Synthesis of dehydrogenation polymers of ferulic acid with high specificity by a purified cell-wall peroxidase from French bean (*Phaseolus vulgaris* L.)

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A cationic (pI8.3) wall-bound peroxidase has been purified to homogeneity from suspension-cultured cells of French bean (*Phaseolus vulgaris* L.). The enzyme was a glycoprotein and its M_r was 46000 as determined by SDS/PAGE and h.p.l.c. gel filtration. It was localized biochemically to microsomes and the cell wall, and the latter subcellular distribution was confirmed by immunogold techniques. The native enzyme showed absorption maxima at 403, 500 and 640 nm, with an RZ (A_{405}/A_{280}) of 3.3. The peroxidase oxidized guaiacol and natural phenolic acids. By desorption-chemical-ionization mass spectrometry the enzyme was found to oxidize the model compound, ferulic acid, into

dehydrodiferulic acid. Kinetics studies indicated an apparent K_m of $113.3 \pm 22.9 \mu\text{M}$ and a V_{max} of $144 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{nmol}^{-1}$ of protein at an H_2O_2 concentration of $100 \mu\text{M}$. In comparison with a second French-bean peroxidase (FBP) and horseradish peroxidase, as a model, it acted with a 6–10-fold higher specificity in this capacity. It is a member of the peroxidase superfamily of bacterial, fungal and plant haem proteins by virtue of its highly conserved amino acid sequence within the proximal and distal haem-binding sites. This is good evidence that this particular FBP may function in constructing covalent cross-linkages in the wall during development and response to pathogens.

INTRODUCTION

Plant peroxidases (donor: H_2O_2 oxidoreductases, EC 1.11.1.7) are a particularly well-studied group of enzymes which are ubiquitous in higher plants and have important functions during development and responses to stress (Gaspar et al., 1991; Edreva, 1991). The simultaneous occurrence of isoforms in the same plant is common, and anionic and cationic forms can be distinguished. They are characterized at the amino-acid-sequence level by several highly conserved sequences found within plant, fungal and bacterial peroxidases, but not among animal peroxidases (Welinder, 1992).

Peroxidases may contribute several different roles in the cell-wall construction and rheology. These include the possible intramolecular cross-linking of tyrosine residues in extensin and possible other wall glycoproteins, the coupling of hydroxycinnamic acids and *p*-hydroxybenzoic acid residues attached to pectins and certain xylans, lignin biosynthesis, lignin bonding to cell-wall glycoproteins and suberization (Gaspar et al., 1991). Of these, the most significant contribution to wall strength is lignin formation (Monties, 1989; Lewis and Yamamoto, 1990), and an anionic peroxidase associated with lignifying tissue from tobacco (*Nicotiana tabacum* L.) has been cloned and subjected to genetic manipulation (Lagrimini, 1991). Stress conditions also lead to the accumulation of phenolics in the wall, often in a highly localized way, which also requires the involvement of wall-bound peroxidase. Anionic peroxidases have been isolated, cloned and genetically manipulated to implicate these in a direct role in the localized suberization in response to wounding or pathogen attack (Kolattukudy et al., 1992). However, cationic peroxidases may also be wall-bound (Zheng and van Huystee, 1992) and may be subjected to modulation by stress-inducing molecules (Bruce and West, 1989).

We report here the isolation and characterization of a cationic peroxidase from French bean (*Phaseolus vulgaris* L.) capable of

the synthesis of diferulic acid *in vitro* with high specificity and which is also wall-bound. It is probably premature to equate only anionic peroxidases with phenylpropanoid metabolism in cell walls.

MATERIALS AND METHODS

Materials

Suspension cultures of French bean were derived and maintained as described by Dixon and Lamb (1979). Endoproteinase Lys-C was obtained from Boehringer, Lewes, East Sussex, U.K.

