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Comparison of Some Kinetic Parameters of Peroxidase and IAA Oxidase in the Course of Growth and Differentiation of Plant Cells

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Abstract. An attempt is made to characterize the functional activity of the protein molecule possessing both peroxidase and IAA oxidase activity by comparing the kinetic parameters for the two types of enzyme activity with regard to the following substrates: H_2O_2, benzidine, guaiacol and IAA. The curves expressing the dependence of the enzyme reaction velocity on the concentration of the enzyme or the substrate are different depending on the enzyme extract origin and the type of the substrate. It is established that the K_m of peroxidase for IAA decreases while its K_m for H_2O_2 increases during cell development. Both types of enzyme activity show similar pH and temperature dependence. The presented data show that IAA oxidase activity of the peroxidase develops as extension and differentiation of the root cells proceed. This is one of the possible mechanisms through which peroxidase may participate in the regulation of growth and differentiation of the primary root cells of maize (*Zea mays* L.)

In our previous investigations (DENCHEVA and KLISURSKA 1982, KLISURSKA and DENCHEVA 1983) we presented evidence that the peroxidase molecules also possess IAA oxidase activity. This conclusion was based on the similarities of ion-exchange properties and electrophoretic mobilities of isoenzymes with peroxidase and IAA oxidase activity. But these similarities are not a sufficient proof of peroxidase and IAA oxidase being two activities of one protein molecule. To elucidate the relationship between peroxidase and IAA oxidase it is also necessary to compare their functional activity and specificity. The characterization of the specific behaviour of the two types of enzyme activity will bring us nearer to elucidating the regulation mechanisms which control the peroxidase — IAA oxidase interrelations.

There is a number of data on peroxidase and IAA oxidase in many plants (GASPAR *et al.* 1982) but few comparative data on the kinetic parameters of both types of enzyme activities (RAY 1960, RAY 1962, SEQUEIRA and MINEO 1966, SANO 1970, SANO and NAGAO 1970). Data also are lacking which characterize the changes of the kinetic parameters of the two types of enzyme activity during the course of plant cell growth and differentiation. The aim of the present investigation is therefore to characterize and to compare some

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Abbreviations used: IAA = indol-3-ylacetic acid; DCP = 2,4-dichlorophenol; *A* = absorbance.

kinetic parameters of these enzyme activities during the extension and differentiation of primary root cells of maize in relation to the following substrates: H_2O_2, benzidine, guaiacol and IAA.

MATERIAL AND METHODS

Investigations were carried out with partially purified enzyme extracts from the meristematic (0—2.0 mm), elongating (2.0—5.0 mm) and differentiating (10.0—25.0 mm) zones of primary roots of 64-h-old maize seedlings. Extraction of the enzyme and its partial purification by gel filtration on Sephadex G-25 were described previously (DENCHEVA and KLISURSKA 1979).

Peroxidase activity assay was performed with two hydrogen donors — benzidine by the method of BOYARKIN (1951) and guaiacol by the method of MAEHLY and CHANCE (1954). IAA oxidase activity was determined according to GORDON and WEBER (1951), in the presence of 0.1 mM 2,4-dichlorophenol (DCP) and 0.1 mM Mn^{2+}-ions as co-factors. The activities were expressed as ΔA min^{-1} ml^{-1} of enzyme extract at 600 and 420 nm for peroxidase and as μg indol-3-ylacetic acid (IAA) destroyed min^{-1} ml^{-1} for IAA oxidase. All measurements of peroxidase activity were carried out at 20 °C and of IAA oxidase activity at 37 °C.

The thermostability assays were carried out by assaying enzyme activity as described after heat-treatment of the samples of enzyme extracts at 50, 75 or 100 °C for 30 min in a water bath.

The values given in the table and figures are averages from three experiments. Standard error was less than 1%. More details about each estimation are given in the explanation of figures.

