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Purification and identification of a Ca^{2+}-pectate binding peroxidase from *Arabidopsis* leaves

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Abstract

A protein fraction was obtained from *Arabidopsis* (*Arabidopsis thaliana*, L.) leaf extract by affinity chromatography through a Ca^{2+}-pectate/polyacrylamide gel. Further purification by preparative isoelectric focusing and SDS PAGE allowed the separation of a peroxidase that was identified as being peroxidase AtPrx34 (AtprxCb, accession number X71794) by N-terminal amino acid microsequencing. AtPrx34 belongs to a group of five *Arabidopsis* sequences encoding putative pectin-binding peroxidases. An expression study showed that it is expressed in root, stem, flower and leaf. It was produced by *Escherichia coli* and tested for its ability to bind to Ca^{2+}-pectate. The identity of the amino acids involved in the interaction between the peroxidase and the Ca^{2+}-pectate structure is discussed.

Keywords: Peroxidase; *Arabidopsis* Ca^{2+}-pectate complex

1. Introduction

Arabidopsis genome harbours 73 genes encoding a class III peroxidase (E.C.1.11.1.7, Tognolli et al., 2002; Welinder et al., 2002). It can be reasonably hypothesized that each of these peroxidases, at least those that are really transcribed in plants, has its own biological role, although functional redundancy among the numerous peroxidases is most likely. The general functions of plant peroxidases have been globally identified, but there are only a few examples relating a particular isoform to a precise reaction *in planta*. It is generally accepted that peroxidases are involved in the formation of cross-links among cell wall polymers (Fry, 1986), in lignin polymerization (Ros Barceló, 1997), in auxin catabolism (Grambow, 1986), in hydrogen peroxide formation (Bolwell, 1996) and in several secondary metabolisms (Awad et al., 2000). Although some of these functions relate peroxidases to cell wall stiffening and growth restriction, it has been recently shown that they can also promote cell wall loosening, by forming hydroxyl radicals within the cell wall (Liszkay et al., 2003). Some cases of involvement of one particular peroxidase in a precise reaction have been reported. For example, a French bean isoperoxidase has been shown to be responsible for the production of hydrogen peroxide in response to a fungal elicitor (Blee et al., 2001) and an *Arabidopsis* peroxidase seemed to be implicated in lignin deposition (Østergaard et al., 2000). The recent literature on plant peroxidases provides numerous examples of works reporting changes in the expression of peroxidase encoding genes in relation to endogenous or exogenous parameters (Cheong et al., 2002; www.arabidopsis.org/tools/bulk/microarray/index.jsp). Such observations bring informations on the regulation of peroxidase genes rather than on the precise function of the encoded enzymes. It must also be kept in mind that assessing the level of expression of a gene does not necessarily provide reliable informations on the amount of enzyme that is really active in tissues (Dunand et al., 2003).

Owing to the great number of genes encoding a peroxidase and the wide spectrum of reactions that these enzymes may catalyze, the prerequisite to the study of a particular isoform should be the identification of both the enzyme and its encoding gene, in order to study the catalytic properties of the former and the expression

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profile of the latter. Reverse and forward genetic approaches are also necessary to understand the function of a member of this multigenic family. *Arabidopsis thaliana* is a good model for such study, because its genome has been entirely sequenced and all peroxidase genes have been identified and analyzed (Tognolli et al., 2002; Welinder et al., 2002). We have focused our attention on a particular group of *Arabidopsis* isoperoxidases, because they contain three cationic amino acids that form a putative binding site to the Ca^{2+}-mediated conformation of polygalacturonate, (Dunand et al., 2002), a pectin structure found in all flowering plants (Carpita and Gibeaut, 1993). Binding assays with recombinant *Arabidopsis* peroxidases produced by the baculovirus/insect cell heterologous expression system showed that one enzyme belonging to this group, AtPrx32 (accession number X98315), actually had an affinity for Ca^{2+}-pectate (Dunand et al., 2002). This was also the case of two other peroxidases, AtPrx12 (accession number X98318) and AtPrx50 (accession number X98314). It has also been possible to purify some isoperoxidases from *Arabidopsis* leaves by affinity chromatography using a Ca^{2+}-pectate/polyacrylamide gel (Dunand et al., 2002). The next step in this study would be to identify the peroxidases that have been purified by this method. For this purpose, we have separated Ca^{2+}-pectate peroxidases from *Arabidopsis* and used microsequencing to identify one of the enzymes.

