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Interactions of a plant peroxidase with oligogalacturonides in the presence of calcium ions

Claude Penel[a,*], Pierre Van Cutsem[b], Hubert Greppin[a]

[a]*Laboratoire de Biochimie et Physiologie végétales, Université de Genève, 3 place de l'Université, CH-1211 Geneve 4, Switzerland*
[b]*Facultés Universitaires Notre-Dame de la Paix, Laboratoire de Biotechnologie Théorique, 61 rue de Bruxelles, B-5000 Namur, Belgium*

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Abstract

The interaction between homogalacturonans and an anionic isoperoxidase purified from zucchini hypocotyls was characterized. The binding of the enzyme to the pectic molecules was studied by gel filtration through Sephacryl S200 and by centrifugation. It took place only in the presence of calcium ions, at pHs ranging from 4.5 to 7.0. Ca^{2+} was necessary because it induced the cross-linking of polygalacturonan chains into a structure which could be recognized by the isoperoxidase. A comparison between mixtures of large (degree of polymerization: DP = 9–22) and small (DP = 2–8) oligogalacturonides showed that only the former ones were able to form the Ca^{2+}-induced structure that could be pelleted upon centrifugation and to bind the isoperoxidase. Large oligogalacturonides (OGAs) and polygalacturonic acid (PGA) had almost the same capacity to retain the isoperoxidase. Gel filtration experiments showed that the binding of the isoperoxidase to large OGAs occurred even at low Ca^{2+} concentrations (0.05 mM). Competition experiments showed that polyanions like dextran sulfate or heparin even at a hundred times higher concentration did not completely prevent the binding of peroxidase to Ca^{2+}-pectate. Alginic acid was also unable to suppress this binding and, although its structure is similar to that of OGAs and although it was also cross-linked by Ca^{2+} ions and formed a pelletable gel, it did not offer a structure suitable for peroxidase binding.

Keywords: Cucurbita pepo; Calcium-pectate; Degree of polymerization; Alginic acid; Gel filtration

1. Introduction

Plant peroxidases (EC 1.11.1.7) are localized mainly in cell walls and in vacuoles (Gaspar, Penel, Thorpe, & Greppin, 1982). They are involved in the assembly of various cell wall constituents by catalyzing the formation of covalent bonds between tyrosine or ferulate residues of different wall polymers (Fry, 1986). They are also generally considered as being responsible for lignin polymerization (Sato, Sugiyama, Górecki, Fukuda, & Komamine, 1993) and could be involved in hydrogen peroxide formation (Bolwell, Butt, Davies, & Zimmerlin, 1995). Owing to the wide spectrum of biochemical reactions they may catalyze, peroxidases are likely to play important roles in the mechanisms of cell elongation, cell differentiation and defence against pathogens (Gaspar, Penel, Castillo, & Greppin, 1985).

It is known that the various polymers forming plant cell wall are spatially arranged to form a highly structured matrix (Carpita, & Gibeaut, 1993). This means that enzymes such as peroxidases which establish cross-links between these polymers should not operate randomly. On the contrary, their spatial distribution can be expected to be precisely determined in order to ensure the proper assembly of the various cell wall constituents. One possibility to achieve this spatial control, is to use one wall polymer as template to set the enzymes in the appropriate position. Such a process would require a specific binding of the enzyme to the wall polymers. We have already shown that a small number of isoperoxidases — and some other proteins — exhibited an affinity for the homogalactur-

* Corresponding author.

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onan parts of pectins, when calcium ions were present (Penel, & Greppin, 1994, 1996). The binding to Ca^{2+}-pectate appeared to be dependent on the presence of cationic amino acids available at the surface of peroxidase molecules. It was observed only with pectins which were not or were moderately esterified. This property was used to perform a kind of affinity chromatography using Ca^{2+}-pectate entrapped in polyacrylamide gel. When applied to a crude preparation of proteins from zucchini hypocotyl, this procedure allowed the separation in one step of a small number of proteins, among which were three isoperoxidases. The majority of the peroxidase isoforms, one of which being the most cationic one, were not retained by the gel. Bound proteins could be released from the gel either with NaCl or with the Ca^{2+} chelator EGTA. This binding was also observed in situ in the cell walls of zucchini hypocotyls (Penel, Crèvecoeur, & Greppin, 1996).