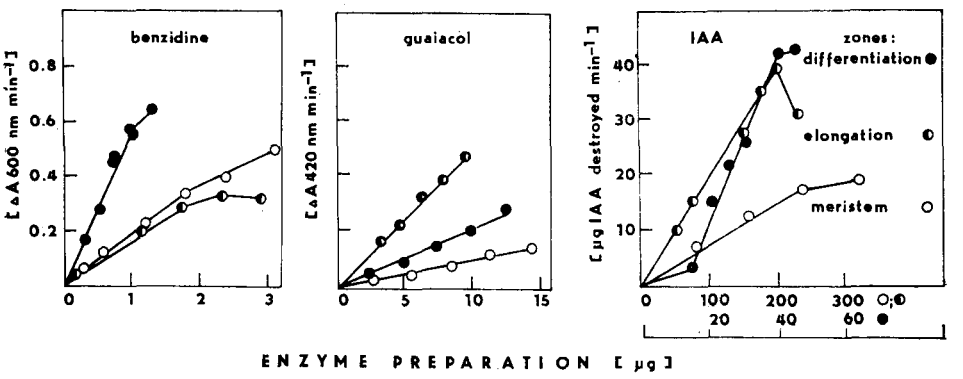

Fig. 1. Effect of enzyme concentration on peroxidase and IAA oxidase reaction velocity. Reaction mixtures for:
benzidine-peroxidase assay — 0—3.0 μg enzyme, 6.0 μM benzidine, 40.0 μM H_2O_2, 0.1 M acetate buffer pH 4.7, for differentiating zone 0—1.5 μg enzyme was used;
guaiacol-peroxidase assay — 0—15.0 μg enzyme, 10.0 μM guaiacol, 40.0 μM H_2O_2, 0.066 M phosphate buffer pH 6.0;
IAA oxidase assay — 0—300.0 μg enzyme for meristematic and elongating zones, 0—50.0 μg for differentiating zone, 100.0 μM IAA, 100.0 μM DCP, 100.0 μM $MnCl_2$, 0.1 M acetate buffer pH 5.0.

RESULTS

Initial experiments were aimed at determining the limits between which peroxidase and IAA oxidase reaction velocities were linearly related to enzyme concentration. It can be seen from the data presented in Fig. 1 that the range of linearity depended upon the type of the substrate as well

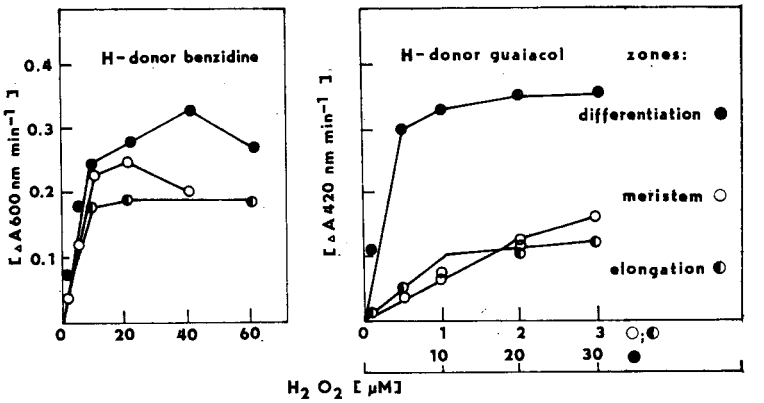

Fig. 2. Effect of H_2O_2 concentration on the peroxidase reaction velocity.
Reaction mixtures for: —
benzidine-peroxidase assay — 1.0 μg enzyme, 6.0 μM benzidine, 0—100.0 μM H_2O_2, 0.1 M acetate buffer pH 4.7;
guaiacol-peroxidase assay — 10.0 μg enzyme, 10.0 μM guaiacol, 0—100.0 μM H_2O_2, 0.066 M phosphate buffer pH 6.0.

as the enzyme origin. Only with guaiacol as H-donor the linearity of the curves was kept with higher concentrations of enzyme preparation over the range from 0 to 15 μg. All further investigations are carried out at enzyme concentrations in linear parts of the curves.

