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A BASIC PEROXIDASE ISOENZYME FROM VACUOLES AND CELL WALLS OF *VITIS VINIFERA*

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Key Word Index—*Vitis vinifera*; vitaceae; basic peroxidase isoenzyme; cell wall; vacuole; kinetic properties; phenolic oxidation.

Abstract—The substrate profile of a basic peroxidase isoenzyme located in vacuoles and cell walls from *Vitis vinifera* and efficacy in oxidizing both vacuolar and cell wall phenolic substrates was studied. The reactivity of this isoenzyme with H_2O_2 [k_1 (CoI formation constant) = 1.1 (quercetin), 1.6 (myricetin), 1.7 (*trans*-resveratrol) and 10.4 (coniferyl alcohol) μM^{-1} sec^{-1}] and with the phenolics [k_3 (CoII reduction constant) = 20.7 (quercetin), 6.9 (myricetin), 11.9 (*trans*-resveratrol) and 8.7 (coniferyl alcohol) μM^{-1} sec^{-1}], suggests that the isoenzyme reacts with H_2O_2 with a similar reactivity to that shown by other peroxidases, and that both vacuolar and cell wall phenolics are excellent substrates for CoII reduction. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Peroxidase (EC 1.11.1.7) is the enzyme responsible for the cross-linking of phenolic moieties during the biosynthesis of lignins in the plant cell wall [1–3]. As may be expected from its specific role in lignin biosynthesis, peroxidase is mainly located in the cell wall, although other subcellular localizations have been reported: for example, in the vacuole. When we have studied peroxidase isoenzyme localization in *Lupinus albus* (Fabaceae) [4, 5], *Vitis vinifera* (Vitaceae) [6, 7], *Capsicum annuum* (Solanaceae) [8]; *Lactuca sativa* (Asteraceae) [9], and *Catharanthus roseus* (Apocynaceae) [10], we found that, in all the studied plant materials, the cell wall contains the complete set of peroxidase isoenzymes present in the plant cell, including both basic (pI > 7.0) and acidic (pI > 7.0) peroxidase isoenzymes; while vacuoles only contain the basic peroxidase isoenzyme group of high pI (pI > 9.2) (BPrx HpI), an isoenzyme group also detected in the cell wall. These results are not surprising, since a similar dual localization for the most basic peroxidase isoenzyme has been found in other plant species [11].

Although some metabolic analogies have been found in plant cells between vacuoles and cell walls, especially regarding both subcellular compartments as lytic compartments [12], the fact that the BPrx HpI isoenzyme may be located in vacuoles and cell walls raises a question on its metabolic plasticity, since the available substrates in these two subcellular compartments are somewhat different.

In the case of grapevines (*Vitis vinifera*), the BPrx HpI isoenzyme is located at vacuole level in hypodermal cells and at cell wall level in the xylem [7, 13, 14]. In this first case, this isoenzyme appears to play a role in the metabolic turnover of certain flavonol glycosides (quercitrin and myricitrin) once the aglycones (quercetin and myricetin) have been released by specific glycosidases [15]; while at the xylem cell wall level it is more probable that it plays a role in the polymerization of cinnamyl alcohol to lignins [16].

For this reason, in this work we have studied the catalytic efficiency of this isoenzyme during the oxidation of the aglycones (i.e. quercetin and myricetin) of vacuolar-type substrates (quercitrin and myricitrin) and of cell wall-type substrates (i.e. coniferyl alcohol). Furthermore, the catalytic efficiency of this isoenzyme for these substrates is compared with its catalytic efficiency during the oxidation of *trans*-resveratrol (a particular phenolic located in both vacuoles and cell walls). From a comparison of the values obtained for the constants of compound I formation (k_1) and compound II reduction (k_3), a strong metabolic plasticity for this BPrx HpI isoenzyme is suggested, which is in accordance with their two different subcellular locations.

