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PEROXIDASES IN THE CELL WALLS OF SEEDS AND SEEDLINGS OF *ARAUCARIA ARAUCANA*

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Key Word Index—*Araucaria araucana*; Araucariaceae; peroxidase cationic neutral gel; conifer; embryo; megagametophyte; *o*-phenylenediamine; dansylhydrazine; horseradish peroxidase.

Abstract—Two major proteins present in the cell walls of the embryo and megagametophyte tissues of *Araucaria araucana* seeds were partially purified, characterized and identified as peroxidases. These two proteins have M_rs of 83 and 145×10^3, a pI of 10.5, an optimal pH of 5.0 and a buoyant density of 1.333 g ml^{-1}. Kinetic studies of these peroxidases define a K_m app. of 13.6 mM for H_2O_2 and 3.4 mM for *o*-phenylenediamine (*o*-PDA). The V_{max} is 525 μmol *o*-PDA oxidized min^{-1} mg^{-1}. After oxidation with periodic acid the peroxidases react on gels with dansylhydrazine, demonstrating that the proteins are glycosylated. The sugar components linked to the proteins are galactose, glucose, xylose and mannose. Galactose comprises more than 60% of the sugar residues in both peroxidases. The other sugar contents show significant quantitative differences between the peroxidases. Expression of the peroxidases increases upon wounding of the seed tissues. The two proteins were differently expressed during seed germination. They also showed a different susceptibility to degradation when the proteins were heated in the presence of 5 M urea.

INTRODUCTION

In plant cell walls there are structural proteins like the hydroxyproline-rich [1], glycine-rich [2] and threonine-rich [3] proteins and enzymes which catalyse important reactions related to cell wall metabolism. Among the enzymes there are peroxidases (EC 1.11.1.7) and different glycosidases such as β-1,4-D-glucanase (EC 3.2.1.4, cellulase), pectinases and hemicellulases [2, 4]. Most of the cell wall enzymes are involved in plant protection against pathogens and wounding [4, 5] and they also seem to have a role in cell elongation control [6]. Peroxidases are the most studied plant enzymes due to their abundance in plant tissues. In cell walls, peroxidases can be soluble or linked by ionic or covalent bonds to other components of cell walls [7]. The presence of peroxidases is induced during cell wall repair [8]. Therefore, the role of peroxidase could be to repair the cell wall, forming an impermeable barrier to water at the site of injury by means of aliphatic and aromatic compounds [9]. Peroxidases also catalyse cross-linking reactions between macromolecules such as those involved in lignin biosynthesis [10], hemicellulose and ferulic acid [11], and cross linking of proteins by isodityrosine [12] or in lysine residues that have been deaminated by oxidation [2].

We have already reported the pattern of proteins present in the cell walls of embryo and megagametophyte tissues of *A. araucana* seeds and seedlings during early growth [13]. *Araucaria araucana* is a conifer tree whose seeds will express oxidative pathways leading to lignification during germination and early development. The seeds are also spread by birds, which do not often eat them but wound them with their beaks. Therefore, the tissues of the seeds need to repair the injury before or during germination. In these two processes, lignification and repair, the cell wall peroxidases may be involved. The purpose of this work was to find out whether there are peroxidases among the cell wall proteins of *A. araucana* seeds. Two major cell wall proteins were identified as peroxidases.

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RESULTS AND DISCUSSION

Cell wall protein pattern

The protein pattern present in cell walls of *A. araucana* seeds was analysed by SDS-PAGE (Fig. 1). The arrows point to the two predominant proteins expressed 24 and 72 hr after seed imbibition in wound and non-wounded seeds. The proteins had M_rs of 145 and 83×10^3. After wounding there was a considerable increase in expression of the same two proteins. This increase was correlated with other physiological events such as the protrusion of the root tips, an increment in the diameter of the hypocotyl (probably a consequence of the ethylene production caused by wounding) and an increment in the total weight of the seed. The weight change may be caused by an

