PERGAMON

Phytochemistry 50 (1999) 711–717

PHYTOCHEMISTRY

Diferulate and lignin formation is related to biochemical differences of wall-bound peroxidases

Luis F. González[a,*], M. Cecilia Rojas[a], Francisco J. Perez[b]

[a]*Departamento de Química, Facultad De Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile*
[b]*Departamento de Ciencias Ecológicas, Facultad De Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile*

Received 29 December 1997

Abstract

Purified cell walls from oat coleoptiles contain ionically and covalently bound peroxidase activity, which correspond to 0.6% of the total peroxidase activity in the coleoptile. Ionically wall-bound peroxidases showed a 2–3-fold higher efficacy than peroxidases in the covalent fraction, in the use of H_2O_2 and phenolic substrates that are precursors of diferulate bridges and lignin. The NADH oxidase activity in both fractions was effectively enhanced by *p*-coumaric acid and the ionic fraction showed a higher efficacy over the covalent one for NADH utilization in the presence of this phenol. Moreover, the isoelectrofocusing pattern revealed marked differences in isoform composition for ionically and covalently bound wall peroxidases. A cationic group of isoperoxidases (pI ~ 9.6) was present only in the ionic fraction while the covalent fraction was enriched with anionic forms (pI ~ 4.0–6.5). In excised coleoptiles incubated for 24 h, the ionically wall-bound peroxidase activity increased by 50% over covalently bound activity for 4 h of incubation. The increase of peroxidase activity preceded the accumulation of diferulic acid and lignin in oat cell walls. Thus, the evidence here reported suggest a possible functional difference of peroxidase wall fractions studied related to diferulate and lignin synthesis in oat coleoptiles.

Keywords: *Avena sativa*; Poaceae; oat; enzymology; peroxidase; diferulic acid; lignin

1. Introduction

Peroxidases (EC 1.11.1.7) are haemoproteins that catalyse the oxidation of a wide variety of substrates using H_2O_2. In plants, much of the peroxidase activity is present in the cell wall of young tissue and of lignifying parts (McDougall, 1993; Li, McClure, & Hagerman, 1989), where it is required to oxidize cinnamyl alcohol precursors to phenoxy radicals which polymerize to form macromolecular lignin (Lewis & Yamamoto, 1990). Moreover, peroxidases are thought to generate *in muro* the H_2O_2 required in the phenolic polymerization reaction, through the oxidation of NADH with molecular oxygen (Elstner & Heupel, 1976; Halliwell, 1978).

Peroxidases have been implicated in the limitation of plant growth because an inverse correlation has been found between enzymatic activity and growth rates at different ages of seedlings and in different regions along the plant axis (Zheng & Van Huystee, 1992; Golderg, Imberty, & Chu-Ba, 1986; MacAdam, Nelson, & Sharp, 1992). This cessation of growth might result from cell wall tightening processes mediated by peroxidase, including formation and deposition of lignin as well as the cross-linking of ferulate residues bound to cell wall hemicellulosic polymers (Fry, 1986; Iiyama, Lam, & Stone, 1994).

In the cell wall, peroxidases can be found either bound by ionic interactions or more tightly linked being released only by enzymatic digestion of the cell wall components (i.e. covalently bound) (Ros Barceló, Muñoz, & Sabater, 1987). Also, various peroxidase isoforms that differ in their electrophoretic mobilities have been described in this compartment (Ros Barceló et al., 1987; Van Huystee, 1987; McDougall, 1992). In spite of intense investigation on plant peroxidases, the functional importance of the different cell wall-bound peroxidases is unknown. In the present report, we show isoenzymatic and kinetic differences between ionically and covalently bound wall peroxidases from oat coleoptiles, both in their ability to peroxidize natural phenolic cell wall components and in NADH oxidation. Furthermore, we found a higher accumulation of ionically wall-bound peroxidases than covalent ones as diferulate (DFA) and lignin were

* Corresponding author.

0031-9422/99/$ - see front matter
PII: S0031-9422(98)00611-6

deposited on cell walls. Our findings suggest that peroxidases contained in the wall fractions studied are functionally different and that ionically bound peroxidases could be more relevant in lignin and DFA synthesis, i.e. in reactions that decrease cell wall plasticity and therefore cellular elongation.

