SUBSTRATE SPECIFICITY OF PLANT PEROXIDASES

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1. Introduction

Plant peroxidases (EC 1.11.1.7; donor: H_2O_2 oxidoreductase) occur as a number of components, which can be separated electrophoretically. They have been isolated in pure form from a few plant species [1]. The isolation of four peroxidase-active hemoproteins (I, III, V and VI) with widely different amino acid compositions has recently been described from the root of horseradish [2]. The term "isoperoxidases" is hardly applicable to such divergent enzymes, I.P. 4.0 –> 11. The reason for this multitude of enzymes with similar functions, at least *in vitro*, may be questioned. Genetic experiments cannot be performed since the plant is sterile and bred in clones. A conversion of one peroxidase into another during the isolation, i.e. an artifact, might occur. The amino acid compositions excluded the interconversions of I, III and V whereas VI could possibly derive from III. The peroxidases might also be localized in different parts of the root, on a gross anatomical or subcellular level. Such a morphological distribution would be likely to parallel a functional specificity, expressed as a substrate preference.

The oxidase activity of cabbage peroxidase [3] towards phloroglucinol appears in basic and neutral solutions and also in acid solutions in the presence of manganous ions, a powerful activator of this reaction.

Guaiacol has previously been found to react faster with peroxidase III than with I at pH 7 [2, 4]. This observation has now been extended to studies of the reaction rates for some other substrates with the above peroxidases.

2. Materials and methods

Peroxidases Ib, IIIb and VI* with I.P. 4.0, 8.8, and > 11 were isolated as described [2] and gave RZ 4.1, 3.5 and 2.0. Peroxidase V was poorly reproducible in some batches and will not be considered in the present report. Concentrations were assayed from the Coret band absorbances.

Solutions of the substrates (hydrogen donors, AH_2) were made up in quartz distilled water immediately before use. All buffers were 10 mM, phosphate being used above and acetate below pH 6.

The enzyme activities, all at 25°, were expressed in two ways. The ratios between the Soret band absorptions of the free enzymes and their complex II (H_2O_2 9–44 μM, ascorbate 200 μM) were found to be essentially the same for the peroxidases I and

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* Named I, III and VI in this paper.

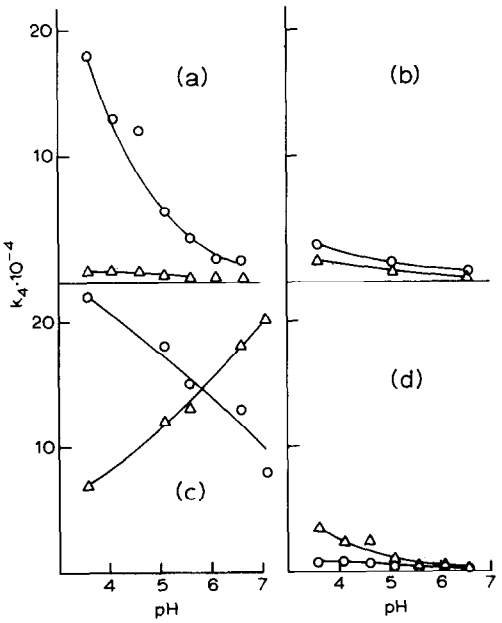

Fig. 1. Effect of pH upon k_4 for peroxidases I (○) and III (△) with (a) ascorbic acid (0.25 – 3.0 μM), (b) uric acid (1.6 – 6.4 μM), (c) guaiacol (0.1 – 1.0 μM), and (d) mesidine (4.9 – 19.6 μM). H_2O_2 0.20 μM, peroxidases 0.17 – 0.86 μM.

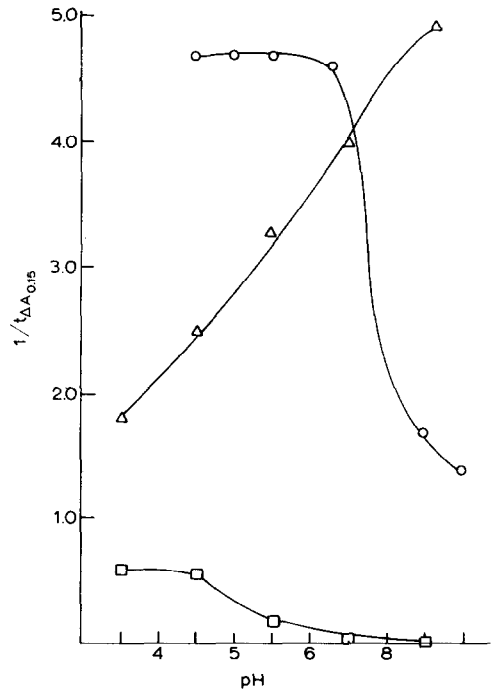

Fig. 2. Relative reaction rates for peroxidases I (○), III (△), and VI (□) with coniferyl alcohol (0.2 μM). All enzyme concentrations 0.01 μM. H_2O_2 0.4 μM