The work presented here was designed to bring some precisions concerning the characteristics of the binding of proteins to polygalacturonides of various lengths in their Ca^{2+}-induced conformation. An anionic isoperoxidases (APRX) was taken as representative of the Ca^{2+}-pectate binding proteins known to exist in zucchini (Penel, & Greppin, 1996). It was chosen rather than the cationic isoperoxidases also exhibiting this binding property, because it has a net negative charge which should not favor a simple ionic interaction with the negatively charged polygalacturonates.

2. Results

Previous work has shown that when incubated in the presence of polygalacturonic acid or pectin from citrus and calcium ions, the APRX from zucchini became pelletable upon centrifugation of the mixture (Penel, & Greppin, 1994, 1996). It bound to the gel formed by pectate and Ca^{2+}. This procedure provided a simple test to assess the affinity of a protein for this particular organization of pectin molecules. It has been routinely performed at pH 7 (Penel, & Greppin, 1994, 1996), but it appeared necessary to do it also at pHs which are known to prevail in vivo in cell walls. For this purpose, binding tests were performed at pHs between 4.5 and 7.0. As shown in Fig. 1, after centrifugation, the peroxidase activity was found mostly in the pellets at all tested pHs. It should be noted that APRX did not sediment when it was centrifuged either alone in the presence of Ca^{2+} or with PGA in the presence of EGTA. A quantification of the pectins in the pellets revealed that 72–83% of PGA sedimented in the presence of Ca^{2+}, whereas only negligible amounts were detected in the bottom of the tubes in the presence of EGTA (data not shown). This experiment showed that the Ca^{2+}-pectate gel was formed at all pHs comprised between 4.5 and 7.0 and that APRX exhibited its affinity for this structure throughout this pH range.

PGA used in the experiment of Fig. 1 had a rather high molecular weight, corresponding to a large degree of polymerization. Previous work has shown that the biological activity of homopolygalacturonate molecules was dependent on their size. Generally, oligogalacturonides were active only if their DP is equal to 9 or greater (Montreuil et al., 1986; Bellincampi et al., 1993; Messiaen, & Van Cutsem, 1994). The existence of receptors, namely proteins exhibiting an affinity for oligogalacturonides, has been postulated (Farmer, Moloshok, & Ryan, 1990). A parallel can be drawn with the binding of proteins to PGA described here. Is there a minimum DP of OGAs necessary for the binding of APRX to occur? To answer this question, two mixtures of pectic fragments were prepared and tested for their capacity to bind APRX. The first one contained oligogalacturonides with DP comprised between 2 and 8 (small OGAs), the second one between 9 and 22 (large OGAs). The binding test showed that large OGAs formed a pellet like PGA when centrifuged after a 60-min incubation in the presence of Ca^{2+}, whereas small OGAs did not (Table 1). APRX was found associated to the pellets formed either by PGA or by large OGAs. The level of pelleted activity was somewhat different, but in each case there was practically no activity remaining in the supernatant. The comparison between small and large OGAs indicated that a minimum size was required for the constitution of the Ca^{2+}-pectate structure and for the binding of APRX. A further comparison of the capacity of PGA and large OGA to bind APRX was performed. For this purpose, a saturation experiment was realized with

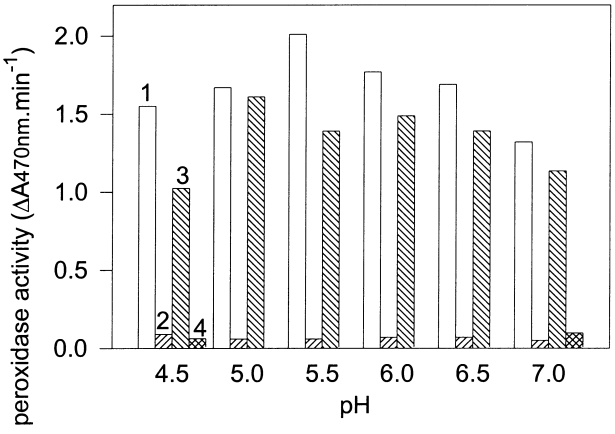

Fig. 1. Effect of pH on the binding of APRX to PGA (1 μg) in the presence of 2 mM $CaCl_2$. Total peroxidase activity was measured before centrifugation (1), in supernatants (2) and pellets (3) after centrifugation. Pellet values obtained in the presence of EGTA are shown for pH 4.5 and 7.0 (4).

