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FEBS Letters 343 (1994) 51–55

FEBS 13883

FEBS LETTERS

Binding of plant isoperoxidases to pectin in the presence of calcium

Claude Penel*, Hubert Greppin

Laboratoire de Biochimie et Physiologie Végétales, Université de Genève, place de l'Université 3, 1211 Genève 4, Switzerland

Received 8 February 1994; revised version received 5 March 1994

Abstract

Some of the isoperoxidases present in an extract from zucchini hypocotyls – one anionic and two cationic – exhibited a Ca^{2+}-dependent pelletability which resulted from an interaction with pectins. Endogenous pectins could be replaced by polygalacturonic acid or pectin extracted from citrus, but not by highly esterified pectin. The interaction between the isoperoxidases and the polysaccharides has been studied by centrifugation, by gel filtration, and with pectins attached in wells of a microtitration plate. These various binding tests have shown that the isoperoxidases had an affinity for the pectins in their Ca^{2+}-induced conformation.

Key words: Hypocotyl; *Cucurbita pepo*; Polygalacturonic acid; Pectin junction zone

1. Introduction

Peroxidases (EC 1.11.1.7) are glycosylated enzymes ubiquitous in higher plants, existing in a single tissue under several different molecular forms [1]. They have attracted much attention because they are very reactive to many external events such as photoperiod changes, attack by pathogens, wounding, and various chemical treatments [1]. Their isoenzyme pattern is also closely dependent on the developmental stage of the plant. Much of the peroxidase activity is present in the cell wall of young tissues and of lignifying parts [2]. Their main known functions are the cross-linking of various wall polymers and the formation of lignin [3,4]. Cell wall peroxidases are either free, either bound by ionic interactions to cell wall polymers, or more tightly linked to the wall. This latter fraction can be released only by enzymatic digestion of the wall and has been considered as 'covalently bound' [5]. In this work, we have investigated the interaction of some isoperoxidases with pectin, a secreted polymer composed of galacturonic acid and some other sugars, which is present both in the cel wall and at the surface of plant cells [6,7]. Our results have shown that the binding of selected isoperoxidases to pectin was dependent on the presence of calcium and on the degree of esterification of pectins.

2. Materials and methods

2.1. Chemicals

Pectin from citrus (degree of esterification 63–66%) and pectinase from mould were obtained from Fluka; polygalacturonic acid (PGA), esterified pectin from citrus (degree of esterification 93%) and poly-L-lysine hydrobromide from Sigma.

2.2. Extraction and purification of peroxidases

Zucchini seedlings (*Cucurbita pepo*, cv. Black Beauty) were grown in the dark on moist paper at 25°C during 4–5 days. Whole hypocotyls were used for extraction. Harvesting and all subsequent manipulations were performed at 0–4°C. Typically, 100 g (F.W.) of hypocotyls were grown in 30 ml of 20 mM HEPES (4-(2-hydroxyethyl)-piperazine-1-ethane sulfonic acid) containing 1 mM EGTA (ethyleneglycol-*O*,*O*'-bis (2-aminoethyl)-*N*,*N*,*N*',*N*'-tetraacetic acid) and adjusted to pH 7 with KOH (hE1), using a domestic blender (Braun, Frankfurt). The extract was filtered through a nylon cloth (100 μm) and centrifuged for 10 min at 4000 × *g* in a ALC 4239R centrifuge. $CaCl_2$ was added to the resulting supernatant to a final concentration of 1 mM. A first centrifugation at 17,000 × *g* for 20 min yielded a pellet which was resuspended in 20 ml of 20 mM HEPES, pH 7, containing 10 mM EGTA. After a second centrifugation at 17,000 × *g* for 20 min, the pellet containing most of the membrane material was discarded and the supernatant containing the molecules released by EGTA was supplemented with $CaCl_2$ to a final concentration of 10 mM and centrifuged during 20 min at 17,000 × *g*. The resulting pellet was dispersed in 10 ml of 20 mM HEPES, pH 7, containing 10 mM EGTA. After a centrifugation for 20 min at 17,000 × *g*, the resulting solution was loaded on a column containing 10 ml of DEAE Sephacel (Pharmacia) equilibrated in hE1. The column was then washed with hE1 and eluted with a linear gradient of 0 to 500 mM NaCl in hE1. The collected fractions were assayed for peroxidase activity using guaiacol/H_2O_2 [8] and for the presence of pectins using the *m*-hydroxy-biphenyl reagent [9]. Fractions containing the peroxidase activity which was not retained by the resin were collected and designated as cationic isoperoxidases, whereas peroxidase activity released by NaCl was named anionic isoperoxidase. Anionic isoperoxidase was mixed with an equal volume of the other collected fractions in order to localize the activity responsible for the Ca^{2+}-dependent pelletability of peroxidases, in presence of 10 mM $CaCl_2$ according to the centrifugation test described below.