2. Results and discussion

2.1. Preparation of cell walls and cell wall-bound peroxidase activity

A cell wall fraction from oat coleoptiles free of contamination with other subcellular components was obtained after five washes of the crude cell wall pellet (1000*g*, 5 min) with a buffered solution of the non-ionic detergent Triton X-100. The purity of the cell wall fragments obtained was evidenced by the absence of enzymatic activities of biochemical markers for mitochondria (cytochrome *c* oxidase, EC 1.9.3.1), endoplasmic reticulum (NADH-cytochrome *c* reductase, EC 1.6.99.3) and plasma membrane (AMP-nucleotidase, EC 3.1.3.5). Also, none of these enzymatic activities were detected in a concentrated and dialyzed fraction containing protein solubilised from the purified cell wall fragments. Thus, any additional peroxidase activity released from our purified cell wall pellet is mainly, if not exclusively, extraprotoplasmic in nature.

Treatment of the cell wall pellet with 1 M KCl and then with cellulase plus pectinase, rendered equivalent amounts of ionically and covalently wall-bound peroxidase activity [15.8–22.0 μkat/l per g fr. wt of tissue, respectively, measured with *o*-phenylenediamine (*o*-PDA) as substrate], and from this point, these pools of peroxidase will be referred to as ionic and covalent fractions. No additional peroxidase activity was present in the remnant cell wall pellet. The two cell wall fractions together represent 0.6% of total peroxidase activity in the oat coleoptile, which was stable for at least three weeks at -20°C.

2.2. Kinetic properties of cell wall-bound peroxidases

Peroxidases contained in the ionic and covalent wall fractions oxidized several precursors of wall phenolic polymers in the presence of H_2O_2, such as coniferyl alcohol (CA), *trans* (*E*) ferulic acid (*E*-FA), and *p*-coumaric acid (*p*-CA). No substrate oxidation was detected in the absence of H_2O_2. Maximum rates (V_{max}) and apparent half saturation substrate concentration (K_m) were calculated from the respective saturation curves at a fixed level of H_2O_2 (Table 1). Because of the presence of cellulase and pectinase in the covalent fraction, kinetic data is reported on a fresh weight basis. The ionic and covalent fractions showed a marked substrate specificity towards CA relative to *E*-FA and *p*-CA, as evidenced for high values of V_{max}/K_m and V_{max} (Table 1). However, the ionic fraction utilized *E*-FA and CA with a higher utilization efficacy (V_{max}/K_m) than the covalent one (3.2- and 2.06-fold, respectively) (Table 1). When H_2O_2 was assayed as substrate in the presence of guaiacol, the utilization efficacy was 1.7-fold higher in the ionic fraction than the covalent fraction. The respective apparent K_m and V_{max} values for the H_2O_2 utilization were 102 μM and 3.87 μkat/l, for the ionic fraction and 187 μM and 4.09 μkat/l for the covalent fraction. As shown below, *E*-FA is present in cell walls from oat coleoptile (and probably also the alcoholic form, CA). Therefore, these results suggest that peroxidases in the ionic fraction could have a more relevant role than those in the covalent fraction in the oxidative polymerization of phenolics rendering DFA bridges or lignin.

Ionic and covalent oat cell wall fractions also catalyze oxidation of NADH with molecular oxygen, a reaction that has been reported to generate H_2O_2 (Elstner et al., 1976; Halliwell, 1978; Mäder & Amberg-Fisher, 1982) which is needed for the oxidative polymerization of phenolics at the cell wall level. NADH oxidation by our wall fractions was catalyzed by peroxidases since it was inhibited by KCN, an inactivator of haemoproteins, by several free radical scavengers (quercetin, gentisic acid or ascorbic acid) and by superoxide dismutase, all being efficient inhibitors of peroxidases (Elstner et al., 1976; Halliwell, 1978; Brouwer, van Valen, Day, & Lambers, 1986) (results not shown). In the absence of effectors, the rate of NADH oxidation by ionic and covalent cell wall fractions is low compared to the rate of peroxidase catalyzed reactions shown in Table 1 (*ca.* 0.64×10^{-2} μkat/l per g fr. wt); however, this reaction was considerably stimulated if hydroxycinnamic acids were added to the reaction mixture. Thus, when the NADH reaction was measured in the presence of *p*-CA (70 μM), the ionic fraction showed an utilization efficacy (V_{max}/K_m) of 1.7-fold higher than the covalent fraction. The respective apparent K_m and V_{max} values for the NADH utilization were 87 μM and 0.60 μkat/l for the ionic fraction and 201 μM and 0.81 μkat/l for the covalent fraction. So then, the supply of H_2O_2 in the cell wall, can be effective and preferentially promoted by ionically bound wall peroxidases through NADH oxidation stimulated by *p*-CA. In fact, we have detected esterified *p*-CA at levels of 0.1 μg per mg cell wall in 6 day old etiolated oat coleoptiles.

