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Oxidase Reactions of Tomato Anionic Peroxidase[1]

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ABSTRACT

Tomato (*Lycopersicon esculentum* Mill) anionic peroxidase was found to catalyze oxidase reactions with NADH, glutathione, dithiothreitol, oxaloacetate, and hydroquinone as substrates with a mean activity 30% that of horseradish peroxidase; this is in contrast to the negligible activity of the tomato enzyme as compared to the horseradish enzyme in catalyzing an indoleacetic acid-oxidase reaction with only Mn^{2+} and a phenol as cofactors. Substitution of Ce^{3+} for Mn^{2+} produced an 18-fold larger response with the tomato enzyme than with the horseradish enzyme, suggesting a significant difference in the autocatalytic indoleacetic acid-oxidase reactions with these two enzymes. In attempting to compare enzyme activities with 2,4-dichlorophenol as a cofactor, it was found that reaction rates increased exponentially with both increasing cofactor concentration and increasing enzyme concentration. While the former response may be analogous to allosteric control of enzyme activity, the latter response is contrary to the principle that reaction rate is proportional to enzyme concentration, and additionally makes any comparison of enzyme activity difficult.

The major peroxidase of tomato fruits, termed tomato anionic peroxidase, differs markedly from horseradish peroxidase in its ability to oxidize IAA (17). The horseradish enzyme catalyzes an autocatalytic IAA-oxidase[2] reaction, which is stimulated by phenols and Mn^{2+}. The IAA-oxidase reaction of the tomato enzyme, however, was found to be completely dependent on the presence of catalytic amounts of H_2O_2 in the assay mixtures (18), and did not appear to respond to Mn^{2+} as a cofactor. Inasmuch as horseradish peroxidase is frequently used in studies of the mechanism of the IAA-oxidase reaction and the fact that this enzyme will catalyze a number of other Mn^{2+}-dependent oxidase reactions, an attempt was made to compare the oxidase activities of the tomato and horseradish enzymes using several different substrates.

The suggestion that tomato anionic peroxidase may act as an IAA-oxidase *in vivo* is speculative (18), and is based on analogy to pear fruit ripening. An extensive series of studies leads to the conclusion that pear fruit ripening is initiated when the level of endogenous auxin is reduced by an H_2O_2-dependent peroxidase-catalyzed IAA-oxidase reaction (3, 7–9, 25). The IAA-oxidase activity of pear fruit extracts, like that of the tomato enzyme, was found to be totally dependent on the presence of peroxides in the reaction mixtures containing low concentrations of IAA (K. Brooks, unpublished observations).

Tomato ripening, like that of pears, bananas, and avocadoes, appears to be at least partly controlled by auxin levels (12); free IAA decreases and peroxides accumulate in tomato ripening.

An isoelectric point of less than 5.5 for tomato anionic peroxidase, as suggested by its column chromatographic behavior, is lower than those of the major horseradish peroxidase isozymes (11) and is in particular lower than the value of 8.9 for horseradish peroxidase isozyme C, which predominates in commercial horseradish peroxidase preparations. This is of interest because of the statement (2) that cationic peroxidases serve as IAA-oxidases *in vivo*. The more cationic members of isozyme sets often show the highest specific activity in the IAA-oxidase reaction or the highest ratio of IAA-oxidase to peroxidase activity (11, 20, 22, 24), though this is not consistently the case (5, 11). In considering the results of *in vitro* comparisons of peroxidases, it should be recalled that IAA-oxidase activity is more closely related to the oxidation-reduction potential of the enzyme than to the isoelectric point (24), that peroxidase isozymes may differ greatly in their peroxidase activity (15), and that relative IAA-oxidase activities may depend greatly on the conditions of assay used (11, 22).