2.3. Electrophoretic methods

Gel isoelectric focussing (IEF) was performed in Servalyt 300-μm Precotes gels pH 3–10 (Serva, Heidelberg) according to the instructions of the manufacturer. Peroxidase activity was revealed with *o*-dianisidine/H_2O_2 [10]. Preparative IEF in column was performed in a glass tube (40 × 1 cm) at 4°C. A gradient made of 1.5–0.15 M sucrose and 3.75–1.3% Servalyt (pH 3–10) and containing the peroxidases desalted by

*Corresponding author. Fax: (41) (22) 329 7795.

SSDI 0014-5793(94)00268-Z

dialysis and concentrated with Centricon-10 (Amicon) was introduced in the column, between 2 electrode solutions consisting of 250 mM phosphoric acid in 2 M sucrose (anode) and 250 mM NaOH (cathode). One thousand volts were applied for 16–18 h. At the end of the separation the gradient was eluted in 1-ml fractions which were assayed for pH and peroxidase activity. The fractions containing peroxidases were pooled and transferred in hE1 using Centricon-10. These purified peroxidases were used for the conversion of the catalytic activity into amounts of enzyme molecules. Peroxidase concentration was determined at 403 nm using $\epsilon = 91\ mM^{-1}$ as extinction coefficient [11]. It was found that an increase of absorbance of 0.1 min^{-1} at 470 m using guaiacol/H_2O_2 corresponded to 9 ng of cationic isoperoxidases and 22 ng of the anionic isoperoxidase.

Electrophoresis in 12.5% SDS-polyacrylamide gel was performed according to [12] and the gels stained with Coomassie blue.

2.4. Binding assays

The ability of cationic and anionic isoperoxidases to bind to pectin was assessed by different tests. A centrifugation test was performed in Eppendorf tubes containing the appropriate amounts of peroxidase, pectin, and $CaCl_2$ in a final volume of 100 µl of hE1. After an incubation of 60 min, the tubes were centrifuged in a Microspin 12S (Sorvall) centrifuge set at maximum speed for 5 min. Peroxidase activity was measured before and after centrifugation. In some cases, the sedimented activity was also determined.

Another binding test was performed in 96-microwell polystyrene plates (Nunc). This procedure was inspired by the immunoassay described by Liners et al. [13]. Briefly, each well was coated with 50 µl of poly-L-lysine HBr (50 µg/ml) for 1 h at 20°C. Then 50 µl of a solution of pectin (0–5 µg) in 20 mM HEPES, pH 7, containing 150 mM NaCl and 2 mM $CaCl_2$ (cationic solution) were incubated overnight at 4°C. Non-specific binding was prevented by the addition of 200 µl of a gelatin solution (200 µg/ml of cationic solution) for 2 h at 25°C. After removal of gelatin, the plate was washed with cationic solution and 50 µl of peroxidase fractions from DEAE Sephacel were added and incubated for 60 min at 20°C. After flicking off the peroxidases, the plate was thoroughly washed with the cationic solution and peroxidase activity remaining bound to the pectin was quantified with *o*-phenylene diamine/H_2O_2 [10].

Gel filtration through a column of Sephacryl S200 (Pharmacia) was also used to assess the association of peroxidases with pectins. A crude preparation of molecules pelletable upon calcium addition was obtained by extraction of 50 g hypocotyls in 50 ml hE1. After centrifugation at $4000 \times g$ for 10 min, the supernatant was centrifuged at $17{,}000 \times g$ for 20 min in presence of 1 mM $CaCl_2$. The resulting pellet was resuspended in 5 ml of HEPES, pH 7, containing 10 mM EGTA. This suspension was centrifuged for 40 min at $54{,}000 \times g$ in a Beckman T50 rotor. The supernatant was supplemented with 1% BSA (bovine serumalbumin) and kept at 4°C until used. One ml was deposited on top of a column of Sephacryl S200 (100 × 1 cm) equilibrated in hE1 containing 0.01% BSA without or with $CaCl_2$. Fractions of 1 ml were collected and assayed for peroxidase activity and pectin content.

