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Action of diverse peroxidases and laccases on six cell wall-related phenolic compounds

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Abstract

Four peroxidases and four laccases were compared as to reaction rates catalysed with six phenolic substrates of relevance to the plant cell wall. When each phenolic substrate was tested at 670 μM and pH 6.0, in the presence of 670 μM H_2O_2 or ~270 μM O_2 as the electron acceptor, all the peroxidases and laccases had similar substrate preferences: reaction rates were in the order sinapyl > coniferyl > *p*-coumaryl alcohols, and feruloyl > *p*-coumaroyl esters. Specific activities were in the order basic peroxidase > acidic peroxidase≫laccase. The data are consistent with the view that peroxidases rather than laccases play a major role in phenolic cross-linking in the cell wall. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Peroxidase; Laccase; Phenol oxidase; Cell wall; Coniferyl alcohol; Ferulic acid; *p*-Coumaric acid; HPLC

1. Introduction

The oxidative coupling of phenolic compounds is important in the architecture of the plant cell wall (Fry, 1986; Wallace & Fry, 1994). From the cross-linking of hemicellulose-bound feruloyl groups (Markwalder & Neukom, 1976; Wallace & Fry, 1995) and of extensin-bound tyrosine groups in primary cell walls (Brady, Sadler & Fry, 1998) to the synthesis of lignin in wood (Sarkanen, 1971), such reactions have a profound effect on the cell wall.

However, despite the biological, dietary and industrial implications of the oxidative coupling of plant cell wall phenolics, many questions remain to be answered about the enzymology of coupling and about the subsequent nonenzymic rearrangements of the immediate reaction products (Ralph & Helm, 1993). For many years the enzyme thought to be principally responsible for the oxidative polymerisation of monolignols to form lignin was peroxidase (donor : H_2O_2 oxidoreductase, E.C. 1.11.1.7) (Harkin & Obst, 1973). More recently, however, an oxidase ('laccase', i.e. benzenediol : O_2 oxidoreductase, E.C. 1.10.3.2), which can catalyse similar coupling reactions of monolignols using O_2 instead of H_2O_2 as the electron acceptor (Dean & Eriksson, 1994; McDougall, Stewart & Morrison, 1994; Savidge & Udagama-Randeniya, 1992; Sterjiades, Dean, Gamble, Himmelsbach & Eriksson, 1993), has been suggested to be responsible for lignification, a possibility first raised by Freudenberg and Neish (1968). There is now confusion as to the relative contributions of peroxidases and laccases in the cell wall.

Several substrates have been used routinely for assaying peroxidase and laccase activities, e.g. guaiacol, *o*-dianisidine, pyrogallol, syringaldazine and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate) (ABTS) (McDougall et al., 1994; Sterjiades et al., 1993). Although such artificial, chromogenic substrates are useful for simple enzyme assays, they give little information about the specificity of the enzymes for particular substrates of relevance to the plant cell wall. Natural substrates yield mainly colourless reaction products and therefore tend not to be favoured for use

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Table 1
Effects of peroxidases and laccases on six cell wall-related phenols

Substrate	Percentage of substrate consumed after 30 min incubation in the presence of							
	plant acidic peroxidases			plant basic peroxidase	plant laccases from		fungal laccases from	
	type A2 (17 ng/ml)	type VII (17 ng/ml)	type VIII (17 ng/ml)	type IX (17 ng/ml)	*Acer* (1.7 μg/ml)	*Populus* (17 μg/ml)	*Rigidoporus* (1.7 μg/ml)	*Pyricularia* (8.3 μg/ml)
Methyl *p*-coumarate	24.2 ± 1.6	27.5 ± 6.6	32.4 ± 3.8	89.2 ± 3.0	0[d] (0)[a]	1.1 ± 3.74	2.2 ± 2.4 (6.2 ± 1.2)[a]	0
Methyl ferulate	83.1 ± 1.5	79.5 ± 1.3	91.6 ± 1.6	97.0 ± 0.9	1.4 ± 5.1 (0.2 ± 0.8)[a]	18.2 ± 4.64	83.2 ± 3.7 (99.2 ± 0.3)[a]	0
Coniferaldehyde	77.5 ± 0.7	73.3 ± 1.2	80.7 ± 1.7	96.3 ± 0.5	3.2 ± 4.1 (4.4 ± 1.0)[a]	0	96.3 ± 0.6 (96.1 ± 0.6)[a]	nd
p-Coumaryl alcohol	7.7 ± 3.1	21.3 ± 6.0	6.4 ± 1.6	87.4 ± 9.3	nd[c] (61.5 ± 17.7)[a]	2.4 ± 3.3	6.2 ± 1.9 (56.5 ± 1.7)[a]	39.3 ± 4.8
Coniferyl alcohol	84.4 ± 3.4 (8.9 ± 6.0)[b]	57.3 ± 2.4	63.9 ± 1.8	94.1 ± 1.3	30.1 ± 2.9 (88.2 ± 3.9)[a]	73.7 ± 9.3	56.5 ± 1.7 (98.7 ± 2.6)[a]	nd
Sinapyl alcohol	70.1 ± 10.0 (77.4 ± 4.8)[b]	73.1 ± 8.4	78.5 ± 8.4	93.0 ± 3.2	61.2 ± 19.0 (63.2 ± 4.7)[a]	100 ± 0	nd (100 ± 0)[a]	nd

