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 (pH 8.3) 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 \( M_t \) was 46000 as determined by SDS/PAGE and d.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 \( R_Z (A_{405}/A_{280}) \) 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 ± 22.9 \( \mu \)M and a \( V_{max} \) of 144 \( \mu \)mol·min\(^{-1} \cdot\)mol\(^{-1}\) of protein at an \( H_2O_2 \) concentration of 100 \( \mu \)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 isomers 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 hydroxyphenylpropionic 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 \textit{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). Endoprotease 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 \( \beta \)-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 \( \alpha \)-methyl mannospyranoside. After concentration and dialysis against a 20 mM Tris/0.2 M NaCl buffer by ultrafiltration 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 ultraconcentration with a PM30 filter.

Abbreviations used: \( R_Z \), absorbance ratio \((A_{405}/A_{280})\); FBP, French-bean peroxidase; d.p.l.c., 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/H$_2$SO$_4$ 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 5% (w/v) solution of sodium alginate. The resultant mixture was spread on the glass slide, covered with 0.05 M CaCl$_2$ 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-$M_c$, 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 μM) 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 and 1 mM ferulic acid, and 0.1 mM H$_2$O$_2$ 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 Rotorofor (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 Rotorofor cell for focusing without further treatment. Focusing in the Rotorofor 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 μg portion of peroxidase, purified to homogeneity, was digested with 5 μg 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 H$_2$O$_2$ in excess for 30 min in water. The reaction was stopped by immersing in liquid N$_2$. 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 $M_c$, 46000 (FBP1) and $M_c$, 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-$M_c$ 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 46000-Mnesota, and 41000-42000-Mnesota peroxidases from French-bean-cell cultures

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Activity (ΔA₄₃₆/min per mg)</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
<th>Activity (ΔA₄₃₆/min per mg)</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
<th>RZ</th>
</tr>
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<tbody>
<tr>
<td>Homogenate</td>
<td>148.1</td>
<td>123.5</td>
<td>100</td>
<td>1</td>
<td>382.8</td>
<td>100</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>10000 g pellet</td>
<td>26.0</td>
<td>262.6</td>
<td>37.2</td>
<td>2.1</td>
<td>533.0</td>
<td>24.4</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>NaCl extract</td>
<td>6.9</td>
<td>840.3</td>
<td>31.7</td>
<td>6.8</td>
<td>1826.3</td>
<td>22.2</td>
<td>4.8</td>
<td>0.28</td>
</tr>
<tr>
<td>Concanavalin A-bound</td>
<td>3.0</td>
<td>1871.7</td>
<td>39.7</td>
<td>15.1</td>
<td>3817.3</td>
<td>20.2</td>
<td>10.0</td>
<td>1.67</td>
</tr>
<tr>
<td>46000-Mnesota FBP</td>
<td>0.5</td>
<td>7572.2</td>
<td>20.7</td>
<td>61.3</td>
<td>14038.9</td>
<td>12.4</td>
<td>35.7</td>
<td>3.26</td>
</tr>
<tr>
<td>41000-42000-Mnesota FBP</td>
<td>0.3</td>
<td>914.5</td>
<td>1.5</td>
<td>7.4</td>
<td>20304.3</td>
<td>10.7</td>
<td>53.0</td>
<td>3.09</td>
</tr>
</tbody>
</table>