RESULTS

Oxidation of the phenolics, quercetin, myricetin, *trans*-resveratrol and coniferyl alcohol, by the grape-

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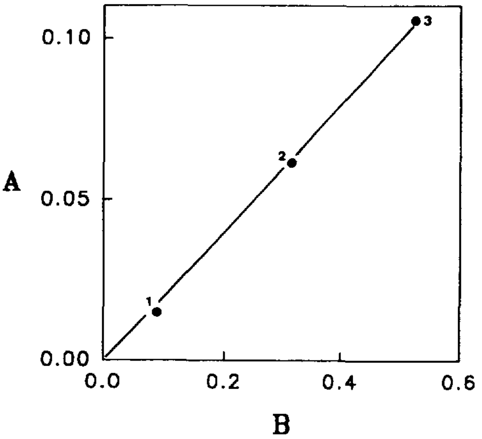

Fig. 1. Plot of parameters A (nmol s^{-1}) vs B (mM) obtained by varying H_2O_2 for three quercetin concentrations [0.01 mM (1), 0.05 mM (2) and 0.15 mM (3)]. From the slope of the straight line (2 [E] k_1), k_1 was calculated to be 1.1 μM^{-1} s^{-1}.

vine BPrx HpI isoenzyme follows Michaelis–Menten type kinetics, with inhibition by substrate at higher concentrations. To calculate the microscopic constants (k_1 and k_3) for the oxidation of phenolic substrates by this basic peroxidase isoenzyme fraction, the rates of oxidation of phenolics were fitted for each concentration of phenolic and H_2O_2, according to the generally accepted mechanism for the peroxidase reaction [17]:

$$P + H_2O_2 \xrightarrow{k_1} CoI + H_2O \quad (1)$$

$$CoI + RH \xrightarrow{k_2} CoII + R^{\cdot} \quad (2)$$

$$CoII + RH \xrightarrow{k_3} P + R^{\cdot} \quad (3)$$

where RH is the particular phenolic and CoI and CoII are the key intermediates in the peroxidase cycle, compound I and compound II.

For non-inhibitory concentrations, the initial rates of the phenolic oxidation may be fitted to the following equation:

$$v = \frac{A[H_2O_2]}{B + [H_2O_2]}$$

where $A = 2$ [E] k_3 [RH] and $B = (k_3/k_1)$ [RH]. Double-reciprocal plots ($1/v$ vs $1/[H_2O_2]$) allow us to calculate A and B values for each phenolic concentration. Fig. 1 shows the plot of A vs B values for three quercetin concentrations. From this straight line ($A = 2$ [E] $k_1 B$), it is possible to calculate the k_1 value (Compound I formation constant) for the peroxidase-mediated quercetin oxidation. The value obtained, as well as those obtained for the peroxidase-mediated myricetin, *trans*-resveratrol and coniferyl alcohol oxidation, are shown in Table 1.

Table 1. k_1 (μM^{-1} sec^{-1}) and k_3 (μM^{-1} sec^{-1}) values at 25° for the oxidation of several phenolic substrates by the basic peroxidase isoenzyme from *Vitis vinifera*

Substrate	pH_{opt}	k_1	k_3
Quercetin	4.0	1.1	20.7
Myricetin	6.0	1.6	6.9
trans-Resveratrol	4.0	1.7	11.9
Coniferyl alcohol	5.0	10.4	8.7

Similarly, the dependence of v on [RH] may be written as:

$$v = \frac{A[RH]}{B + [RH]}$$

where $A = 2$ [E] k_1 [H_2O_2] and $B = (k_1/k_3)$ [H_2O_2]. Double reciprocal plots ($1/v$ vs $1/[RH]$) allow us to calculate A and B values for each H_2O_2 concentration. Figure 2 shows the plot of A vs B values for three H_2O_2 concentrations during the oxidation of myricetin. From this straight line ($A = 2$ [E] k_3 B), it is possible to calculate the k_3 value (Compound II reduction constant) for the peroxidase-mediated myricetin oxidation. The value obtained, as well as those obtained for quercetin, *trans*-resveratrol and coniferyl alcohol, are shown in Table 1. In all the cases, k_1 and k_3 values were calculated at the optimum pH for the peroxidase-catalysed oxidations, these values also being listed in Table 1.

