

RESEARCH PAPER

Cell wall-bound cationic and anionic class III isoperoxidases of pea root: biochemical characterization and function in root growth

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Received 25 January 2012; revised 19 April 2012; accepted 20 April 2012

Abstract

Cell wall isolated from pea roots was used to separate and characterize two fractions possessing class III peroxidase activity: (i) ionically bound proteins and (ii) covalently bound proteins. Modified SDS–PAGE separated peroxidase isoforms by their apparent molecular weights: four bands of 56, 46, 44, and 41 kDa were found in the ionically bound fraction (iPOD) and one band (70 kDa) was resolved after treatment of the cell wall with cellulase and pectinase (cPOD). Isoelectric focusing (IEF) patterns for iPODs and cPODs were significantly different: five iPODs with highly cationic pI (9.5–9.2) were detected, whereas the nine cPODs were anionic with pI values between pH 3.7 and 5. iPODs and cPODs showed rather specific substrate affinity and different sensitivity to inhibitors, heat, and deglycosylation treatments. Peroxidase and oxidase activities and their IEF patterns for both fractions were determined in different zones along the root and in roots of different ages. New iPODs with pI 9.34 and 9.5 were induced with root growth, while the activity of cPODs was more related to the formation of the cell wall in non-elongating tissue. Treatment with auxin that inhibits root growth led to suppression of iPOD and induction of cPOD. A similar effect was obtained with the widely used elicitor, chitosan, which also induced cPODs with pI 5.3 and 5.7, which may be specifically related to pathogen defence. The differences reported here between biochemical properties of cPOD and iPOD and their differential induction during development and under specific treatments implicate that they are involved in specific and different physiological processes.

Key words: Auxin, cell wall peroxidase, elicitor, IEF–PAGE, pea root, *Pisum sativum*.

Introduction

The primary cell wall is a complex extracellular structure mostly made of cellulose, hemicellulose, and pectin, with proteins embedded in the polysaccharide matrix. The most abundant enzymes among extracellular proteins are class III peroxidases (EC 1.11.1.7). They are normally involved in the dissipation of

hydrogen peroxide (H₂O₂); however, they also possess a capacity to produce H₂O₂ via one-electron reduction of oxygen while oxidizing reductants [NAD(P)H, ascorbate, or auxin]. H₂O₂ participates in oxidative reactions in the apoplast both as a substrate in polymerization processes of the cell wall and also

Abbreviations: cPOD, covalently bound peroxidase; DAB, 3,3'-diaminobenzidine; DEPMPO, spin-trap (5-diethoxy-phosphoryl-5-methyl-1-pyrroline-n-oxide); EPR, electron paramagnetic resonance; HRP, horseradish peroxidase; IAA, indole-3-acetic acid; HRP, horseradish peroxidase; IEF, isoelectric focusing; iPOD, ionically bound peroxidase; NAA, naphthalene acetic acid; PNGase F, peptide N-glycosidase F; PR, pathogen-related; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SHAM, salicylhydroxamic acid; TMB, tetramethyl benzidine; WGA, wheat germ agglutinin.

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as an intermediary molecule in the process of cell wall loosening. In addition, H₂O₂ participates in defence reactions and signalling processes in the apoplast and at the plasma membrane (Mika *et al.*, 2010). Various peroxidase reactions are linked to the cell wall, such as those involved in lignin and suberin synthesis (Espelie *et al.*, 1986; Christiansen *et al.*, 1998; Brownleader *et al.*, 2000; Quiroga *et al.*, 2000), indole-3-acetic acid (IAA) catabolism (Hinnman and Lang, 1965; Nakajima and Yamazaki, 1979; Brooks, 1986; Alba *et al.*, 1998), and/or oxidative cross-linking of cell wall components (Cooper and Varner, 1984; Lopez-Serrano *et al.*, 2004). Although certain peroxidase functions could be related to the specific isoforms, such as those in wounding (Bernards *et al.*, 1999; Minibayeva *et al.*, 2009) or during pathogen attack (Lamb and Dixon, 1997; Bolwell *et al.*, 1999), it is generally difficult to assign a specific function to the individual peroxidase isoforms *in vivo*. Numerous studies have examined the functions of peroxidases and tried to attribute some function *in vivo* to the anionic, cationic, neutral, ionically or covalently bound, or soluble peroxidase isoforms (Brooks, 1986; Narita *et al.*, 1995; Sanchez *et al.*, 1995; Groppa *et al.*, 1999; Quiroga *et al.*, 2000). Alternatively, there are several reasons in favour of factors other than enzyme structure being important in determination of peroxidase activity: microenvironment, a high redundancy found in peroxidase genes, very similar immunological properties of different isoenzymes, and failure to relate down-regulated specific peroxidases to phenotype (Cosio and Dunand, 2009). The aim of this work was to elucidate if localization within the cell wall and the type of binding to polysaccharide or isoform structure determine peroxidase function during plant growth.

Based on the type of their interaction with cell wall constituents, several groups of proteins may be distinguished. Soluble proteins extracted with an intercellular washing fluid are probably loosely or not bound to cell wall constituents and move freely in apoplastic space. Some cell wall peroxidases are weakly bound to a polysaccharide matrix by Van der Waals interaction, hydrogen bonds, or hydrophobic interactions (Jamet *et al.*, 2006). Finally, proteins can be strongly bound to the cell wall using either ionic bonds or cross-linking by covalent bonds (Jamet *et al.*, 2006). Peroxidase activity is found in all cell wall protein fractions. One of the most common classifications of extracellular peroxidases is based on their isoelectrophoretic mobilities, being classified as anionic, neutral, and cationic. Also one of the classifications that is often used is soluble peroxidase found in the apoplast, and forms which are ionic or covalently bound to the cell wall (Ros Barcelo *et al.*, 1998). As a working hypothesis, a specific role for covalently bound cell wall peroxidase in the process of cleavage of cell wall polymers during growth has been proposed. Recent work demonstrated that peroxidases covalently bound to the cell wall may spontaneously generate hydroxyl radicals (Kukavica *et al.*, 2009) that have a specific role in the process of cleavage of cell wall polymers.

In the present study, peroxidases that are either ionically (iPOD) or covalently bound (cPOD) to the cell wall of pea roots were extracted and their structural and biochemical characteristics studied. Changes in isoperoxidase induction were analysed after auxin or chitosan treatment and also during plant growth and along root zones.

Materials and methods

Plant materials and growth condition

Pea seeds (*Pisum sativum* L. Mali Provansalac) were washed under tap water and germinated at 18 °C in the dark for 3 d. Seedlings were then placed in Hoagland solution, which was changed after a week, and grown hydroponically for 3, 6, 10, 17, and 23 d in a growth chamber with a photoperiod of 16 h/8 h (light/darkness) at 24 °C and 18 °C. Irradiance of 80 μmol m⁻² s⁻¹ was provided by white fluorescent tubes. For auxin treatments, plants were grown in the presence of 10 μM 1-naphthaleneacetic acid (NAA) that was added to the Hoagland solution for 11 d. Elicitation was performed with chitosan using plants of different ages (3, 6, 10, 17, and 23 d). Plants were treated with 1 g l⁻¹ chitosan (Sigma, Deisenhofen, Germany) before harvesting and cell wall isolation from the root for 16 h.

Cell wall isolation

The cell wall fraction was isolated from roots by a method modified from Kukavica *et al.* (2009). Roots were cut and kept in TRIS buffer [50 mM TRIS pH 7.2, 50 mM NaCl, 0.05% Tween-20, 1 mM phenylmethylsulphonyl fluoride (PMSF)] at 4 °C and immediately homogenized in a Waring blender (VWR, Darmstadt, Germany) for 2 min. The homogenate was filtered through two layers of cloth and centrifuged at 1000 g for 20 min. The pellet with cell wall fragments was washed four times in 50 mM TRIS (pH 7.2). To extract the ionically bound protein fraction, the pellet was suspended in 1 M NaCl, incubated for 30 min at 4 °C, and then centrifuged at 1000 g for 15 min. The supernatant was used for analysis of iPOD. After salt treatment, the pellet was washed four times with TRIS buffer. The covalently bound protein fraction was released after incubation of cell wall isolate with 0.5% cellulase (Sigma, Taufkirchen, Germany) and 2.5% pectinase (Fluka, Taufkirchen, Germany) in a cold room for 24 h. After centrifugation of the suspension at 1000 g for 15 min, the supernatant was used to analyse cPOD. Both iPODs and cPODs were extracted from roots of 3-day-old pea seedlings divided into four zones: I, 5 mm from the root tip; II, 1.5 cm; III, 2 cm; and IV, 2 cm according to Cordoba-Pedregosa *et al.* (2003) (Fig. 6A). For other experiments, whole roots were used to isolate the cell wall and the ionically and covalently bound fractions. For enzyme analysis, 3–4 cell wall isolates were used.

Extraction of apoplastic fluid

Freshly cut roots were vacuum infiltrated for 10 min in distilled water, blotted, placed into tips mounted on top of Eppendorf tubes, and centrifuged for 20 min at 500 g. No significant contamination of the isolated apoplastic fluid and cell wall fragments was detected using glucose-6-phosphate dehydrogenase activity as a cytoplasmic marker. The reaction mixture consisted of 5 mM MgSO₄, 5 mM glucose-6-phosphate, and 0.1 mM NADH in 0.1 M K-phosphate buffer pH 8, and an absorbance decrease at 340 nm indicated NADH oxidation ($\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$).

Extraction of soluble proteins

For extraction of soluble proteins from roots, frozen roots (0.5 g) were powdered in a mortar containing liquid N₂ and suspended in 100 mM potassium phosphate buffer (pH 6.5). The homogenate was centrifuged at 10 000 g for 15 min at 4 °C. The supernatant was used for electron paramagnetic resonance (EPR) measurements.