[a] Data in parentheses are for enzyme assayed at 3.3 μg/ml.
[b] Data in parentheses are for enzyme assayed at 3.3 ng/ml.
[c] nd = not determined.
[d] 0 = substrate not measurably consumed.

in routine spectrophotometric assays. Although several studies have used individually optimised assays, with 'natural' phenolics as peroxidase substrates, e.g. coniferyl alcohol (Calderon, Muñoz, Morales & Ros Barceló, 1992; Ferrer & Ros Barceló, 1994a, 1994b; Rasmussen, Dunford & Welinder, 1995; Takahama, 1993), ferulate (Gelinas, 1973; Pickerling, Powell, Wender & Smith, 1973; Rasmussen et al., 1995; Whitmore, 1976; Zimmerlin, Wojtaszek & Bolwell, 1994) and caffeate (Rasmussen et al., 1995), such studies are in the minority. In addition, peroxidases tend to have lower affinities for many artificial substrates than for wall-related phenolics; for example, K_M values for pyrogallol or guaiacol of 2–7 mM (Kwak et al., 1995; Zimmerlin et al., 1994) compare with values of 20–460 μM for coniferyl alcohol, *p*-coumaroyl esters and ferulic acid (Otter & Polle, 1997; Takahama & Oniki, 1997; Zimmerlin et al., 1994).

We present here a direct quantitative comparison of the specificity of several peroxidases and laccases on a range of cell wall-related phenolic substrates.

2. Results and discussion

2.1. Internal standardisation

For the analysis of phenylpropanoid reaction products, methyl benzoate fulfilled all the requirements of an internal standard: it is chemically similar to the analytes, it was stable and unreactive to peroxidase and laccase under the conditions used, and it could be completely resolved from all substrates and products (Snyder & Kirkland, 1979). Addition of up to 6 mM methyl benzoate did not affect the oxidation of guaiacol by any of the peroxidases or laccases used in this study when assayed spectrophotometrically (results not shown).

2.2. Action of peroxidases and laccases on cell wall-related substrates

Each enzyme was assayed (Table 1) on a range of cell wall-related phenolic substrates at a standardised concentration (670 μM) well above reported K_M values of peroxidases for natural substrates (Otter & Polle, 1997; Takahama & Oniki, 1997). None of the peroxidases exhibited detectable oxidase activity on any of the six phenolic substrates tested. In the presence of H_2O_2, the basic iso-peroxidase was more active than the acidic isoenzymes, which are often proposed to be associated with lignification (Ferrer & Pedreño, 1992; Mäder, Nessel & Bopp, 1977). Laccases, when assayed at 100× higher enzyme concentrations (mg protein basis), had activities similar to or lower than those of the peroxidases. H_2O_2 was used at 670 μM whereas O_2