2. Results and discussion

A column filled with polyacrylamide particles containing a Ca^{2+}-polygalacturonate gel allows the separation of a small number of Ca^{2+}-pectate binding isoperoxidases from a crude extract of *Arabidopsis* leaves (Dunand et al., 2002). These peroxidases have all a basic isoelectric point and need further purification steps before they can be subjected to microsequencing. This was achieved with the use of isoelectric focusing in column, a preparative electrophoresis performed in a liquid medium that was already used to purify isoperoxidases from zucchini (Penel and Greppin, 1994). In the present case, ampholytes establishing a pH gradient from 9 to 11 were used. Fig. 1 shows that the slope of the pH gradient was very progressive between pH 8.8 and 10.6. This technique yielded two major peroxidase peaks at pH 9.4 and 9.9 and a third one at pH 11.1. The fractions corresponding to the three peaks were separately collected and concentrated with Centricon-10. A SDS PAGE separation of the proteins present in the first peak (pH 9.4) was performed (Fig. 2), showing the presence of two main protein bands with an apparent molecular mass (36,300 and 38,500 Da) compatible with the usual size of plant peroxidases. The two other peaks were also resolved into two bands (data not shown).

The two main protein bands were transferred to a membrane and used for microsequencing. Despite the unblocking treatment with pyroglutamate aminopeptidase, the microsequencing of the 38500-Da protein gave no result. The N-terminal sequence obtained for the 36300-Da band is shown in Fig. 3. It corresponds to peroxidase AtPrx34 (accession number X71794). The predicted molecular mass and pI of this peroxidase calculated from the deduced amino acid sequence of the mature proteins using ProtParam (Swiss-Prot) are 35624

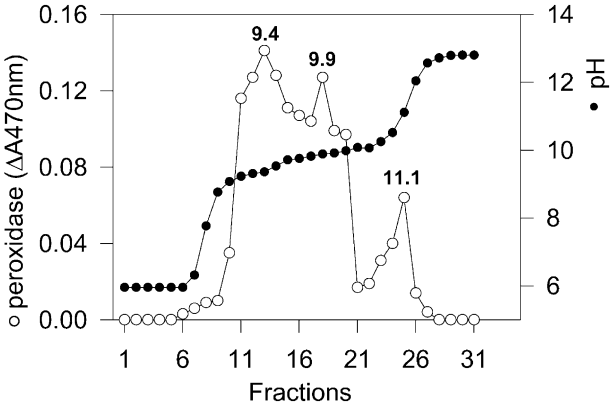

Fig. 1. Preparative isoelectric focusing separation of the peroxidases obtained by chromatography through a column of Ca^{2+}-pectate/polyacrylamide. After elution, the fractions were assayed for peroxidase activity with guaiacol/H_2O_2 and pH.

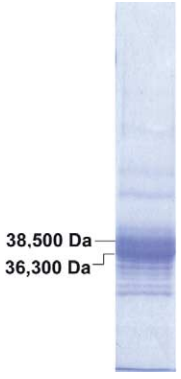

Fig. 2. Protein blot of the SDS PAGE gel loaded with the proteins present in the pH 9.4 peak obtained by preparative isoelectric focusing (Fig. 1). The membrane was stained for proteins with amido black.

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microsequence:  QLTPTFYDRS?PNVTNI
AtPrx32         QLTPTFYDNTCPSVFTI
AtPrx33         QLTPTFYDTSCPTVTNI
AtPrx34         QLTPTFYDRSCPNVTNI
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Fig. 3. N-terminal amino acid sequence of the 36.3-kDa protein aligned with the amino acid sequences of *Arabidopsis* AtPrx32, AtPrx33 and AtPrx34 peroxidases. Q represents the putative pyroglutamate.