Modified SDS-PAGE

Modified SDS-PAGE was used to separate peroxidase isoforms by molecular weight using the prosthetic haem group according to Mika and L uthje (2003). The final concentration of SDS was 0.1% (w/v) in all solutions and gels. Samples were diluted in loading buffer to final concentrations of 62.5 mM TRIS-HCl, 0.1% (w/v) SDS, 10% (w/v) glycerol, and 0.002% (w/v) bromophenol blue without reducing compounds

and loaded onto the gels without heating. It was shown that all isoforms stayed active after separation on modified SDS–PAGE by staining with 0.01% α -chloro-naphthol used as a substrate for peroxidase reaction and 0.03% H_2O_2 in 0.1 M Na-phosphate buffer pH 6.5. This enabled the determination of the apparent molecular weights of peroxidase isoforms using molecular mass standards (Broad Range, Bio-Rad, Munich, Germany) according to Laemmli (1970). The other half of the gel was used for haem staining with 6.3 mM tetramethyl benzidine (TMB) and 30 mM H_2O_2 (Thomas *et al.*, 1976).

Isoelectric focusing of peroxidase isoforms

Isoelectric focusing (IEF) was performed with mini gels [7.5% (w/v) acrylamide with 3 M urea and 2% ampholyte; Serva, Heidelberg, Germany] at 4 °C for 3 h. IEF was performed at a gradually increasing voltage: 90 min at 100 V; 60 min at 250 V; and 30 min at 500 V. The anode buffer was 10 mM phosphoric acid and the cathode buffer was 20 mM NaOH. For more precise resolution of iPOD isoforms, a pH gradient from 9 to 11 and for cPODs a pH gradient from 3 to 7 were used. Before loading on the gel, samples were mixed with loading buffer containing 40% glycerol and 20% ampholyte.

The thermostability of iPOD and cPOD was determined on the IEF gel by staining for peroxidase activity after incubation of aliquots at 25, 50, 60, and 80 °C for 15 min.

Determination of IAA oxidase activity

IAA oxidase peroxidase activity on the gel was visualized according to the protocol of Hoyle (1977). Staining solutions were: (A) Fast Blue Salt BB (4 mg ml⁻¹) dissolved in ethanol; (B) 2 μM *p*-coumaric acid, 2 μM IAA, and 2 μM H_2O_2 were diluted in 50 ml; (C) 2 M sodium acetate buffer (pH 4.2). Final staining solution consisted of 25 ml of solution A, 50 ml of solution B, and 25 ml of solution C.

Peroxidase activity

The peroxidase activity of iPOD and cPOD was measured spectrophotometrically in a reaction mixture consisting of 100 mM Na-phosphate buffer pH 6.5, 0.01 M pyrogallol, and aliquots of fractions bound ionically and covalently to the cell wall. The reaction was started by addition of 3.3 mM H_2O_2 , and an increase in absorbance at 430 nm was followed. Peroxidase activity was calculated using the extinction coefficient for purpurogallin ($\epsilon = 12 \text{ mM}^{-1} \text{ cm}^{-1}$).

Oxidation of the hydroxycinnamic acids (chlorogenic, caffeic, or ferulic) by iPOD or cPOD was measured in a reaction mixture (3 ml) containing 50 μl of a suspension of cell wall isolates or ionic fraction, 3.3 mM H_2O_2 , and 4 mM acids in 100 mM potassium phosphate buffer (pH 6.5). An increase in absorbance was followed at 410, 450, and 356 nm, respectively (Bestwick *et al.*, 1998).

To investigate the effect of lectins on peroxidase activity, aliquots of ionically and covalently bound cell wall fractions were incubated for 3 min with different concentrations (1 $\mu\text{g ml}^{-1}$ and 5 $\mu\text{g ml}^{-1}$) of concanavalin A (Con A) and wheat germ agglutinin (WGA).

For deglycosylation treatment, endoglycosidases F1, F2, and F3 (Endo F1, F2, and F3) and peptide *N*-glycosidase F (PNGase F) were used. Aliquots of samples were incubated with different proteolytic enzymes for 2 h and peroxidase activity was measured.

Protein concentrations were determined according to Bradford (1976).

NADH-POD oxidase activity

Measurement of NADH-POD oxidase activity in ionic and covalent cell wall fractions was done according to Fecht-Christoffers *et al.* (2006) with some modifications. The reaction mixture contained 100 mM sodium acetate buffer, pH 5, aliquot of samples, 0.15 mM NADH, 1.6 mM *p*-coumaric acid, and 16 mM MnCl_2 . Enzyme activity was calculated using the extinction coefficient for NADH ($\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$).

During NADH-POD oxidase activity, the formation of H_2O_2 occurs (Fecht-Christoffers *et al.*, 2006). After 1 min of NADH oxidation by peroxidase, 10 mM pyrogallol was added and an increase in absorbance at 430 nm was followed for 2 min. The amount of H_2O_2 generated by oxidase activity was estimated according to the enzyme activity determined under the same conditions in the presence of a known H_2O_2 concentration.

EPR spectroscopy

EPR measurements were performed using a custom-made Teflon flat cell with one cell side made of oxygen-permeable thin Teflon foil. The spectra were recorded at room temperature using a Varian E104-A EPR spectrometer operating at X-band (9.51 GHz) and using the following settings: modulation amplitude, 2 G; modulation frequency, 100 kHz; microwave power, 10 mW; centre of magnetic field, 3410 G; scan range, 200 G, the same in all EPR spin-trap measurements. Spectra were recorded and analysed using EW software (Scientific Software). DEPMPO spin-trap (5-diethoxy-phosphoryl-5-methyl-1-pyrroline-*n*-oxide) (Alexis Biochemicals, Switzerland) was added to a final concentration of 42.5 mM. Distilled and deionized 18 M Ω water was used in all experiments.

Statistic analysis

All data were subjected to analysis of variance (ANOVA), and means were compared by the Holm–Sidak test (SigmaPlot 11.0, Systat Software, Inc., USA). The level of significance was set at $P < 0.05$. Two-way ANOVA was carried out to assess the difference in means from various concentrations of inhibitors and from various inhibitors at the same concentration, followed by multiple comparisons using the Holm–Sidak test ($P < 0.05$) test.

Results

Modified SDS–PAGE and IEF separation of iPOD and cPOD

After the cell wall had been isolated from roots of 2-week-old pea plants, it was used to extract two protein fractions: ionically and covalently bound to the cell wall. Ionically bound cell wall proteins were salt extracted (1 M NaCl) and covalently bound proteins were released with 0.5% cellulase and 2.5% pectinase. Peroxidase isoforms were separated by modified SDS–PAGE and stained with α -chloro-naphthol for detection of peroxidase activity. According to the work of Mika and L uthje (2003) modified SDS–PAGE with a low SDS concentration (0.1%) can be used for estimation of enzyme molecular weight. Four peroxidase isoforms with apparent molecular masses of 56, 46, 44, and 41 kDa were identified in the ionic fraction (Fig. 1A). In the covalent fraction, one peroxidase isoform was detected with an apparent molecular mass of 70 kDa (Fig. 1B). When the same gels were stained with TMB (an indicator of the haem prosthetic group) no other haem enzyme was obtained in the covalent fraction. However, in the ionic fraction, two bands in addition to those with peroxidase activity were separated (Fig. 1A). Modified SDS–PAGE with a low SDS concentration (0.1%) can also be used for detection of peroxidase isoforms on the gel and can distinguish peroxidase isoforms from other oxidases stained with TMB (Fig. 1). IEF of peroxidase isoforms extracted from either ionic or covalent cell wall protein fractions showed that iPODs had pI values distinct from those of cPOD (Fig. 2A). Isoenzyme profiles obtained in the pH gradient 2–11 showed that iPODs and cPODs had significantly different pI values: iPODs were highly

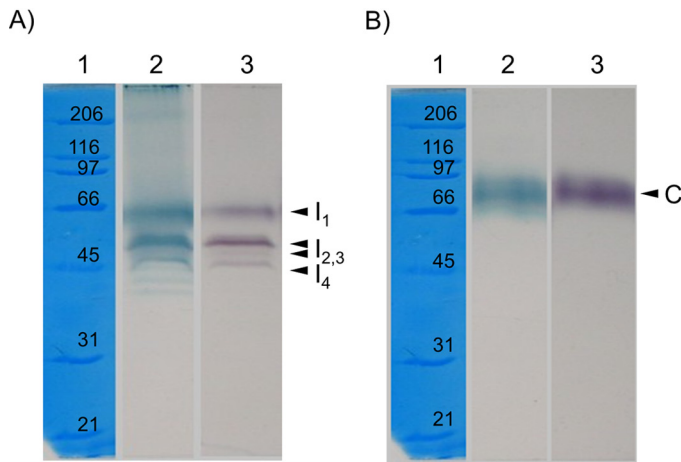


Fig. 1. Separation of peroxidase isoforms from ionic (A) and covalent (B) cell wall fractions by modified SDS-PAGE. Lane 1, protein standards with their corresponding molecular weight. Gels were stained for haem with TMB (lane 2) and with α -chloro-naphthol for peroxidase activity (lane 3). Arrows indicate different peroxidase isoforms. Four ionic peroxidases isoforms (I_1 – I_4) and one covalent isoform (C) were identified.

cationic, while cPODs were all anionic (Fig. 2A). Using ampholite with a narrow range of pH gradient, acidic for cPODs or alkaline for iPODs, five iPOD isoforms (Fig. 2B) and nine cPOD isoforms (Fig. 2C) could be separated.