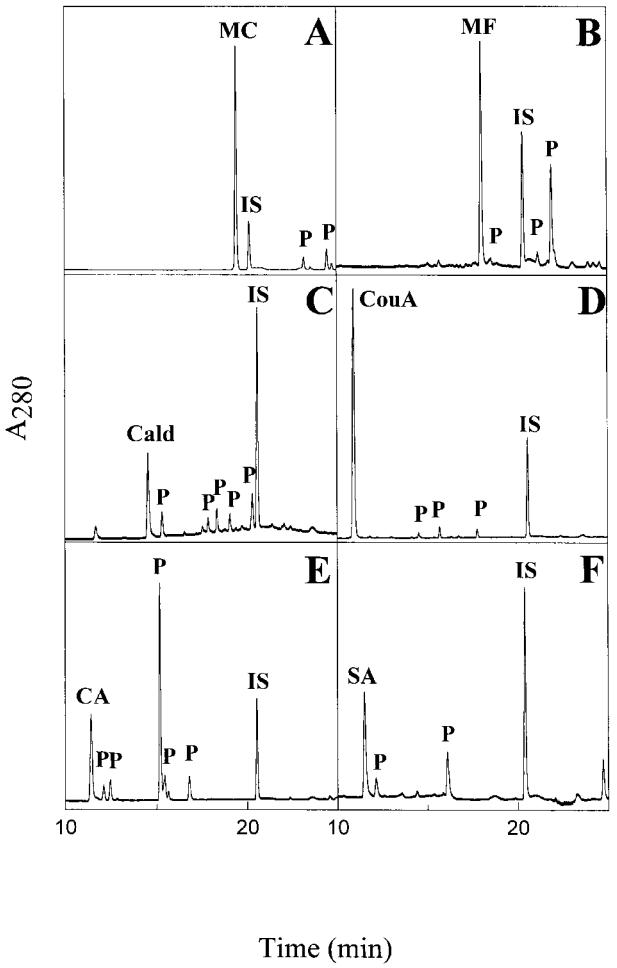

Fig. 1. HPLC profiles of oxidation products formed by the action of horseradish peroxidase A2 on (A) methyl *p*-coumarate (MC), (B) methyl ferulate (MF), (C) coniferaldehyde (Cald), (D) *p*-coumaryl alcohol (CouA), (E) coniferyl alcohol (CA) and (F) sinapyl alcohol (SA). IS = internal standard (methyl benzoate), P = oxidation product, not present in substrate or enzyme preparations alone. A_{280} is shown in arbitrary units.

was supplied as air-saturated buffer (which provides ~270 μM O_2 at 25°C); however, this difference in electron acceptor concentration between the peroxidases and the laccases is small compared with the difference in enzyme concentrations. Thus, under the conditions employed, the peroxidases had a far higher specific activity than the laccases.

All enzymes tested followed the same pattern of substrate preference. The reaction rates were in the order: sinapyl > coniferyl > *p*-coumaryl alcohols, and feruloyl > *p*-coumaroyl esters. These results are in accordance with the findings of Russell, Forrester and Chesson (1996) but disagree with those of Takahama (1995) and Ros Barceló, Muñoz and Sabater (1987), who reported that peroxidases oxidise coniferyl alcohol much faster than sinapyl alcohol, and *p*-coumarate faster than ferulate. None of the enzymes tested showed a strong discrimination between ester, aldehyde or alcohol substrates (e.g. methyl ferulate ≈ coniferaldehyde ≈ coniferyl alcohol; Table 1).

2.3. Product profiling

Peroxidase A2 oxidised each substrate to yield several products (Fig. 1). It has been reported that different peroxidases may yield different products (Zimmerlin et al., 1994); such a phenomenon could have profound effects on lignin synthesis. However, all the enzymes studied here yielded product profiles very similar to those shown in Fig. 1.

2.4. Conclusions

We have established a simple and effective method for assaying oxidase and peroxidase activities on diverse cell wall-related substrates. Most previously described routine assays for laccase and peroxidase have involved the measurement of either one arbitrarily selected product or an ill-defined mixture of products. The present HPLC-based assay monitors the disappearance of substrate and thus records total enzyme activity on the substrate of interest whether or not all the diverse reaction-products can be efficiently detected. Our assay therefore enables a reliable estimate of the ability of a given enzyme to oxidise a given phenolic component of the cell wall under the conditions tested.

Numerous iso-peroxidases and several phenol oxidases are present in plants. Although it remains possible that other isoforms, not yet tested, will turn out to exhibit different catalytic preferences or to yield different reaction products, all the peroxidases and laccases tested by us followed the same pattern, preferring dimethoxy > monomethoxy > nonmethoxy phenols. There was relatively little difference between alcohols, aldehydes and esters.

The basic peroxidase was considerably more active on all the substrates used in this study than the acidic peroxidases. Acidic peroxidases are often held to be those most directly involved in lignification (Ferrer & Pedreño, 1992; Mäder et al., 1977), although there is relatively little lignin in horseradish roots and thus the predominant physiological role of the acidic isoenzymes studied in the present work may be other than in lignification. At comparable enzyme concentrations (expressed either per mg of protein or per nmol of protein), and at the mildly acidic pH and submillimolar phenolic substrate concentrations likely to be encountered in vivo, laccases were much less effective than peroxidases. Several independent studies, using diverse methods, have indicated that H_2O_2 is produced in the plant apoplast in vivo (Frahry & Schopfer, 1998; Liu, Eriksson & Dean, 1995; Olson & Varner, 1993;

Patterson, Macrae & Ferguson, 1984; Schopfer, 1994; Warm & Laties, 1982) and in solutions mimicking apoplastic conditions in vitro (Elstner & Heupel, 1976; Frahry & Schopfer, 1998; Federico & Angelini, 1986; Halliwell, 1978; Ishida, Ookubo & Ono, 1987). If these studies are correct in implying that H_2O_2 is available in the cell wall in vivo, and/or if some peroxidases can under certain circumstances effectively act as 'oxidases' (Grambow & Langenbeck-Schwich, 1983), the results presented here support the view of Ros Barceló (1995) that peroxidase and not laccase has the major role in lignification.