Table 1
Sedimentation of APRX after incubation and centrifugation with 12 μg of various pectins in the presence of 1 mM EGTA (E) or 2 mM $CaCl_2$ (Ca)

Pectins	EGTA/$CaCl_2$	Pellet content	
		pectins (μg)	peroxidase activity (ΔA470 nm·min^{-1})
None	E	–	0.006
	Ca	–	0.004
Small OGAs	E	0.7	0.013±0.003
	Ca	1.0	0.044±0.003
Large OGAs	E	0.1	0.016±0.002
	Ca	10.1	0.260±0.008
PGA	E	0.7	0.014±0.004
	Ca	8.9	0.191±0.008

increasing concentrations of APRX mixed to 1 μg of PGA or large OGAs (Fig. 2). The maximum binding capacity of both kinds of pectic molecules was reached for a peroxidase concentration of around 0.8 μg per 100 μl. This result emphasized the fact that the Ca^{2+}-pectate structure offered a limited number of binding sites to APRX. Since the molecular weight of APRX was shown to be 41.4 kDa (Penel, & Greppin, 1994), the K_m value of APRX binding can be estimated to be around 0.1 μM.

The role of Ca^{2+} ion concentration was also assessed. Usually, binding experiments were performed in the presence of a rather high concentration of $CaCl_2$ (2 mM). Fig. 3 shows the behavior of APRX and either large OGAs or PGA when centrifuged in the presence of lower concentrations. After centrifugation, the pellets were assayed for their peroxidase activity and uronic acid content. It appeared that OGAs formed a gel that can be pelleted only at Ca^{2+} concentrations higher than 0.2 mM, while a pellet could already be detected at this Ca^{2+} concentration with PGA. The size of the pellets was roughly proportional to the concentration of Ca^{2+} ions for the two kinds of pectic molecules. APRX binding was observed at Ca^{2+} concentrations above 0.2 and 0.4 mM for PGA and large OGAs respectively and reached a plateau at 0.4 and 1.0 mM $CaCl_2$. Time course experiments have also revealed that this binding was almost immediate when APRX was added to a preformed gel obtained by incubation of PGA or large OGAs in the presence of 2 mM $CaCl_2$ (data not shown).

The relation between APRX and oligogalacturonides was investigated further using gel filtration through a column of Sephacryl S200. This technique had already been used to demonstrate the association of peroxidases to pectins from zucchini hypocotyls (Penel, & Greppin, 1996). Mixtures containing small or large oligogalacturonides and APRX were preincubated in the presence of 0.2 mM $CaCl_2$. They were then submitted to a gel filtration through Sephacryl S200 (Fig. 4). The elution volume of APRX was quite different, depending on whether the peroxidase was chromatographed with small (51 ml) or large (32 ml) fragments. In the

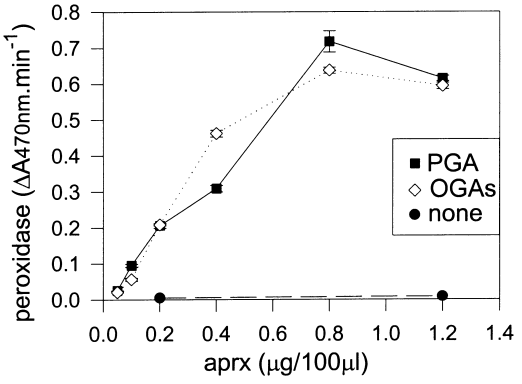

Fig. 2. Effect of APRX concentration on its binding to 1 μg PGA or large OGAs. Peroxidase activity was measured in the pellets after incubation and centrifugation in the presence of 2 mM $CaCl_2$.