Substrate and inhibitor studies

Peroxidases are enzymes with a wide spectrum of substrates, capable of oxidizing hydroxycinnamic derivatives and other phenolic

compounds with different specificities. Besides pyrogallol as an artificial electron donor, common endogenous hydroxycinnamic acids present in the pea root (Kukavica *et al.*, 2009) were used to analyse the total activity of soluble and cell wall-bound peroxidases (Table 1). The oxidation rate of chlorogenic, caffeic, and ferulic acids was expressed as an absorbance increase at 410, 450, and 356 nm, respectively. An absorbance increase, rather than a absorbance decrease, was measured (Bestwick *et al.*, 1998; Hadži-Tašković Šukalović *et al.*, 2003; Mika and Lühje, 2003) for two reasons: (i) the substrate concentration was above the measured K_m values; and (ii) there is no possibility of overlapping in the absorbance spectra of substrate and products, as found to be the case for chlorogenic acid (data not shown). The analyses of enzyme affinity for different substrates by determining the K_m (Table 1) showed that all peroxidases had a similar K_m for pyrogallol that differs from that of horseradish peroxidase (HRP). It was determined that only cPOD had a lower affinity for H_2O_2 ($K_m=2.4$ mM) when compared with all other fractions and HRP (K_m 1.1–1.4 mM; Table 1). The lowest activity for all peroxidases was measured with ferulic acid as reducing substrate; cPOD had the highest affinity for ferulic acid compared with all others peroxidases (Table 1). Compared with HRP, all pea root peroxidases had twice as high K_m values for caffeic acid.

IEF of peroxidase isoforms extracted from either ionic or covalent cell wall protein fractions showed that both fractions consisted of several isoforms (Fig. 2), which might differ in their substrate specificity. To test this possibility, IEF gels with separated iPODs and cPODs were stained for peroxidase activity using different substrates: α -chloro-naphthol, 3,3'-diaminobenzidine (DAB), guaiacol, or IAA (Fig. 3). Four iPOD isoforms (pI 9.5, 9.3, 9.24, and 9.2) had similar activity with each of the substrates, while the isoform with pI 9.34 had

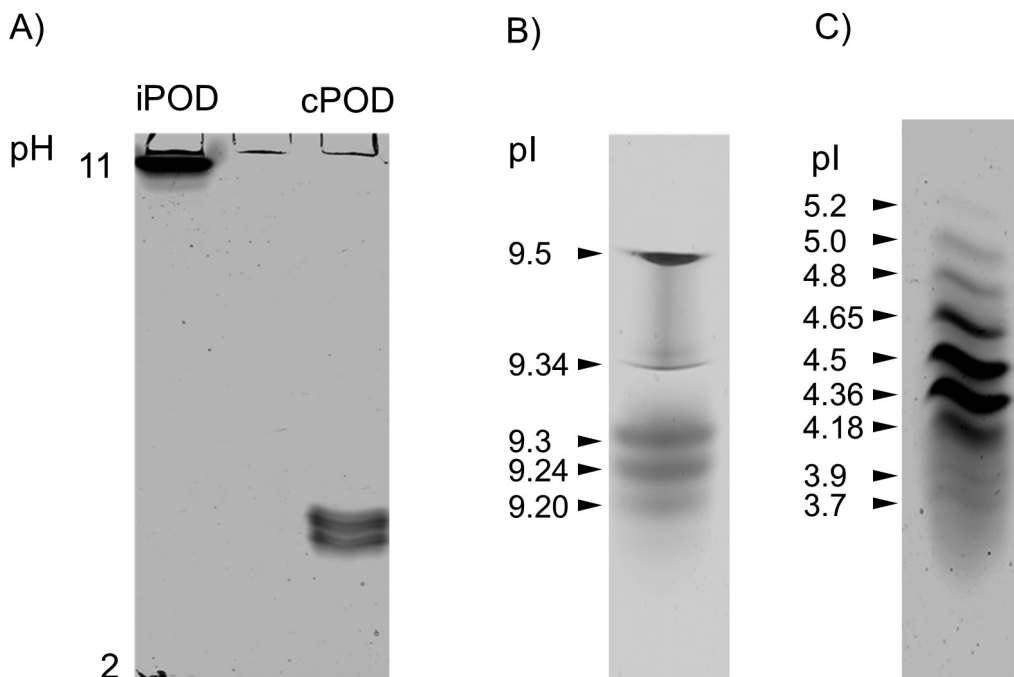


Fig. 2. IEF-PAGE of peroxidase isoforms of ionic and covalent cell wall fractions. Separation of ionically and covalently bound peroxidases obtained using a wide pH range (A), an alkaline pH range (9–11) for iPOD (B), and an acidic pH range (3–7) for cPOD (C). Peroxidase isoforms on the IEF gel were visualized with α -chloro-naphthol.

Table 1. K_m values of apoplastic (aPOD), ionic (iPOD), and covalent (cPOD) cell wall-bound peroxidases from pea roots and HRP for different reducing substrates (pyrogallol, chlorogenic, caffeic, and ferulic acids) in the presence of 3.3 mM H_2O_2 . K_m values for H_2O_2 were obtained with pyrogallol as a substrate.

CGA, chlorogenic acid; CA, caffeic acid; FA, ferulic acid.

Fraction	K_m (mM)				
	H_2O_2	Pyrogallol	CGA	CA	FA
aPOD	1.4 ± 0.2	2.6 ± 0.4	0.6 ± 0.2	2.7 ± 0.5	4.0 ± 0.7
iPOD	1.3 ± 0.1	2.5 ± 0.3	1.0 ± 0.2	2.4 ± 0.3	1.2 ± 0.3
cPOD	2.4 ± 0.4	2.7 ± 0.3	0.7 ± 0.12	2.1 ± 0.2	0.6 ± 0.2
HRP	1.4 ± 0.3	1.1 ± 0.1	1.6 ± 0.3	0.9 ± 0.2	1.1 ± 0.4

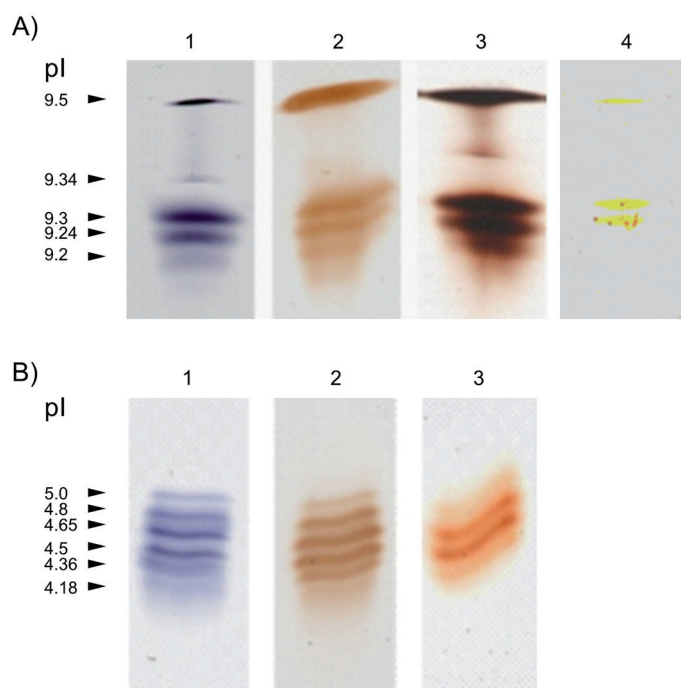


Fig. 3. Substrate affinity of iPOD (A) and cPOD (B) isoforms on an IEF gel. After separation of the isoforms, gels were stained with 18 mM α -chloro-naphthol (lane 1), 1 mM 3,3-diaminobenzidine (DAB; lane 2), 20 mM guaiacol (lane 3), and 1 μ M IAA (lane 4).

no activity with DAB (Fig. 3A). Two cPOD isoforms (pI 4.5 and 4.65) had the highest activity with guaiacol and α -chloro-naphthol, while four isoforms (pI 4.8, 4.65, 4.5, and 4.36) had similar activity with DAB (Fig. 3B). The results suggested that only the protein with a pI 9.34 differed from all other iPODs regarding its substrate specificity, while among six cPODs, only

two had similar affinity for all three substrates. For staining of IAA oxidase activity on the IEF gel, the procedure was carried out as described by Hoyle (1977). Comparison of staining patterns of IAA oxidase and peroxidase activities showed that three of five iPOD isoforms (pI 9.5 and 9.3, and 9.24) had also oxidizing activity, while none of the cPOD isoforms had IAA oxidase activity (Fig. 3). It was also shown that iPODs and cPODs differed in their K_m for H_2O_2 (Table 1), with significantly higher affinity for H_2O_2 of cPODs.

Catalase-like activity was measured in all cell wall fractions, with the highest rate obtained in the cell wall fraction with cPODs, compared with HRP, soluble peroxidases, and iPODs (Table 2). The catalase-like activity of peroxidase was proposed to act as a major protective mechanism for HRP-C against inactivation by H_2O_2 , being optimal at pH > 6.5 (Hernandez-Ruiz *et al.*, 2001). The present study showed that the highest rate of O_2 evolution coincided with the highest resistance to H_2O_2 -induced inhibition of peroxidase reaction (Table 2).