Preparation of wall-bound peroxidase

The purification procedure was carried out at 4°C . Suspension-cultured cells of French bean were homogenized in 100 mM Tris buffer, pH 7.2, containing 5 mM β -mercaptoethanol and 0.25 M sucrose. The cell-wall-enriched fraction was pelleted by centrifugation at $10000 g$ for 20 min. The pellet was then resuspended in 20 mM Tris, pH 7.2, containing 1 M NaCl and 1 mM each of MnCl_2 , CaCl_2 and MgCl_2 . After 2 h incubation the cell-wall debris was again pelleted ($10000 g$ for 20 min) and the supernatant was applied to a column (10 cm $\times$ 1 cm) of concanavalin A-Sepharose equilibrated with the same buffer. Bound proteins were eluted with column buffer containing 500 mM α -methylmannopyranoside. After concentration and dialysis against a 20 mM Tris/0.2 M NaCl buffer by ultracentrifugation using PM30 filters (Amicon, Stonehouse, Glos., U.K.), the eluted proteins were separated by size-exclusion h.p.l.c. [TSK G2000SW; 7.5 mm (internal diameter) $\times$ 60 cm; Anachem, Luton, Beds., U.K.] using a linear gradient of 0–0.5 M NaCl in 20 mM Tris buffer, pH 7.2, over 80 min at room temperature. Fractions were collected and analysed for peroxidase activity as described below. The volume was reduced by ultracentrifugation with a PM30 filter.

Abbreviations used: RZ, absorbance ratio (A_{405}/A_{280}); FBP, French-bean peroxidase; d.c.i.-m.s., desorption-chemical-ionization mass spectrometry.

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Determination of cell-wall localization

Cell-wall localization was determined biochemically and by immunolocalization. The cell-wall pellet was resuspended in 0.1 M sodium phosphate buffer, pH 7.8, and sequentially washed three times in the same buffer containing 0.1 % (v/v) Triton X-100 and once in the same buffer without Triton X-100 followed by two washes with 0.1 M sodium phosphate buffer, pH 7.8, containing 1 M NaCl. Microsomal fractions were subjected to a similar regime of washings. Pellets and supernatants were analysed for peroxidase activity and protein was determined by the BCA assay (Pierce, Chester, U.K.), because of the presence of detergent, and carbohydrate by the phenol/H₂SO₄ method.

In addition, immunogold localization was carried out on suspension-cultured cells of French bean. Cells were harvested by gentle centrifugation at 1000 *g* for 2 min and fixed in freshly prepared 4 % (w/v) paraformaldehyde/0.1 % (v/v) glutaraldehyde in 0.1 M Pipes buffer, pH 7.2, for 1 h. Fixed cells were collected by centrifugation at 1000 *g* for 2 min, washed with 0.1 M Pipes buffer, pH 7.2, and mixed with an equal volume of aq. 5 % (w/v) solution of sodium alginate. The resultant mixture was spread on the glass slide, covered with 0.05 M CaCl₂ in 0.1 M Pipes buffer, pH 7.2, and allowed to set for 15 min at room temperature. The alginate gel was cut to small pieces, which were subsequently dehydrated in a graded series of ethanol solutions and placed in a 1:1 mixture (v/v) of ethanol/LR White (London Resin Co., Woking, Surrey, U.K.) for 4 h. After overnight infiltration with pure LR White resin, the cells were embedded in gelatin capsules (size 00) and polymerized for 24 h at 50 °C. Ultrathin sections of bean cells were collected on nickel grids and immunostained. Immunogold localization was carried using anti-(bean 46000-*M_r* cationic peroxidase) serum as described by Smith et al. (1993), except that, as a secondary antibody, goat anti-rabbit IgG conjugated with colloidal gold (Biocell, Cardiff, U.K.; 15 nm particle size) was used without subsequent silver enhancement. Following thorough washing with distilled water the grids were counterstained with uranyl acetate and lead citrate and examined under Hitachi H600 electron microscope.