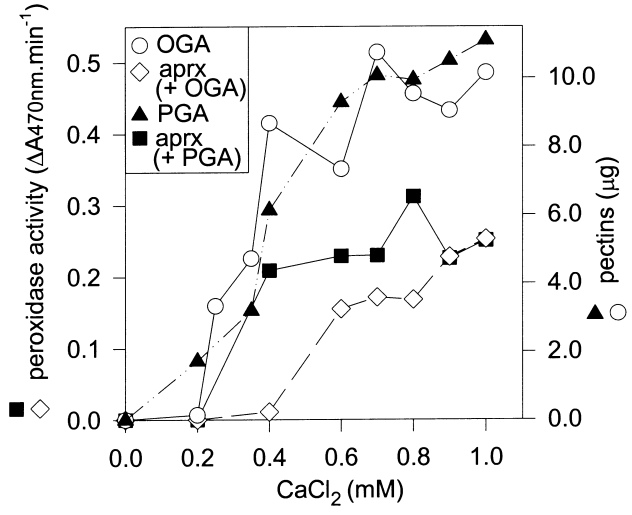

Fig. 3. Amounts of uronic acids and peroxidase activity measured in pellets after centrifugation of samples containing 10 μg PGA or large OGAs, 60 ng APRX and increasing concentrations of $CaCl_2$.

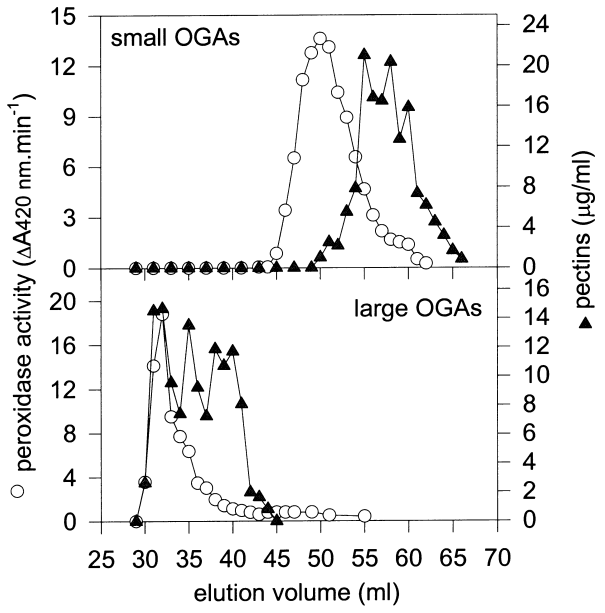

Fig. 4. Elution profiles of peroxidase activity and uronic acids after a chromatography through a column of Sephacryl S200 of samples containing small or large OGAs, APRX and 0.2 mM $CaCl_2$.

presence of the small pectic fragments, APRX eluted from the column almost at its own elution volume (see Fig. 5), while, in the presence of large pectic fragments, the same APRX eluted in the void volume of the column. This change of elution volume resulted from the fact that APRX was carried through the column by large OGAs cross-linked by Ca^{2+}. The large fragments were separated into three peaks, corresponding to aggregates of different sizes. According to the results presented in Fig. 4, it may be concluded that, in the presence of Ca^{2+}, APRX exhibited an affinity for the large oligogalacturonides but not for the small ones. This observation was done using a Ca^{2+} concentration of 0.2 mM. The separation profiles obtained in the presence of other Ca^{2+} concentrations are shown in Fig. 5. The distribution of the pectic fragments was clearly shifted towards smaller elution volumes when Ca^{2+} concentration was increased, going from 44 ml in the presence of EGTA to 32 ml in the presence of 0.4 mM $CaCl_2$. Intermediate concentrations induced the formation of aggregates of different molecular weights, exhibiting elution volumes comprised between these two values. APRX appeared always associated with the largest aggregate. It should be noted that the elution volume of the small oligogalacturonides was much less affected by the concentration of Ca^{2+} (data not shown).

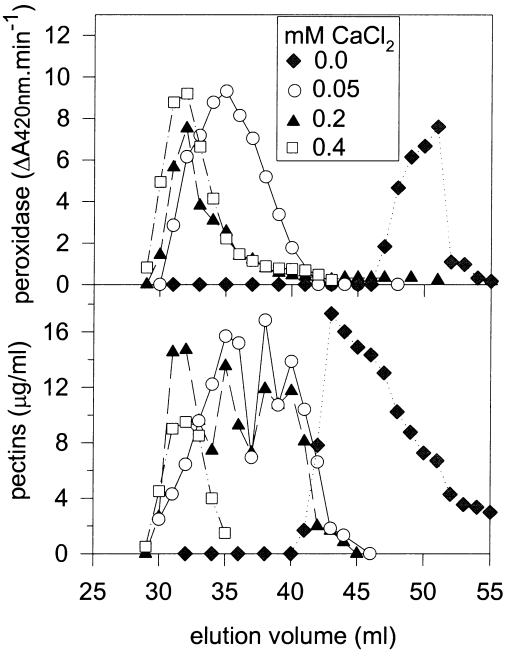

Fig. 5. Elution profiles through a column of Sephacryl S200 of samples containing APRX and large OGAs in the presence of different $CaCl_2$ concentrations.