Fig. 2 shows the data for the dependence of peroxidase reaction velocity on the H_2O_2 concentration studied with two H-donors: benzidine and guaiacol. The curves resemble rectangular hyperbolae for both substrates and enzyme extracts from all three zones of the primary root with the possible exception of the meristematic zone with guaiacol as the H-donor. With benzidine as H-donor, the range of linearity of the curves was the same (0 to 10.0 μM) for each of the three root zones. With guaiacol as H-donor, however, the range of linearity was from 0 to 2.0 μM H_2O_2 for the meristematic zone, from 0 to 1.0 μM H_2O_2 for the elongating zone and from 0 to 5.0 μM H_2O_2 for the differentiating zone.

The dependence of peroxidase reaction velocity on the H-donor concentration and of IAA oxidase on the IAA concentration is given in Fig. 3. The peroxidase activity showed linearity from 0 to 1.0 μM with benzidine as H-donor with enzyme extracts from all three root zones. The linear parts of the curves showing the effect of IAA concentration on IAA oxidase activity for the enzyme isolated from the three root zones were also localized within the same limits of concentration from 0 to 0.35 μM IAA. However

differences between the activities of enzymes of different origin at similar substrate concentrations were observed.

The kinetic curves for peroxidase activity were different for guaiacol as the H-donor compared with those for benzidine. In this case, however, no appreciable differences could have been observed between the enzyme reaction

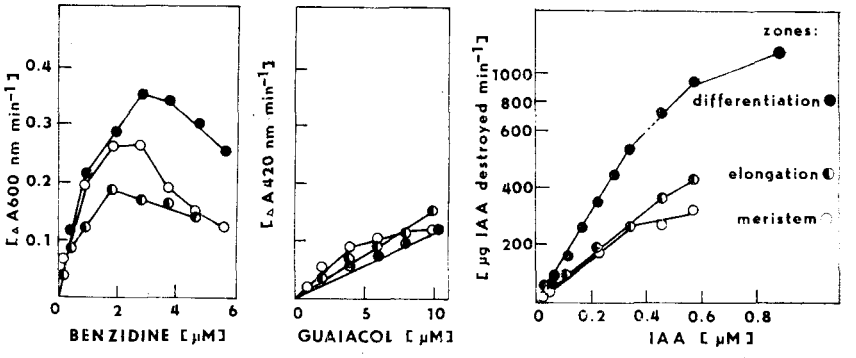

Fig. 3. Effect of H-donor concentration on the peroxidase reaction velocity and of IAA on the IAA oxidase reaction velocity.
Benzidine-peroxidase assay — 1.0 μg enzyme, 5.0 μM H_2O_2, 0—6.0 μM benzidine, 0.1 M acetate buffer pH 4.7. For differentiating zone 10.0 μM H_2O_2 were used;
guaiacol-peroxidase assay — 10.0 μg enzyme, guaiacol 0—100.0 μM, 0.066 phosphate buffer pH 6.0 and 0.2 μM H_2O_2 for meristematic zone, 0.5 μM H_2O_2 for elongating zone and 10.0 μM H_2O_2 for differentiating zone.
IAA oxidase assay — 100.0 μg enzyme, 0—6.0 μM IAA for meristematic and elongating zones, 25.0 μg enzyme, 0.8 μM IAA for differentiating zone, 0.1 M acetate buffer pH 5.0.

velocities at the same concentration with enzyme extracts from the three root zones.

Table 1 summarizes the values of Michaelis constants (K_m) for peroxidase and IAA oxidase determined according to the method of LINEWEAVER and BURK (1934) with H_2O_2, benzidine, guaiacol and IAA as substrates. The K_m data show that there were considerably greater differences between the enzyme's affinity for IAA depending on the enzyme origin than between its affinity for H_2O_2, benzidine or guaiacol. For example, K_m-values for H-donors were similar for the three root zones, whereas the enzyme's affinity for IAA increases 830times in the differentiating zone compared with that of the meristematic zone.

Fig. 4 shows pH-optima of peroxidase and IAA oxidase isolated from the three root zones. There are greater differences between the substrates tested than between the three root zones. The pH-optimum for benzidine is between pH 4.5 and pH 5.5, while for guaiacol the values are between pH 5.5 and pH 7.0. The pH-optima of IAA-oxidase coincide with those of guaiacol-peroxidase activity.