Da and pI, 7.04, respectively. The pI was relatively different from the pH value of the peak containing AtPrx34 (Fig. 1). However, the isoelectric focusing in column is a preparative method that is not appropriate for precise pI determination. *Atprx34* cDNA was first identified by Intapruk et al. (1994) and it belongs to the group of three homologous enzymes already mentioned (AtPrx32, AtPrx33, AtPrx34, Tognolli et al., 2002). The predicted molecular weights of Atprx32 and Atprx33 are 32,952 and 35,712 Da, respectively and their pI 9.06 and 6.11. These three enzymes were suspected to have a particular affinity for the Ca^{2+}-pectate structure, because they bear an amino acid motif similar to the binding domain of the anionic peroxidase from zucchini (Dunand et al., 2002). The expression of the genes encoding these peroxidases was assessed in the various organs of *Arabidopsis* plants (Fig. 4). It appeared that *AtPrx34* was expressed strongly in roots and rather weakly in flower, leaf and stem. Transcripts corresponding to the paralogous genes, *AtPrx32* and *AtPrx33*, were detected only in roots. *AtPrx32* and *AtPrx34* expression profiles were in agreement with previous results obtained by RT-PCR (Welinder et al., 2002).

The encoded proteins contain all a cationic amino acid motif similar to the Ca^{2+}-pectate binding site of the zucchini peroxidase APRX (Carpin et al., 2001). In *Arabidopsis* peroxidases, this motif consisted of three lysine residues with a particular spacing, KXXXXX-KXXK, resembling the RXXXXXRXXR motif of APRX. These three lysines would form a site that could have three positive charges suitably distributed to interact with the polygalacturonate chains cross-linked by calcium ions. A 3-D model of the AtPrx34 protein was generated, using the Swiss-PDB viewer software (Guex and Peitsch, 1997) and the crystal structure of a peanut peroxidase as a template (Schuller et al., 1996). Fig. 5 shows a close-up on the AtPrx34 zone containing the three lysine residues suspected to be responsible for the binding to Ca^{2+}-pectate. Two of them (K233 and K239) are located in α-helix G. It appeared that they are not along a line with K242, like the three arginines of zucchini APRX, but rather form a triangle. However, an arginine residue (R225) placed between K233 and K242 could play a role in the interaction with Ca^{2+}-pectate, since it forms with the two lysines a cationic amino acid motif similar to the arginine cluster of APRX (Fig. 4, R262, R268, R271) known to confer a strong affinity for Ca^{2+}-pectate. The distances between the three amino acids, estimated using the Swiss-PdB Viewer, were somewhat different in AtPrx34 and APRX (Fig. 5), but it is known that the side chain of arginine exhibit enough flexibility to allow its positive charges to fit on the negative parts of anionic polysaccharides (Fromm et al., 1995). It would be necessary to replace the cationic amino acids by site-directed mutagenesis to identify without doubt the residues involved in the interaction with Ca^{2+}-pectate, as already done for APRX (Carpin et al., 2001).

An important feature of zucchini APRX is that it binds strongly to polygalacturonic acid chains cross-linked by calcium, but not to isolated chains (Carpin et al., 2001; Penel et al., 2000). This property was also observed in cell walls. In order to see whether AtPrx34 binding to polygalacturonate is also dependent on the presence of calcium ions, an assay was performed, using recombinant AtPrx34 peroxidase and nitrocellulose pieces coated with polygalacturonate. Active AtPrx34 peroxidase was produced with *E. coli*, according to the