It has been shown that isolated cell wall fragments containing cPOD spontaneously generated $\cdot OH$ radicals as demonstrated by the spectrum of the DEPMPO/OH adduct signal (Kukavica *et al.*, 2009). In addition to cPOD and iPOD, the apoplastic and soluble fractions have a capacity to generate $\cdot OH$ (Fig. 4), although to a much lesser extent (10% of the signal being induced by cPOD). In all of the cases studied, the generation of the DEPMPO/OH adduct signal was oxygen dependent, with measurements in a nitrogen atmosphere abolishing the DEPMPO/OH adduct signal (as in Veljović-Jovanović *et al.*, 2005). It was also previously demonstrated that purified commercial HRP did not generate either of the two adducts, DEPMPO/OOH or DEPMPO/OH, while addition of H_2O_2 induced the generation of both adducts (Kukavica *et al.*, 2009). Also the addition of H_2O_2 to cell wall fragments led to increased production of $\cdot OH$ signal, while the signal $O_2^{\cdot -}$ can

Table 2. Catalase-like activity of the cell wall fraction with cPODs, iPODs, soluble extract from pea roots (sol. ex.), and commercial peroxidase from horseradish (HRP). Differences in peroxidase sensitivity on inactivation with H_2O_2 were determined by measurements of pyrogallol-dependent peroxidase activity as described in the Materials and methods after aliquots of all fractions were incubated for 30 min in 10 mM H_2O_2 in phosphate buffer, pH 6.5 at 30 °C. Catalase-like activity was determined by measuring oxygen production with a Clark-type electrode in the presence of 3 mM H_2O_2 at 30 °C.

	cPOD	iPOD	sol. ex.	HRP
O_2 evolution (nmol min ⁻¹ g ⁻¹ protein)	230 ± 38	43 ± 6	40 ± 3	17 ± 0.6
Inactivation of POD activity (%)	9 ± 3	64 ± 11	76 ± 9	81 ± 4

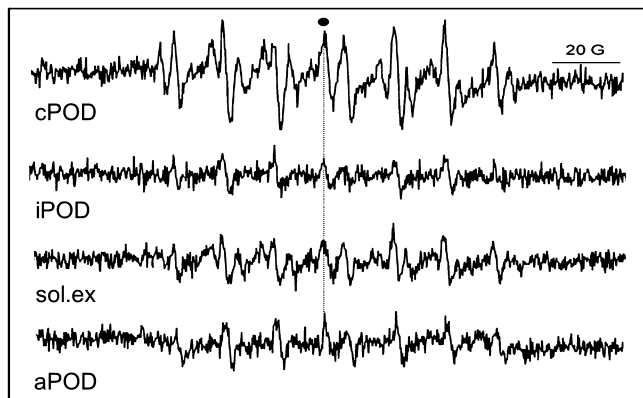


Fig. 4. EPR spectra of the DEPMPO/OH adduct obtained from cPOD bound to the cell wall, iPOD, soluble protein extract from roots (sol. ex.), and apoplastic fluid isolated from intact pea roots (aPOD). Aliquots with the same peroxidase units in different isolates were used for EPR measurements.

only be seen after the addition of very high concentrations of H_2O_2 (10 mM) (data not shown).

The effects of three common peroxidase inhibitors KCN, salicylhydroxamic acid (SHAM), and NaN_3 , on the activity of iPOD and cPOD isoforms were tested on IEF gels. After separation of peroxidase isoforms, IEF gels were incubated in different inhibitor concentrations and, after thorough washing, gels were stained for peroxidase activity as described in the Materials and methods. The effects of inhibitors on the activity of peroxidase isoforms were similar among isoforms of the same type of binding to the cell wall. The inhibition of total peroxidase activity

in the ionic fraction increased with increasing concentration of inhibitor, which is especially pronounced in the case of SHAM (Fig. 5A). All iPODs were more sensitive to the inhibition by SHAM compared with cPODs (Fig. 5B). The iPOD with pI 9.3 and iPOD with pI 9.24 were the least sensitive to all three inhibitors, whereas the isoforms at pI 9.34 and pI 9.2 were the most sensitive (data not shown). The two isoforms among cPODs (pI 4.5 and 4.65) were the most resistant to inhibition with azide.

When aliquots of ionic and covalent cell wall protein fractions were pre-incubated at various temperatures (4–80 °C) and peroxidase isoforms separated on an IEF gel, a distinctive sensitivity of iPODs and cPODs to high temperatures was obtained (Supplementary Fig. S1 available at *JXB* online). At extremely high temperatures (80 °C), only the two iPOD isoforms (pI 9.5 and 9.34) were completely inhibited, while all cPODs were inhibited at 80 °C and significantly inhibited also at 60 °C.

Glycosylation and binding properties of iPOD and cPOD isoforms

Class III peroxidases are haem-containing glycosylated proteins. Carbohydrates may modulate the physicochemical and biochemical properties of the enzyme and may change its activity, resistance to protease attack, or thermal stability. The effects of deglycosylation on iPOD and cPOD activity were compared to determine if they also differed in a binding site for the carbohydrate moiety. For deglycosylation treatments, Endo F1, F2, and F3 and PNGase F were used. After the deglycosylation treatment lasting for 24h, the activity of cell wall peroxidases was measured (Table 3). The results showed that the treatment with endoglycosidases caused inhibition of cPOD activity (from 26%

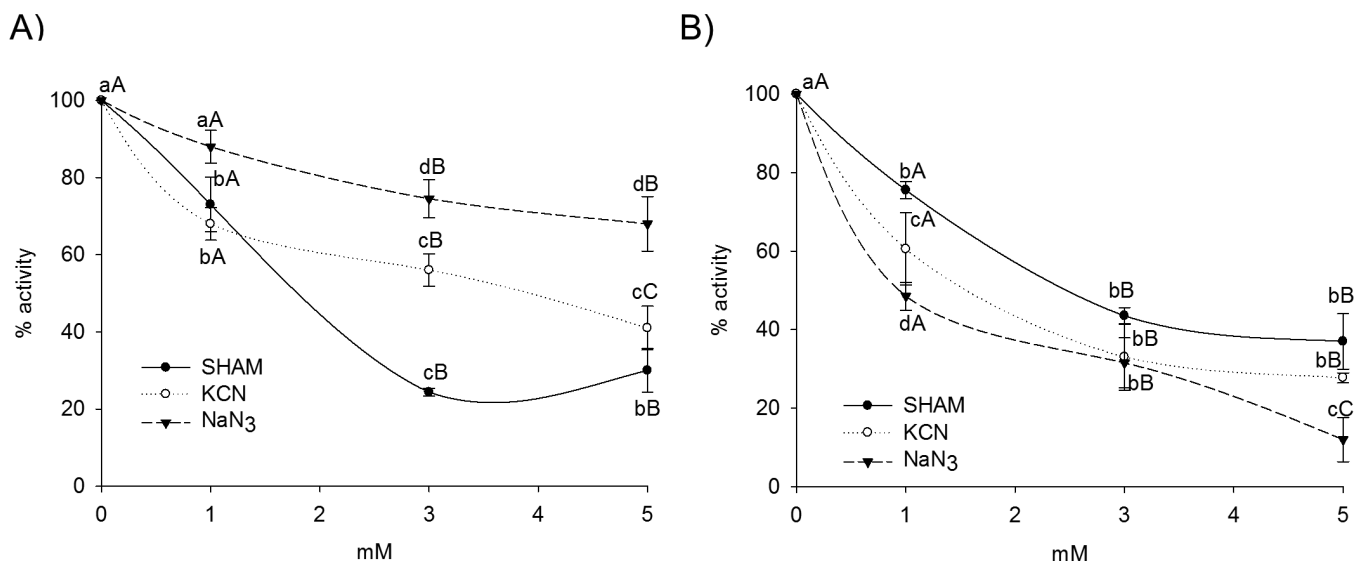


Fig. 5. Inhibition of iPOD (A) and cPOD (B) activity after 15 min incubation in solution with different concentrations of inhibitors (SHAM, KCN, and NaN_3). The peroxidase activity after treatment with inhibitor is expressed as a percentage of the control. Peroxidase activity represents the sum of the relative activity of each peroxidase isoform calculated by using the program TotalLab. Two-way ANOVA was carried out to assess the difference of means from various concentration of inhibitors and from various inhibitors at the same concentration, followed by multiple comparisons using the Holm–Sidak test ($P < 0.05$). Different lower case letters denote the results for the same concentration of different inhibitors (and controls), which are significantly different ($P < 0.05$, Holm–Sidak), and different upper case letters denote the results for the different concentration of the same inhibitors (and controls), which are significantly different ($P < 0.05$, Holm–Sidak).

Table 3. The effect of native protein deglycosylation on the peroxidase activity in ionic and covalently cell wall-bound fractions. Means followed by an asterisk are significantly different from control at $P < 0.05$, according to Holm–Sidak test.

	iPOD		cPOD	
	Activity ($\Delta A \text{ min}^{-1}$)	% of control	Activity ($\Delta A \text{ min}^{-1}$)	% of control
Control	0.2697 \pm 0.0197	100	0.01113 \pm 0.001	100
+F1	0.3068 \pm 0.056	114	0.0083 \pm 0.0014*	74
+F2	0.3132 \pm 0.03	116	0.0071 \pm 0.0003*	64
+F3	0.318 \pm 0.036	118	0.0069 \pm 0.0002*	62
+PNGase	0.1 \pm 0.014*	37	0.0118 \pm 0.0023	106

Table 4. Effects of the lectins, concanavalin A (Con A), and wheat germ agglutinin (WGA) on iPOD and cPOD activity. Peroxidase activity was determined by absorbance change measurement at 430 nm in the presence of 20 mM pyrogallol. Means followed by an asterisk are significantly different from control at $P < 0.05$, according to Holm–Sidak test.

	iPOD		cPOD	
	Activity ($\mu\text{mol min}^{-1} \text{ mg}^{-1} \text{ protein}$)	% of control	Activity ($\mu\text{mol min}^{-1} \text{ mg}^{-1} \text{ protein}$)	% of control
Control	102 \pm 2	100	4.1 \pm 0.12	100
+1 $\mu\text{g ml}^{-1}$ ConA	107 \pm 3.4	105	3.6 \pm 0.3*	88
+5 $\mu\text{g ml}^{-1}$ ConA	104 \pm 1	101	3.1 \pm 0.5*	76
+1 $\mu\text{g ml}^{-1}$ WGA	106.6 \pm 1.6	104	2.9 \pm 0.15*	71
+5 $\mu\text{g ml}^{-1}$ WGA	103 \pm 1.6	101	7 \pm 1*	173

to 38%), while they did not affect the activity of iPOD. In contrast to this, PNGase F inhibited iPODs ~63% and even slightly increased cPODs activity (Table 3). Additionally, to test a possible protein glycosylation, the changes in peroxidase activity caused by binding of lectins to sugar moieties were determined. The result showed different effects of the lectins Con A and WGA on the activity of iPOD and cPOD (Table 4). While the activity of cPOD was inhibited, that of iPOD was even slightly increased after treatment with both molecules, Con A and WGA. Increasing concentrations of Con A and WGA further inhibited cPOD activity (Table 4).