The thermostability data for enzyme activities with the different substrates are given in Fig. 5. The highest degree of inhibition at 50 °C was observed for IAA oxidase activity in the meristematic zone of the root. This temperature did not decrease the activity of IAA oxidase or benzidine-

TABLE 1

K_m values for peroxidase isolated from the three root zones for the substrates — H_2O_2, benzidine, guaiacol and IAA

Root zone	K_m [μM]				
	H_2O_2		benzidine	guaiacol	IAA
	benzidine as H-donor	guaiacol			
Meristematic	10.0	0.15	1.17	12.5	310.0
Elongating	5.5	0.26	0.40	11.0	1.4
Differentiating	3.8	3.50	1.42	40.0	0.37

Reaction mixtures:

1) for K_m (H_2O_2) determination —
with H-donor benzidine: 1.0 μg enzyme, 6 μM benzidine, 0—15.0 μM H_2O_2, 0.1 M acetate buffer pH 5.0;
with H-donor guaiacol: 10.0 μg enzyme, 10.0 μM guaiacol, 0—60.0 μM H_2O_2 for meristematic and elongating zones and 0—100.0 μM H_2O_2 for differentiating zone, 0.066 M phosphate buffer pH 6.0;
2) for K_m (benzidine) —
1.0 μg enzyme, 40.0 μM H_2O_2, 0—6.0 μM benzidine, 0.1 M acetate buffer pH 4.5 for meristematic and elongating zones and pH 5.0 for the differentiating zone;
3) for K_m (guaiacol) —
10.0 μg enzyme, 40.0 μM H_2O_2, 0—100.0 μM guaiacol, 0.066 M phosphate buffer pH 6.0;
4) for K_m (IAA) —
100.0 μg enzyme for meristematic and elongating zones and 25.0 μg for differentiating zone, 0—0.8 μM IAA for meristematic and elongating zone and 0—8.0 μM IAA for differentiating zone, 0.1 M acetate buffer pH 5.0.

-peroxidase from the differentiating zone, and it even slightly stimulated them. At 75 C and 100 °C activities were completely inhibited for enzyme extracts from the meristematic and elongating zones for all substrates tested. Residual enzyme activities of about 25% for IAA oxidase and about 40% for benzidine-peroxidase were observed in the extracts from the differentiating zone treated at 75 °C.

DISCUSSION

The data for the dependence of peroxidase and IAA oxidase reaction velocity on the enzyme concentration show that irrespective of enzyme origin, the benzidine and guaiacol oxidations are more active than IAA oxidation (Fig. 1).

The curves for benzidine and IAA oxidation deviated from the theoretical linear course with higher enzyme concentrations. According to DIXON and WEBB (1959) the deviation of the experimental curves from the "straight" line can be explained in several ways: disadvantage of the method used, presence of inhibitors, *etc.* A non-enzymic binding of benzidine with hemoproteins (HAIS and MACEK 1959) probably explains the deviations at higher enzyme concentrations that we have observed with our partially purified

extract for the peroxidase assay. In the case of IAA oxidation we consider that the most probable reason is the presence of high molecular weight--inhibitors (such as auxin protectors) which co-eluate with enzyme protein (STONIER and YONEDA 1967) or some protein-protein interactions in the partially purified extracts (RICARD *et al.* 1977).

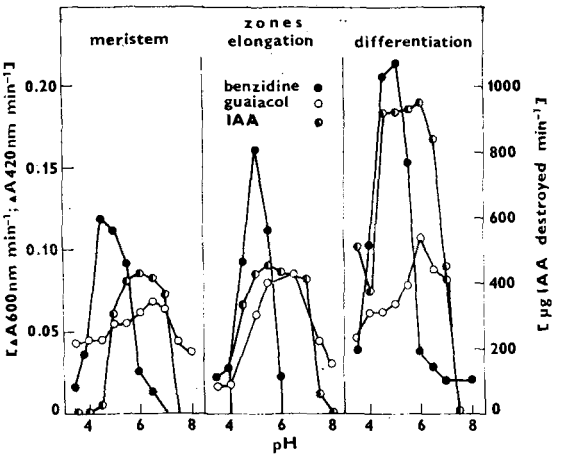

Fig. 4. Effect of pH on peroxidase and IAA oxidase reaction velocity.