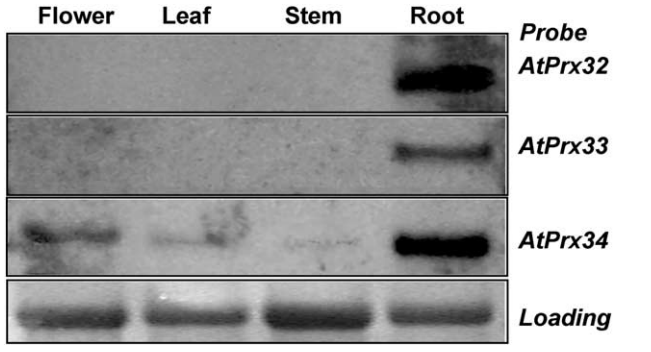

Fig. 4. Northern blot analysis of *AtPrx32*, *AtPrx33* and *AtPrx34* transcripts in different organs of *Arabidopsis*. Total RNA was extracted from flowers, leaves, stems and roots. RNA loading (10 μg) was visualized with methylene blue.

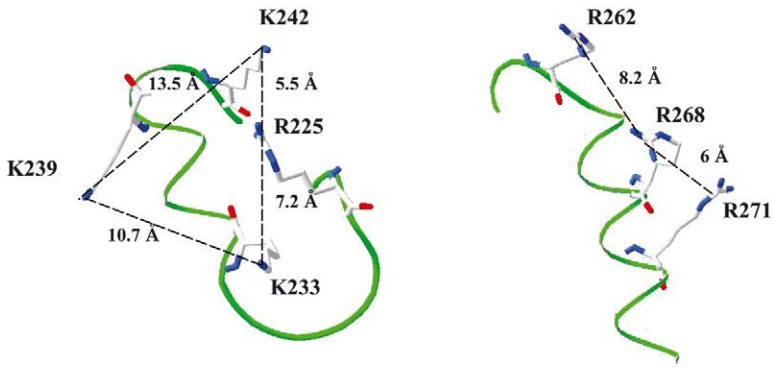

Fig. 5. 3-D Homology models showing the Ca^{2+}-pectate binding site of zucchini APRX and the putative binding site of *Arabidopsis* AtPrx34.

procedure of Teilum et al. (1999). Nitrocellulose coated with polygalacturonate offers the advantage that Ca^{2+} can be added or withdrawn at various steps to assess its role (Fig. 6). It turned out that if it was present during the whole experiment, AtPrx34 bound specifically to the polygalacturonate-coated nitrocellulose (Fig. 6, 5). If Ca^{2+} was absent throughout the experiment, no binding occurred (Fig. 6, 2). This was not due to a weak adsorption of isolated polygalacturonate chains to nitrocellulose, since the addition of Ca^{2+} just prior to the incubation of the nitrocellulose with AtPrx34 was sufficient to allow the binding of the enzyme (Fig. 6, 4), although the incubation of nitrocellulose in polygalacturonic acid solution was carried out in the absence of Ca^{2+}. On the other hand, the chelation of Ca^{2+} by EGTA during the washing step following the incubation with AtPrx34 released the peroxidase from polygalacturonate. Staining of the nitrocellulose pieces for peroxidase activity was performed in the presence of Ca^{2+} ions, to ensure optimal conditions for the determination of activity. This experiment demonstrated that polygalacturonate chains bound AtPrx34 molecules only when they were cross-linked by Ca^{2+}.

The results presented here confirm the hypothesis previously made that *Arabidopsis* AtPrx34 peroxidase has a particular affinity for the Ca^{2+}-pectate structure (Dunand et al., 2002). The role of this affinity for the peroxidase function in plant cell wall remains unclear. The Ca^{2+}-pectate structure is abundant in middle lamella and at cell corners (Jarvis et al., 2003), where it could retain or even attract proteins having an affinity for it. What could be the function of the peroxidases associated to this particular pectic structure? It has been recently observed that the gene encoding the zucchini pectin binding peroxidase APRX is mainly expressed in elongating zones of hypocotyls and roots (Dunand et al., 2003), suggesting that APRX could be involved in growth mechanism. It is known that peroxidases may promote the elongation of tissues through the formation of hydroxyl radicals which loosen cell walls (Liszkay et al., 2003). It would be interesting to know if AtPrx34 and other pectin binding peroxidases are able to promote this reaction. Pectin binding peroxidases, such as AtPrx34 or zucchini APRX, are likely to be mainly localized in middle lamella and cell corners, two sites known to contain much Ca^{2+}-pectate (Jarvis et al., 2003). By this way, they could be involved in the formation of reinforcing zones that carry the turgor-imposed stress occurring during the cell growth process, which avoid the separation of cells (Parker et al., 2001). These various possibilities are currently investigated, using different transgenic *Arabidopsis* plants.