MATERIALS AND METHODS

Enzymes. Tomato anionic peroxidase was prepared from tomato pericarp using extraction, $(NH_4)_2SO_4$ fractionation, and QAE-Sephadex column chromatography with gradient elution as previously described (17), except that column chromatography was done at pH 5.5 rather than pH 6.5. The peak peroxidase fractions were pooled, dialyzed against 1.0 M $(NH_4)_2SO_4$ in 10 mM K-phosphate (pH 6.5), and rechromatographed on a Phenylsepharose 4B column (14). The enzyme was eluted with a linear $(NH_4)_2SO_4$ gradient, the initial and final concentrations being 1.0 M and 0.0 M $(NH_4)_2SO_4$, respectively in 10 mM K-phosphate (pH 6.5). The pooled fractions from the enzyme peak were dialyzed against distilled H_2O, concentrated with aquacide II, adjusted to 5 mM K-phosphate with 1.0 M K-phosphate (pH 7.4), and either lyophilized or stored frozen. The RZ values of the preparations were usually in the range of 1.0 to 1.5.

Commercial hrp used was obtained from Nutritional Biochemicals (RZ 1.0) and from Boehringer-Mannheim (RZ 3.0).

Spectrophotometric Assays. A Perkin Elmer Coleman 124 spectrophotometer with thermostated cell holder and chart recorder was used. Assays were performed at 24°C and reaction volumes were 3.0 ml, except for the guaiacol peroxidase assay where a 2.5 ml final volume was used. Peroxidase activity with *p*-phenylenediamine was assayed by the method of Lück (19) modified to give a final pH of 6.8, and with guaiacol as described by Evans (6). In assays with guaiacol as a substrate, premixing the substrates 10 min prior to use gave higher rates and more consistent results. Peroxidase activities with ferrocyanide (4), ascorbate (26), and iodide (26) as substrates were followed at 420, 263, and 350 nm, respectively. Reaction mixtures for these assays contained 33 mM citrate buffer (pH 4.0), 240 μM H_2O_2, and 1.1 mM $K_4Fe(CN)_6$, 200 μM potassium L-ascorbate, or 5 mM

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[2] The term oxidase reactions is used here to indicate reactions using molecular oxygen as an oxidant.

KI. Peroxidase NADH-oxidase activity (1) was assayed at 340 nm. Reaction mixtures contained 200 μM NADH, 100 μM DCP,[3] 20 μM $MnSO_4$, and 67 mM phosphate buffer (pH 6.4). Hydroquinone-oxidase activity (16) was assayed at 294 nm. Reaction mixtures contained 30 μM hydroquinone, 1.0 mM $MnSO_4$, and 50 mM succinate buffer (pH 6.1). Citrate buffer (16) was found to be unsuitable in the hydroquinone-oxidase assay.

Oxygraph Assays. O_2 uptake in oxidase reactions was followed using a Gilson oxygraph with a Clark type electrode, with reaction mixtures of 1.6 ml volume. In reactions involving very low rates of O_2 uptake, a tapered glass rod was inserted into the removable glass tube in the oxygraph sample addition port to reduce or eliminate the back-diffusion of O_2 into the reaction mixture. Glutathione-oxidase (28) reaction mixtures contained 400 μM neutralized reduced glutathione, 100 μM $MnSO_4$, 100 μM DCP, and 94 mM K-phosphate (pH 5.8). DTT-oxidase (23) reaction mixtures contained 3.1 mM DTT, 100 μM $MnSO_4$, 100 μM DCP, 50 mM sodium citrate, and 50 mM K-phosphate (pH 6.8). The NADH-oxidase (1) reaction mixtures contained 200 μM NADH, 100 μM DCP, 20 μM $MnSO_4$, and 67 mM K-phosphate (pH 6.4). Oxaloacetate-oxidase (15) reaction mixtures contained 1.0 mM potassium hydrogen oxaloacetate, 750 μM $MnSO_4$, and 50 mM acetate buffer, pH 4.7. Note that the oxaloacetate-oxidase reaction would proceed without added H_2O_2, but was preceded by a lag of 20 to 30 min. The IAA-oxidase reaction mixtures contained 800 μM IAA, 75 mM succinate buffer (pH 4.0), 200 μM DCP or pCA, and 100 μM $MnSO_4$, $CeCl_3$, or H_2O_2 unless otherwise stated. Assays were carried out at 25°C with IAA and at 24°C with DTT as substrates, and at 23°C with the other substrates.