2.3. Isoenzymatic pattern on isoelectrofocusing

When oat cell wall-bound peroxidases were characterized for isoperoxidase forms by isoelectrofocusing in a pH range of 3 to 10, different patterns of activity were obtained for the ionic and covalent fractions (Figure 1). The covalent fraction showed mainly a group of neutral (pI, *ca.* 6.0), anionic (pI, 4.6) and cationic isoforms

Table 1
Apparent kinetic parameters in the oxidation of phenolic substrates by ionically and covalently cell wall-bound peroxidases

Substrate	Parameter	Ionic wall peroxidases	Covalent wall peroxidases
Ferulic acid	V_{max} (μkat/l)	0.34±0.03	0.35±0.05
	K_m (μM)	15.0±3.2	49.6±1.3
	$V_{max}/K_m \times 10^2$ (s^{-1})	2.25	0.70
p-Coumaric acid	V_{max} (μkat/l)	0.15±0.01	0.40±0.01
	K_m (μM)	54.9±4.3	117.2±0.12
	$V_{max}/K_m \times 10^2$ (s^{-1})	0.27	0.33
Coniferyl alcohol	V_{max} (μkat/l)	5.38±0.4	4.13±0.16
	K_m (μM)	42.0±5.0	66.5±4.8
	$V_{max}/K_m \times 10^2$ (s^{-1})	12.38	6.22

Assay conditions as described in Section 3 with 0.5 mM H_2O_2. Rate values are normalized per gram of fresh tissue.

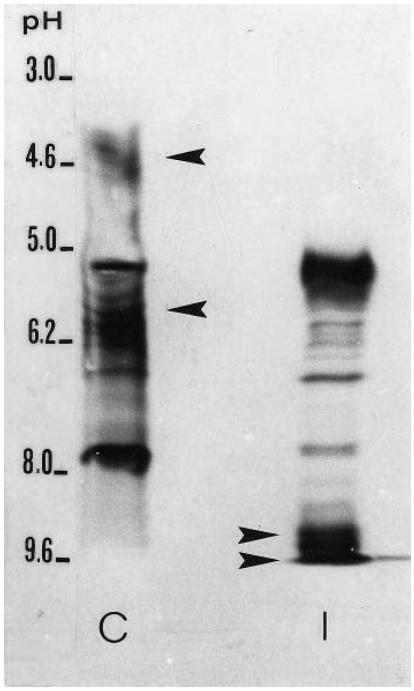

Fig. 1. Isoenzyme pattern of ionically (I) and covalently (C) cell wall-bound peroxidases on isoelectrofocusing in a 3–10 pH gradient. Stained with 4-methoxy-1-naphthol and H_2O_2.

(pI, 8.0), whereas acidic (pI, *ca.* 5.0) and strongly cationic isoforms (pI, 9.6) were present in the ionic fraction. The extreme cationic band present in the ionic fraction (pI, 9.6) was absent or negligible in the covalent fraction, even loading the gel with 3-fold higher peroxidase activity than the ionic fraction. This isoelectrofocusing pattern of peroxidase activity was observed when gels were stained either with 4-methoxy-1-naphthol, 4-chloro-1-naphthol or *o*-PDA in the presence of H_2O_2. The differences in isoperoxidase pattern of the wall fractions, were not due to partial deglycosylation of the peroxidases in the covalent fraction through the action of glycanases present in the cellulase and pectinase preparations, since the kinetic constants and isoperoxidase patterns of the ionic fraction were not altered by treatment with cellulase and pectinase for 12 h. Moreover, the cellulase and pectinase preparations did not show any peroxidase activity. In the monocot *Festuca arundinacea* Schreb., cationic isoforms have been related to the cell wall tightening process (MacAdam, Sharp, & Nelson, 1992) and in peanut seedlings, inhibition of cationic isoforms of cell wall resulted in an increase of hypocotyl elongation (Zheng et al., 1992). Positively charged cationic isoforms could be attached to the negatively charged matrix of cell wall and hence effectively oxidize *E*-FA to render DFA bridges and covalent cross-linking of wall polymers (Fry, 1986; MacAdam et al., 1992). Thus, the abundance of extreme cationic isoforms in the ionic fraction, reported here, also suggests an important role related to cell wall tightening for ionically bound wall peroxidases in etiolated oat coleoptiles.