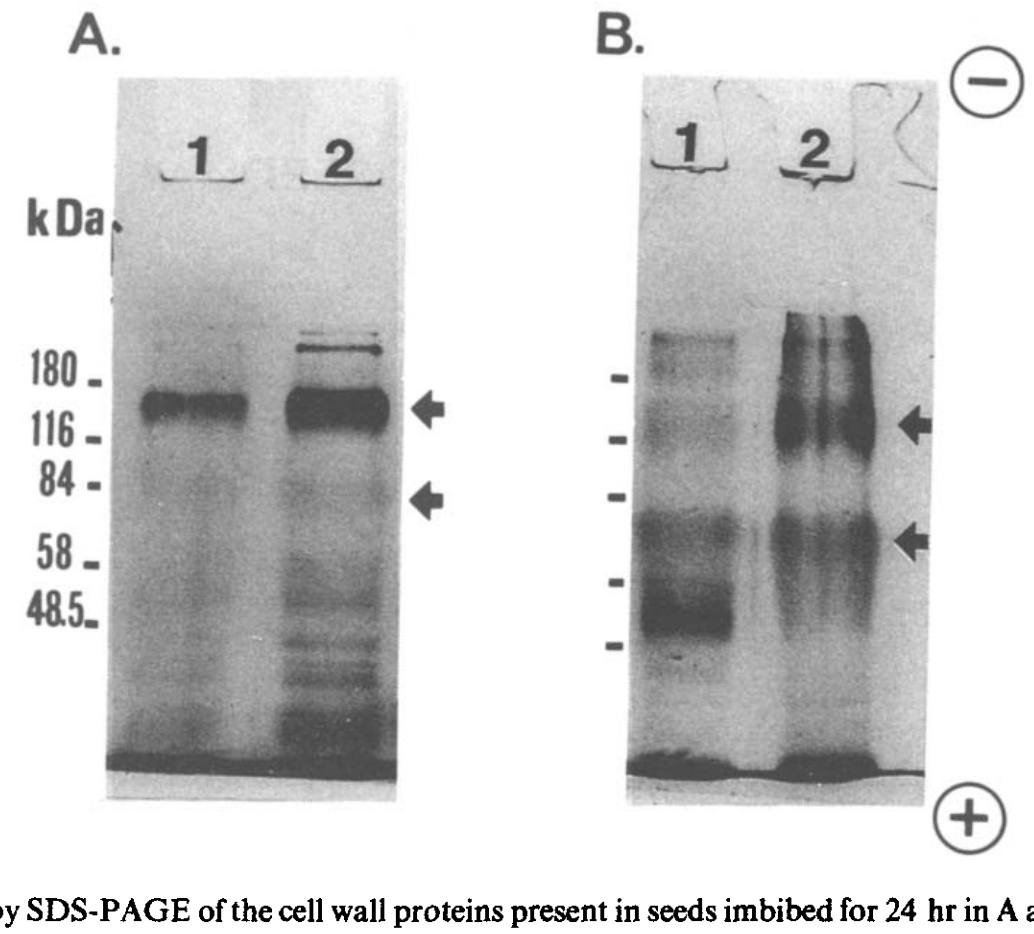

Fig. 1. Analysis by SDS-PAGE of the cell wall proteins present in seeds imbibed for 24 hr in A and for 72 hr in B. In A and B: Lane 1, loaded with cell wall proteins of non-wounded seeds. Lane 2, loaded with cell wall proteins of wounded seeds. Each lane was loaded with amount of proteins present in 250 mg of dried walls.

increase in the water content of the tissues or by the increase of other proteins not present in the cell wall of non-wounded seeds [14, 15].

Cationic neutral electrophoresis of these proteins demonstrated that they have a basic nature at a gel pH 6.8. The proteins could run on cationic neutral gels and be analysed by electrophoresis of their native forms due to their basic nature [2, 16, 17].

Purification and separation of the two major cell wall proteins

The proteins were partially purified by chromatofocusing using a column made of the polybuffer exchanger PBE 118. The chromatofocusing was run in a pH gradient from 11 to 8.5 (Fig. 2). The two proteins elute together in fractions 8 to 15 at a pI of 10.5. In the insert, a photograph of a SDS-PAGE analysis corroborates the degree of purification. The specific activity and the yield per cent of this purification were also determined (Table 1).