Previous work has shown that the interaction between APRX and the polygalacturonic acid in its Ca^{2+}-induced conformation involved the positive charges of lysine or arginine residues exposed at the surface of APRX (Penel, & Greppin, 1996). In addition, APRX, as the other pectin binding proteins, exhibited an affinity for heparin, an anionic polysaccharide consisting of iduronic acid and sulfated glucosamine dimers (Casu, 1989). This showed that PGA was not the only polyanion able to retain this protein. In the present work, we have tested heparin, dextran sulfate and alginic acid for their ability to inhibit the binding of APRX to polygalacturonic acid (Table 2). As $CaCl_2$ was added to the samples after the other components, APRX was in contact with the competing polyanions before the formation of the Ca^{2+}-pectate structure. However, neither heparin, nor dextran sulfate or alginate were able to suppress completely the binding of the peroxidase to Ca^{2+}-pectate, even when added at a much higher concentration than PGA. In addition, the pelletable gel formed by alginic acid (polymannuronic acid) in the presence of Ca^{2+} did not exhibit the capacity to bind APRX.

3. Discussion

The molecular interactions between the various cell wall constituents play certainly a major role in many processes like plant cell growth or differentiation. These interactions may occur between the structural wall polymers, allowing the plant cell wall to get the appropriate rheological and mechanical properties (Carpita, & Gibeaut, 1993; Cosgrove, 1997), but the numerous enzymes and functional proteins involved in

Table 2
Effect of the presence of polyanions on the binding of 250 ng APRX to 1 μg PGA in the presence of 2 mM $CaCl_2$ [a]. Peroxidase activity was measured in pellets after incubation and centrifugation

Additions	$\Delta A470$ nm·min^{-1}
PGA	0.382 ± 0.014
+ 5 μg heparin	0.287 ± 0.004
+ 100 μg heparin	0.230 ± 0.005
+ 5 μg dextran sulfate	0.094 ± 0.012
+ 100 μg dextran sulfate	0.140 ± 0.005
+ 5 μg alginic acid	0.412 ± 0.001
+ 100 μg alginic acid	0.360 ± 0.020
5 μg alginic acid alone	0.025 ± 0.005
100 μg alginic acid alone	0.025 ± 0.001

[a] Order of additions: PGA–polyanions–hepes–APRX–$CaCl_2$.

the assembly of the wall constituents, in the maintenance of the wall structure and in the defence against pathogens are probably also concerned. These proteins should neither move freely within the network of polymers, nor bind randomly to them through unspecific linkages. One can rather expect that the spatial distribution of most of them is strictly controlled through associations to some cell wall polymers acting as guiding structures. Among cell wall components, pectins appeared as being particularly suitable as protein binding structure. They exhibit a great chemical diversity, their structure is continuously modified through esterification/deesterification reactions, or in function of the apoplastic Ca^{2+} concentration and they can form a matrix network independently of the other wall polymers (Carpita, & Gibeaut, 1993; McCann, & Roberts, 1996). Until now, some isoperoxidases (Ros Barceló, Pedreño, Muñoz, & Sabater, 1988; Penel, & Greppin, 1994; Penel, & Greppin, 1996; Penel et al., 1996), a pectinesterase (Glover, & Brady, 1995) and remorin, a protein associated to plasma membrane (Reymond et al., 1997), have been reported to exhibit an affinity for pectins. The present work brings some new informations concerning the interaction between Ca^{2+}-pectate and APRX. It provides a model to study the mechanisms of recognition of oligogalacturonides occurring in apoplast. These mechanisms are known to have an influence on several aspects of plant physiology and plant development (Van Cutsem, & Messiaen, 1994).

