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THE ROLE OF CALCIUM IN THE CONTROL OF PEROXIDASE ACTIVITY

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Introduction

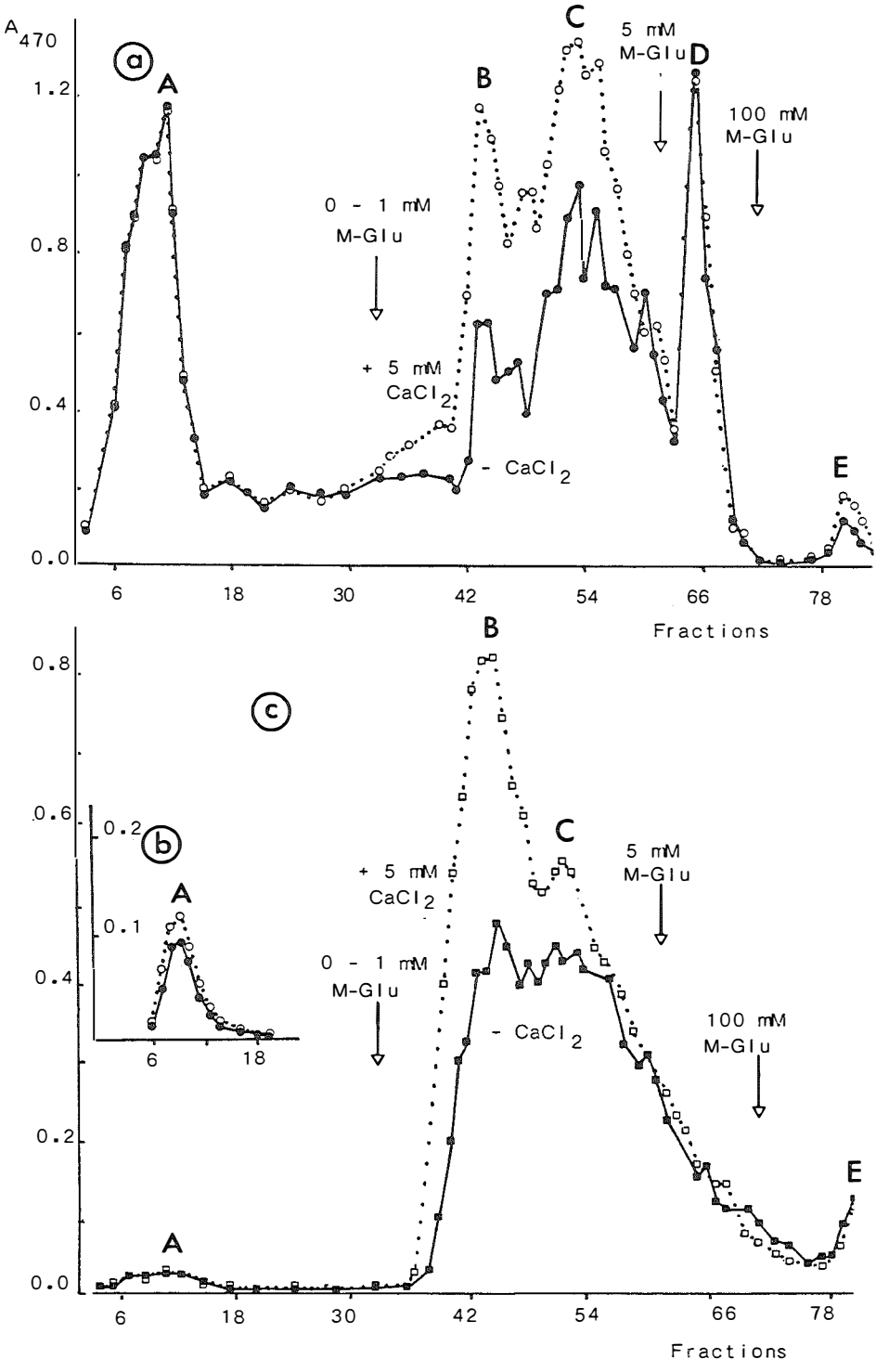
Peroxidases are used as markers by plant physiologists in a wide variety of experimental situations: following chemical treatments, in relation with growth or differentiation, for genetic studies, to show the effects of environmental factors on plants. In most cases, quantitative or qualitative variations of peroxidases are reported . The great sensitivity of these enzymes to many endogenous or exogenous factors explains why peroxidases are so often chosen. But the numerous publications devoted to them rarely concern the molecular reasons which could explain their high reactivity.