Enzyme Activity. Enzyme activity is expressed throughout this paper in terms of nmol O_2 used/min·nmol peroxidase for oxygraph assays and absorbance change/min·nmol peroxidase for spectrophotometric assays. The peroxidase concentration (μmol/ml) of stock enzyme solutions was determined from their 403 nm *A*, assuming a mM extinction coefficient of 107 for both horseradish and tomato peroxidases (18).

RESULTS

The data in Table I indicate that tap and hrp have comparable specific activities with *p*-phenylenediamine and guaiacol as substrates. The tomato enzyme is shown to catalyze the NADH-, oxaloacetate-, glutathione-, DTT- and hydroquinone-oxidase reactions with specific activities ranging from 0.24 to 1.8 times that of the horseradish enzyme, as compared to previously obtained values (14) in the range of 0.005 to 0.01 for the IAA-oxidase reaction. The assays used in Table I were tested and roughly optimized with the horseradish enzyme before the tomato enzyme was tested; thus, the values presented represent a comparison under a preselected set of conditions possibly favoring the horseradish enzyme. The data on the peroxidative oxidation of ferrocyanide, iodide, and ascorbate are included for the sake of completeness; the reaction rates for both the horseradish and the tomato enzymes were larger at pH 4.0 than at any higher pH tested with these substrates (data not shown).

The data in Table I also indicate that the oxidase reactions catalyzed by the tomato enzyme are stimulated by Mn^{2+}. However, the response is generally less than the almost total dependence on Mn^{2+} previously reported for hrp in several oxidase reactions (1, 12, 13). In the NADH-oxidase reaction, omission of Mn^{2+} decreased the rates of reaction with the tomato and horseradish enzymes 83 and 99% respectively; the reaction rate for the tomato enzyme in the absence of Mn^{2+} was perhaps four times that of the horseradish enzyme and was not further reduced by 10 mM EDTA. Alternately, omission of Mn^{2+} from DTT-oxidase reaction mixtures containing the tomato enzyme and buffered at pH 7.8 resulted in a 50% reduction in reaction rate, while a 97% reduction was found in reaction mixtures buffered at pH 4.0.

Table I. *Activity of tap Relative to that of hrp in Various Peroxidative and Oxidase Reactions*

Assays were performed as described in "Materials and Methods." The NADH-oxidase activity was determined by O_2 uptake in determining specific activities and spectrophotometrically in Mn^{2+} dependence measurements. All oxidase reaction mixtures contained $MnSO_4$ and DCP, except for the hydroquinone-oxidase reaction where DCP is omitted. Specific activities were determined from measurements at low enzyme concentration where reaction rates were proportional to enzyme concentration.

Reaction and Substrate	Relative[a] Activity	Activity[b] minus $MnSO_4$
Peroxidase reactions		
p-Phenylenediamine	1.16	
Guaiacol (pH 7.75)	0.74	
Guaiacol (pH 5.50)	1.28	
Ferrocyanide	0.10	
Iodide	0.07	
Ascorbate	17.3	
Oxidase reactions		
NADH	0.33	0.17
Oxaloacetate	0.73	
Glutathione	0.33	0.29
Dithiothreitol	0.24	0.50
Hydroquinone	1.80	0.03

[a] Specific activity of tomato enzyme/specific activity of horseradish enzyme. [b] Reaction rate minus Mn^{2+}/reaction rate with Mn^{2+} for the tomato enzyme under the specified assay conditions.

Table II. *Cofactor Response for tap in the IAA-Oxidase Reaction at pH 4.0*

Phenol Added	IAA-Oxidase Reaction With[a]			
	H_2O_2	Ce^{3+}	Mn^{2+}	
	nmol O_2 used/min			
PCA (200 μM)	283	76	2.1	0.4
DCP (200 μM)	7	51	2.5	
DCP (400 μM)	23	85	6.2	
DCP (800 μM)	109	67	5.8	

[a] Reaction mixtures initially contained 258 μM O_2 and 5.2 nM enzyme.