Figure 1  SDS/PAGE analysis of FBP purification

Lane 1, M₀ markers; lane 2, purified 41000-42000-Mnesota peroxidase; lane 3, purified 46000-Mnesota 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 46000-Mnesota 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 Kₘ for the 46000-Mnesota 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 46000-Mnesota 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 10000 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.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>10² x Activity (ΔA₄₃₆ - min⁻¹)</th>
<th>Protein (mg)</th>
<th>Carbohydrate (mg)</th>
<th>10² x Activity (ΔA₄₃₆ - min⁻¹)</th>
<th>Protein (mg)</th>
<th>Carbohydrate (mg)</th>
<th>% Extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer (0.1 M, pH 7.8)</td>
<td>595.0 ± 12.0</td>
<td>45</td>
<td>8.7</td>
<td>116.0 ± 13.6</td>
<td>23</td>
<td>4.3</td>
<td>19.5</td>
</tr>
<tr>
<td>+ Triton X-100 (1 %)</td>
<td>461.1 ± 7.7</td>
<td>18</td>
<td>4.7</td>
<td>22.0 ± 9.5</td>
<td>2.7</td>
<td>0.4</td>
<td>4.7</td>
</tr>
<tr>
<td>+ Triton X-100 (1 %)</td>
<td>402.8 ± 19.6</td>
<td>16</td>
<td>4.6</td>
<td>0</td>
<td>1.2</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>+ Triton X-100 (1 %)</td>
<td>348.6 ± 42.2</td>
<td>15</td>
<td>4.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Phosphate buffer (0.1 M, pH 7.8)</td>
<td>343.9 ± 9.5</td>
<td>15</td>
<td>3.8</td>
<td></td>
<td>0</td>
<td>0.9</td>
<td>0.1</td>
</tr>
<tr>
<td>+ NaCl (1 M)</td>
<td>82.7 ± 4.1</td>
<td>12</td>
<td>3.6</td>
<td>265.9 ± 13.6</td>
<td>3.2</td>
<td>0.1</td>
<td>77.3</td>
</tr>
<tr>
<td>+ NaCl (1 M)</td>
<td>65.4 ± 7.7</td>
<td>10</td>
<td>3.6</td>
<td>25.6 ± 5.9</td>
<td>0.9</td>
<td>0.05</td>
<td>30.1</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pellet</th>
<th>Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10^3 \times$ Activity ($A_{492}$ min$^{-1}$)</td>
<td>Protein (mg)</td>
</tr>
<tr>
<td>Phosphate buffer (0.1 M, pH 7.8)</td>
<td>210.1 ± 11.0</td>
<td>50</td>
</tr>
<tr>
<td>+ Triton X-100 (1%)</td>
<td>80.0 ± 7.7</td>
<td>24</td>
</tr>
<tr>
<td>+ Triton X-100 (1%)</td>
<td>65.6 ± 6.5</td>
<td>15</td>
</tr>
<tr>
<td>+ Triton X-100 (1%)</td>
<td>52.2 ± 3.3</td>
<td>10</td>
</tr>
<tr>
<td>Phosphate buffer (0.1 M, pH 7.8)</td>
<td>41.8 ± 7.4</td>
<td>8.5</td>
</tr>
<tr>
<td>+ NaCl (1 M)</td>
<td>11.2 ± 2.1</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Figure 2  Immunolocalization of 46 000$\times$M, 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 × 50 000; the scale bar represents 0.2 μm). (b) Typical level of labelling in primary cell wall (magnification × 80 000; the scale bar represents 0.1 μm). (c) Lower power of typical primary wall labelling (magnification × 25 000; the scale bar represents 0.5 μm). Arrowheads indicate examples of gold particles.

showed by the rapid decrease of their typical $\lambda_{\text{max}}$ when incubated with peroxidase and $\text{H}_2\text{O}_2$. Owing to the occurrence of non-enzymic side reactions with α-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 ± 22.9 μM. This compares with values of 928 ± 31 μM and 629 ± 27 μM for the 42 000/41 000-M$_r$ peroxidase and horseradish peroxidase respectively. The reaction catalysed for the more specific 46 000-M$_r$ peroxidase occurred with a pH optimum of 4.4. This reaction was inhibited 60% by 2 μM KCN when ferulic acid was at 1 mM and $\text{H}_2\text{O}_2$ at 0.1 mM.

Identification of reaction products

When the 46 000-M$_r$ peroxidase was incubated with ferulic acid and $\text{H}_2\text{O}_2$, polymeric metabolites were formed. Reaction products yielded a molecular ion and fragmentation pattern typical of dehydrodiferulate 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 + H]$^+$ = 387), and the decarboxylated molecular ions (m/z 360, 343 or 299) of dehydrodiferulate.