Table 1
Distribution of peroxidase activity during the various steps of the preparation of peroxidases pelletable upon calcium addition

Centrifugation at $17{,}000 \times g$ no.	Addition before centrifugation	Total peroxidase activity (ΔA_{470} min^{-1})	
		Pellet	Supernatant
1	1 mM $CaCl_2$	1290*	5170
2	10 mM EGTA	323	1128*
3a	10 mM $CaCl_2$	923*	120
3b	10 mM $CaCl_2$ + pectinase (500 µg/ml)	236	828
4	10 mM EGTA	–	672*

Fraction used for the next step (for details, see section 2).

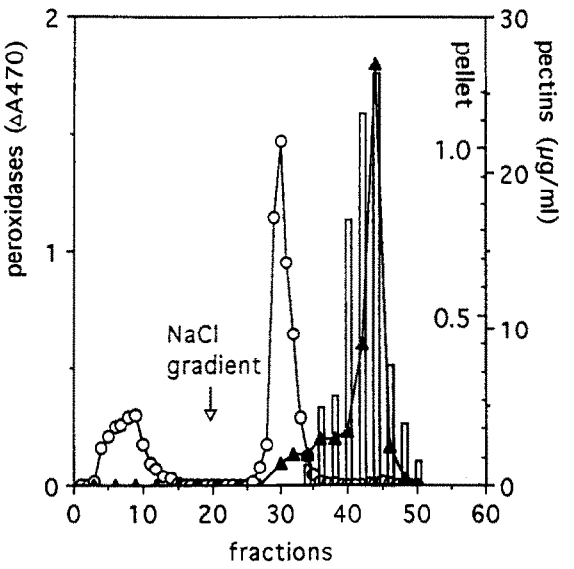

Fig. 1. Separation of a preparation of peroxidases pelletable in presence of Ca^{2+} by ion exchange chromatography through DEAE Sephacel. Peroxidase activity (○) and pectin (▲) were assayed. Pellet (bars) shows the capacity of the fractions to restore the pelletability of anionic peroxidase (ΔA_{470}/pellet).

3. Results

A particular fraction of the peroxidase activity was obtained from an extract of zucchini hypocotyls by successive sedimentations in the presence of calcium and solubilisations in the presence of EGTA (Table 1). This procedure led to a preparation containing about 10% of total peroxidase activity and exhibiting a peroxidase specific activity about four times higher than the original extract. The addition of pectinase during the preparation lowered considerably the Ca^{2+}-dependent pelletability of peroxidases, indicating that the presence of pectins was necessary. The preparation obtained by the successive centrifugations with and without calcium contained 2 of the 3 cationic isoperoxidases and 1 of the 2 main anionic isoperoxidases present in a crude extract (Fig. 2). This preparation was fractionated further by ion exchange chromatography through a column of DEAE Sephacel (Fig. 1). An analysis of the resulting fractions showed a first peak of peroxidase activity not retained by the resin, consisting of 2 cationic isoperoxidases (Fig. 2d). A second peak, released at about 130 mM NaCl, corresponded to an anionic isoperoxidase (Fig. 2e). Pectic material was mostly released by higher NaCl concentrations. When centrifuged alone in the presence of calcium, cationic and anionic isoperoxidases could not be spun down (Table 2). This observation indicated that a cofactor was necessary for the pelletability to occur. As suggested by the effect of the addition of pectinase (Table 1), this cofactor could be a pectin. Effectively, the pelletability of the anionic isoperoxidase was restored by the addition of the pectic material eluted from DEAE Se-

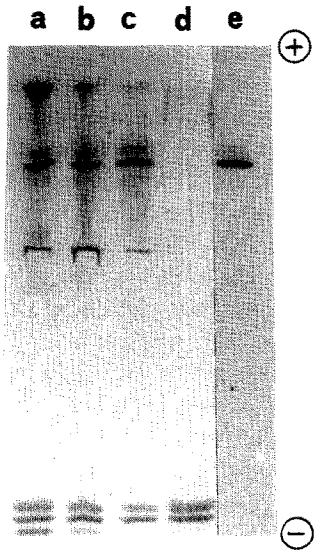

Fig. 2. IEF separation showing the isoperoxidase profile at various steps of the preparation. (a) Crude extract; (b) pellet after centrifugation no. 1 (Table 1); (c) supernatant of centrifugation no. 4; (d) cationic isoperoxidases after DEAE Sephacel (Fig. 1); (e) anionic isoperoxidase after DEAE Sephacel.