It is now evident that peroxidases are dependent on calcium ion through several mechanisms. Calcium mediates many different cellular processes, acting as second messenger and regulating many functions of plant cells (for recent reviews, see 7, 12). One reason why peroxidases are so reactive could lie in the fact that they are closely dependent on calcium. This means that cellular redistributions of this ion occurring in response to hormones, light or other factors could change peroxidase activity. There are apparently three distinct mechanisms by which calcium can control peroxidases rather directly. These mechanisms, which are described with some details below, are the direct activation of peroxidases, their binding to membranes and their secretion.

Peroxidase activation

In many instances, it was reported that the addition of calcium activated plant peroxidases (1, 5, 22). Generally, this activation only affects some of the isoperoxidases present in a tissue. In order to study further this property, peroxidases extracted from spinach leaves were separated through a column of concanavalin A (con A) Sepharose and their activation by calcium was measured. Taking advantage of the fact that only manganese is really necessary for the binding of glycoprotein to con A (21), the affinity chromatography was performed in the absence of added calcium. In addition , as a progressive elution by the competing sugar was used, a microheterogeneity of peroxidases appeared, as already with other glycoproteins (2). The total peroxidase activity present in a crude extract may be separated into several peaks (Fig.1a): the first peak (A) corresponds to peroxidases which are not retained by con A, then there are several fractions corresponding to peroxidases with increasing affinity for the lectin and, finally, a peak with high affinity. Among all these fractions, only some can be activated by the addition of calcium in the assay medium. They exhibit a low affinity for con A. Two particular fractions were also prepared from spinach leaves and submitted to the same chromatography procedure. The peroxidases present in free spaces of leaves and collected by vacuum infiltration had no affinity for con A (Fig.1b) and are not activated by calcium. On the contrary, peroxidases ionically bound to cell walls were all retained by con A (Fig.1c) and were partly

Fig. 1. Separation of peroxidases by affinity chromatography through a column of concanavalin A Sepharose (20 x 0.8 cm). The resin was equilibrated in 100 mM acetate buffer pH 6 containing 1M NaCl and 1mM $MnCl_2$. Elution of the glycoproteins bound to con A was obtained by a linear gradient of methyl-α -D-glucopyranoside (M-Glu: 0-1 mM) followed by two washings with 5 and 100 mM M-Glu. Peroxidase activity was assayed in 20 mM acetate buffer containing 8 mM guaiacol and 2 mM H_2O_2, without or with 5 mM $CaCl_2$. The absorbance at 470 nm was read after 5 min. a) Crude extract: spinach leaves were ground in chromatography buffer and the resulting extract centrifuged at 10000 g for 10 min. b) free space peroxidases: obtained by vacuum infiltration of leaves in phosphate buffer pH 7 followed by centrifugation c) peroxidases ionically bound to cell walls: leaves were ground in 25 mM Mes-tris pH 7.2. The resulting extract was centrifuged at 1000 g, the pellet washed in the same buffer and then the bound proteins were detached with chromatography buffer.


a
A_{470}
1.2
0.8
0.4
0.0
A
B
C
D
E
5 mM M-Glu
100 mM M-Glu
0 - 1 mM M-Glu
+ 5 mM $CaCl_2$
- $CaCl_2$
6
18
30
42
54
66
78
Fractions
c
b
0.2
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0.4
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activated by calcium. A separation of isoperoxidases present in these different peaks by isoelectric focusing in agarose gels is shown in Fig.2. It can be observed that isoperoxidases exhibiting a high affinity for con A and no activation by calcium are mainly acidic, while the activable isoperoxidase with low affinity for con A is cathodic. The isoperoxidase from free spaces has the most basic isoelectric point. The activable cathodic isoperoxidase apparently exhibits the greater microheterogeneity, but this microheterogeneity is not apparent on gel after isoelectric focusing.