3. Experimental

3.1. Materials

Methyl benzoate, ferulic and *p*-coumaric acids were from Aldrich, Poole, Dorset. Sinapyl alcohol was from Dr W.R. Russell (Rowett Research Institute, Aberdeen). Coniferaldehyde, horseradish peroxidases (acidic types VII [RZ 3.0] and type VIII [RZ 3.0]; basic type IX [RZ 3.2]), bovine liver catalase, and crude laccase from the fungus *Pyricularia oryzae* were purchased from Sigma, Poole, Dorset. A highly purified prepn of horseradish peroxidase A2 (acidic; RZ 3.67) (Østergaard, Abelskov, Mattsson & Welinder, 1996) was from Professor K. Welinder (University of Aalborg). Highly purified laccases from the white rot fungus *Rigidoporus lignosus* (Bonomo et al., 1998) and the trees *Acer pseudoplatanus* and *Populus euramericana* (Ranocha et al., 1999) were from Dr D. Goffner, Dr R. Sterjiades and Professor A.-M. Boudet (University of Toulouse). All laccase preparations were stored in Eppendorf tubes previously treated with 0.1% polylysine soln (Sigma) to prevent adsorption of the enzyme.

3.2. Synthesis of methyl ferulate, methyl p-coumarate and coniferyl and p-coumaryl alcohols

Methyl ferulate and methyl *p*-coumarate were prepd as described (Wallace & Fry, 1995). Ethyl ferulate and ethyl *p*-coumarate were prepd by the same method using EtOH instead of MeOH. Ethyl ferulate and ethyl *p*-coumarate (5 g) were dissolved in 150 ml dry Et_2O and stirred under a stream of N_2 at $-10°C$. $LiAlH_4$ (1.5 g) in 100 ml dry Et_2O was then slowly dripped into the soln, over 1 h, to reduce the ester to the corresponding alcohol. The mixture was stirred for 18 h at 0°C. H_2O was slowly added to the mixture until all the $LiAlH_4$ was destroyed. The Et_2O phase was then extracted 3× with satd aq. NaCl, dried with Na_2SO_4 and evapd to dryness. The alcohols were recrystallised from $CHCl_3$.

3.3. Effect of methyl benzoate on peroxidase activity

To 0.5 ml of 30 mM guaiacol, with and without 18 mM methyl benzoate, in 50 mM P_i buffer (pH 6.8), was added 0.5 ml 5 mM H_2O_2 and 0.5 ml of an appropriate concentration of enzyme soln. The reaction was monitored by A_{436} for 5 min.

3.4. HPLC assays

3.4.1. Peroxidase

To 20 μl of 2 mM phenolic substrate (in 50 mM succinate (Na^+) buffer, pH 6.0, containing 6 mM methyl benzoate), was added 20 μl of 2 mM H_2O_2 and 20 μl of peroxidase solution (typically 50 ng/ml). Controls containing H_2O in place of the enzyme or H_2O_2 were used for time zero. The mixture was incubated at 25°C for 30 min and the reaction stopped by the addition of 60 μl MeOH. All assays were performed in quadruplicate. The choice of pH for the assays was necessarily arbitrary because the precise natural pH of mature cell walls during lignification or other phenolic cross-linking is unknown. A value of 6.0 was selected bearing in mind that the mature cell wall is not likely to be as acidic as the rapidly growing wall (Schopfer, 1993).

3.4.2. Laccase

The assay was as above but the enzyme solns added typically contained 10 μg/ml, and 20 μl of a 5 mg/ml soln of catalase was added instead of the H_2O_2.

3.5. Product profiling

HPLC was performed on a 25 × 0.46 cm column of Spherisorb 5ODS2 using a linear 30-min gradient (0–100%) of H_2O/BuOH/HOAc (98.3:1.2:0.5) to MeCN/BuOH/HOAc (98.3:1.2:0.5) with detection by A_{280}.

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