It was already known that positive charges exposed by APRX were necessary for the binding to Ca^{2+}-pectate (Penel, & Greppin, 1996). This was not a sufficient condition, since for example some cationic isoperoxidases from zucchini did not bind even if they exhibited a high isoelectric point, exposing therefore many positive charges at their surface (Penel, & Greppin, 1996). In the absence of Ca^{2+}, isolated OGAs were unable to retain APRX (Fig. 5), although all their negative charges were free. Ca^{2+} is known to organize the homogalacturonate chains into complex supramolecular structures which have been described by different models such as the "egg-box" (Rees, 1982) or the "cable" (Goldberg, Morvan, Jauneau, & Jarvis, 1996) model. The differences observed in the present work in the Ca^{2+} concentration required for a complete binding of APRX to PGA or OGAs (Fig. 3) could be explained by the fact that long pectic chains formed more stable structures at low Ca^{2+} concentration than short chains (Messiaen, & Van Cutsem, 1994). It is not easy to explain why isolated polygalacturonan did not bind APRX, while chains organized by Ca^{2+} allowed the binding. It may be hypothesized that, in the presence of Ca^{2+}, some negative charges became distributed in such a manner that they fitted exactly with the positive charges exposed at the surface of APRX. The size of the OGAs was also important. Small ones were unsuitable for the binding of APRX in the presence of Ca^{2+}. This inability could be explained either by the absence of the Ca^{2+}-induced conformation in small OGAs or because the distance between negative charges was too short. The observation that OGAs with a DP lower than 9 were unsuitable to bind the peroxidase can be compared with the observations that showed that OGAs must have a size above DP = 8 to be active as elicitors (Jin, & West, 1984; Bellincampi et al., 1993; Messiaen, & Van Cutsem, 1994).

Finally, the interaction between APRX and pectins occurred only with PGA molecules partially or totally unesterified (Penel, & Greppin, 1994). As the level of pectin esterification is developmentally controlled within plant tissues (Knox, Linstead, King, Cooper, & Roberts, 1990; Jauneau, Quentin, & Driouich, 1997) it could be hypothesized that pectins can influence the distribution of proteins such as APRX within the apoplast through three parameters: their degree of polymerization, their conformation depending of the Ca^{2+} concentration and their level of esterification.

4. Experimental

The anionic isoperoxidase (APRX) from zucchini hypocotyls (*Cucurbita pepo*, L., cv. Black Beauty) was purified and quantified as described previously (Penel, & Greppin, 1994). Polygalacturonic acid, alginic acid, heparin (MW 6000) and all other chemicals were obtained from Sigma (Buchs, Switzerland), except dextran sulfate which was from Meito Sangyo Co (Japan). The mixtures of small and large oligogalacturonides were prepared as described by Messiaen and Van Cutsem (1994).

Gel filtration was performed through a column (100 × 1 cm) containing Sephacryl S200 (Pharmacia,

Dübendorf, Switzerland) equilibrated in 20 mM Hepes pH 7 and EGTA (ethyleneglycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid) or $CaCl_2$ at a concentration specified in each case. The sample (500 µl), which contained the same concentration of Ca^{2+} as the column, was incubated during 60 min before being loaded onto the gel. Fractions of 1 ml were collected. The void volume of the column, determined with Blue Dextran (Pharmacia), was 31 ml. Peroxidase activity was assayed in fractions, using 2 mM ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and 5 mM H_2O_2 in acetate buffer pH 4.5 (ΔA at 420 nm). Uronic acids were quantified with the *m*-hydroxy diphenyl reagent (Montreuil et al., 1986).

The binding of APRX to different polygalacturonates was assessed by centrifugation according to a procedure already described (Penel, & Greppin, 1996). Briefly, 100 µl of Hepes pH 7 containing peroxidase, polygalacturonates and either $CaCl_2$ or EGTA (added in this order) were centrifuged in a Sorvall Microfuge at 10000*g* for 5 min, after an incubation of 60 min at room temperature. The resulting supernatants were carefully removed and the pellets resuspended in 100 µl Hepes containing 10 mM EGTA and allowed to stand for several h at 4°C before peroxidase activity or pectin content were assayed. The concentration of the various components is specified in each case. Peroxidase activity in supernatants and resuspended pellets was determined with 8 mM guaiacol, 2 mM H_2O_2 in 50 mM phosphate buffer pH 6.1 by following the increase of absorbance at 470 nm.

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