Spectrophotometric measurements

Enzymic assays of peroxidases were performed spectrophotometrically by monitoring the oxidation of guaiacol at 436 nm (ϵ 25.5 mM⁻¹·cm⁻¹). For the measurement of the kinetic parameters of ferulic acid oxidation, the consumption of ferulic acid (10–200 μ M) was monitored at 310 nm (ϵ 16 mM⁻¹·cm⁻¹) during the first 15 s of the reaction (less than 10 % transformed). The peroxidase activity was determined in 1 ml of 0.1 M acetate buffer, pH 4.4, containing 10 mM guaiacol or 1 mM ferulic acid, and 0.1 mM H₂O₂ as substrates. For routine analysis and comparative measurement, ferulic acid oxidation can be monitored by the increase of absorbance at 380 nm. The assays were carried out at room temperature. Protein was determined using the Bio-Rad microassay system.

Liquid-phase isoelectric focusing

Purified peroxidase was subjected to liquid-phase isoelectric focusing using a Rotofor (Bio-Rad, Hemel Hempstead, Herts., U.K.) system. The sample was diluted to 50 ml with water and 1 ml (2 %) Biolyte solution (pH range 3–10; 40 %, w/v) was added and the solution loaded onto the Rotofor cell for focusing without further treatment. Focusing in the Rotofor cell was carried out at 12 W constant power for 3 h at 4 °C. The initial

conditions were 600 V and 20 mA. At equilibrium, the values were 2500 V and 4 mA. Fractions were collected and their pH values measured. Peroxidase was detected enzymically using guaiacol as substrate as described above.

Gel electrophoresis

SDS/PAGE was carried out as described by Laemmli (1970) on 10 % (w/v) acrylamide discontinuous gels. Proteins were detected by silver staining.

Preparation and analysis of peptides

A 500 μ g portion of peroxidase, purified to homogeneity, was digested with 5 μ g of endoproteinase Lys-C for 16 h. Peptides were separated by microbore h.p.l.c. on an Applied Biosystems (Foster City, CA, U.S.A.) model 140 system. The separated peptides were subjected without further treatment to amino acid sequencing on a pulsed-liquid sequencer (model 477; Applied Biosystems).

Product analysis

Ferulic acid (1 mM) was allowed to react with enzyme and H₂O₂ in excess for 30 min in water. The reaction was stopped by immersing in liquid N₂. After freeze-drying, the products were dissolved in ethanol and subjected to desorption-chemical-ionization mass spectrometry (d.c.i.-m.s.). D.c.i.-m.s. was performed on a VG Analytical ZAB, with ammonia as the d.c.i. gas, using a standard d.c.i. probe with platinum wire and a source temperature at 150 °C.

RESULTS

Purification of French-bean peroxidase (FBP)

Wall-bound peroxidase isoenzymes were purified in a four-step procedure from suspension-cultured French-bean cells (Table 1). Cells were homogenized and a wall-enriched pellet prepared. A simple salt wash removed much of the ferulic acid oxidase activity, and this could be extracted by passage through immobilized concanavalin A. Following desorption this activity could be resolved into two major peroxidase species of *M_r* 46000 (FBP1) and *M_r* 41000–42000 (FBP2) by size-exclusion h.p.l.c. utilizing a salt gradient, which was necessary to maintain solubility and to obtain an optimum separation of the proteins. These procedures led to a 60-fold purification of the higher-*M_r* peroxidase (Table 1), the preparation of which appeared to be homogeneous when analysed by SDS/PAGE and silver staining (Figure 1).

Ionic binding of peroxidases to cell walls

These two peroxidases were previously shown to be present in a microsomal preparation from similar cells where they were found to interfere with the purification of cytochrome P-450 proteins (Rodgers et al., 1993). However, they were in higher abundance in the cell-wall fraction (Tables 2 and 3). Both wall-enriched and microsomal fractions were subjected to a sequential series of extraction and the peroxidase activity determined in the pellet and the supernatant. Table 2 shows that, for the cell-wall-enriched pellet, following a first extraction with Triton X-100, which would remove solubilized membrane-bound enzyme, 80 % of the ferulic acid peroxidase activity remained in the particulate fraction. Subsequent extractions with Triton X-100 released very little of the activity. NaCl extraction of the Triton X-100-insoluble fraction then allowed solubilization of 77 % of the

Table 1 Purification of 46 000- M_r and 41 000–42 000- M_r peroxidases from French-bean-cell cultures

Purification of two peroxidases is shown for two substrates, guaiacol (measured spectrophotometrically at 436 nm) and ferulate (measured at 380 nm). Note that, although the RZ values as a measure of purity are high, the 46 000- M_r peroxidase shows relatively higher specificity for ferulic acid.