Benzidine-peroxidase assay — 1.0 μg enzyme, 0.5 μM benzidine for meristematic and elongating zones, 1.0 μM benzidine for differentiating zone, 5.0 μM H_2O_2 for meristematic and elongating zones and 10.0 μM H_2O_2 for differentiating zone, 0.1 M acetate buffer pH 3.5—5.5 or 0.066 phosphate buffer pH 5.0—8.0;
guaiacol-peroxidase assay — 10.0 μg enzyme, 3.0 μM guaiacol, 0.1 M acetate buffer pH 3.5—5.5 or 0.066 M phosphate buffer pH 5.0—8.0, 0.2 μM H_2O_2 for meristematic zone, 0.5 μM H_2O_2 for elongating zone and 10.0 μM H_2O_2 for differentiating zone.
IAA-oxidase assay — 100.0 μg enzyme 0.3 μM IAA for meristematic and elongating zones and 25.0 μg enzyme for differentiating zone, 0.1 M acetate buffer pH 3.5—5.5 or 0.066 M phosphate buffer pH 5.0—8.0.

Unusual kinetic curves were observed at low concentrations of enzyme preparation (from 0 to 15.0 μg) for IAA oxidase in the differentiating zone. Activity appeared to be inhibited in this range. According to DIXON and WEBB (1959) this may be due to the presence of small amounts of some highly toxic impurity in one of the components of the reaction mixture other than the enzyme extract itself. It is possible in this case that the effect is due to the excess of the Mn^{2+}-ions (GALSTON and BAKER 1951).

By comparing the velocities of oxidation of a single H-donor by enzyme extracts from the meristematic, elongating and differentiating zones of the root cells, it can be seen that higher specific enzyme activities are developed as cell elongation and differentiation proceed (see Figs. 1—3) with the exception of benzidine oxidation in the elongating zone. IAA oxidase activity increases many times more than the peroxidase activity during cell growth and differentiation. The impression arises that in meristematic cells the basic function of the enzyme is peroxidative oxidation of substrates. With progressive cell development, the oxidase function of the enzyme increases and is fully revealed in the differentiated cells.

Differences can also be observed in the peroxidase functions depending on the enzyme origin. Thus, the data in Fig. 1 show that the highest activity with guaiacol was revealed with enzyme extracts from the elongating zone. It is possible that this high activity reflects the enzyme's role in the oxidation of phenolics during cell wall formation.