phacel. As shown in Fig. 1, the distribution of the pelleting activity closely corresponded to the level of pectins present in the fractions. Attempts made to replace endogenous pectins by commercial preparations showed that PGA was active (Table 2). In order to know whether this phenomenon resulted from a direct interaction between the enzymes and the molecules of pectin, the isoperoxidases separated by ion exchange chromatography were purified further by preparative IEF in lipuid medium (Fig. 3). The anionic isoperoxidase was found in the gradient at a pH value of about 4.3, whereas the two cationic isoperoxidases formed a single peak at the upper limit of the pH gradient around 12. Polyacrylamide-SDS

Table 2
Binding of anionic and cationic isoperoxidases to 200 ng of PGA

Isoperoxidases	Additions	ng isoperoxidases in solution	
		Before centrifugation	After centrifugation
After DEAE separation			
Anionic	$CaCl_2$	184 ± 7*	178 ± 7
	PGA + $CaCl_2$	176 ± 5	10 ± 1.7
Cationic	$CaCl_2$	150 ± 6	154 ± 6
	PGA + $CaCl_2$	189 ± 4	41 ± 1
After isoelectric focussing			
Anionic	PGA + $CaCl_2$	224 ± 9	5.2 ± 1.2
Cationic	PGA + $CaCl_2$	200 ± 12	8.2 ± 1.0
Cl	PGA + $CaCl_2$	232 ± 8	232 ± 9

The isoperoxidases used were separated with DEAE Sephacel or purified by isoelectric focussing. Spinach isoperoxidase C1 was also tested. The amounts of isoperoxidases were estimated as described in section 2. *Each value is the mean ± S.E.M. of 3 replicates.

gel electrophoresis showed that the two peroxidase fractions obtained by separation through DEAE Sephacel exhibited a limited number of protein bands (Fig. 4). After purification by IEF, the cationic isoperoxidases appeared as two close and relatively diffuse bands with molecular weights comprised between 35 and 40.8 kDa. The anionic isoperoxidase was found as a single band of 41.4 kDa and co-purified with a protein of higher molecular weight. However, this latter protein did not precipitate with the isoperoxidase when centrifuged in the presence of PGA and calcium (Fig. 4e). The almost pure isoperoxidases, anionic as well as cationic ones, did sediment when centrifuged in the presence of PGA and Ca^{2+} (Table 2), showing the ability of Ca^{2+}-pectate gel to carry peroxidase molecules in a w/w ratio of about 1.

The association of the isoperoxidases to pectins in the presence of Ca^{2+} was also illustrated by gel filtration through a column of Sephacryl S200. In absence of Ca^{2+}, the peroxidase activity came out of the column at an elution volume corresponding to the molecular weight of the isoperoxidases (Fig. 5A). In the presence of a low concentration of free Ca^{2+}, the peroxidases were eluted at the void volume, indicating the formation of an aggregate with a molecular weight higher than 250 kDa (Fig. 5B). Pectic material formed a broad peak coming out of the column after peroxidases in the absence of Ca^{2+} and was distributed in 3 peaks in the presence of Ca^{2+}, the first one corresponding to the peak of peroxidase activity (Fig. 5B). If the preparation was incubated with pectinase prior to chromatography in the presence of Ca^{2+},

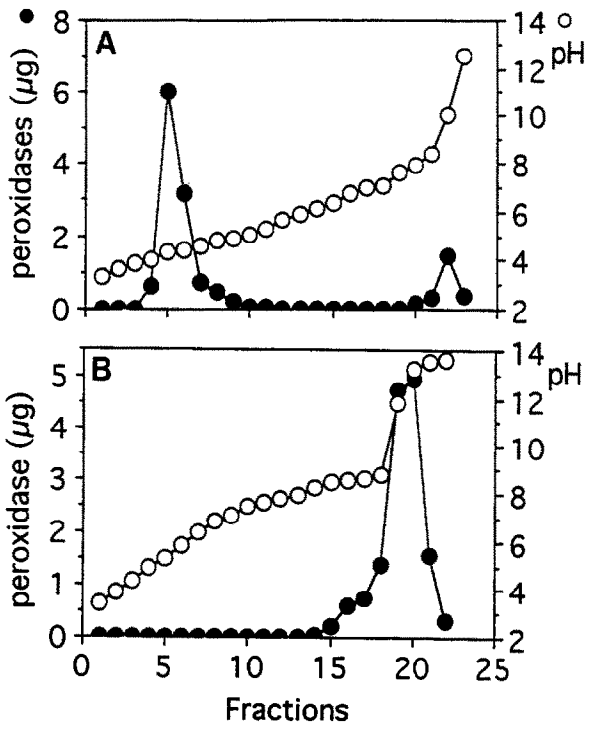

Fig. 3. Preparative IEF of the anionic (A) and the cationic (B) isoperoxidases.