Step	Protein (mg)	Ferulate oxidase			Guaiacol oxidase			RZ
		Activity ($\Delta A_{380}/\text{min per mg}$)	Recovery (%)	Purification (fold)	Activity ($\Delta A_{436}/\text{min per mg}$)	Recovery (%)	Purification (fold)	
Homogenate	148.1	123.5	100	1	382.8	100	1	
10 000 g pellet	26.0	262.6	37.2	2.1	533.0	24.4	1.4	
NaCl extract	6.9	840.3	31.7	6.8	1826.3	22.2	4.8	0.28
Concanavalin A-bound	3.0	1871.7	30.7	15.1	3817.3	20.2	10.0	1.67
46 000- M_r FBP	0.5	7572.2	20.7	61.3	14 038.8	12.4	36.7	3.26
41 000–42 000- M_r FBP	0.3	914.5	1.5	7.4	20 304.3	10.7	53.0	3.09

Figure 1 SDS/PAGE analysis of FBP purification

Lane 1, M_r markers; lane 2, purified 41 000–42 000- M_r peroxidase; lane 3, purified 46 000- M_r peroxidase. A 10%-acrylamide gel was used.

remaining ionically-wall-bound peroxidases. This probably represents a relatively wall-enriched pellet which was carbohydrate-rich, indicating the presence of wall polysaccharides. In contrast, the bulk of the peroxidase activity was readily solubilized from

the microsomal fraction with detergent (Table 3). These properties were consistent with a cell-wall localization for both peroxidases, and their presence in the microsomal fraction representing the proportion being secreted.

Direct evidence for a wall localization was obtained by immunogold localization. The 46 000- M_r peroxidase has been immunolocalized previously to cell wall in sections of intact bean hypocotyl (Smith et al., 1993). Using the same antibody the peroxidase was immunolocalized in suspension-cultured cells, and Figure 2 shows the localization in the primary wall. Localization was particularly dense in the corners of cells associated in small clumps (Figure 2a). In other parts of the wall, localization was more intense towards the plasmalemma side (Figures 2b and 2c). Taken together, both sets of evidence indicate a significant proportion of the peroxidase as being cell-wall-localized.

Comparative properties of the peroxidases

Both enzymes showed high activity towards the universal substrate guaiacol. The K_m for the 46 000- M_r peroxidase was measured at 3 mM. However, there were found to be considerable differences with other substrates and in comparison with the model peroxidase from horseradish. The 46 000- M_r peroxidase showed much higher activity towards phenylpropanoids and their conjugates (ferulic and caffeic acids, chlorogenic acid), as

Table 2 Ferulate oxidase activity, protein and carbohydrate content of six successive supernatants and pellets obtained during the extraction of a 10 000 g pellet from 200 g of French-bean cells under standard conditions: cell-wall-enriched fraction

The first pellet refers to the original particulate fraction. Subsequent values refer to successive extractions, and for each pair the supernatant value depicts the material removed in each wash, and the pellet gives the amounts in the residual material before the next wash. '% Extraction' refers to the proportion of the enzyme activity arising in the wash relative to the activity in the previous pellet. Note the slight activation of the peroxidase on solubilization and the highest specific activity in the initial salt wash.