Protein analysis

The 46 000-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 ($\lambda_{\text{max}}$ 500 and 640 nm). When 10 μM $\text{H}_2\text{O}_2$ is added, the spectrum changes showing the absorption maxima of compound II ($\lambda_{\text{max}}$ 529 and
The buffer in enzyme NH₃ Spectrum (B) peroxidase M, including compound (A) Spectrum 4 Figure 3 Figure 3 Conversion to spectrum of compound II (λₘₐₓ 529 and 556 nm), including compound P670 (λₘₐₓ 670 nm), after addition of 500 μM H₂O₂ to the buffer and the enzyme in the spectral state depicted in (B).

556). In conditions of excess H₂O₂ (500 μM), the spectrum of compound III is distinguished (λₘₐₓ 545 and 580 nm), showing also a characteristic peak absorbing at 670 nm.

It differed from the 42000/41000-Mₚ 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.
Peptide A containing the proximal haem-binding histidine (*):

\[ \text{FB1} \quad ^{1} \text{TDLVALSGAHFT}^{15} \text{R} \]
\[ \text{FR2} \quad \text{X} \quad \text{. . . . . . . . . . .} \quad \text{X} \quad \text{. . . . . . . . . . .} \]
\[ \text{TUR} \quad \text{R} \quad \text{M} \quad \text{. . . . . . . . . . .} \quad \text{I} \quad \text{Q} \quad \text{S} \]
\[ \text{TOB} \quad \text{. . . . . . . . . . .} \quad \text{. . . . . . . . . . .} \quad \text{. . . . . . . . . . .} \]
\[ \text{POT} \quad \text{R} \quad \text{E} \quad \text{M} \quad \text{A} \quad \text{. . . . . . . . . . .} \quad \text{V} \quad \text{F} \]
\[ \text{HOR} \quad \text{S} \quad \text{. . . . . . . . . . .} \quad \text{G} \quad \text{. . . . . . . . . . .} \quad \text{K} \quad \text{N} \]

Peptide B containing the distal haem-binding histidine (*):

\[ \text{FB1} \quad ^{1} \text{AALIRLHFDXVF}^{22} \text{Q} \]
\[ \text{FR2} \quad \text{. . . . . . . . . . .} \quad \text{X} \quad \text{. . . . . . . . . . .} \]
\[ \text{TUR} \quad \text{. . . . . . . . . . .} \quad \text{C} \quad \text{. . . . . . . . . . .} \quad \text{N} \]
\[ \text{TOB} \quad \text{. . . . . . . . . . .} \quad \text{C} \quad \text{. . . . . . . . . . .} \quad \text{N} \]
\[ \text{POT} \quad \text{. . . . . . . . . . .} \quad \text{C} \quad \text{. . . . . . . . . . .} \quad \text{D} \]
\[ \text{HOR} \quad \text{. . . . . . . . . . .} \quad \text{C} \quad \text{. . . . . . . . . . .} \quad \text{N} \]

Figure 5 Amino acid sequences of two lysyl endopeptidase-digested peptides from 46000-Mr and 42000/41000-Mr FBPs and their alignment with the regions of proximal (peptide A) and distal (peptide B) histidine residues (*) from turnip (TUR; Mazza and Welinder, 1980), Tobacco (TOB; Lagrimini et al., 1987), potato (POT; Roberts and Kolattukudy, 1989) and horseradish (HOR; Welinder, 1979).

The identical amino acids are designated by a bold point (*).

DISCUSSION

A novel cationic peroxidase (FBP1) has been isolated from cell-wall fractions of suspension-cultured cells of French bean. It was purified to homogeneity, has an Mr of 46000, and showed high specificity towards the polymerization of ferulic acid relative to the general substrate guaiacol and to the properties of a second FBP and the model peroxidase from horseradish.