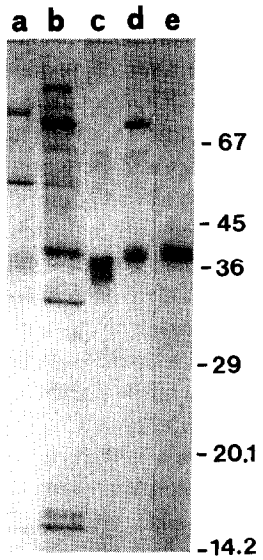

Fig. 4. SDS-PAGE separation of proteins at various steps of peroxidase separation. (a) Cationic isoperoxidases after DEAE Sephacel (Fig. 1). (b) Anionic isoperoxidase after DEAE Sephacel. (c) Cationic isoperoxidases after IEF (Fig. 3). (d) Anionic isoperoxidase after IEF. (e) 'd' pelleted in presence of PGA and Ca^{2+}. Molecular masses are indicated in kDa.

peroxidase activity elution was shifted from the void volume to a volume corresponding to its molecular weight (data not shown). The main peak of absorbance at 280 nm was due to the presence of BSA (MW = 67 kDa), which was added because preliminary experiments had shown that, in presence of Ca^{2+}, peroxidase activity remained blocked in Sephacryl column unless BSA was added.

The affinity of zucchini isoperoxidases for pectins could also be demonstrated by measuring their binding to commercially available pectins previously attached to the bottom of wells of a microtitration plate (Fig. 6). The results showed that a binding did occur in such conditions, provided Ca^{2+} was present at each step of the procedure (incubations of pectins, incubation of peroxidases, and washings). The anionic peroxidases bound more efficiently to PGA than to the partially esterified pectin from citrus. The affinity of the cationic isoperoxidases for the 2 pectins was similar. On the other hand, a pectin esterified at 93% was unable to bind the peroxidases. If calcium was omitted during one step, the binding of either type of isoperoxidases to every kind of pectins was completely prevented, although isolated pectic chains remained attached to the bottom of the wells. With this method also no binding of the spinach highly cationic isoperoxidase, used as control, could be observed (data not shown).

4. Discussion

The results presented here show that some isoperoxidases have an affinity for pectins. This affinity was unchanged between pH 5 and 7.5 (data not shown). Although the molecular mechanism of the interaction is not known, the features reported here point to a specific attachment of peroxidases to pectins in their calcium-induced conformation. It is known that in the presence of calcium ions pectic chains associate as dimers or oligomers through the formation of a particular structure named 'egg-box' or junction zone [6,14]. That this structure is necessary for the binding of peroxidases to occur was demonstrated here by the absence of binding of the