A separation of the two proteins was attempted by isopycnic centrifugation in a CsCl gradient. Both proteins migrated together in fractions 28–35. They have a buoyant density of 1.33 g ml^{-1}. This result suggested that these proteins are not structural proteins, namely extensin-like, because they have a density of 1.44 g ml^{-1} [6, 16, 17]. The high M_r of the proteins also indicates that they could not be arabinogalactans [18]. Furthermore, the β-glucosyl Yariv antigen [19] has demonstrated that the arabinogalactans are not present in the cell wall extracts of *A. araucana* [13].

Identification of the two major proteins as peroxidases

The peroxidases are enzymes frequently present in the plant cell wall of woody plants. Identification of these proteins as peroxidases was performed after cationic neutral electrophoresis of the native proteins incubating the gel with *o*-PDA and H_2O_2. The two major proteins gave a positive peroxidase reaction.

Kinetic characterization of the peroxidases

A saturation curve with increasing concentration of *o*-PDA at a constant concentration of 20 mM H_2O_2 was obtained together with the Lineweaver–Burk plot for *o*-PDA. The effect of the increment of H_2O_2 in the velocity of the reaction at a constant concentration of *o*-PDA of 5.5 mM, with the corresponding derived Lineweaver–Burk plot was also obtained. These results give a K_m app. of 3.4 mM for *o*-PDA and V_{max} of 525 μmol of *o*-PDA oxidized min^{-1} mg^{-1} with a K_m app. of 13.6 mM for H_2O_2. The kinetic constant for the cell wall peroxidases of *A. araucana* seeds in the *o*-PDA oxidation suggests that the binding of H_2O_2 to the active site is the rate-limiting step because a four-fold higher concentration of H_2O_2 than of *o*-PDA is needed to get 1/2 of the V_{max}.

The two peroxidases studied were inhibited at high concentration of H_2O_2 (over 64 mM). Other peroxidases like those isolated from cell walls of lupins are inhibited with 0.75 mM H_2O_2 and peroxidases of tomato cell culture are inhibited with 0.1 mM [20]. It is known that the efficiency of peroxidase oxidation is