and III both at pH 7.0 and 4.7. The rate constant k_4 for the reaction between peroxidase–peroxide complex II and the hydrogen donor (AH_2) was determined according to Chance [5] by means of a Beckman DB spectrophotometer, connected to a linear Servogor RE 511 recorder (full deflection 200 mm, paper speed 30 mm per min). At the actual peroxidase concentrations in the cuvettes the changes in transmission at 427 nm caused pen deflections of 2.5 – 15 mm. The mole fraction of peroxidase, present as complex II, was calculated from the observed absorbance changes upon the addition of enzyme to (buffer + donor) and the subsequent addition of hydrogen peroxide; the ratio between the molar absorbances for complex II and free peroxidase was given by Chance as 2.7 at pH 7.0 [6] and with two techniques, 2.2 and 3.0 at pH 4.7 [7]. We found 2.6 – 2.8 at pH 7.0 for peroxidase I and III and at pH 4.5 for peroxidase III. The reaction with peroxidase I at pH 4.5 was too fast for our equipment, and the ratio 2.7 was used. The "k_4 method" does not register changes in substrate or product concentrations but only shifts between (free enzyme + complex I) and complex II. The presumption is made that k_1 is not rate-limiting for either peroxidase.

This technique was unsuitable with benzidine and phloroglucinol that yielded intensely coloured products. With coniferyl alcohol well-shaped complex II plateaus could not be obtained. In these cases, and with the other donors for the sake of comparison, the relative velocities were expressed as $1/t_{0.10}$ or $1/t_{0.15}$, where t stands for the time required for an absorbance shift of 0.10 or 0.15 in either direction at a suitable wave-length (ascorbate 268, coniferyl alcohol 262, mesidine 490, guaiacol 470, leuco malachite green 550, phloroglucinol 340, and benzidine 620 nm; the absorbances of ascorbate, urate, and coniferol decreased during the reaction). Conditions were chosen so as

Table 1
Relative rates for the reactions of peroxidases I and III with three hydrogen donors. k_4-values from fig. 1.

Donor (AH_2) μM	pH	H_2O_2 μM	HRP μM		$1/t_{0.10}$ min^{-1}		Ratio I:III from	
							$1/t$	k_4
			I	III	I	III		
Ascorbate								
0.11	4.7	2.0	0.20	2.0	6.7	4.0	17	20
Mesidine								
5.3	4.9	2.0	0.09	0.03	1.1	1.7	0.2	0.4
Guaiacol								
1.2	3.6	0.4	0.01	0.01	2.5	1.0	2.5	3.1
	7.1	0.4	0.01	0.01	1.0	2.5	0.4	0.4

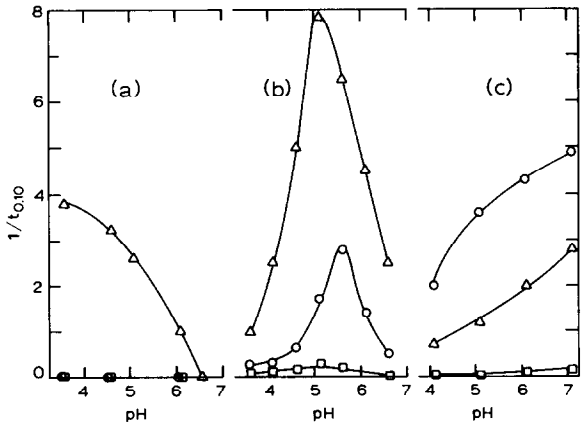

Fig. 3. Relative reaction rates for peroxidases I (○), III (△), and VI (□) with (a) leuco malachite green, (b) benzidine, and (c) phloroglucinol. The rates for the different substrates with a given enzyme are not comparable.