2.4. Peroxidase activity and phenolic accumulation in cell walls from excised coleoptiles

Peroxidase activity from the ionic and covalent fractions increased when excised 6 day old coleoptiles were incubated in aerated Mes buffer (pH 5.8) for 24 h (Figure 2). After 4.5 h of incubation, the increase of peroxidase activity in the ionic fraction was 50–60% higher than in the covalent fraction (Fig. 2). Similar behavior was observed using NADH or *o*-PDA as peroxidase substrates. DFA, *E*-FA and lignin also showed a pronounced accumulation in the cell wall of excised coleoptiles, but the activity increase in the ionic fraction preceded the increase in wall phenolics (Fig. 3 and not shown). The peroxidase activity in the ionic fraction levelled off around 10 h and was kept constant until 24 h incubation, when the maximum DFA and lignin levels accumulated in the cell wall (Figures 2–3). The level of DFA accumulated at a higher rate than *E*-FA as evidenced by the decrease of the *E*-FA/DFA ratio (Fig. 4). These results suggest that DFA and lignin synthesis are preferentially associated to ionically bound wall peroxidase activity in

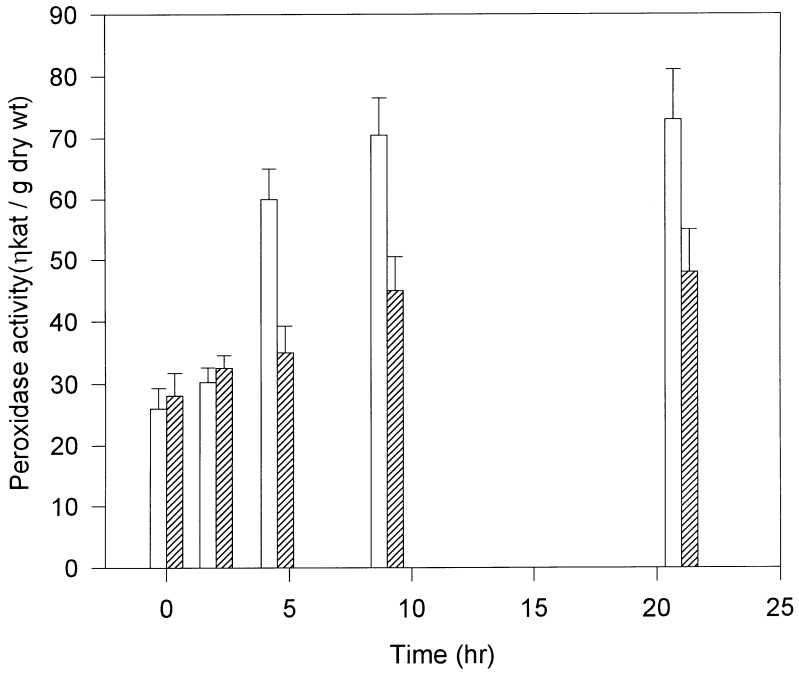

Fig. 2. Peroxidase activity in ionic (blank area) and covalent (diagonally striped area) wall fractions at different times after excision of oat coleoptiles. Peroxidase activity was assayed with coniferyl alcohol as described in Section 3. Data are means ± SE from three independent replicates.

oat coleoptiles, supporting our previous proposal based on both kinetic and isoenzyme differences.