	PGA →	3W →	BSA →	3W →	P34 →	3W →	staining
1	-	Ca	Ca	Ca	Ca	Ca	Ca
2	E	E	E	E	E	E	Ca
3	E	E	E	E	E	Ca	Ca
4	E	E	E	Ca	Ca	Ca	Ca
5	Ca	Ca	Ca	Ca	Ca	Ca	Ca
6	Ca	Ca	Ca	Ca	Ca	E/Ca	Ca
7	Ca	Ca	Ca	Ca	E	Ca	Ca

Fig. 6. Staining of nitrocellulose pieces for peroxidase activity after seven different treatments. The pieces were successively dipped in polygalacturonate solutions (PGA), washed three times (3W), blocked in bovine serum albumin (BSA), washed three times, incubated in a solution containing AtPrx34, washed three times and stained for peroxidase activity. These various steps were performed in the presence of 2 mM EGTA (E) or 2 mM $CaCl_2$ (Ca). PGA was omitted in the first treatment (−). The picture in the right column shows a nitrocellulose piece for each treatment, after staining for peroxidase activity using *o*-dianisidine/H_2O_2.

3. Experimental

3.1. Arabidopsis peroxidase identification

Peroxidases are identified according to the numbering proposed by Tognolli et al. (2002), with the addition of At for *Arabidopsis thaliana* and Prx for peroxidases (Pn becomes AtPrxn).

3.2. Extraction and isolation

Proteins were extracted from leaves of *Arabidopsis thaliana* L. (ecotype Columbia) in 20 mM Hepes pH 7 containing 1mM EGTA and 10 mM ascorbic acid. After filtration through cheesecloth, the pH of the extract was adjusted to 7 with 10 M KOH and centrifuged at 12,000×*g* for 10 min. Proteins were precipitated with ammonium sulphate at 20–90% saturation. The pellet was resuspended in 20 mM Hepes pH 7, containing 1 mM EGTA and 0.1% Tween 20 and dialysed against the same buffer. $CaCl_2$ (2 mM) was added to the resulting solution that was submitted to affinity chromatography through a column filled with a Ca^{2+}-polygalacturonate-polyacrylamide gel prepared as already explained (Penel and Greppin, 1996). The column was washed with Hepes pH 7 containing 2 mM $CaCl_2$ and 0.1% Tween prior to elution. Bound proteins were eluted using 0.5 M NaCl in 20mM Hepes. The fractions were assayed for peroxidase activity with guaiacol/H_2O_2 and active fractions were pooled and concentrated using Centricon-10 (Amicon).

3.3. Preparative IEF column

The isoperoxidases obtained by chromatography through the column of Ca^{2+}-pectate were dialysed before further separation using preparative isoelectric focusing in column (Penel and Greppin, 1994). Several solutions were successively introduced into the main compartment of the column: an anode solution (6.9 g

sucrose in H_2O containing 114 μl H_3PO_4 (final volume: 9 ml); 2 ml containg 40 mg glycine and 1.4 g sucrose; a linear gradient made of 10 ml of H_2O containing 5 g sucrose, 400 μl Servalyt (Serva, Heidelberg, Germany) pH 9-11, 0.05% Tween 20 and the protein solution to be separated, and 10 ml of H_2O containing 0.5 g sucrose, 100 μl Servalyt and 0.05% Tween; 2 ml of a cathode solution containing 0.1 g sucrose, 8 mg arginine, 8 mg lysine, 240 μl ethylene diamine; and finally 5 ml 1% NaOH. Sucrose was added in the five layers at different concentrations to prevent any mixing. A constant voltage of 1000 V was applied during 18 to 24 h. The column content was eluted into 500-μl fractions that were each assayed for enzyme activity with guaiacol/H_2O_2 and pH. The different peaks of peroxidase activity obtained were pooled and concentrated with Centricon-10.