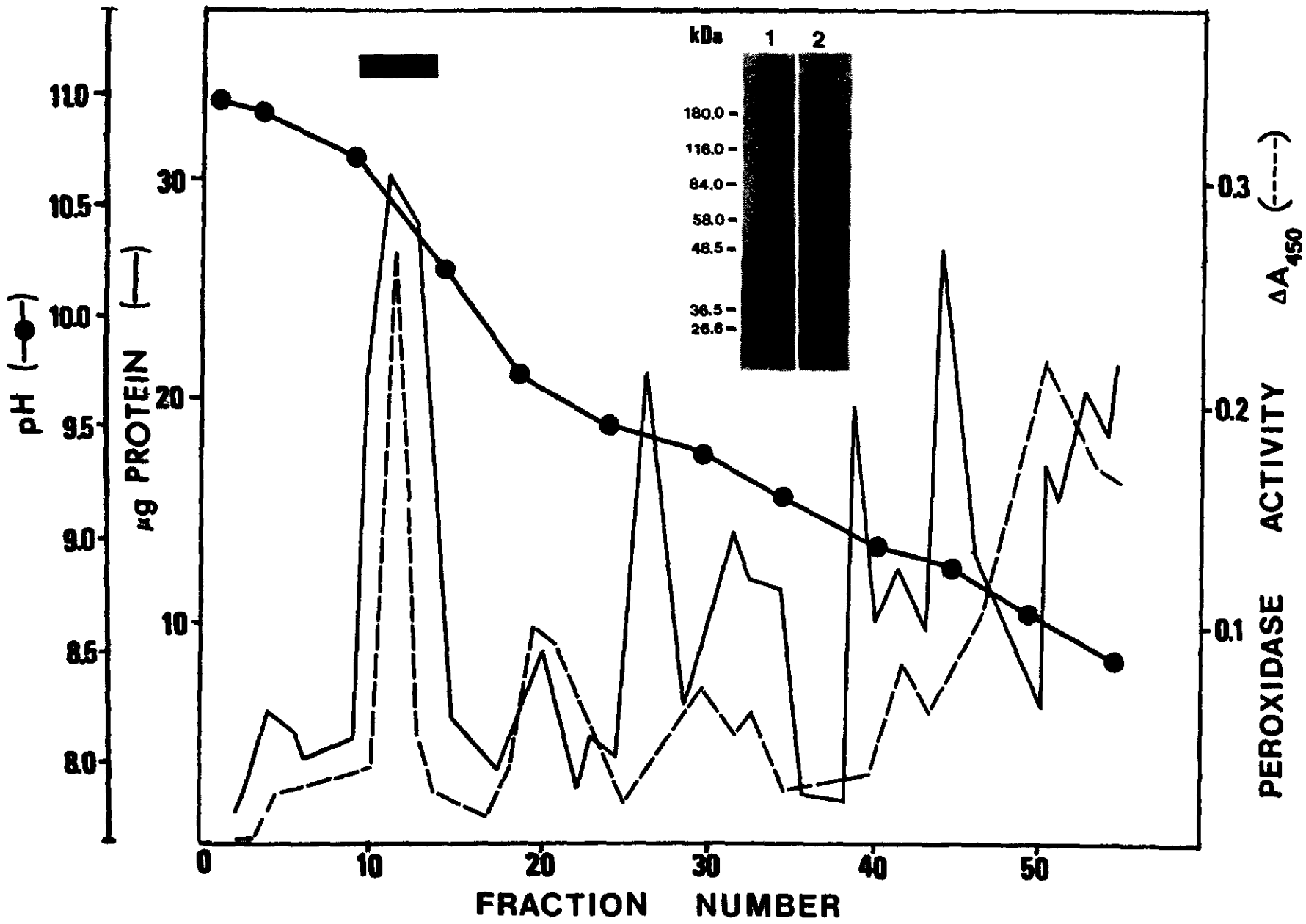

Fig. 2. Chromatofocusing profile of the cell wall proteins after elution from the PBE 118 column. The column was loaded with 600 μg of crude extract of cell wall proteins.——Amount of proteins present in each fraction. - - - - - Peroxidase activity. -●- pH. ▬ Indicates the fraction where the two proteins elute. The insert is a SDS-PAGE of the proteins present in fractions 10–15 (lane 2) and compared with those present in the crude extract (lane 1).

Table 1. Partial purification by chromatofocusing in PBE 118 of the cell wall peroxidases

					% Yield	
Step	Volume (ml)	Protein (mg)	Units of activity*	Specific activity†	Protein	EU
Crude extract	50	0.576	92.8	161	100	100
PBE-118	3	0.078	27.4	351	14	30

The PBE 118 column was loaded with 600 μl of cell wall extract after equilibration with 0.025 M of triethylamine at pH 11. In the fractions collected the amount of protein and the peroxidase activity was determined.

*EU, enzyme units: μmol *o*-PDA oxidized min^{-1}.

†Specific activity: μmol *o*-PDA oxidized min^{-1} mg^{-1} of protein.

affected by the type of substrate used [21]. Therefore, the two cell wall peroxidases of *A. araucana* seeds could have a different oxidation efficiency with their natural biological substrates present in the cell wall, which are unknown.

Optimal enzyme pH

The pH profile studied for these peroxidases showed an optimal pH of 5.0. At this pH the activity of the enzyme is 0.059 μmol of *o*-PDA oxidized min^{-1}. Half maximal

activity occurs at pH 4.6 and 5.6. This optimal pH might suggest that these enzymes are not responsible for the synthesis of molecular bonds which probably contributes to decrease the cell wall plasticity or they are not present or not active in the walls of those cells which elongate [6].

Characterization of the peroxidases as glycoproteins

The wide migration pattern of these proteins in the SDS-PAGE suggested that these peroxidases are glycoproteins. The glycoprotein nature of these enzymes was demonstrated by incubating the gel after SDS-PAGE with periodic acid and dansylhydrazine. Both proteins gave positive fluorescence bands with these reagents.