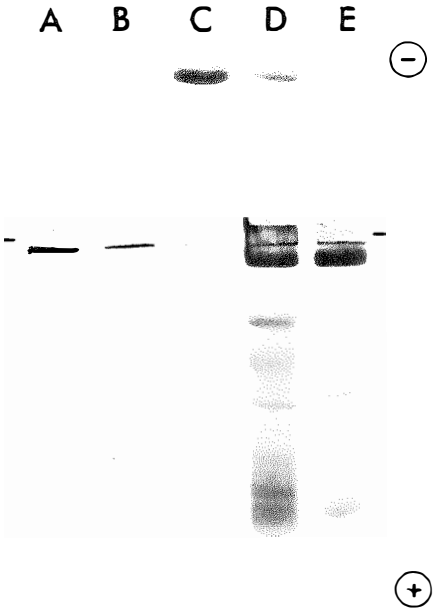

Fig. 2. Isoelectric focusing in agarose gel of the various fractions obtained by affinity chromatography (Fig. 1). Fractions were desalted through Sephadex G 25 before electrophoresis. The gradient of pH was provided by 1.50% Ampholine (LKB) 3.5-10.0 and 0.75% Ampholine 9.0-11.0. The electrophoresis was run for about 90 min at 6 W. Bands were revealed with benzidine- H_2O_2.

The activable cathodic isoperoxidase obtained by affinity chromatography (fraction B in Fig.1) was used to characterize further the effect of calcium. Fig.3 shows the rate of guaiacol oxidation at two different concentrations of hydrogen peroxide. It is evident from this simple experiment that calcium increases the velocity of the reaction and the activation is greater at the lower hydrogen peroxide concentration (after a 1-min incubation the ratio A_{470}+Ca / A_{470}-Ca is 6.2 and 1.8 respectively). Measurements performed at various calcium concentrations showed that the ion is active at low concentrations (Fig.4).

The effect of calcium was also tested on peroxidases preparations which were not purified through con A Sepharose

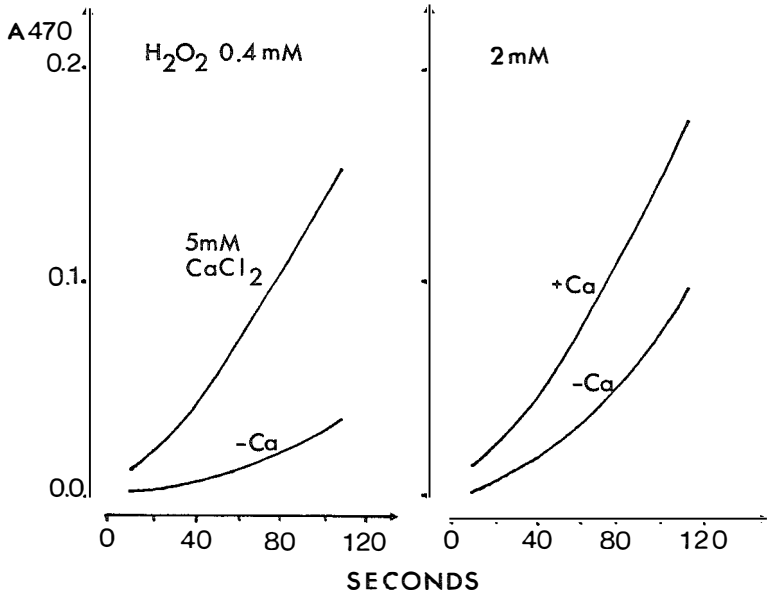

Fig. 3. Rate of oxidation of guaiacol (4 mM) in the presence of H_2O_2 (0.4 or 2 mM) by peroxidase from fraction B (Fig. 1), measured with a DU 5 Beckman spectrophotometer.

