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EFFECT OF CALCIUM ON SUBCELLULAR DISTRIBUTION OF PEROXIDASES

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Key Word Index—*Cucurbita pepo*; Cucurbitaceae; bound peroxidases; subcellular fractionation; calcium.

Abstract—About 40% of the peroxidase activity extracted from hypocotyl hooks of etiolated *Cucurbita pepo* was pelletable at 20000 *g*. The activity in the pellet was partially solubilized by the addition of 5 mM EGTA. This effect of EGTA was reversed by Ca^{2+}, but not by Mg^{2+}. Reassociation of EGTA-solubilized peroxidases to 4 cellular fractions obtained by centrifugation on discontinuous sucrose gradients was assayed. It appeared that the enzymes could be linked to ribosomes or RNP particles through Ca^{2+}. Two fractions, identified as smooth and rough endoplasmic reticulum, also bound peroxidases and, in this case, the Ca^{2+}-mediated binding involved a loss of the enzyme activity. The fraction containing mitochondria and plasmalemma exhibited a slight binding capacity. Isoelectric focusing in thin layer polyacrylamide gel showed that only 5 out of the 10 isoperoxidases present in hypocotyl hooks had their pelletability level changed by Ca^{2+}.

INTRODUCTION

Several authors have studied the localization of peroxidases (donor: H_2O_2, oxidoreductase: EC 1.11.1.7.) within plant cells. By cytochemical methods Poux [1] and Czaninski and Catesson [2] found them associated to small vacuoles, endoplasmic reticulum, Golgi apparatus, cell wall and plasma membrane. The chromosomes and the nucleoli also exhibited a peroxidase activity [3]. On the other hand, it was shown, after cell fractionation, that some peroxidase activity occurred on the cell wall [4], ribosomes [5], provacuole membrane [6] and on other unidentified membranes [7, 8]. The amount of pelletable peroxidases appeared highly dependent on the composition of the extraction medium. A high ionic strength [9], an alkaline pH [9] or the use of a chelating agent such as EDTA [10] generally released a great part of the pelletable activity.

The solubilization of peroxidases by EDTA indicated that a divalent cation was involved and preliminary experiments showed us that Ca^{2+} was very effective in overcoming the effect of EDTA. We describe in this paper the effect of Ca^{2+} on the binding of peroxidases to various subcellular fractions.

RESULTS

Hypocotyl hooks of etiolated zucchini seedlings contained a high peroxidase activity, *ca* 40% of which was found pelletable at 20000 *g*, after an extraction at neutral pH and weak ionic strength. Fig. 1 shows that the level of peroxidases found in a 1500–20000 *g* pellet was reduced by the addition of 5 mM ethyleneglycol-bis (*β*-aminoethyl ether) *N*,*N*′-tetraacetic acid (EGTA). At the same time, the specific activity of the peroxidase in the 20000 *g* supernatant was increased. The effects of calcium and magnesium chloride were examined in order to see if the effect of EGTA could be reversed. For this purpose, samples of a 1500 *g* supernatant containing 5 mM EGTA were given varying concentrations of the two salts and, after centrifugation at 20000 *g*, the peroxidase was determined in both the pellets and the supernatants. It is evident from Fig. 1 that Ca^{2+} overcame the effect of EGTA and increased the pelletability of an additional part of the peroxidase activity with respect to the untreated control (without EGTA). However, Mg^{2+}

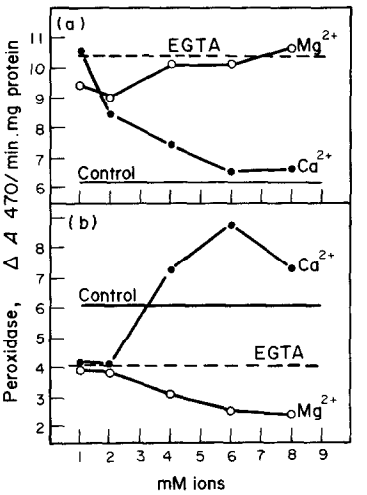

Fig. 1. Effect of Ca^{2+} or Mg^{2+} on the pelletability of peroxidases. Aliquots of a 1500 *g* supernatant containing 5 mM EGTA were centrifuged at 20000 *g* during 30 min in the presence of increasing ion concentrations. Peroxidase specific activity (ΔA_{470}/min mg protein) was measured in the supernatants (a) and in the resuspended pellets (b). The activity of a control without EGTA was plotted.

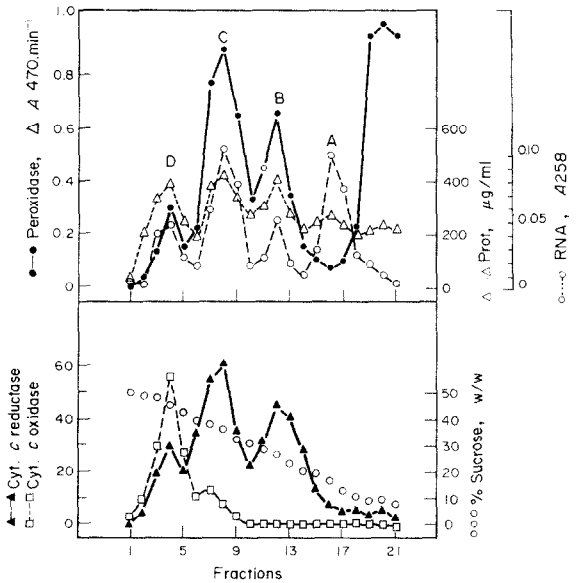

Fig. 2. Distribution profiles of various components in a 1500–20000 *g* pellet following centrifugation on a discontinuous sucrose gradient containing 3 mM EGTA and 50 mM KCl. Cytochrome *c* oxidase and reductase: arbitrary units.