Identification of the sugar residues

The sugar residues linked to the proteins were identified by HPLC after hydrolysis of the proteins electroeluted from the gel after SDS-PAGE (Table 2). The sugar components were compared with those present in a horseradish peroxidase [22]. In both cell wall peroxidases, galactose was the predominant sugar, making more than 60% of the sugar residues in both peroxidases. Glucose was the second most common sugar in the *A. araucana* peroxidases (36% in that of 83×10^3 and 19% in that of 145×10^3). D-*N*-Acetyl glucosamine is the major sugar in horseradish peroxidase (44.3%), mannose being the second most abundant sugar residue in the horseradish enzyme (32.1%). Arabinose and fucose were not found in *A. araucana* peroxidases, although they are present in horseradish peroxidase with 2% of arabinose and 9.4% of fucose.

Expression of peroxidases during development

The expression of the enzymes during seed germination, from quiescent seeds to 96 hr after imbibition, was quantified by scanning SDS-PAGE separations of the proteins (Fig. 3). The lanes of the gel were loaded with the amount of proteins contained in 100 mg of dried weight

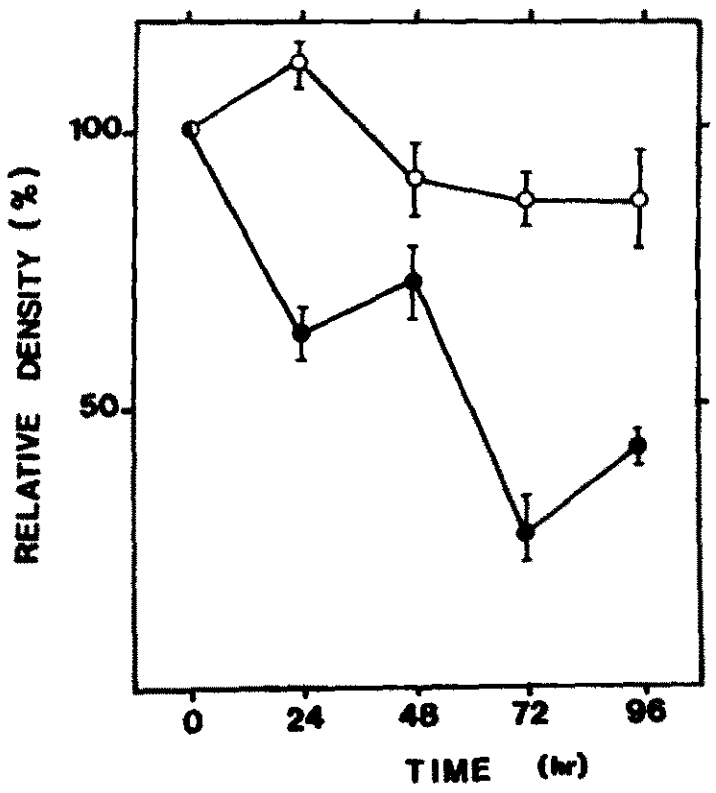

Fig. 3. Expression of cell wall peroxidases after 96 hr of seed imbibition. The plot is a densitometric analysis of the protein bands run on SDS-PAGE. The wells of the gel were loaded with the proteins present in 100 mg of dried walls of quiescent seeds (0 hours) and of seeds imbibed for 24, 48, 72 and 96 hr. –●– Expression of the $145 \times 10^3 M_r$ peroxidase. –○– Expression of the $83 \times 10^3 M_r$ peroxidase.

Table 2. Sugar composition of cell wall peroxidases

Sugar	$83 \times 10^3 M_r$ protein	$145 \times 10^3 M_r$ protein	Horseradish* peroxidase
	(μg sugar mg^{-1} protein)		
D-Galactose	72.4 ± 2.9 (61.1%)	76.8 ± 1.6 (75.1%)	0.0 (0.0%)
D-Glucose	42.8 ± 2.0 (36.1%)	19.6 ± 3.6 (19.2%)	5.7 (3.8%)
D-Xylose	1.4 ± 0.3 (1.2%)	1.3 ± 0.5 (1.3%)	12.8 (8.5%)
D-Mannose	2.0 ± 0.6 (1.6%)	4.5 ± 0.9 (4.4%)	48.2 (32.1%)
L-Arabinose	0.0 (0.0%)	0.0 (0.0%)	2.9 (1.9%)
D-*N*-Acetyl glucosamine	N.D.	N.D.	66.5 (44.3%)
% Carbohydrate in the protein	12	10	15