It has been proposed that dehydrodimer formation in primary cell walls is limited by H_2O_2 production rather than by abundance of wall-bound peroxidases or wall feruloylation (Grabber, Hatfield, Ralph, Zon, & Amrhein 1995). However, our results indicate that the highest induction of ionically bound peroxidase should also be functionally important in DFA or lignin formation, because a more effective H_2O_2 supply in the cell wall is associated with this fraction by NADH oxidation, as stated above. In agreement with our results, in oat leaves during the hypersensitive response to *Puccinia coronata* f.sp. *avenae*, an increase in ionically-bound cell wall peroxidase activity has been observed together with an increase in DFA levels (Ikegawa, Mayama, Nakayashiki, & Kato, 1996).

The plant growth process is dependent on wall extensibility, which is related inversely to the ester-linked *E*-FA and DFA levels in oat and rice coleoptiles (Kamisaka, Takeda, Takahashiki, & Shibata, 1990; Tan, Hoson, Masuda, & Kamisaka, 1992; 1991). Thus, the enhancement of ionically wall-bound peroxidase activity associated with the accumulation of wall phenolics in excised coleoptiles here reported, suggests a possible role for this kind of wall peroxidase in the growth limitation through phenolic cross-linking. Indeed, it has been reported that ionically wall-bound peroxidase activity increased in slower growing segments along etiolated mung bean hypocotyls (Golderg et al., 1986). This correlation was not observed with peroxidase activity present in the covalent wall fraction (Golderg et al., 1986). However, further work is needed to bring this idea forward.

3. Experimental

3.1. Plant material and coleoptile incubations

Oat seeds (*Avena sativa* L. cv. Nehuen) were grown on vermiculite at 22–25°C in the darkness. After six days, coleoptiles were excised 3 mm under the tip in segments of 1.0–1.5 cm and used for cell wall preparations. In experiments on phenolics and peroxidase activity accumulation in cell walls, 1.5–3.0 g of the excised coleoptiles were incubated for 24 h in aerated plastic tubes and shaken gently with 25 ml of 5 mM Mes, pH 5.8. The tubes were kept in the dark with the soln renewed each 6 h. After each time, the coleoptiles were processed for the cell wall preparation and peroxidase or phenolic extraction as described in Sections 3.2, 3.3 and 3.4.

3.2. Extraction and purification of cell walls for enzymatic extraction

Oat coleoptiles were ground with a mortar and pestle in 50 mM Tris buffer, pH 7.2 containing 0.1 mM PMSF, 5% insoluble PVPP, 5 mM $MgCl_2$ and sand (5g/100 g fw). The homogenate was filtered through gauze and centrifuged at 1000*g* for 5 min. The ppt. was washed ×5 by resuspension in 25 ml of 50 mM Tris buffer pH 7.2 containing 1% Triton X-100 (w/v) with centrifugation at

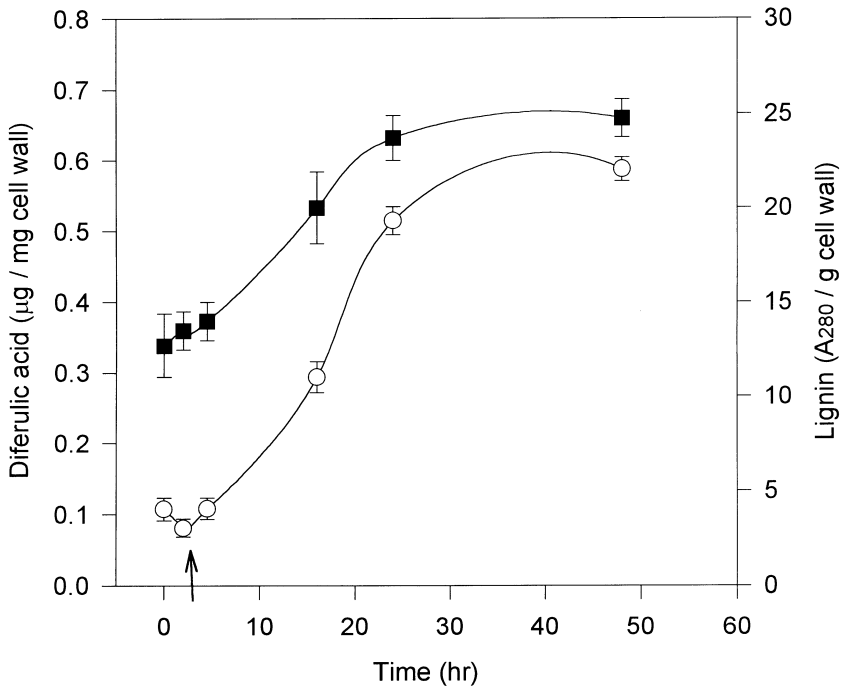

Fig. 3. Accumulation of lignin (■) and diferulic acid (○) in oat cell walls at different times after excision of the coleoptiles. Arrow indicates the start of increase of ionically bound peroxidase activity described in Fig. 2. Data are mean ± SE from three independent replicates.