Zonal distribution of iPOD and cPOD along the root

Root growth is divided into phases with several zones along the root moving upwards towards the stem: the root tip, the zone of active division, the zone of cell elongation, and the zone of maturation or differentiation. Cell wall peroxidases are involved in elongation and also in differentiation processes: cell wall loosening is necessary for elongation and cell wall stiffening for differentiation. In the present study, the cell wall was extracted from four zones along the root of 3-day-old seedlings and the distribution of iPODs and cPODs in the samples was analysed by separation of the isoforms by IEF–PAGE (Fig. 6). Isoperoxidase profiles of iPOD did not change significantly along the root (Fig. 6B), whereas a significant increase in abundance of cPODs was obtained in zone II and in the last zone (IV) with differentiated cells (Fig. 6C). In root tips, only three cPODs (pI 4.5, 4.36, and 4.18) were detected with the lowest activity (Figs 6B, 7B). Induction of new cPOD isoforms with pI 4.6, 4.8, and those with pI 3.9–3.7 occurred in the root base consisting of differentiated

vascular tissue (Supplementary Fig. S3 at *JXB* online). The total activity of iPODs and cPODs was measured with pyrogallol as substrate (Fig. 7A, B) and corresponded to the intensity of bands from the gel. In addition to peroxidase activity, iPODs and cPODs were analysed for their ability to oxidize NADH and generate H_2O_2 in the oxidative cycle of peroxidase (Elstner and Heupel, 1976; Halliwell, 1978). Although there is no proof for the occurrence of NADH in the apoplast under standard conditions, release of NADH into the apoplast could be demonstrated after wounding (Zhang and Mou, 2009); NADH-POD oxidase activity of iPOD decreased, while that of cPOD increased along the root from the tip to the base (Fig. 7C, D).

Induction of iPODs and cPODs in root during plant growth

Root growth begins as soon as seedling germinate by division of meristemic cells in the root tip and by elongation of cells in zone II of the primary root. After 6 d, secondary roots started to branch from the original primary root and continued with growth during a 3 week period (Supplementary Fig. S2 at *JXB* online). Induction of specific cell wall peroxidases during root growth was determined using IEF separation of iPODs and cPODs extracted from the root of pea seedlings and the whole root system of plants 6, 10, 17, and 23 days old (Figs 8, 9). Total iPOD activity increased 3-fold with the appearance and elongation of secondary roots (after 6 d and 10 d). The induction of a new ionic isoform with pI 9.34 and an increase in abundance of one with pI 9.5 accompanied elongation of primary and secondary roots (Fig. 8A).

In the covalent fraction, high peroxidase activity was measured in the seedlings, and then it decreased during elongation of

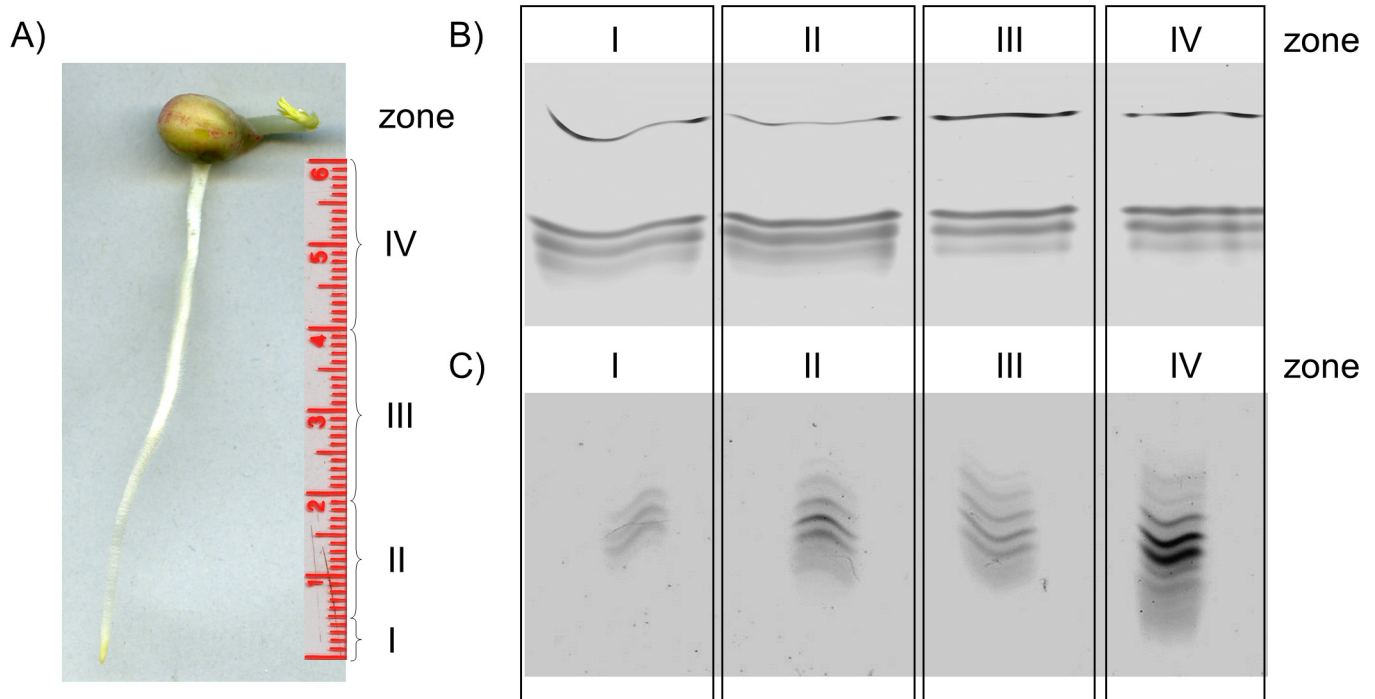


Fig. 6. Zonal distribution of cell wall-bound isoperoxidases. Allocation of root zones (A) used for peroxidase analysis. Distribution of iPOD (B) and cPOD (C) isoforms along the root zones separated on an IEF gel and stained with α -chloro-naphthol. Pea seedlings were used for cell wall isolation.

primary and secondary roots (Fig. 9B). After 17 d, lignification of endodermal cells started, as well as formation of the Casparian strip (data not shown). Only on 23rd day, the central cylinder was almost fully formed and lignified (Supplementary Fig. S3 at *JXB* online) which was accompanied by the highest activity of cPODs (Fig. 9A, B). The peroxidase activity was also visualized by staining with α -chloro-naphthol of the root cross-section in the area of the central cylinder (Supplementary Fig. S3). However, the Casparian strip was not completely formed, implying that the root was still growing.

NADH oxidase activity in both protein fractions increased significantly with plant growth. The ratio of NADH oxidase and peroxidase activity for the ionic fraction was the highest for 6-day-old plants, when secondary roots started to develop (Supplementary Fig. S2 at *JXB* online). Although in the covalent fraction the ratio of oxidase/peroxidase activity increased with the age of the plants, the NADH oxidase reaction was not accompanied by H_2O_2 production. Only iPODs had the capacity to produce H_2O_2 during NADH oxidation (Fig. 8C).

Auxin and chitosan induced changes in the peroxidase profile

Treatment of pea plants with auxin (10 μ M NAA) for 11 d led to a significant inhibition of root growth, which was similar to the observations of Kukavica *et al.* (2007). The inhibition and an intensified lignification were accompanied by a decrease in iPOD and an increase in cPOD activities (Fig. 10). Two iPOD isoforms with pI 9.5 and 9.3 almost disappeared, while NAA

induced a new cPOD isoform with pI 5.2 along with a >2-fold increase obtained in the intensity of all cPODs (Fig. 10B).

Chitosan is known to have eliciting activities leading to a variety of defence responses in host plants to microbial infections, including the accumulation of phytoalexins, pathogen-related (PR) proteins, and proteinase inhibitors, lignin synthesis, and callose formation. In maize and pea plants, chitosan led to slightly increased total peroxidase activity of the membrane fraction, while the total activity of soluble peroxidase did not change (Mika *et al.*, 2010; Meisrimler *et al.*, 2011). The present study shows a remarkable effect of chitosan on cell wall-bound peroxidases, both iPODs and cPODs, compared with the control (Fig. 11). In Fig. 12, the effects of 1 g l⁻¹ chitosan on peroxidase patterns in the ionic and covalent fraction of the cell wall isolated from plants of different ages are shown. Plants were grown in Hoagland solution till the treatment was applied on the third, sixth, 10th, 17th, and 23rd day, lasting for 16 h.

Chitosan induced significant inhibition of activity of all iPODs regardless of plant age (Fig. 11, left panels; Fig. 12A). However, the effect of chitosan on the activity of cPODs was dependent on the plant age: it decreased in the youngest and oldest plants. When chitosan was applied on the 10th day, cPOD isoforms (pI 5.2 and pI 5.0) were induced (Fig. 11, right panels; Fig. 12B). In other words, an opposite effect of chitosan was obtained that depends on plant age: a decrease in number and intensity of cPOD isoforms in root of 3- and 23-day-old plants and an increase in number and intensity of cPODs extracted from 10- and 17-day-old roots (Figs 11, 12).