The data in Table II summarize the rates of the IAA-oxidase reaction catalyzed by tap under various assay conditions. The low activity of the tomato enzyme relative to that of hrp in reaction mixtures unsupplemented with H_2O_2 is indicated by reaction rates of 0.1 and 1.5 nmol of O_2 used/min with reaction mixtures containing either 200 μM pCA or 100 μM Mn^{2+} and 400 μM DCP, respectively; under the same conditions, 5.2 nM hrp gave rates of 438 and 194 nmol O_2 used/min, respectively. Two other significant features are seen in these data. First, while the tomato enzyme does respond to Mn^{2+}, Ce^{3+} gives 12 to 36 times the rate of O_2 uptake seen with Mn^{2+} under the conditions of assay used. For comparison, the IAA-oxidase reaction of hrp was stimulated a maximum of 5- and 10-fold by Mn^{2+} and Ce^{3+} when assays were carried out at low enzyme concentrations and under a variety of conditions (data not shown). This is consistent with literature reports of a 2-fold stimulation for Mn^{2+} (13) and a 3-fold stimulation for Ce^{3+} (21) with the latter enzyme. The data in Table II also show that rates of oxygen uptake with H_2O_2 as an activator increase exponentially with DCP concentration

[3] Abbreviations: DCP, 2,4-dichlorophenol; pCA, *p*-coumaric acid; hrp, horseradish peroxidase; tap, tomato anionic peroxidase; RZ, Reinheitszahl.

over the range of concentrations tested, while higher DCP concentrations tend to inhibit oxygen uptake in the metal ion supported reactions. It should be noted that the rates indicated, even for the very slow reactions, are steady state rates.

The data in Table III indicate a pH optimum about pH 4.0 for the tomato enzyme with pCA and either Ce^{3+} or H_2O_2. Previously published data (18) indicate that the tomato enzyme is somewhat more active at pH 3.0 than at pH 4.0 in reaction mixtures containing DCP, H_2O_2, and citrate buffer. Hoyle and Routley (13) reported that both hrp and yellow birch leaf peroxidase have pH optima about pH 3.6 in the IAA-oxidase reaction when pCA was used as a cofactor.

Figure 1 compares the activities of tap and hrp in the IAA-oxidase reaction with pCA. The data indicate that at the lowest enzyme concentration for which data are available, the ratio of activity of the tomato enzyme to that of the horseradish enzyme is 0.20 and 0.14 with H_2O_2 and Ce^{3+}, respectively, as cofactors; due to the nonlinear nature of the curves, these values increase with increasing enzyme concentration. These data are presented over a range of enzyme concentrations in view of the data in Figure 2. Figure 2 demonstrates that over a limited range of enzyme concentration in what are perhaps typical assay mixtures, the rate of O_2 uptake in the IAA-oxidase reaction will increase exponentially, rather than linearly or hyperbolically, with increasing enzyme concentration.

DISCUSSION

The fact that tap will catalyze a number of oxidase reactions, albeit with an activity somewhat lower than that of hrp, indicates that the two enzymes are not totally dissimilar in oxidase activity; the striking difference in the IAA-oxidase activity of the two

Table III. *PH Response for tap in the IAA-Oxidase Reaction with pCA*

pH	Buffer	IAA-Oxidase Reaction with[a]	
		Ce^{3+}	H_2O_2
3.0	Succinate	8	120
3.5	Succinate	34	167
4.0	Succinate	65	192
4.5	Succinate	69	196
5.0	Succinate	53	145
5.5	Succinate	37	108
6.0	Succinate	0	49
6.0	Phosphate	0	34
6.5	Phosphate	0	16
7.0	Phosphate	0	9

[a] Reaction mixtures contained 258 μm O_2 and 5.2 nM enzyme.

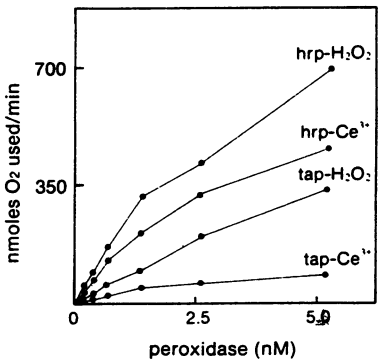

FIG. 1. Rates of the IAA-oxidase reaction with tap and hrp at pH 4.0 with pCA. Reaction mixtures contained 200 μM pCA, 100 μM $CeCl_3$ or H_2O_2, 258 μM O_2 and enzyme at the concentrations indicated.