The sugar composition was obtained after hydrolysis of the protein bands electroeluted from polyacrylamide gels after SDS-PAGE. HPLC was performed in Knauer HPLC type 364 equipped with a CARBOPACK PAl column using 5.25 mM NaOH, pH 11.5, as solvent. The gas chromatograph was provided with a gold electrode present in the Dionex pulsed amperometric detector.

N.D. not determined.

*From ref. [22].

± s.d.

of cell walls. After 72 hr of seed imbibition, the 145×10^3 peroxidase decreased to 27% of the original level in the quiescent seeds, while the 83×10^3 peroxidase increased 20% after 24 hr of seed imbibition and remained stable up to 96 hr. The maximum peroxidase activity in the cell walls was observed after 48 hr of imbibition. At this time the seeds have already germinated [23]. The increase in peroxidase activity at this time could be related to the protective role of this enzyme (cell wall repair) at the time of seed dispersal.

EXPERIMENTAL

Biological material. Uncoated seeds of *A. araucana* (Mol.) Koch were sterilized in a 5% soln of commercial bleach for 15 min at room temp. The seeds were germinated in dark at 20°. Three groups of seeds were transversally sectioned with a razor blade [13], and three groups of intact seeds were used as control.

Purification of cell walls and proteins extraction. The cell walls were purified and extracted for proteins after purification with 0.2 M $CaCl_2$, in 2 mM $Na_2S_2O_5$ from the wounded and non-wounded seeds using the method of refs [13, 16, 17]. After extraction, the proteins were pptd with 5 vol of Me_2CO and the protein pellet sepd by centrifugation at 15 000 *g*. The proteins were resuspended in 0.1 M Tris–HCl, pH 6.8. The salt free soln containing the proteins was obtained by ultrafiltration using an Amicon membrane PM-10. The proteins were quantified by the method of ref. [24].

Electrophoretic analyses. The cell wall proteins were analysed by SDS-PAGE, in a 7% gel as has been described [13].

SDS-PAGE in the presence of urea, at 5 M in the concn gel and 8 M in the sepn gel, was performed to determine if the major proteins of the wall could have two or more peptides. Before loading the gel, the samples were heated in the presence of 5 M urea at 90° for 5, 10 and 20 min. Cationic neutral gel electrophoresis analysis of the native proteins was performed as described in ref. [25]. In all types of electrophoresis, the protein bands were visualized with silver stain reagent using the method of ref. [26] or 0.25% Coomassie blue R-250 in 50% MeOH and 10% HOAc.

Protein purification by chromatofocusing. A glass column (18 × 1 cm) was packed with a Polybuffer Exchanger (PBE 118) (Pharmacia) to perform isofocusing chromatography. The column was equilibrated with 0.025 M triethylamine, pH 11. Fractions of 1 ml were collected. Aliquots of each fraction were used for protein determination by the method of ref. [24] and for peroxidase activity as described below.

Isopycnic centrifugation on CsCl *gradient.* CsCl (3 g) was dissolved in 3 ml of deionized H_2O and placed in a centrifuge tube. On top of the CsCl a soln of 0.5 μl of cell wall protein extract containing 500 μg of protein was layered. Deionized H_2O was added to make 5 ml. The tubes with the samples were centrifuged in a Beckman L5-50 centrifuge at 45 000 rpm for 72 hr at 4°. After centrifugation the tube bottoms were punctured to obtain fractions of 6 drops each. Five μl from each 5 fractions was used to measure the refractive index using a Bausch & Lomb refractometer. The amount of protein was determined in the fractions collected.