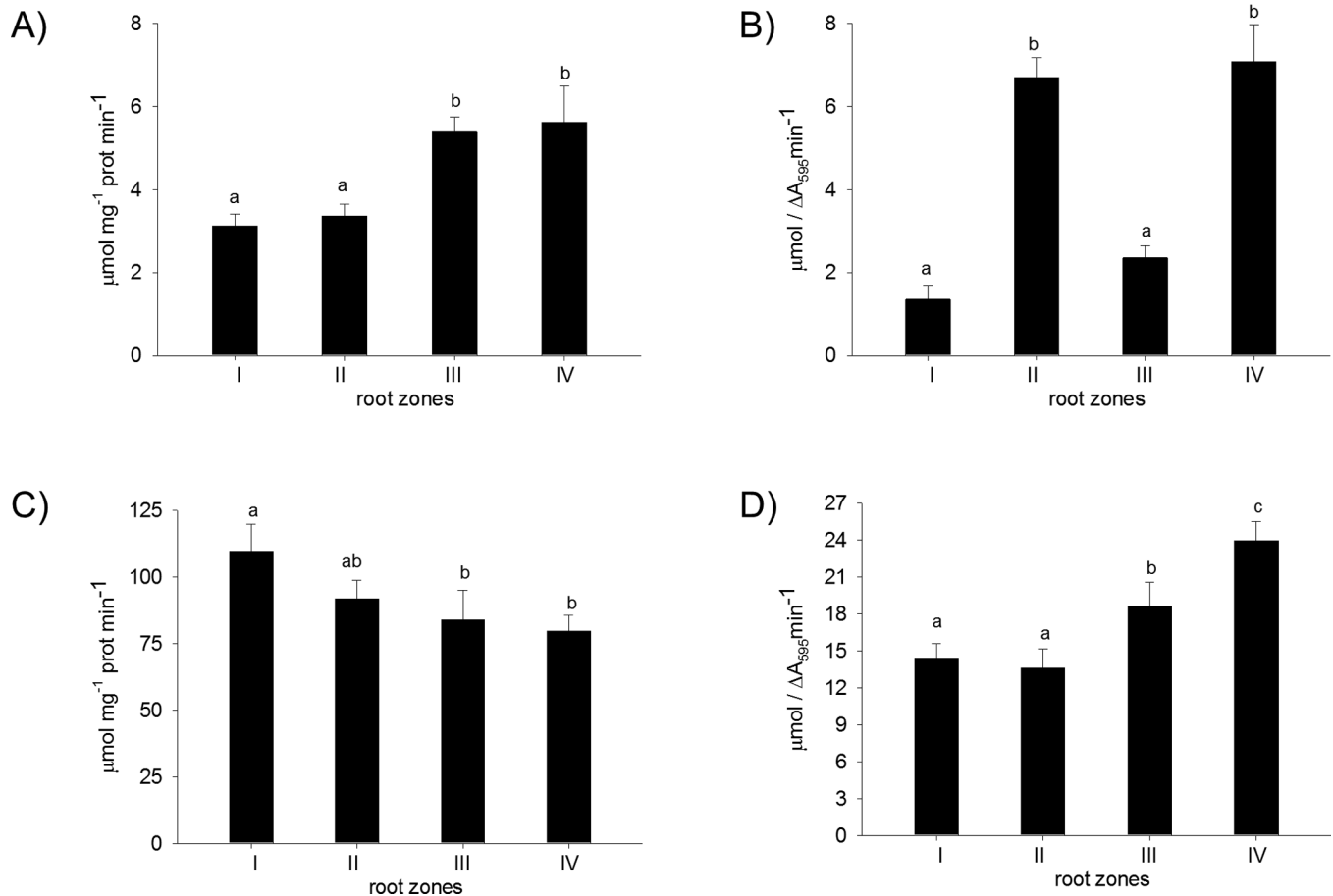


Fig. 7. Total peroxidase (A, B) and NADH-POD oxidase activity (C, D) of ionic (A, C) and covalent (B, D) cell wall fractions. Cell wall fractions were isolated from different root zones of 3-day-old pea seedlings. Peroxidase activity was measured using pyrogallol as substrate as described in the Material and methods. Oxidizing activity of peroxidase was measured with NADH in the presence of catalytic concentrations of Mn and *p*-coumaric acid. Values with different letters are significantly different at $P < 0.05$, according to Holm-Sidak test.

Discussion

Biochemical and binding properties of cell wall peroxidases

The cell wall of pea root contains two groups of peroxidases according to their isoelectrophoretic mobility: cationic peroxidases that are retained within the polysaccharide matrix by ionic interactions and anionic peroxidases that are cross-linked by covalent bonds. In the present study, five cationic isoforms were detected with pI values between 9.2 and 9.5 (apparent mol. wts from 41 kDa to 56 kDa) and nine anionic isoforms with pI values ranging from 3.7 to 5.2 with apparent mol. wts of ~70 kDa (Figs 1, 2). A wide range of peroxidase isoforms regarding pI (from pI 2 to 11.6) were found in many species (Welinder and Mazza, 1975; Heidrich *et al.*, 1983; Floris *et al.*, 1984; Quiroga *et al.*, 2001; Dicko *et al.*, 2006). HRP extract from commercial sources contained 42 isoforms with a different range of pI values, from 2 to 10 (Hoyle, 1977). The present study showed that isoform patterns differ depending on the type of binding to the cell wall constituents. Among cell wall polysaccharides, with most of them being neutral, only pectins provide negative

charges for interactions with basic proteins. As such interactions may be modified by pH, Ca^{2+} concentration (Penel and Greppin, 1996), or pectin esterification (Ros Barcelo *et al.*, 1988; Penel and Greppin, 1994), pectin may be involved in the regulation of peroxidase distribution within the apoplast. A distinct charge of these two groups of isoforms, which are bound to the cell wall of pea root, might imply different domains within the cell wall and, accordingly, a specific physiological function. Indeed, the results from the literature are in favour of the hypothesis that anionic and cationic isoforms have specific and distinct physiological functions *in situ*. There are numerous articles regarding the specific roles of either cationic (Church and Galston, 1988; Abeles and Biles, 1991; Sato *et al.*, 1995; Ros Barcelo *et al.*, 1998) or anionic isoperoxidase (Ferrer *et al.*, 1991; McDougall, 1992; Mohan *et al.*, 1993; Ros Barcelo, 1997) in lignification and suberization (Espelie and Kolattukudy, 1985; Bernards *et al.*, 1999). However, Quiroga *et al.* (2001) demonstrated that both the basic isoform with pI 9.6 and the acidic isoform with pI 3.6 from tomato root showed a similar catalytic efficiency with coniferyl alcohol as substrate. It was also shown that both iPODs and cPODs have a similar K_m for pyrogallol, chlorogenic acid, and

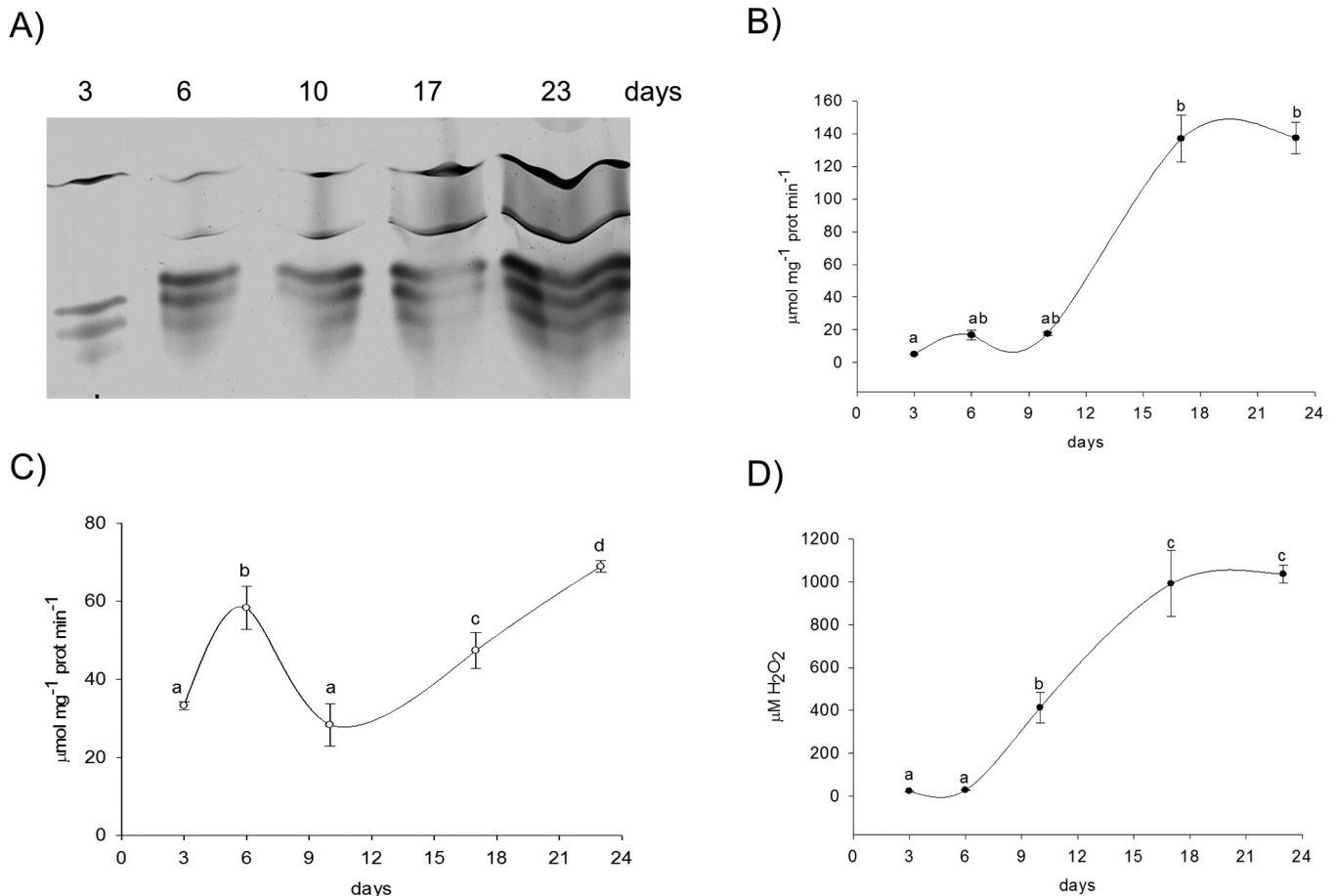


Fig. 8. IEF pattern of iPOD (A), total peroxidase (B), and NADH-POD oxidase activity (C), and capacity of iPOD to produce H_2O_2 (D) in root cell wall isolated from plants of different ages as indicated. Whole roots were used for cell wall isolation. Peroxidase activity was measured spectrophotometrically with pyrogallol as a substrate. Measurement of NADH-POD oxidase activity and capacity for formation of H_2O_2 was done according to Fecht-Christoffers *et al.* (2006). Values with different letters are significantly different at $P < 0.05$, according to Holm–Sidak test.

cumaric acid (Table 1). The only difference in substrate affinity between iPODs and cPODs was found for H_2O_2 , with the K_m of cPODs being 2-fold higher than that of iPODs.