Ferulic acid occurs in many plants (Graf, 1982). It is covalently conjugated with mono- and di-saccharides, plant-cell-wall polysaccharides, glycoproteins, lignin, betacyanins, and other insoluble carbohydrate biopolymers of cell walls. Several physiological roles of ferulic acid have been proposed. It cross-links vicinal pentosan chains, arabinoxylans, and hemicelluloses in cell walls (Markwalder and Neukom, 1976; Whitmore, 1976; Tachibana et al., 1992; MacAdam et al., 1992). Cross-linking is essential to the formation of barriers to invading pathogens (Mansfield, 1990; Kolattukudy et al., 1992) and has been implicated in the cessation of elongation (Fry, 1986; Hoson and Matsuda, 1991). It is thus a useful compound for the study of the formation of dehydrogenation polymer phenolics in vitro in order to understand the nature of these reactions in the wall (Ralph et al., 1992).

High-ionic-strength-buffer treatment of detergent-washed French-bean cell walls solubilized almost all the 46000-Mr peroxidase, suggesting that a significant proportion of the peroxidase activity was ionically wall-bound. Part of the ferulic acid peroxidase activity remaining in the 10000 g supernatant can be subsequently pelleted at 100000 g, but is all released from microsomes by detergent washings. The wall peroxidases are identical with those extracted from the microsomes (Rodgers et al., 1993), where they are probably in transit and undergoing glycosylation. However, some of this additional localization may be also due to the fact that positively charged cationic isoenzymes could be attracted to the net negatively charged membranes during the homogenization procedure (Schloss et al., 1987).

The 46000-Mr protein (FBP1) appears as a single band on SDS/PAGE (Figure 2) and its RZ value of greater than 3 denotes a pure peroxidase. It was found to be an abundant protein in suspension-cultured cells; its initial content was about 0.3% of total protein. The 41000-42000-Mr protein (FBP2) purified during the same process had a similar guaiacol oxidase activity to FBP1, but was much less effective in oxidizing ferulic acid. This illustrates that other peroxidases are able to polymerize this phenylpropanoid, but with far less efficiency than FBP1. The product of this polymerization in vitro was shown to be dehydroferulic acid by d.c.i.-m.s. This compound occurs extensively in primary cell walls (Fry, 1986), and its contribution to cross-linking in more extensively lignified walls during differentiation and response to stress are probably underestimated (Ralph et al., 1992). The present study is one of the first demonstrations of its formation by a known cell-wall peroxidase in vitro. The specificity is certainly higher for phenylpropanoids and model substrates than other classes of enzymes of the laccase-type recently implicated in lignification and the formation of other dehydrogenation polymers (Savidge and Udagama-Radeniya, 1992; Bao et al., 1993).

Cyanide inhibited the activity of the enzyme. This suggests that the enzyme contains a metal prosthetic group. A typical peroxidase prosthetic group was further evidenced by the absorption spectra of the enzyme in the oxidized form and in the presence of H2O2 (Figure 4).

Enzymically, at least, the 46000-Mr bean peroxidase is implicated in the formation of phenolic cross-linkages in the wall. The pH optimum of 4.4 suggests that the enzyme can function in an acidic environment such as the cell wall. An antibody has been raised against the peroxidase and used to immunolocalize it to the wall in the suspension-cultured cells. In intact tissue (Bolwell, 1993; Smith et al., 1993), it has been found associated with secondary thickenings in terminally developing xylem vessels and extracellularly at bacterial and fungal infection sites. All these data indicate that this particular cationic peroxidase can perform similar functions to those previously ascribed to anionic peroxidases in tobacco (Lagrimini et al., 1987), potato (Roberts and Kolattukudy, 1989), and tomato (Lycopersicon esculentum L.) (Kolattukudy et al., 1992). Preliminary results also indicate that elicitation of the French-bean-cell cultures increases the ratio FBP1/FBP2. Further work is needed to understand the role of FBP1 in defence mechanisms.

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