	μM		
	(a)	(b)	(c)
Peroxidase	0.065	0.005	.0.007
H_2O_2	0.20	0.40	0.40
H-donor	0.15	0.58	2.0

to give convenient absorbances and linear relations between enzyme concentration and $1/t$. Corrections were made for non-enzymatic oxidations. This procedure follows a decrease in the hydrogen donor (ascorbate, coniferol) or an increase in late appearing derivatives of the primary product. Hence it permits comparisons of the overall reaction velocities of the three peroxidases with the same substrate but not of the reaction rates of an enzyme with various substrates.

3. Results and discussion

Ascorbic acid and uric acid react more rapidly with complex II of peroxidase I than with complex II of peroxidase III, whereas the base mesidine shows the reversed ratio (fig. 1). The reaction rates with all three substrates for both peroxidases are enhanced by increasing acidity. The rate of reaction with guaiacol (fig. 1) increases for peroxidase I and decreases for peroxidase III when pH is lowered; at pH 7 the ratio between the k_4-values with guaiacol is close to the ratio between the previously reported overall velocities with this substrate [2, 4].

Coniferyl alcohol gives relative reaction rates with essentially the same pH-dependence as guaiacol (fig. 2). The good parallelism between the results from the two methods (table 1) suggests that k_4 also for the reactions between both peroxidases and coniferyl alcohol and guaiacol varies with pH in the same way. Coniferyl alcohol never produced a brown colour, but occasionally a white precipitate was observed.

The differences between the two peroxidases are

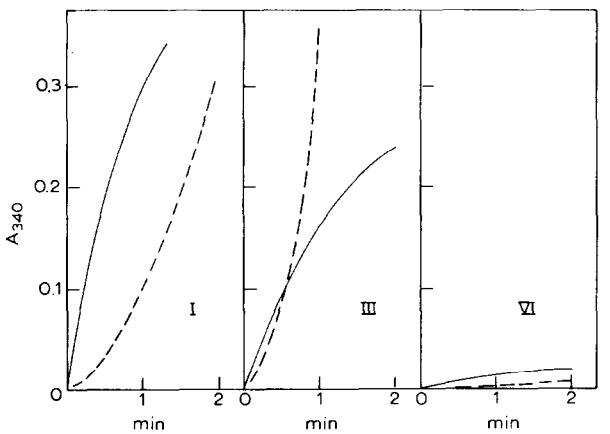

Fig. 4. The peroxidatic (–) and oxidatic (- - -) reactions at pH 6.1 with 0.0066 μM HRP (I, III, VI), 0.4 μM H_2O_2 or 0.46 μM $MnCl_2$, and 2.0 μM phloroglucinol as obtained from the recorder.

most pronounced at low pH, but there is no obvious connection to substrate pK_a (ascorbic acid 4.1, uric acid 3.9, mesidine 4.7, guaiacol 10.0). The involvement of other factors as well is demonstrated by the pH-dependence of the peroxidatic reactions with guaiacol and phloroglucinol (pK_{a1} 8.9) (fig. 3). As pH is increased the reaction rates between the trihydric phenol and both peroxidases increase. Thus, the reaction velocities with peroxidases I and III correlate positively with phloroglucinol and negatively with guaiacol as substrate.

The two peroxidases give different reaction rates with benzidine of essentially the same pH-dependence. On the other hand leuco malachite green, a dialkylated aromatic amine and a triphenylmethane dye, behaves fundamentally different towards the peroxidases I and III, the former giving no spectrophotometrically detectable reaction.

Both peroxidases I and III are active as oxidases towards phloroglucinol in the presence of manganous ions (fig. 4). The present data reveal a low activity of VI towards all tested substrates (fig. 2–4), supporting the artifact hypothesis. Earlier [2] fraction VI was found to equal III and V in activity towards guaiacol. At present no explanation can be given for this discrepancy but for the possibly occurring fragmentation.

The results presented here establish the two main peroxidases I and III as separate enzymes with different substrate preferences or even specificities, likely to handle separate functions *in vivo*. The observations, with leuco malachite green in particular, suggest a method for their histochemical differentiation *in situ*.

Fraction III accounts for half, and I for a fourth, of the total amount of peroxidase in horseradish. Most data in the literature on reactivity, rate constants etc. have been obtained on enzyme preparations containing more than one of the peroxidases, and they may therefore have to be redetermined, at need. Plant peroxidases are usually stated to have a fairly low substrate specificity, but this idea may have to be revised.

Acknowledgements

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