3.4. Microsequencing

Fractions obtained by preparative IEF were separated by SDS PAGE. The gel was electroblotted onto a PVDF membrane (Sequi-Blot, BioRad, Reinach, Switzerland) that was stained with 0.1% Amido Black in 1% acetic acid and 40% methanol. Protein bands were excised and soaked for 30 min in 0.5% soluble polyvinylpyrrolidone at 37 °C for blocking free sites. The pieces of membrane were then rinsed in ultrapure water and incubated overnight at 37 °C in 100 μl of 50 mM phosphate buffer pH 7.0 containing 10 mU pyroglutamate aminopeptidase from *Pyrococcus furiosus* (E.C. 3.4.19.3, BioWhittaker, Verviers, Belgium) to unblock the protein N-terminus (LeGendre et al., 1993). The membrane was then used for peroxidase microsequencing by Edman degradation.

3.5. RNA gel analysis

Total RNA for northern blot analysis was extracted from the main *Arabidopsis* organs using the extraction kit from Sigma (Buchs, Switzerland). RNA (10 μg) was electrophoresed through 1% formaldehyde-containing agarose gels and transferred onto Hybond-N membranes (Amersham, Dübendorf, Switzerland) with 10x SSC, as described by the manufacturer. RNA deposition and transfer were estimated by ethidium bromide staining and by coloration with a solution containing 0.5 M sodium acetate and 0.04% methylene blue. Hybridization was performed at 50 °C in DIG easy hyb buffer (Boehringer Mannheim) using *AtPrx32*, *AtPrx33* and *AtPrx34* probes obtained by PCR from the whole cDNA sequences and labeled with a DNA labeling kit (DIG, Boehringer Mannheim). Stringent washing temperature (55 °C) was used to compensate the relatively high nucleotide homologies between the three cDNAs (*AtPrx32*/*AtPrx33*: 80% homology, *AtPrx32*/*AtPrx34*: 84% homology, *AtPrx33*/*AtPrx34*: 96% homology). Each experiment was repeated at least three times with similar results.

3.6. Expression in E. coli

The cDNA sequence encoding AtPrx34 without the signal peptide has been cloned into restriction site KpnI and BamHI of pET29a vector (Stratagen, USA) and expressed in *E. coli* (BL21. Codon plus, RIL strain, Stratagen,). A single colony from a LB plate was transferred to 5 ml LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. The culture was diluted 50x in LB medium and the production of the recombinant protein was induced for 4 h at 37 °C with 0.4 mM isopropylthiogalactoside. Isolation of inclusion bodies, extraction and refolding of peroxidase polypeptide were conducted following the protocol described by Teilum et al. (1999).

3.7. Binding assay

The ability of recombinant AtPrx34 peroxidase to bind to Ca^{2+}-polygalacturonate complex was assessed using nitrocellulose (Optitran BA-S 85, Schleicher & Schuell, Dassel, Germany) according to a procedure already described (Penel et al., 2000). The buffer used throughout the experiment was 20 mM Hepes pH 7, containing 0.1% Tween 20 and either 2 mM EGTA or 2 mM $CaCl_2$ (see Fig. 5 for details). Pieces of nitrocellulose (5×5 mm) were dipped for 60 min in solutions of polygalacturonic acid (1 mg/1 ml) in the presence of either EGTA or $CaCl_2$. After three washings in the same buffer, the pieces were transferred to 2% bovine serumalbumin for 60 min. After 3 washings, they were incubated in a solution containing recombinant AtPrx34 for 60 min. The nitrocellulose pieces were washed again and stained for peroxidase activity, using 2 mM *o*-dianisidine in 100 mM acetate buffer pH 4.5, containing 15 mM H_2O_2 and 5 mM $CaCl_2$.

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