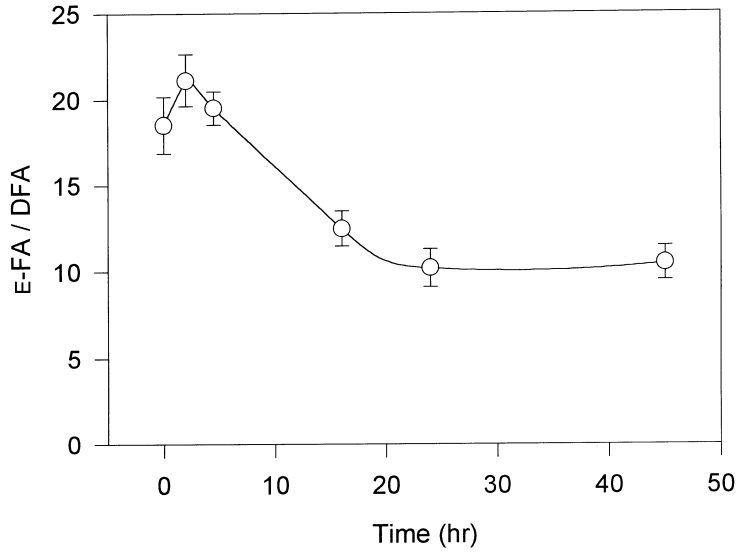

Fig. 4. Ferulic (*E*-FA) to diferulic acid (DFA) ratio in oat coleoptiles cell walls at different times after excision. Data are means ± SE from three independent replicates.

2000*g* for 5 min each time. The resultant pellet was washed × 3 with the same buffer without detergent giving a pellet considered to be a purified cell wall fraction. The assays for cytochrome *c* oxidase, antimycin A–insensitive NADH–cytochrome *c* reductase and 5′-AMP nucleotidase, as enzymatic markers, were carried out as described in (Briskin, Leonard, & Hodges, 1987).

3.3. Extraction of cell wall-bound peroxidases

The purified cell wall fraction was incubated in 1 M KCl on ice with gentle stirring. After 4 h the mixture was centrifuged at 5000*g* for 10 min and the supernatant was called the ionic cell wall fraction. The remnant cell walls after four washes with 1 M KCl, were resuspended in

0.1 M acetate buffer pH 5.0 containing 25 U/ml of cellulase [EC 3.2.1.4; 1,4-(1,3-1,4)-β-D-glucan-4-glucanohydrolase] and 3 U/ml of pectinase [EC 3.2.1.15; poly-(1,4-α-D-galacturonide glycanohydrolase], both from *Aspergillus niger* (Sigma). One unit of pectinase or cellulase is defined as the enzyme amount required to liberate 1.0 μmol of galacturonic acid equivalents from polygalacturonic per min at pH 4.0 at 25° or 1.0 μmol of glucose equivalents from cellulose per hr at pH 5.0 at 37°, respectively. Also, as protease inhibitors, 1 mM PMSF, 2 mM benzamidine and 1 μM pepstatin A were included in the reaction mixture. Incubation was carried out for 18 h with shaking at 25° and the soln was centrifuged at 5000*g* for 10 min. The supernatant was designated as the covalent cell wall fraction.

3.4. Preparation of cell walls for phenolic extraction

All the procedures were carried out under fluorescent light. Oat coleoptiles were ground in a mortar with liquid nitrogen. The resulting fine powder was suspended and transferred to Eppendorf vials and stirred vigorously for 15 min in 1.5 ml of 2% (w/v) Triton X-100, 50 mM azide and 1 M KCl. After centrifugation (14 000 rpm for 5 min in a bench-top centrifuge), the pellet was consecutively treated for 15 min (followed by centrifugation as in Section 3.3) with MeOH (twice), H_2O, 1% (w/v) SDS, H_2O (twice), EtOH, MeOH/$CHCl_3$ (1:1 v/v), EtOH and H_2O (twice). The remnant insoluble material was incubated with 5 ml α-amylase (1 mg/ml) in 50 mM Tris pH 7.0 for 6 h and then with 5 ml pronase E (0.5 mg/ml) in the same buffer overnight. Finally, cell walls were washed with H_2O, MeOH, MeOH/$CHCl_3$ (1:1, v/v) and dried at 40°.