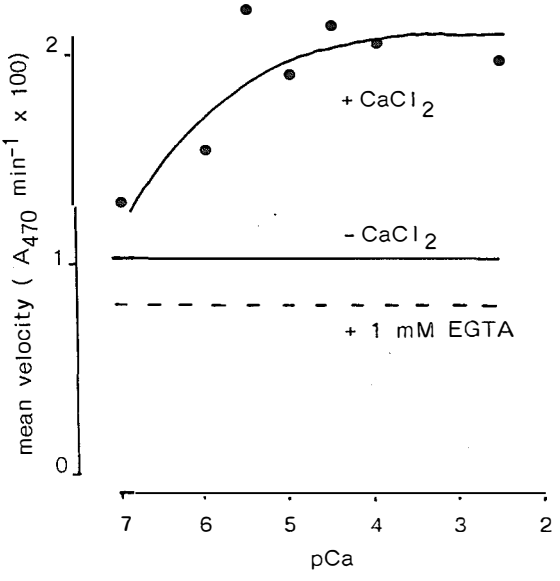

Fig. 4. Effect of EGTA or various concentrations of $CaCl_2$ on the oxidation of guaiacol (1 mM) in the presence of 20 µM H_2O_2 by peroxidase from fraction B (Fig. 1).

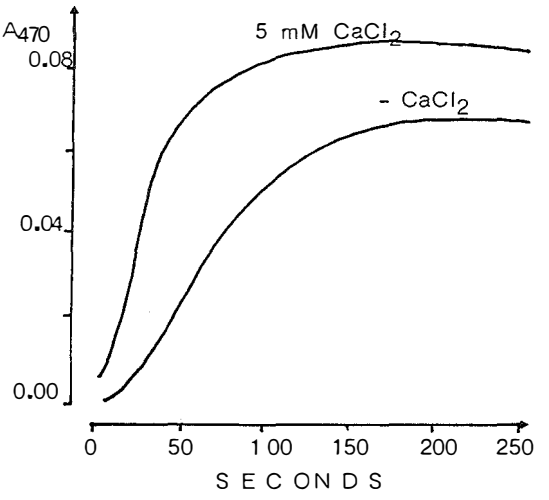

Fig. 5. Effect of 5 mM $CaCl_2$ on the rate of oxidation of guaiacol (1 mM) in the presence of 20 µM H_2O_2 by peroxidases ionically bound to walls prepared as described for Fig. 1.

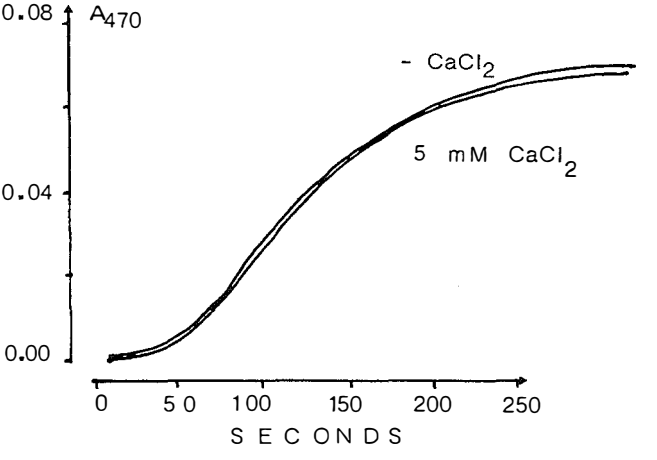

Fig. 6. Effect of 5 mM $CaCl_2$ on the rate of oxidation of guaiacol (1 mM) in the presence of 20 µM H_2O_2 by peroxidases from free spaces prepared as described for Fig. 1.