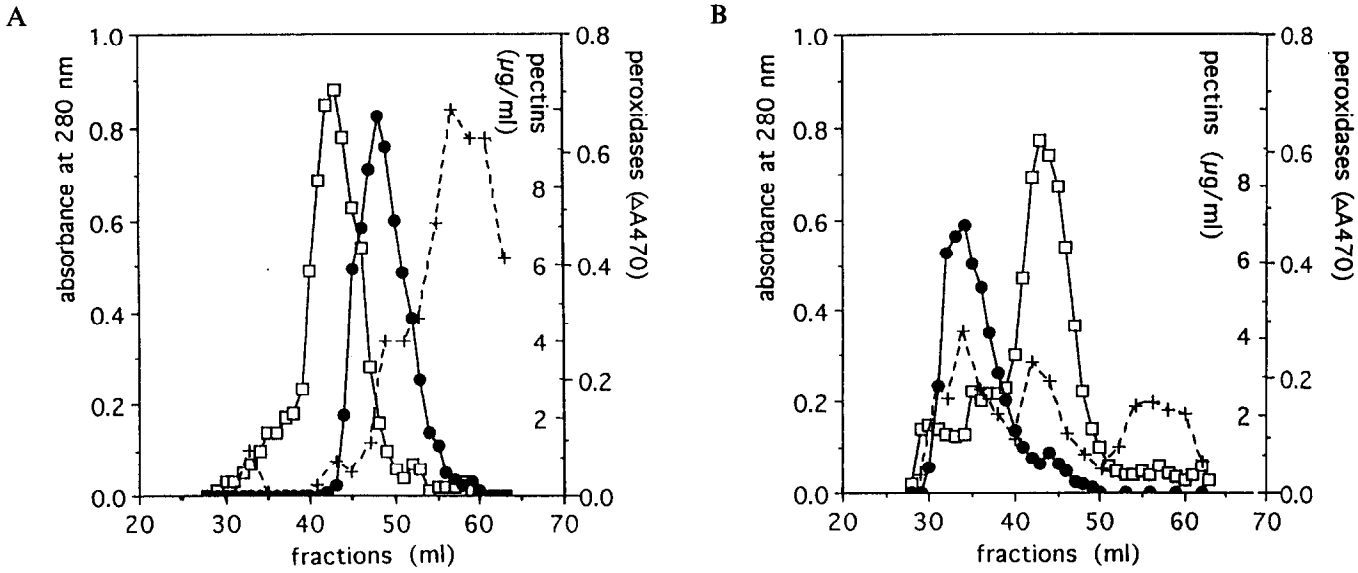

Fig. 5. Gel filtration chromatography of a crude preparation of pelletable peroxidases on Sephacryl S200. A_{280} (□), peroxidase activity (●) and pectin content (+) were determined in each fraction. (A) In presence of EGTA (10 mM in the preparation and 1 mM in the column); (B) As (A) plus $CaCl_2$ (7 mM in the preparation and 0.7 mM in the column).

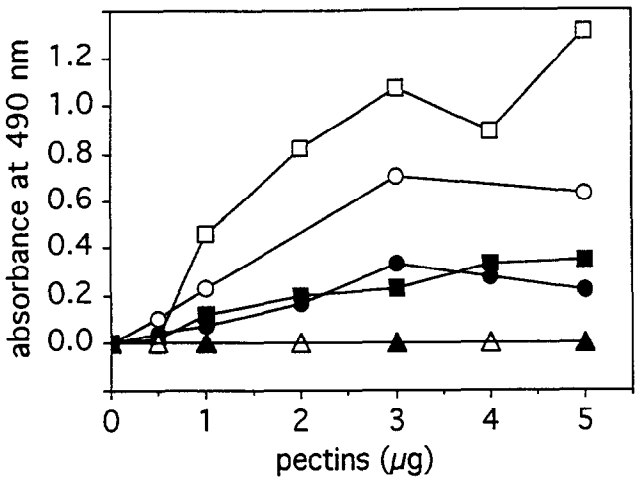

Fig. 6. Binding of peroxidases to increasing amounts of pectins fixed in the wells of a microtitration plate. Bound activity of anionic/cationic isoperoxidases to PGA (■/□), citrus pectin (○/●) and esterified pectin (△/▲) was quantified with *o*-phenylenediamine/H_2O_2 (A_{490}).

enzymes to isolated chains of pectins. This was shown either by gel filtration (Fig. 4a) or by the binding assay in microtitration plates. Also, the inability of highly esterified pectins to associate peroxidase molecules in presence of Ca^{2+} (Fig. 6) can be explained by the fact that they do not form junction zones [6]. The possibility of an ionic interaction between pectins and peroxidases can be completely ruled out because: (1) ionic binding should also occur with isolated pectic chains; (2) a very cationic isoperoxidase from spinach did not bind; and (3) the negatively charged anionic isoperoxidase, which should not stick to anionic pectate molecules, was bound efficiently. Additional work will be necessary to know if the three isoperoxidases, and the eventual other proteins able to bind to Ca^{2+}-pectate gel, bear a common structure necessary for the interaction with the junction-zone structure. A former work has shown that the presence of the glycan moiety of a cationic isoperoxidase from *Pharbitis* was essential for its Ca^{2+}-induced pelletability [8].

Owing to the properties described above, the binding of the isoperoxidases to pectins in their Ca^{2+}-induced conformation is likely to occur in vivo. This binding could take place in cell walls, which contain peroxidases that can be released after a treatment with a pectinase [15], but also within the Golgi apparatus, which may carry pectins and peroxidases together. Pectins and peroxidases were found also tightly associated in an extracellular macromolecular complex released by soybean cells in suspension culture [16]. Work is underway to determine the possible physiological function of this association.

Acknowledgements: We thank Lucette Von Tobel, Anne-Marie Monat, and Lydie Chane-Favre for their excellent technical assistance. This work was supported by a grant from the Swiss Foundation for Scientific Research (no. 31-32456.91).

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