From these results, it may be concluded that oxidation of these phenolics by the grapevine BPrx HpI isoenzyme follows the accepted model for peroxidase oxidation, in which CoI and CoII appear as the main intermediates in the catalytic cycle [eqns (1)–(3)]. This observation is supported by the good fits to a straight line which passed through the coordinate origin when the parameters A vs B were represented for varying phenolics (Fig. 1) and H_2O_2 (Fig. 2) concentrations.

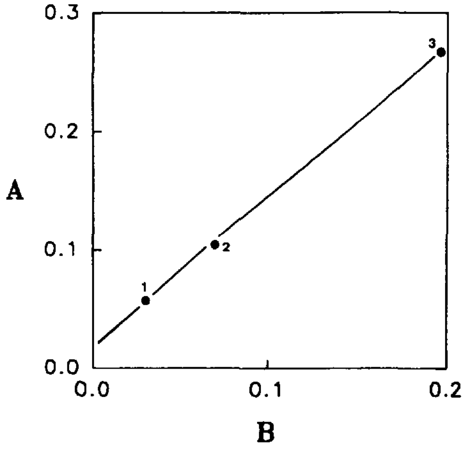

Fig. 2. Plot of parameters A (nmol s^{-1}) vs B (mM) obtained by varying myricetin for three H_2O_2 concentrations [0.25 mM (1), 0.50 mM (2) and 2.0 mM (3)]. From the slope of the straight line (2 [E] k_3), k_3 was calculated to be 6.9 μM^{-1} s^{-1}.

DISCUSSION

From the rate constant values (k_i) shown in Table 1, it can be deduced that the grapevine BPrx HpI isoenzyme is capable of oxidizing vacuolar phenolic substrates (i.e. quercetin, myricetin, and *trans*-resveratrol) as well as cell wall phenolic substrates (i.e. *trans*-resveratrol and coniferyl alcohol) without strong substrate discrimination.

Examination of the CoI formation constant (k_1) for the four substrates assayed reveals that it varies from 1.1 to 1.7 μM^{-1} sec^{-1} for polyhydroxy phenolic compounds (i.e. quercetin, myricetin and *trans*-resveratrol), while it reaches the value of 10.4 μM^{-1} sec^{-1} for a guaiacyl-type phenolic compound (i.e. coniferyl alcohol). Although, *a priori*, it is difficult to explain at molecular level how the nature of the substrate influences the rate constant of CoI formation, this is a fact described in the literature [17]. Moreover, this value is usually greater for guaiacyl-type structures than for polyhydroxy phenolic compounds [17].

As has been discussed above, k_1 values depend on the nature of the reducing substrate. For this reason, it is misleading to compare the k_1 values obtained in this work using the grapevine BPrx HpI isoenzyme (see Table 1) with those obtained with different substrates for other peroxidase preparations. A previous study using a moderately basic peroxidase isoenzyme from barley [17] at pH 3.96 showed a value for k_1 of 6.7 μM^{-1} sec^{-1} using coniferyl alcohol as substrate. If we compare this value with that obtained for the grapevine BPrx HpI isoenzyme (Table 1), one may conclude that the reactivity of the grapevine BPrx HpI isoenzyme with H_2O_2 [whose magnitude is evaluated through the constant of CoI formation (k_1)] is greater than that shown by barley peroxidase during the oxidation of coniferyl alcohol oxidation.