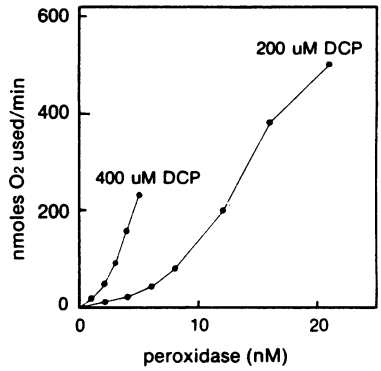

FIG. 2. Effect of enzyme concentration on the rate of the IAA-oxidase reaction with hrp at pH 4.0 and suboptimal concentration of DCP. Reaction mixtures contained 100 μM $MnSO_4$, 258 μM O_2, and DCP and enzyme at the concentrations indicated.

enzymes remains. This difference in IAA-activity may be related to observations suggesting that the autocatalytic oxidation of IAA by hrp proceeds by a mechanism or mechanisms different from other oxidase reactions; the IAA-oxidase reaction of hrp is relatively insensitive to inhibition by superoxide dismutase as compared to other oxidase reactions (29), and may use skatole hydroperoxide, a reaction product, as an oxidant (22). A difference in the rate of reaction of hrp isozymes A and C with skatole hydroperoxide (22) has been used to explain the difference in rates of autocatalytic oxidation of IAA by these isozymes, while differences in the autocatalytic rate of oxidation of IAA have been related to enzyme oxidation-reduction potentials in a turnip peroxidase isozyme system (24).

The mechanism of stimulation of the IAA-oxidase reaction and other peroxidase oxidase reaction by metal ions has been studied, but a definitive picture does not seem to have been developed; the metal ions may potentially play more than one role. However, the 180-fold rate increase observed with the tomato enzyme in response to Ce^{3+}, as compared to the 10-fold or smaller response to Ce^{3+} found with hrp, suggests that the metal ion-dependent autocatalytic oxidation of IAA by the tomato enzyme proceeds by a pathway distinctly different from the autocatalytic mechanism of IAA oxidation by hrp. Metal ions may be used as a tool in studying the mechanisms of peroxidase oxidase reactions, but no physiological significance is implied to the stimulation observed; the NADH-oxidation reaction, which was originally described as a totally Mn^{2+} dependent reaction (1), probably proceeds *in vivo* by a mechanism involving malate dehydrogenase-bound NADH free radicals (10).

The exponential increase in rate of the IAA-oxidase reaction at suboptimal (200 μM) DCP concentrations seen in Figure 2 differs greatly from the hyperbolic responses seen with 200 μM pCA in Figure 1 or with 750 μM DCP and tap (15). This unexpected result was perhaps foreshadowed by the comment of Gove and Hoyle (11) that suboptimal concentrations of DCP gave unreliable results in the same reaction. In investigating the mechanism of the IAA-oxidase reaction of hrp in the absence of both metal ions and phenols, Smith *et al.* (27) demonstrated that a 5.5-fold increase in enzyme concentration resulted in a 35- or 55-fold increase in the reaction rate, depending on the IAA concentration. This effect was demonstrated to be due to a shift in enzyme mechanism with enzyme concentration. While a direct comparison of the results might be made, other factors may be involved in the data presented in Figure 2. Decreasing enzyme concentration increased the lag period in the reaction and decreased the steady state rate of the reaction; reaction rates were measured at the point where 30% of the O_2 had been consumed, assuming that the measured rate would be propor-

tional to the final steady state rate. This may not be strictly true. Moreover, no account is made of possible enzyme inactivation during the lag period, the rate of which may be dependent on DCP concentration. Tap with Mn^{2+} and DCP in the IAA-oxidase reaction gave rate *versus* enzyme concentrations that were hyperbolic (data not shown), so that even at 55 nM enzyme the rate of reaction remained low compared to the horseradish enzyme with these cofactors.

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