3.5. Extraction and determination of wall-bound ferulic and diferulic acids

The phenolics ester-linked to wall polysaccharides were liberated by alkaline hydrolysis. Dried cell walls (20–40 mg) were treated at room temp. for 20 h by shaking with 2 M NaOH (5 ml degassed) in vials sealed under N_2. A soln of *E*-3,4-dihydroxi-cinnamic acid (caffeic acid) was added to the vials as internal standard. After hydrolysis, the mixture was centrifuged and the alkaline fraction was acidified to pH 2.0 with HCl and then extracted with Et_2O (4 × 10 ml). The combined ethereal extracts were vacuum-dried and stored in the dark. Then the samples were analyzed by HPLC on a Waters model 600E system controller fitted with a Waters Ultra Wisp model 715 sample processor and a photodiode array detector (Waters, model 990$^+$). The HPLC method was carried out with detection at 310 nm using a analytical reversed phase Waters Nova-Pak C_{18} (150 × 3.9 mm i.d. stainless steel) column. The flow rate was 0.9 ml/min and the elution was performed with a MeCN convex gradient against 3% HOAc. After sample injection, the MeCN was kept at 5% for 5 min, increased to 10% in 15 min and then to 20% in an additional 25 min. The proportion of MeCN was held at 20% for 10 min, then increased linearly to 50% in 10 min and after 2 min the MeCN proportion was brought back to 5% for an additional 13 min. The identity of *E*-FA and DFA were carried out by comparison with the authentic standard (retention time and UV spectra), whereas the quantity of each phenolic was calculated according to the yield of the internal standard and the calibration curve obtained with the respective standard.

3.6. Determination of lignin

The content of lignin was assayed in the saponified cell walls by the method of Iiyama (Iiyama & Wallis, 1990). The dried cell walls (5–10 mg) were dissolved in 2 ml of 25% w/w acetyl bromide in glacial acetic acid and 80 μl of $HClO_4$ in a sealed tube at 70° for 30 min. The tubes were shaken gently at 10 min intervals. After complete digestion, the samples were cooled on ice and an aliquot was transferred to glass tubes and diluted with 2 M NaOH (1 ml) and HOAc (4 ml). The lignin content was estimated measuring the absorbance of the samples at 280 nm against a blank soln which was run in conjunction with the samples. Under the conditions of this acetyl bromide procedure, the contribution in the *A* at 280 nm of ether-linked phenolics to lignin and hemicellulose remnants after saponification, is negligible as established in Ref. (Iiyama et al., 1990).

3.7. Peroxidase assays

All assays were carried out in 1 ml cuvettes in a Shimadzu UV-120 spectrophotometer. Typically peroxidase activity was assayed with 5 mM of *o*-PDA or 0.1 mM CA and 0.5 mM H_2O_2 in citrate buffer (0.1 M, pH 4.5) at 24°. The absorbance increase due to *o*-PDA oxidation was monitored at 450 nm ($\varepsilon = 1.05\ mM^{-1}\ cm^{-1}$). The molar extinction coefficient for *o*-PDA product was obtained from the A_{450} of the complete oxidation of *o*-PDA in the presence of a excess of horseradish peroxidase (type X, Sigma) and 30 mM H_2O_2 in citrate buffer (0.1 M, pH 4.5). The assays with *E*-FA, *p*-CA or CA were carried out in the same buffer with 0.5 mM H_2O_2 and the absorbance decrease measured at 310 nm ($\varepsilon = 16\ mM^{-1}\ cm^{-1}$), 286 nm ($\varepsilon = 19\ mM^{-1}\ cm^{-1}$) and 260 nm ($\varepsilon = 2.2\ mM^{-1}\ cm^{-1}$), respectively. The catalytic efficacy for H_2O_2 was assayed with guaiacol 5 mM as reductant in K-Pi buffer (0.1 M, pH 6.0) following the increase in absorbance at 470 nm ($\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$). NADH oxidase activity was measured by monitoring the decrease in absorbance at 340 nm ($\varepsilon = 6.02\ mM^{-1}\ cm^{-1}$). The assay mixture contained 50 μM $MnSO_4$, 70 μM *p*-CA and 0.16 mM NADH in K-Pi buffer (0.1 M, pH 7.0). Rates were calculated from the slope of the progress curves and K_m and V_{max}

values were obtained fitting the data to hyperbolic functions by non-linear regression analysis.