Treatment	Pellet			Supernatant			% Extraction
	$10^3 \times \text{Activity}$ ($\Delta A_{380} \cdot \text{min}^{-1}$)	Protein (mg)	Carbohydrate (mg)	$10^3 \times \text{Activity}$ ($\Delta A_{380} \cdot \text{min}^{-1}$)	Protein (mg)	Carbohydrate (mg)	
Phosphate buffer (0.1 M, pH 7.8)	595.0 $\pm$ 12.0	45	8.7				
+ Triton X-100 (1%)	461.1 $\pm$ 7.7	18	4.7	116.0 $\pm$ 13.6	23	4.3	19.5
+ Triton X-100 (1%)	402.8 $\pm$ 19.6	16	4.6	22.0 $\pm$ 9.5	2.7	0.4	4.7
+ Triton X-100 (1%)	348.6 $\pm$ 42.2	15	4.0	0	1.2	0.3	0
Phosphate buffer (0.1 M, pH 7.8)	343.9 $\pm$ 9.5	15	3.8	0	0.9	0.1	0
+ NaCl (1 M)	82.7 $\pm$ 4.1	12	3.6	265.9 $\pm$ 13.6	3.2	0.1	77.3
+ NaCl (1 M)	65.4 $\pm$ 7.7	10	3.6	25.6 $\pm$ 5.9	0.9	0.05	30.1

Table 3 Ferulate oxidase activity, protein and carbohydrate content of six successive supernatants and pellets obtained during the extraction of a 100 000 g pellet from 200 g of French-bean cells under standard conditions: microsomal fraction

The sequential extraction of this microsomal pellet was as described in Table 2. Abbreviation: nd, not detected.

Treatment	Pellet			Supernatant			% Extraction
	$10^3 \times \text{Activity}$ ($\Delta A_{380} \cdot \text{min}^{-1}$)	Protein (mg)	Carbohydrate (mg)	$10^3 \times \text{Activity}$ ($\Delta A_{380} \cdot \text{min}^{-1}$)	Protein (mg)	Carbohydrate (mg)	
Phosphate buffer (0.1 M, pH 7.8)	210.1 $\pm$ 11.0	50	8.5				
+ Triton X-100 (1%)	80.0 $\pm$ 7.7	24	2.0	120.0 $\pm$ 3.6	25	8.2	60
+ Triton X-100 (1%)	65.6 $\pm$ 6.5	15	1.2	23.4 $\pm$ 1.5	5.4	1.0	26.1
+ Triton X-100 (1%)	52.2 $\pm$ 3.3	10	0.8	11.9 $\pm$ 2.2	3.6	0.4	18.6
Phosphate buffer (0.1 M, pH 7.8)	41.8 $\pm$ 7.4	8.5	0.6	7.8 $\pm$ 1.2	0.8	nd	15.9
+ NaCl (1 M)	11.2 $\pm$ 2.1	4.0	0.6	36.6 $\pm$ 7.3	4.8	nd	76

Figure 2 Immunolocalization of 46000- M_r cationic peroxidase in suspension-cultured cells of French bean

Abbreviations: CW, cell wall; CYT, cytoplasm; ML, middle lamella; PW, primary wall. (a) Shows an interface between three cells; the highest abundance of immunogold particles was found in this area (magnification $\times 50\,000$; the scale bar represents $0.2\ \mu\text{m}$). (b) Typical level of labelling in primary cell wall (magnification $\times 80\,000$; the scale bar represents $0.1\ \mu\text{m}$). (c) Lower power of typical primary wall labelling (magnification $\times 25\,000$; the scale bar represents $0.5\ \mu\text{m}$). Arrowheads indicate examples of gold particles.

showed by the rapid decrease of their typical λ_{max} when incubated with peroxidase and H_2O_2 . Owing to the occurrence of non-enzymic side reactions with *o*-dihydrophenols and cinnamoyl alcohols, which makes detailed determination of kinetics difficult, ferulate oxidation was chosen as the model reaction and further analysed. K_m was determined for ferulate at $113.3 \pm 22.9\ \mu\text{M}$. This compares with values of $928 \pm 31\ \mu\text{M}$ and $629 \pm 27\ \mu\text{M}$ for the 42000/41000- M_r peroxidase and horseradish peroxidase respectively. The reaction catalysed for the more specific 46000- M_r peroxidase occurred with a pH optimum of 4.4. This reaction was inhibited 60 % by $2\ \mu\text{M}$ KCN when ferulic acid was at 1 mM and H_2O_2 at 0.1 mM.