(Fig. 5 and 6). In these cases, the rate of reaction was less linear. This can be explained by the presence of cofactors such as phenolics, which affect the kinetic properties of peroxidases (15). It appeared that peroxidases ionically bound to walls can be activated by calcium, with a maximum activation at the beginning of the reaction (Fig.5), when free-space peroxidases, which correspond to fraction A in Fig.1, are not activated (Fig.6).

Several facts can be emphasized. i) Only a cathodic isoperoxidase is sensitive to calcium addition. This is consistent with a study on horseradish isoperoxidases (6). ii) The effect of calcium is stronger at low hydrogen peroxide concentrations. As one can imagine that this concentration is low in cells or cell walls, this mechanism could have a great importance in vivo. This importance is also suggested by the observation by Geiger and Goujon (5) that healthy tissues of Hevea contained a calcium-activable isoperoxidase which disappeared in tissues infected by a fungi. iii) The calcium-dependent isoperoxidase from spinach leaves is sensitive to low calcium concentrations and may be separated by affinity chromatography.

Binding of peroxidases to membranes

It was observed several years ago that some isoperoxidases bind to membranes upon addition of calcium. This occurred in extracts of hypocotyl hooks of Cucurbita pepo (17), of lentil roots (16), of sugarbeet cells (18) and of Pharbitis cotyledons (10). In this latter case, manganese was shown to be also active. Generally, the isoperoxidase which binds is cathodic. However, the calcium-dependent binding of acidic isoperoxidases was also reported (20). The binding of the cathodic isoperoxidase is saturable by increasing the calcium (manganese) concentration or isoperoxidase concentration; it depends on the presence of the carbohydrate moiety of peroxidase; its mechanism seems common to several plants since, for example, zucchini peroxidases can be associated to Pharbitis microsomes (10). Preliminary studies have shown that plasmalemma, tonoplast and other cellular membranes are able to bind the cathodic isoperoxidase upon addition of calcium or manganese.

The maximum effect of calcium requires an ion concentration in the millimolar range (10, 17). This concentration, which can appear rather high, could mean that the binding only occurs in cell compartments which contain such concentrations. It can be noticed that receptors for glycoproteins in animal cells exhibit the same calcium requirement to be active (19). It is tempting to hypothesize that the binding of peroxidases is due to calcium (and/or manganese) dependent receptors involved in the control of the subcellular localization of the cathodic isoperoxidase. As a next step, the existence of such receptors should be demonstrated.

Peroxidase secretion

Cell wall usually contains a great part of the peroxidase activity of a plant cell, another part being found in vacuoles (14). The role of wall peroxidases could be important, owingto their ability to cross-link or polymerize many molecules (3, 11). These peroxidases must be transported across the plasmalemma. Several works have given indications that this transport is, at least partly, achieved by a calcium-dependent secretory mechanism (1, 8, 9, 22).

Conclusion

The experimental facts presented above emphasize the complexity of the effects of calcium on peroxidases. It is too early to consider the role of these mechanisms in the control of peroxidase activity in cells. However, regarding the cathodic isoperoxidase(s), we can imagine that, following biosynthesis, its transfer toward cell wall or vacuole could be determined by the activity of the putative calcium-dependent receptors, and by the secretory machinery which is under the control of cytosolic calcium. Once they have reached their final destination, these isoperoxidases would be dependent on the local calcium concentration for their activity. As it was reported that free calcium readily exchanges with the cathodic isoperoxidase of horseradish (6) and is essential for maintaining the protein structure in the heme environment (13), the modulation of their catalytic activity by changes in calcium concentrations cannot be ruled out. On the contrary, acidic isoperoxidases, which cannot exchange their calcium and regain activity upon calcium addition if calcium has been removed (6), have another mode of regulation. Some of them, however, are likely to be controlled by calcium either through binding to membranes (20), or through secretion. This difference in the control of the two groups of isoperoxidases has already been discussed (4).

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