3.8. Isoelectrofocusing

This technique was carried out in a pH range of 3–10 with 2% Pharmalyte ampholytes (Pharmacia), using a Bio-Rad 111-mini IEF chamber under the manufacturer specifications. Prior to the run, wall fraction samples were desalted and concentrated in centricon tubes (cut off 10,000, from Amicon®) and 16.7–25 μkat of peroxidase activity measured with *o*-PDA, were applied to the gels. After the run, the gels were stained with 5 mM 4-methoxy-1-naphthol and 0.04% (v/v) H_2O_2 in 0.1 M citrate buffer pH 4.5.

Acknowledgements

The authors wish to thank especially Dr Norman G. Lewis and Dr Laurence B. Davin (Washington State University) for the facilities given to L. F. G. to carry out the phenolic analyses at their laboratory and for financial assistance through the E_{isig}-TODE foundation. A sample of authentic DFA was kindly synthesized by Dr Zhi-Quiang Xia. L. F. G. is grateful to FONDECYT for financial support through 2950050/95 research grant.

References

Briskin, D., Leonard, R., & Hodges, T. (1987). *Methods in Enzymology*, *148*, 542.

Brouwer, K., van Valen, T., Day, D., & Lambers, H. (1986). *Plant Physiology*, *82*, 236.

Elstner, E. F., & Heupel, A. (1976). *Planta*, *130*, 175.

Fry, S. (1986). *Annual Review of Plant Physiology*, *37*, 165.

Golderg, R., Imberty, A., & Chu-Ba, J. (1986). *Phytochemistry*, *25*, 1271.

Grabber, J. H., Hatfield, R. D., Ralph, J., Zon, J., & Amrhein, N. (1995). *Phytochemistry*, *40*, 1077.

Halliwell, B. (1978). *Planta*, *140*, 81.

McDougall, G. (1993). *Journal of Plant Physiology*, *142*, 631.

Ikegawa, T., Mayama, S., Nakayashiki, H., & Kato, H. (1996). *Physiological and Molecular Plant Pathology*, *48*, 245.

Iiyama, K., & Wallis, A. F. (1990). *Journal of Science and Food Agriculture*, *51*, 145.

Iiyama, K., Lam, T. B.-T., & Stone, B. A. (1994). *Plant Physiology*, *104*, 315.

Kamisaka, S., Takeda, S., Takahashiki, K., & Shibata, K. (1990). *Physiologia Plantarum*, *78*, 1.

Li, Z.-C. H., McClure, J. W., & Hagerman, A. E. (1989). *Plant Physiology*, *90*, 185.

Lewis, N. G., & Yamamoto, E. (1990). *Annual Review Plant Physiology and Plant Molecular Biology*, *41*, 455.

Mäder, M., & Amberg-Fisher, V. (1982). *Plant Physiology*, *70*, 1128.

MacAdam, J. M., Nelson, C. J., & Sharp, R. E. (1992). *Plant Physiology*, *99*, 872.

MacAdam, J. M., Sharp, R. E., & Nelson, C. J. (1992). *Plant Physiology*, *99*, 879.

McDougall, G. (1992). *Phytochemistry*, *31*, 3385.

Ros Barceló, A., Muñoz, R., & Sabater, F. (1987). *Physiologia Plantarum*, *71*, 448.

Tan, K.-S., Hoson, T., Masuda, Y., & Kamisaka, S. (1992). *Plant Cell Physiology*, *33*, 103.

Tan, K.-S., Hoson, T., Masuda, Y., & Kamisaka, S. (1991). *Physiologia Plantarum*, *83*, 397.

Van Huystee, R. B. (1987). *Annual Review of Plant Physiology*, *38*, 205.

Zheng, X., & Van Huystee, R. B. (1992). *Plant Science*, *81*, 47.