In the case of the cell wall-located phenolic compound, coniferyl alcohol, in which the k_3 value may be compared with that shown in literature, the reactivity of the grapevine BPrx HpI isoenzyme for coniferyl alcohol is higher than that shown by other peroxidases. Thus, k_3 values reported for coniferyl alcohol are 2.80 μM^{-1} sec^{-1} for the moderately basic peroxidase isoenzyme from horseradish at pH 4.5 [18], and 2.4 μM^{-1} sec^{-1} for the moderately basic peroxidase isoenzyme from barley at pH 3.96 [17], values clearly below that shown by the grapevine BPrx HpI isoenzyme for coniferyl alcohol (8.3 μM^{-1} sec^{-1}, Table 1).

On the other hand, coniferyl alcohol is frequently recognized as one of the best substrates for CoII reduction [17, 18]. However, the grapevine BPrx HpI isoenzyme oxidizes some vacuolar phenolic substrates (i.e. quercetin, myricetin and *trans*-resveratrol) with rate constants comparable, and even greater, than that shown for coniferyl alcohol (Table 1). These results illustrate the great reactivity and metabolic plasticity for oxidizing phenolic substrates of an isoenzyme found in two different subcellular localizations (vacuole and cell wall), where the availability of particular substrates, conditioned by its tissue-specific localization, appears to be the main factor delimiting its metabolic function.

EXPERIMENTAL

Chemicals. Quercetin (3,3′,4′,5,7-pentahydroxyflavone) and myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone) were purchased from Extrasynthese (Genay, France). Coniferyl alcohol (3-methoxy, 4-hydroxycinnamic) and H_2O_2 from Sigma *Trans*-resveratrol [(E)-3′,4,5′-trihydroxystilbene] was synthesized by a Wittig reaction linking 3,5-dimethoxybenzyltriphenyl phosphine bromide and *p*-anisaldehide [19]. Methylated precursors were used to protect the OH groups, which were removed by boron tribromide. The Wittig products were a mixture of (E) and (Z) isomers. The (E)/(Z) mixture was isomerized to the pure (E) isomer by heating with a catalytic amount of diphenyl disulfide in tetrahydrofuran. The identities of the structures were confirmed from their ^{1}H-NMR spectra: the coupling constants of the olefinic protons were 16–17 Hz for the (E)-isomer and 12 Hz for the (Z)-isomer. Confirmation of *trans* conformation was deduced from their UV-spectral characteristics (λ_{max}^{EtOH} = 307 nm), IR absorption peak at 965 (w) cm^{-1} (*trans* form of the double bond), δ [^{1}H-NMR ($CDCl_3$, CD_3COCD_3, 300 MHz)] = 6.79–6.84 (*m*, 3H, H_3, $+H_a/H_b$) and 6.97 (*d*, 1H, J = 16.5 Hz, H_a/H_b) and δ [^{13}C-NMR ($CDCl_3$, CD_3COCD_3, 75 MHz)] = 125.07 (C_a/C_b) and 127.38 (C_a/C_b). All the other chemicals used in this work were obtained from various commercial suppliers and were of the highest purity available.

Plant material and peroxidase isoenzyme purification. The *Vitis vinifera* cultivar, Gamay rouge, was grown in field and sampled in August at veraison. Fruit clusters were transported to the laboratory and frozen immediately at −30°. From this material, the homogenization and purification of the BPrx HpI isoenzyme by prep. isoelectric focusing have been described in refs [15, 16]. Examination by analytical isoelectric focusing of the purified isoenzyme revealed that it migrates as a sole peroxidase isoenzyme band during electrophoresis [16].

Spectrophotometric studies. The spectrophotometric assays for the oxidation of phenolics by the grapevine BPrx HpI isoenzyme were performed at 25° in a reaction medium containing variable concs of phenolics [0.01, 0.05 and 0.15 mM for quercetin; 0.025, 0.05 and 0.1 mM for myricetin and coniferyl alcohol; and 4.5, 45 and 90 μM for *trans*-resveratrol] and H_2O_2 [0.1–5.0 mM] in 0.1 M Tris-acetate buffer. The reaction was initiated by the addition of grapevine peroxidase. Oxidation of quercetin, myricetin, *trans*-resveratrol and coniferyl alcohol were monitored by the changes in absorbance using a $\Delta\varepsilon_{365}$ = 13200, $\Delta\varepsilon_{365}$ = 7200, $\Delta\varepsilon_{306}$ = 26800 and $\Delta\varepsilon_{260}$ = 2200 M^{-1} cm^{-1}, respectively. The concn of peroxidase was determined at 403 nm using a $\varepsilon_{403} = 1.02\ 10^5\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$ for the Soret band.