The differences in biochemical properties between isoperoxidases ionically and covalently bound to the cell wall are summarized in Table 5. Besides their different electrophoretic mobility and apparent molecular weights, comparative analysis of their oxidizing and catalase-like activities, sensitivity to deglycosylation, and the effects of inhibitors or high temperature indicate that iPODs and cPODs possess characteristic and specific structure and also different binding domains within the cell wall. Taken together, the results implicate their specific role in root physiology during development and defence response.

Peroxidases are bifunctional enzymes, possessing the capacity to catalyse either oxidative or peroxidative reactions, which *in vitro* depends on the presence of activators (diphenols and Mn^{2+} and NADH as reductant). A novel function of peroxidase in the hydroxylic cycle has been postulated (Chen and Schopfer, 1999; Liszczay *et al.*, 2003). It was found that both cell

wall fractions had oxidizing activity when NADH was used as reductant (Fig. 7). However, no H_2O_2 production during NADH oxidation was detected with anionic isoforms. Also when IAA was used as a reductant to stain the oxidizing reaction on the gel, the activity was obtained only with cationic isoforms. On the other hand, it was shown that anionic or cPODs bound to the cell wall were the most efficient in $\cdot\text{OH}$ production compared with iPOD, apoplastic peroxidase, or HRP (Fig. 4). A failure of iPODs or commercial HRP (VI and II type) to promote $\cdot\text{OH}$ production in the absence of NAD(P)H even when added to the deproteinated cell wall may implicate that a specific property of cPODs is inevitable for this reaction (Kukavica *et al.*, 2009). In addition to that, it was also shown that cPODs had the highest catalase-like activity (Table 2), being the most stable against H_2O_2 inactivation. Besides that, the cPOD isoforms possess a different affinity for H_2O_2 compared with the peroxidases in all other fractions (Table 1). Since the K_m can be used to approximate the value of the intercellular level of the substrate (Segal, 1976); a higher K_m of H_2O_2 for cPODs ($K_m = 2.36 \text{ mM}$) than for all other fractions may indicate their specific

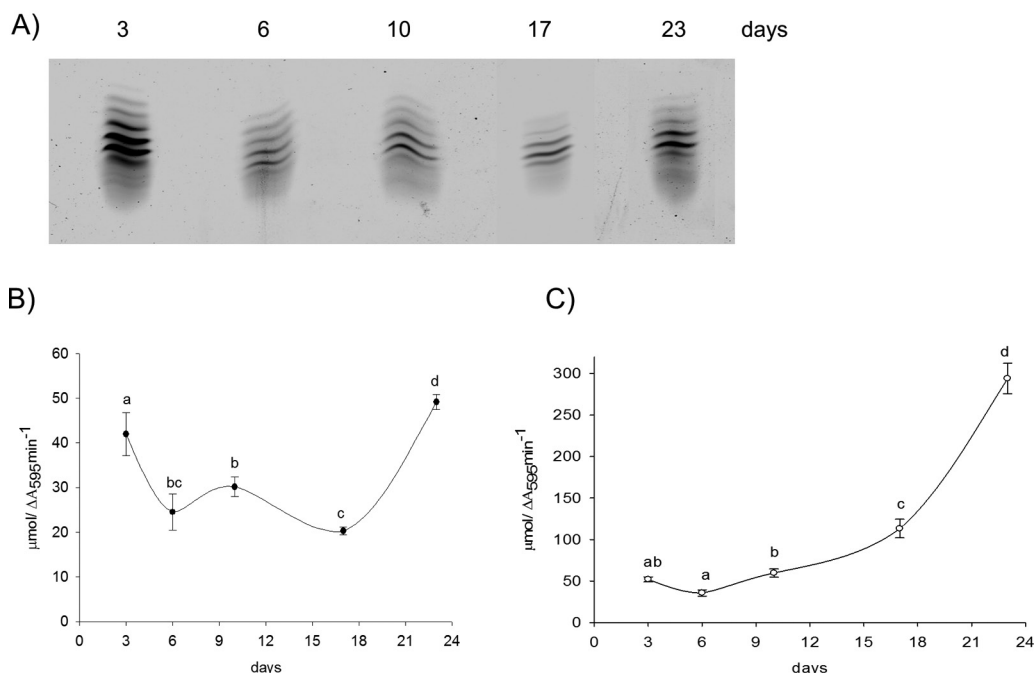


Fig. 9. IEF pattern of cPOD (A), total peroxidase (B), and NADH-POD oxidase activity (C) in root cell wall isolated from plants of different ages as indicated. Whole roots were used for cell wall isolation. Peroxidase activity was measured spectrophotometrically with pyrogallol as a substrate. Measurement of NADH-POD oxidase activity was done according to Fecht-Christoffers *et al.* (2006). Values with different letters are significantly different at $P < 0.05$, according to Holm–Sidak test.

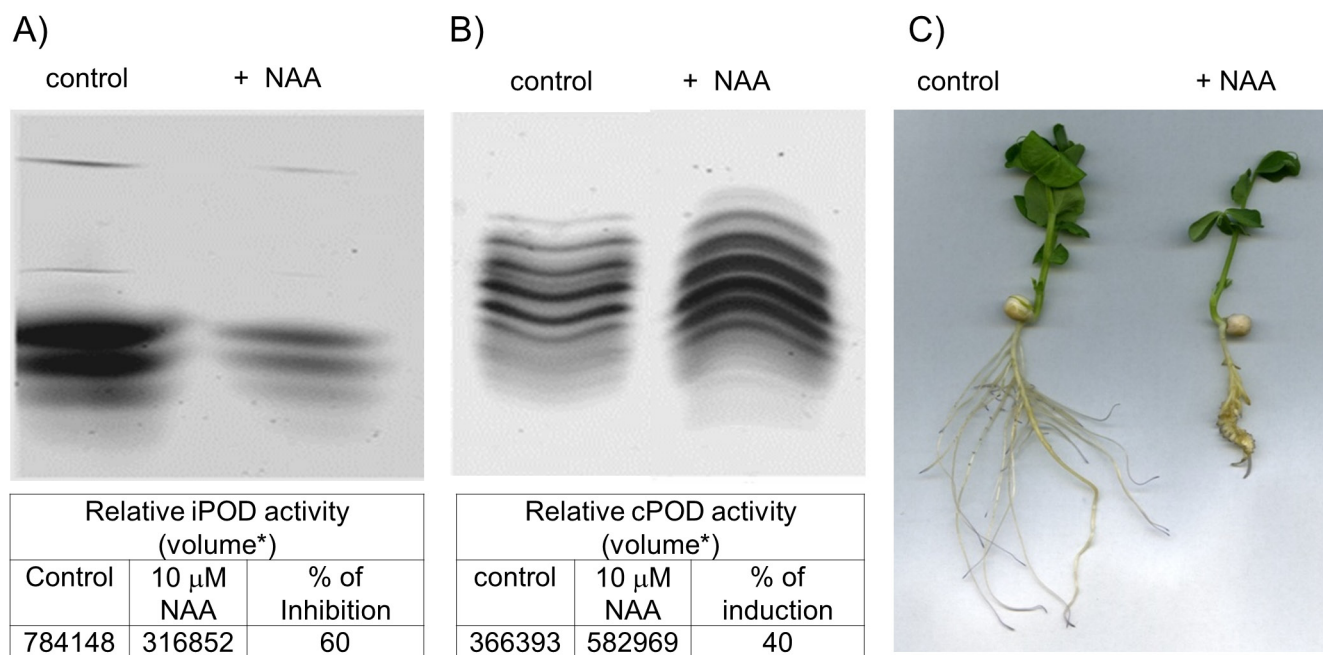


Fig. 10. Inhibitor of root growth of 10-day-old plants induced by NAA (10 μM) was accompanied by a decrease in iPOD (A) and an increase in cPOD (B) isolated from root cell wall. NAA was present in hydroponic solution from the beginning of the experiment. The tables show the total peroxidase activity that represents the sum of the relative activity of each peroxidase isoform. Data were calculated by TotalLab, and the percentage of inhibition of iPOD and induction of cPOD activity is given. *The raw volume of the uncalibrated quantity of material in the image feature after the background intensity has been removed (the units of the numbers are expressed as relative activity units). (C) Roots of control and treated pea plants. Roots were incubated for 15 min in 0.1 M Na-phosphate buffer pH 7.5 containing 0.005% NBT to indicate *in vivo* production of superoxide anion radical.

