the anionic isoenzymes of the peroxidase, stained with phenolic compounds as hydrogen donors: A — guaiacol; B — catechol; C — pyrogallol; D — phloroglucinol.

Fig. 2. Densitometric scans of the anionic peroxidase isoenzymes stained with aromatic amines as hydrogen donors: A — benzidine; B — *o*-dianisidine; C — *o*-tolidine; D — *o*-phenylenediamine; E — *p*-phenylenediamine.

isoenzyme-4 does not stain with phloroglucinol. Besides this, isoenzyme-1 stained with phloroglucinol is not identical in R_m with the corresponding isoenzymes stained with the other phenolic compounds; the same applied to isoenzyme-5 stained with pyrogallol. All t -values in Table 1 are calculated with respect to guaiacol. An intensive oxidation of the pyrogallol takes place and at the same time a fast diffusion of the colour compounds formed begins in the incubation medium, on account of which the intensity of the bands on the gels decreases, if not recorded in an optimal time.

If we compare the isoenzyme spectra stained with the bivalent phenols — guaiacol and catechol with those stained with the trivalent phenols — pyrogallol and phloroglucinol, we notice that the peroxidase activity is disturbed in a different way in the different groups of isoenzymes. With the bivalent phenols as hydrogen donors, the more active appear to be the isoenzymes moving slowly towards the anode, whilst with the trivalent phenols, the activity of individual isoenzymes is distributed comparatively more uniformly. Densitometric scans of the anionic isoenzymes of the peroxidase with aromatic amines as hydrogen donors (Fig. 2) show the difference in the total number and the relative activity of the individual isoenzymes with the investigated compounds. Most isoenzymes (6) are stained with benzidine (A) and *o*-dianisidine (B); the least (3) with *o*-phenylenediamine (D) and *p*-phenylenediamine (E); 4 isoenzymes are stained with *o*-tolidine (C). The isoenzymes which stain with all used hydrogen donors, are not in all cases identical in R_m values (Table 2). All t -values in Table 2 are calculated in respect to the benzidine. A comparison can be made for the relative activity of the isoenzymes stained with one and the same hydrogen donor. In this case, one may notice that the isoenzymes moving fast to the anode, are the most active at staining with benzidine, *o*-dianisidine and *p*-phenylenediamine; on the contrary, in staining with *o*-tolidine and *p*-phenylenediamine, those moving slowly to the anode are more highly active.

From the presented densitometric scans, no quantitative assessment can be made of the relative activity of the isoenzymes stained with different hydrogen donors, as a different quantity of protein had been applied (from

TABLE 2
 R_m values of the anionic isoenzymes of peroxidase with aromatic amines as hydrogen source

Iso-enzymes	Benzidine 	<i>O</i> -di-anisidine 	<i>O</i> -tolidine 	<i>O</i> -phenylene-diamine 	<i>P</i> -phenylene-diamine 	S_d	t	S_d	t	
1	0.0202	0.0997	0.0054	$t_{1r}^* = 3.56$	$t_{1r} = 0.51$	0.0850	-	-	0.0843	$t_{13} = 0.91$
2	0.1423	0.1630	0.0036	$t_{12} = 5.64$	-	-	-	-	-	-
3	0.2170	0.2073	0.0109	$t_8 = 0.84$	$t_6^{***} = 2.81$	0.1823	0.0127	0.2170	0.2652	$t_7^{**} = 4.00$
4	0.4160	0.4208	0.0101	$t_7 = 0.27$	$t_1 = 1.11$	0.4330	0.0134	-	-	-
5	0.5480	0.5412	0.0098	$t_{10} = 0.69$	-	-	-	-	-	-
6	0.6330	0.6412	0.0094	$t_5 = 0.97$	$t_3 = 0.78$	0.6400	0.0089	0.6195	$t_{10}^{***} = 2.64$	0.5587 $t_{12} = 4.60$

* - significant at probability 0.001

** - significant at probability 0.01

*** - significant at probability 0.1

5 to 20 μg with benzidine, *o*-dianisidine, *o*-tolidine, *o*- and *p*-phenylenediamine, catechol and guaiacol and 100 μg with phloroglucinol per gel), so that the fullest possible spectrum of the isoenzyme composition should be obtained.