Identification of protein bands by peroxidase activity. Peroxidase activity was detected in native proteins run on cationic neutral gel electrophoresis using a 0.1% soln of *o*-PDA and 0.012% H_2O_2 as substrates. The buffer was 0.1 M Na citrate, pH 5 [27]. After visualization of the bands, the reaction was stopped using successive washes with deionized H_2O. The gels were dried under vaccum.

Peroxidase assay. The peroxidase activity was detected using 5 μl of each fraction and 1 ml of 0.1% *o*-PDA and 0.012% H_2O_2 in 0.1 M Na citrate, pH 5. After 10 min at 25° the reaction was stopped by addition of 10 μl of β-mercaptoethanol to the reaction media. *A* was measured at 450 nm. One enzyme unit (EU) was defined as the amount of enzyme which oxidizes 1 μmol of *o*-PDA min^{-1}.

Standard curve to quantify the oxidized o-*phenylendiamine.* Known quantities of *o*-PDA in the range of 10–100 μg were added to 1 ml of the assay made up to 32 mM H_2O_2 excess of horseradish peroxidase (Sigma type VI) and 0.1 M Na citrate, pH 5. The reaction mixt. was incubated at 20° until *A* reached a maximum and remained stable at 450 nm. At this point it is assumed that all the substrate is converted into product. In the standard curve, *A* at 450 nm was plotted against the concn of oxidized *o*-PDA.

Determination of the optimal pH of the cell wall peroxidases. The reaction mixt. used was 5.5 mM *o*-PDA and 4 mM H_2O_2 made up in the corresponding buffer to which 0.42 μg of protein was added. For the pH range 3–5 the buffer used was 0.1 M Na citrate; for pH 6 0.6 M Bis–Tris; for pH 7 0.7 M MOPS; for pH 8–9 0.7 M Tris–Base and for the pH 10–11 0.5 M CAPS. The reaction was monitored for 10 min at 20°. The activity of the enzyme for the *o*-PDA oxidation was calculated from the plot A_{450}/time and was expressed in μmol of *o*-PDA oxidized min^{-1}.

Kinetic studies. Initial velocity as a function of the H_2O_2 *concentration.* The standard conditions of the reactions were: 0.42 μg of purified proteins added to 1 ml of a mixt. containing 5.5 mM *o*-PDA and from 1 to 64 mM H_2O_2 in 0.1 M Na citrate, pH 5 incubated at 20°. The initial velocity was obtained as described before. The kinetic parameters were calculated taking the average values of the Lineweaver–Burk, Hanes and Eadie–Hofstee plots.

Initial velocity as function of the o-*PDA.* The standard conditions of the reaction were: 0.42 μg of purified proteins added to 1 ml of a mixt. containing 20 mM H_2O_2 and from 2.75 to 27.5 mM of *o*-PDA in 0.1 M Na citrate, pH 5, incubated at 20°. The initial velocity was obtained as described above. The kinetic parameters were calculated taking the average values of the Lineweaver–Burk, Hanes and Eadie–Hofstee plots.

Identification of the peroxidases as glycoproteins. The glycosylation of the peroxidases was detected by the reaction periodic acid/dansylhydrazine [28], on gels after

SDS-PAGE, or electrophoresis of the native proteins under cationic neutral conditions. The protein bands were visualized under UV light.

Identification of sugar residues present in the glycoproteins. To obtain 0.5 mg of each peroxidase, the enzymes were electroeluted from gels after SDS-PAGE. The proteins were hydrolysed with 300 μl of 2 N TFA heating for 1 hr at 121°. The TFA was evapd under vacuum and the product of the hydrolysis was resuspended in deionized H_2O. The sugar analysis was performed in a Knauer HPLC type 364 equipped with a column of CARBOPACK PA1 (Dionex Corporation, U.S.A.) and using 5.25 mM NaOH as solvent. Sugars were detected with a gold electrode present on the Dionex pulsed amperometric detector.

Determination of the peroxidase expression during development. Cell wall proteins of quiescent seeds and from seeds imbibed for 24, 48, 72 and 96 hr were extracted and analysed by SDS-PAGE, loading the gel with the amount of proteins present in 100 mg of dried cell walls. After electrophoresis, the gel was stained with silver reagent and vacuum-dried.

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