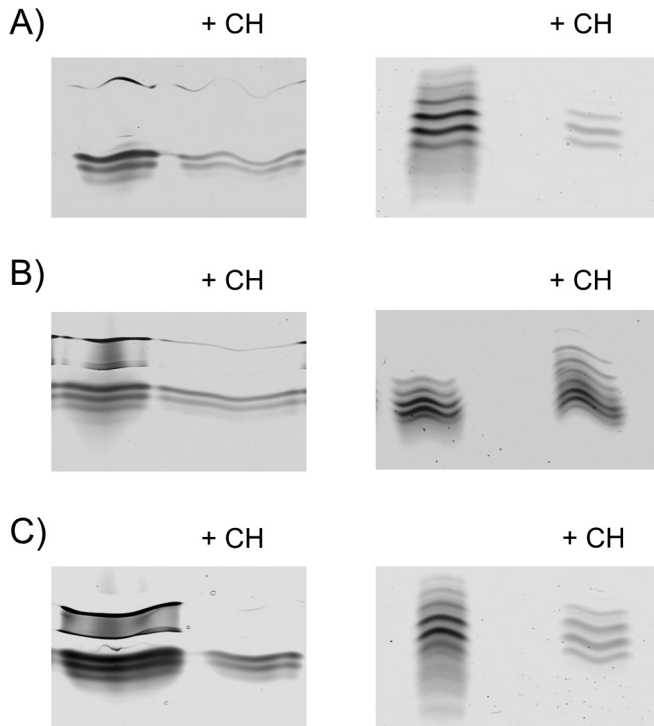


Fig. 11. Effect of chitosan (CH) on the IEF pattern of iPOD (left) and cPOD (right) isolated from root cell wall. Plants of different ages, 3 (A), 10 (B), and 23 (C) d, were treated with 1 g l^{-1} of chitosan for 16h.

role in physiological processes dependent on H_2O_2 concentration (Schopfer, 1994). It is hypothesized that iPODs and cPODs differ in their capacity to gain an oxidative or hydroxylic cycle, with iPODs that favour the oxidative cycle coupled to the peroxidative, while cPODs are preferentially involved in the hydroxylic cycle.

Developmental- and elicitor-induced changes in anionic and cationic cell wall peroxidases

A significant increase in the activity of anionic isoforms was obtained only in the fourth zone of the root with differentiated cells, while cationic isoforms did not significantly change along the 3-day-old root (Fig. 6). On the other hand, growth of pea plants during 3 weeks was accompanied by an increase in the abundance and activities of cationic isoforms, both peroxidative and oxidative. Additionally, a new isoform with pI 9.34 appeared on the sixth day and increased with root elongation (Fig. 8). The capacity of cationic peroxidases to oxidize NADH in the presence of diphenol and Mn^{2+} and to produce H_2O_2 also increased with time. However, the anionic isoforms were most abundant in the roots of seedlings and in the roots of the oldest plants used in the experiment. The results suggest that cationic isoforms with pI 9.34 and 9.5 are specifically involved in root elongation, and anionic isoforms are involved in the formation of the cell wall and lignification. It has been shown that peroxidases covalently

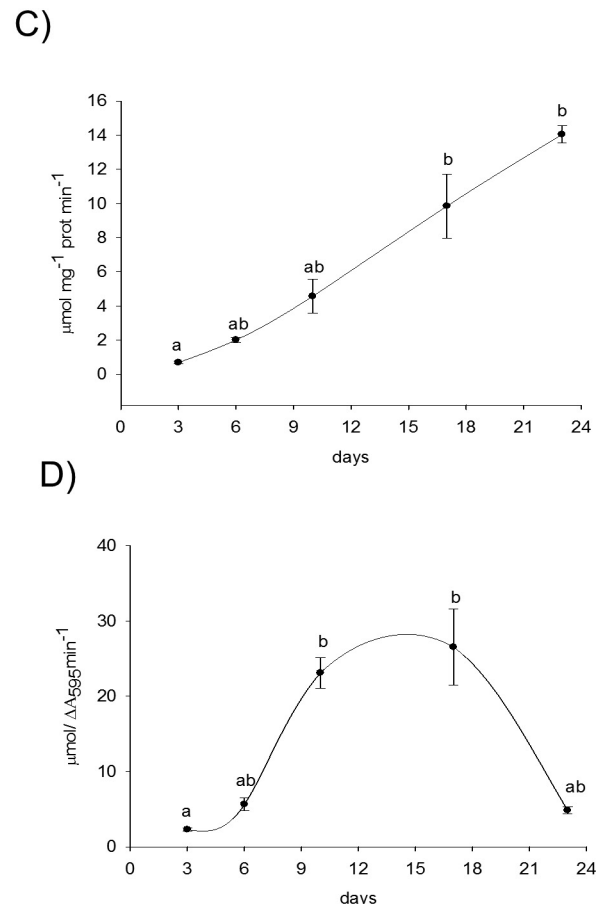
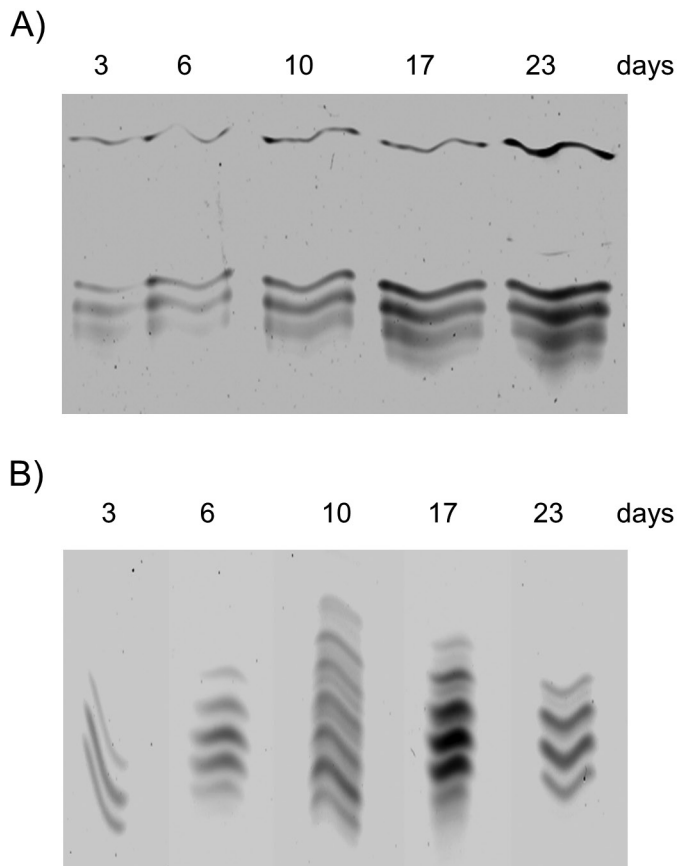


Fig. 12. Chitosan induced distinct changes in iPOD (A) and cPOD (B) isoform activities in roots of plants of different ages as indicated. Data on iPOD (C) and cPOD (D) activity obtained spectrophotometrically with pyrogallol as a substrate. Values with different letters are significantly different at $P < 0.05$, according to Holm-Sidak test.

Table 5. Biochemical properties of pea cell wall peroxidases.

+++ , highest inhibition; + , lowest inhibition.

	iPOD	cPOD
pI	Cationic (9.2–9.5)	Anionic (5.2–3.7)
Thermostability	Very high (80 °C)	High (60 °C)
Deglycosylation (F1, F2, F3)	No effect	+++
Deglycosylation (PNGase)	+++	No effect
Binding lectins (Con A, WGA)	No effect	+++
OH production	No	Yes
Catalase-like activity	No	Yes
IAA oxidase activity	Yes	No
Capacity for H ₂ O ₂ production	Yes	No
K _m for H ₂ O ₂	1.3±0.1 mM	2.4±0.4 mM
SHAM	+++	+
KCN	++	++
NaN ₃	+	+

bound to the cell wall are preferentially involved in generation of •OH, when endogenous H₂O₂ is produced by MnSOD (superoxide dismutase), which is also bound to the cell wall (Kukavica *et al.*, 2009). In addition to the roles in the process of lignification, extracellular anionic and cationic peroxidases are associated with other physiological processes such as defence reaction in response to pathogen attack (Young *et al.*, 1995; Morimoto *et al.*, 1999). The changes in the peroxidase profile after two treatments, with the phytohormone auxin on root growth, and chitosan, a potent elicitor of plant resistance against fungal pathogens, are in favour of the specific roles of anionic and cationic peroxidases in root physiology. Exogenous auxin or auxin-like substances usually inhibit root growth (Pilet and Elliot, 1981; Lüthen and Böttger, 1993). There are two hypotheses for the mechanism of auxin-induced growth inhibition and accompanying cell wall stiffening: one involves alkalization of the apoplast (Evans *et al.*, 1980) and the other is an acid-independent mechanism affected by auxin (Lüthen and Böttger, 1993). The present study showed that a cessation of pea root growth by auxin was accompanied by a significant decrease in cationic peroxidase and induction of the activity of anionic peroxidase (Fig. 10). This is in agreement with the hypothesis that cationic peroxidases are responsible for elongation of root cells and anionic peroxidases for stiffening of the cell wall. Similar to the auxin effect, applying chitosan to plants for 16h induced strong inhibition of cationic isoperoxidase, with the most pronounced effect on isoforms with pI 9.34 and 9.5, isoforms that increased during development (Fig. 11). It has been reported that chitosan, which enhances plant defence, also induced lignification (Barber *et al.*, 1989). Taken together, the results of the present study strongly indicate that the ratio of cationic and anionic peroxidases in the cell wall may be an important parameter for determination of root growth. Developmental and environmental factors induced differences in the level of peroxidase activity of those enzymes bound either ionically or covalently to the cell wall. The different stability of the particular isoforms presented in this work implies a different regulation mechanism of their physiological roles. The results further confirm the heterogeneity of the peroxidase system including specific *in muro* localization (Gibson and Liu, 1978). The results on differentially induced or inhibited peroxidase isoforms during root development and under the

effects of auxin or elicitor indicate that cationic isoperoxidases are responsible for cell wall loosening during root elongation, while anionic isoperoxidases are involved in cell wall stiffening and lignification or suberization.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Thermostability of cell wall peroxidases.

Figure S2. Appearance of pea roots dependent on plant development.

Figure S3. Cross-sections of zone IV of 3- and 23-day-old pea roots.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (DFG Lu 668/4-4, Lu 668/8-1, Lu 668/9-1) and by the Ministry of Education and Science of the Republic of Serbia (to SVJ, Project no. III43010). BK and SVJ gratefully acknowledge Miloš Mojić for EPR measurements, Jovana Glušac (University of Banja Luka) for statistical analysis and Dino Hasanagić (University of Banja Luka) for histological analysis. The authors are grateful to Hartwig Lüthen (University of Hamburg, Germany) for stimulating discussions.

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