DISCUSSION

KAY *et al.* (1967) consider that in order to understand the physiological significance of the peroxidase isoenzymes, the nature and the degree of heterogeneity between the individual forms of the enzyme should be explained. In view of this, all information concerning the similarities and the differences in the physical and catalytic properties of the isoenzymes are of essential importance. It was well known that the activity of the peroxidase isoenzymes depends to a great extent on the nature of the hydrogen donor (MACKO and NOVÁČKÝ 1966) and that the substrate specificity of this enzyme varies considerably among the individual isoenzymes (EVANS and ALLDRIDGE 1965).

The present investigation treats the question of the substrate specificity of the anionic peroxidase isoenzymes for phenolic compounds and aromatic amines as hydrogen donors. The chosen representatives differ structurally in the number, type and position of the substituents in the phenyl ring. These structural differences between the substrates used afford the possibility of substrate preference of the peroxidase isoenzymes if such differences exist *in vivo*.

When phenolic compounds were used as hydrogen donors, we ascertained that those isoenzymes moving slowly towards the anode catalyze the oxidation of all investigated substrates. This is probably due to the low substrate specificity. The isoenzymes that move at medium rate and fast towards the anode show greater differences in the substrate specificity. For example: isoenzyme-3 does not stain with pyrogallol, and isoenzyme-4 with phloroglucinol. There is evidence that phloroglucinol is a preferred substrate in reactions of oxidase oxidation with the participation of the peroxidase, on account of which it should be admitted that in our assays, where conditions were created for a peroxidase oxidation of the substrate, some of the isoenzymes would not be active. In contrast to PRUIDZE *et al.* (1976), we ascertained that the peroxidase oxidation of the bivalent phenols (guaiacol, catechol) is more intensive than that of the trivalent ones (pyrogallol, phloroglucinol). The anionic peroxidase isoenzymes manifest substrate preferences towards the aromatic amines as hydrogen donors, too. For example: isoenzymes-2 and -5 stain with benzidine and *o*-dianisidine and do not stain with the other investigated aromatic amines; isoenzyme-4 stains with benzidine, *o*-dianisidine and *o*-tolidine, and does not stain with *o*- and *p*-phenylenediamine. Therefore, this isoenzyme appears substrately specific for compounds which contain biphenyl radical in the molecule. The results obtained clearly show that the type or the position of the substituents in the phenyl ring or the size of the hydrogen donor molecule, influence the rate of the reaction in which the peroxidase isoenzymes participate. Contrary to phenolic compounds, the three groups of isoenzymes moving slowly at medium rate and fast towards the anode show substrate specificity for the aromatic amines as hydrogen donors.

In a series of investigations of this type, a comparison is made between the substrate specificity of the peroxidase isoenzymes for aromatic amines and phenolic compounds (MACKO and NOVACKÝ 1966, GORDON and HENDERSON 1973, MARKLUND *et al.* 1974) and especially for the guaiacol and the benzidine, as traditional hydrogen donors.

The isoenzymes moving fast towards the anode are highly active when stained with benzidine (Fig. 2A) and slightly active when stained with guaiacol (Fig. 1A). We have the opposite result with the slowly moving isoenzymes, which are comparatively more highly active when stained with guaiacol. These data are in agreement with those of GORDON and HENDERSON (1973), *i.e.* that guaiacol is a more effective hydrogen donor for staining the slowly moving isoenzymes, in comparison with benzidine.

With the guaiacol and also with the rest of the investigated phenolic compounds, but not with benzidine, is stained the isoenzyme, fast moving towards the anode, with R_m 0.6993 to 0.7100. Consequently, this isoenzyme is substrately specific for phenolic compounds. It is very possible that the presence of this isoenzyme is associated with the intensified metabolism of the phenols in the zone of differentiation of the cells. This assumption is also based on the data of RUBIN and LUSHINSKAYA (1969), who found that the two peroxidase isoenzymes synthesized *de novo* as a result of an infection, take part in the oxidation of pyrogallol but not in that of benzidine. It is known that in infection, the metabolism of the phenols is activated. In such a case, it may be admitted that the high substrate specificity of some isoenzymes for a definite type of compound, ensures the realization of a new metabolic function of the enzyme.

It is possible that the differences in the substrate specificity would express the different biological function of the peroxidase isoenzymes. In other words, with the availability of multiple substrates for the individual peroxidase isoenzymes, the many diverse metabolic functions of the peroxidase in higher plants may be explained.

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