To calculate the microscopic constants (k_1 and k_3) for the oxidation of phenolic substrates by this basic peroxidase isoenzyme fraction, the rates of oxidation of phenolics were fitted for each concn of phenolic and H_2O_2, according to the generally accepted mechanism for the peroxidase reaction [17]:

$$\mathrm{P} + \mathrm{H_2O_2} \xrightarrow{k_1} \mathrm{CoI} + \mathrm{H_2O} \qquad (1)$$

$$\mathrm{CoI} + \mathrm{RH} \xrightarrow{k_2} \mathrm{CoII} + \mathrm{R}^{\cdot} \qquad (2)$$

$$\mathrm{CoII} + \mathrm{RH} \xrightarrow{k_3} \mathrm{P} + \mathrm{R}^{\cdot} \qquad (3)$$

where RH is the particular phenolic and CoI and CoII are the key intermediates in the peroxidase cycle, compound I and compound II. Assuming that $k_2 > k_3$, as occurs for most peroxidases [17], the steady-state equation rate may be written as:

$$v = \frac{2[\mathrm{E}]k_3[\mathrm{RH}][\mathrm{H_2O_2}]}{(k_3/k_1)[\mathrm{RH}] + [\mathrm{H_2O_2}]} \qquad (4)$$

from which the dependence of v on $[H_2O_2]$ may be written as:

$$v = \frac{A[\mathrm{H_2O_2}]}{B + [\mathrm{H_2O_2}]} \qquad (5)$$

where $A = 2\ [\mathrm{E}]\ k_3\ [\mathrm{RH}]$ and $B = (k_3/k_1)\ [\mathrm{RH}]$. Double-reciprocal plots ($1/v$ vs $1/[H_2O_2]$) allow us to calculate A and B values for each phenolic concn. Plotting A vs B values gives a straight line ($A = 2\ [\mathrm{E}]\ k_1\ B$), and from its slope it is possible to calculate the k_1 value.

Similarly, and starting from eqn (4), the dependence of v on [RH] may be written as:

$$v = \frac{A[\mathrm{RH}]}{B + [\mathrm{RH}]} \qquad (6)$$

where $A = 2\ [\mathrm{E}]\ k_1\ [\mathrm{H_2O_2}]$ and $B = (k_1/k_3)\ [\mathrm{H_2O_2}]$. Double reciprocal plots ($1/v$ vs $1/[\mathrm{RH}]$) allow us to calculate A and B values for each H_2O_2 concn. Plotting A vs B values gives a straight line ($A = 2\ [\mathrm{E}]\ k_3\ B$), and from its slope it is possible to calculate the k_3 value. These A/B plots allows us to calc. true reaction constants (k_i) from steady-state measurement of the oxidation rate avoiding their dependence on substrate concn. [17].

From eqn. 4, the catalytic efficacy ($k_{\mathrm{cat}}/K_M^{\mathrm{obs}}$) for the utilization of H_2O_2 would be given by:

$$k_{\mathrm{cat}}/K_M^{\mathrm{obs}} = \frac{2k_3[\mathrm{RH}]}{(k_3/k_1)[\mathrm{RH}]} = 2k_1$$

while the catalytic efficacy ($k_{\mathrm{cat}}/K_M^{\mathrm{obs}}$) for the utilization of the phenolic would be given by:

$$k_{\mathrm{cat}}/K_M^{\mathrm{obs}} = \frac{2k_1[\mathrm{H_2O_2}]}{(k_1/k_3)[\mathrm{H_2O_2}]} = 2k_3$$

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