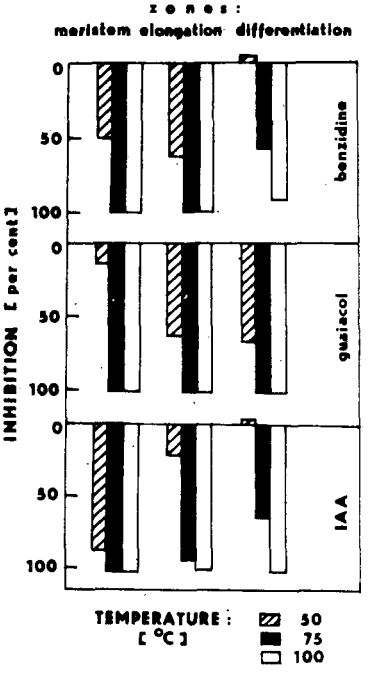

Fig. 5. Effect of heat treatment on peroxidase and IAA-oxidase reaction velocity.
Benzidine-peroxidase assay — 1.0 μg enzyme, 0.5 μM benzidine, 5.0 μM H_2O_2 for meristematic and elongating zones and 1.0 μM benzidine, 10.0 μM H_2O_2 for differentiating zone, 0.1 M acetate buffer pH 4.7 for meristematic zone and pH 5.0 for elongating and differentiating zones;
guaiacol-peroxidase assay — 10.0 μg enzyme, 3.0 μM guaiacol, 0.066 M phosphate buffer pH 6.0, 0.2 μM H_2O_2 for meristematic zone, 0.5 μM H_2O_2 for elongating zone and 10.0 μM H_2O_2 for differentiating zone.
IAA oxidase assay — 100.0 μg enzyme for meristematic and elongating zone and 25.0 μg enzyme for differentiating zone, 0.3 μM IAA, 0.1 M acetate buffer pH 5.0.

The data showing the dependence of peroxidase and IAA oxidase reaction velocities on the concentration of H_2O_2, H-donors and IAA show that the velocity is dependent upon the type of substrate and only in a few cases it depends on the enzyme origin. The enzyme isolated from the elongating zone revealed the highest sensitivity to H_2O_2 concentrations, whereas the least sensitivity was observed for the enzyme isolated from the differentiating zone (Fig. 2). Differences also exist with respect to enzyme sensitivity to guaiacol concentrations. The most sensitive in this case was the enzyme from the meristematic zone (Fig. 3).

K_m-values varied widely, depending upon enzyme origin. With progressive cell development the enzyme affinity for H_2O_2 decreased while its affinity for IAA increased. Thus, with guaiacol as H-donor the affinity of the enzyme from the differentiated cells for H_2O_2 decreases 23times and its affinity for IAA increases 830times compared with that of the meristematic zone enzyme. We suppose that this is due to the allosteric effect of supra-optimal concentrations of IAA (ELKINAWY and RAA 1973) on the activity of some basic peroxidase isoenzymes in this root zone. The electrophoretic data indicating that a greater number of cationic peroxidase isoenzymes begin to function as IAA oxidases in the course of cell development support this view (DENCHEVA and KLISURSKA 1982).

It must be emphasized in passing that all investigations reported in this work were carried out with H-donors which do not occur naturally. This must be taken into account when these conclusions are applied to living cells.

The high thermostability of peroxidase is well known (KRUGER and LABERGE 1974). The present investigation indicates that the degree of this thermostability depends on the type of H-donor used for the enzyme assay and on the cell development stage. The most thermostable is the enzyme isolated from the differentiating zone. Our data show, however, a similar behaviour of both types of enzyme activity under the various heat treatments, contrary to the statement of FRIČ (1974), who reported that IAA oxidase is more thermolabile than peroxidase.

Summarizing all the present data we may conclude that they support our previous results, which showed that both activities — peroxidase and IAA oxidase are connected with the same protein molecule (DENCHEVA and KLISURSKA 1982, KLISURSKA and DENCHEVA 1983). The data showing similar behaviour of both types of enzyme activity as affected by increasing enzyme concentration and during the course of cell development, the coincidence of pH-optima for guaiacol-peroxidase and IAA oxidase and the similarity of benzidine-peroxidase and IAA oxidase thermostability may be considered as further proofs of our previous conclusions. However, at the same time both types of enzyme activity reveal a specificity in their behaviour. In particular, the degree of the enhancement of both types of enzyme activity during the course of cell development is different. Differences also exist with respect to enzyme sensitivity to increasing H_2O_2, H-donor and IAA concentrations.

It is interesting to note that, with respect to the dependence on pH and heat treatment, there are greater differences between benzidine-peroxidase and guaiacol-peroxidase than between peroxidase and IAA oxidase activities. This suggests that peroxidase and IAA oxidase reactions are realized by the same protein molecule but at different active sites. Most likely, the oxidation of benzidine and guaiacol is carried out at different active sites and the active site responsible for benzidine oxidation is localized close to or coincides with the active site responsible for IAA oxidation. A similar assumption regarding the presence of two active sites in the peroxidase molecule has been made by SIEGEL and GALSTON (1967) and SRIVASTAVA and VAN HUYSTEE (1977). The data presented thus support and help to explain the polyfunctionality of the peroxidase enzyme.

Acknowledgements

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