Identification of reaction products

When the 46000- M_r peroxidase was incubated with ferulic acid

and H_2O_2 , polymeric metabolites were formed. Reaction products yielded a molecular ion and fragmentation pattern typical of dehydrodiferulic acid in d.c.i.-m.s. The main peaks in Figure 3 represent the ammoniated molecular ion ($m/z\ 404$), the protonated molecular ion ($[M+1]^+ = 387$), and the decarboxylated molecular ions ($m/z\ 360, 343$ or 299) of dehydrodiferulate.

Protein analysis

The 46000- M_r peroxidase has been further identified as such by its absolute spectra and protein-sequence analysis. It has an RZ (A_{405}/A_{280}) value of 3.3 and analysis by non-denaturing isoelectric focusing showed it to be cationic with a pI of 8.3. It has a typical visible spectrum (Figure 4) of native peroxidase (λ_{max} 500 and 640 nm). When $10\ \mu\text{M}$ H_2O_2 is added, the spectrum changes showing the absorption maxima of compound II (λ_{max} 529 and

Figure 3 D.c.i.-m.s. spectrum of dehydrodiferulic acid

The NH_3 reagent gas [m/z 404 corresponds to $(M + \text{NH}_4)^+$].

Figure 4 Spectra in the visible region of different enzymic forms of 46 000- M_r peroxidase

(A) Spectrum of ferriperoxidase (λ_{max} 500 and 600 nm) in 20 mM Tris/HCl buffer, pH 7.2. (B) Spectrum of compound II (λ_{max} 529 and 556 nm) obtained by addition of 10 μM H_2O_2 to the buffer as in (A). (C) Conversion to spectrum of compound III (λ_{max} 545 and 580 nm), including compound P670 (λ_{max} 670 nm), after addition of 500 μM H_2O_2 to the buffer and the enzyme in the spectral state depicted in (B).

556). In conditions of excess H_2O_2 (500 μM), the spectrum of compound III is distinguished (λ_{max} 545 and 580 nm), showing also a characteristic peak absorbing at 670 nm.

It differed from the 42 000/41 000- M_r bean peroxidase in its peptide map, and peptides from both isoforms were subjected to

amino acid sequencing. The peptides that contained sequences typical of the highly conserved proximal and distal haem-binding sites of the bacterial, fungal and plant peroxidase superfamily were identified (Figure 5). The partial FBP isoform sequences of portions of the proteolytic peptides can be aligned with the residues of turnip (*Brassica napus* L.) (TUR; Mazza and Welinder, 1980), tobacco (TOB; Lagrimini et al., 1987), potato (*Solanum tuberosum* L.) (AP; Roberts and Kolattukudy, 1989) and horseradish (*Armoracia rusticana*) (HOR; Welinder, 1979) showing sequence similarity. These two sequences are known as the most conserved in plant peroxidases because of their involvement in haem binding and acid/base catalysis (Welinder, 1991). Residue 10 (histidine) of FBP1 peptide A is known to be the fifth haem ligand in all other peroxidases. Comparison of peptide B sequence with other peroxidases suggests that residue 9 (histidine) of this peptide is the distal co-ordination amino acid of the haem iron. The 'X' symbol in position 11 of peptide B denotes a blank cycle in the sequencing process. This position corresponds to a highly conserved cysteine residue in the plant peroxidases involved in a disulphide bridge. Such a residue would not appear in a sequencing cycle, and it suggests that X is indeed a cysteine residue in the FBP1 and FBP2 sequences. However, further extensive sequence data (A. Zimmerlin and G. P. Bolwell, unpublished work) reveals the FBP as having much lower sequence similarity to other peroxidases, including FBP2, in other regions of the polypeptide chain.

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