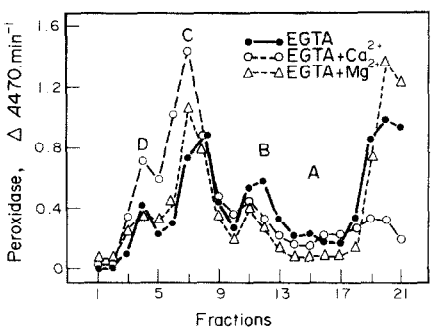

Fig. 3. Effect of Ca^{2+} or Mg^{2+} on the distribution profile of peroxidases in 1500–20000 *g* pellets following centrifugation on discontinuous sucrose gradients (without EGTA and KCl). The resuspended pellets contained 5 mM EGTA, 5 mM EGTA + 6 mM Ca^{2+} or 5 mM EGTA + 6 mM Mg^{2+}.

exhibited a slight detaching effect, probably due to the increase of the ionic strength. This experiment confirmed the data of Macnicol [10]; i.e. the addition of a chelating agent solubilized some peroxidase activity. Moreover, it appeared that Ca^{2+}, but not Mg^{2+}, was involved in the binding of peroxidases to a sedimentable fraction of zucchini hypocotyl hooks. Experiments performed with spinach leaves or lentil roots gave similar results, indicating that the effect of Ca^{2+} probably is a general phenomenon (unpublished data).

The pelletability of peroxidases in the presence or absence of Ca^{2+} was tested at different pH values ranging from 6 to 8. Aliquots of an EGTA-containing 1500 *g* supernatant were brought to the desired pH by the addition of 0.2 N HCl or NaOH. They were then divided: one part was centrifuged without Ca^{2+}, the other one with 6 mM Ca^{2+}. It appeared that Ca^{2+} exhibited almost the same effect over the range of pH 6 to 8.

The distribution of peroxidases among the various constituents of the 1500–20000 *g* pellet was studied by layering resuspended pellets on discontinuous gradients consisting of 5 layers of sucrose solutions (50, 38.5, 30, 21 and 10% w/w) all containing 3 mM EGTA and 50 mM KCl. After 3 hr at 27000 rpm, the gradients contained 4 bands at the interfaces of the sucrose solutions (Fig. 2). For convenience, these 4 fractions were designated by the letters A (21/10%), B (30/21%), C (38.5/30%) and D (50/38.5%). Three of the 4 fractions (B, C and D) exhibited a peroxidase activity. The less dense one (A), devoid of any detectable activity, was essentially composed of ribonucleoprotein particles (RNP). The other fractions were identified as smooth endoplasmic reticulum (B), rough endoplasmic reticulum (C) and mitochondria plus plasmalemma (D) by Quail and Hughes [11] using the same gradient and the same plant material. This identification seemed to be confirmed by the distribution of the marker enzymes (cytochrome *c* oxidase and antimycin-insensitive NADH cytochrome *c* reductase) used here, as well as by the sucrose densities. However, RNA was present in all the 4 fractions which were probably somewhat cross-contaminated.

The effect of Ca^{2+} and Mg^{2+} was studied on the distribution of peroxidases in discontinuous gradients without the uniform presence of EGTA and KCl. For this purpose, 3 samples of a 1500–20000 *g* pellet were used, which were resuspended in 5 mM EGTA. One of these aliquots received in addition 6 mM Mg^{2+} and another one 6 mM Ca^{2+} (Fig. 3). Despite the absence of EGTA and KCl in the gradients, the profile of peroxidases was similar to that obtained in Fig. 2. The suspension with EGTA alone and that with added Mg^{2+} gave a rather similar distribution. The main difference was the greater soluble activity in the Mg^{2+}-containing suspension. This observation was in accordance with the results presented in Fig. 1. The distribution profile exhibited by the Ca^{2+}-treated suspension also confirmed the results of Fig. 1: almost all the activity was found associated to the 3 more dense bands and there was practically no soluble activity remaining at the top of the gradient. The addition of Ca^{2+} or Mg^{2+} also shifted some protein and RNA towards the 2 more dense bands (C and D). These data confirmed that Ca^{2+} promoted the association of peroxidases to cellular structures.

A more accurate identification of these structures was attempted by reassociating the peroxidases solubilized from a 1500–20000 *g* pellet to the 4 fractions separated by a density gradient centrifugation. The solubilized enzymes were purified through DEAE Sephadex to eliminate contaminating RNA and mixed to the 4 fractions. Aliquots of the resulting mixtures were centrifuged in the presence of increasing Ca^{2+} concentrations (Table 1). It was observed that Ca^{2+} had only little influence on the pellet size, except for the A fraction in which the amount of sedimentable material increased with the Ca^{2+} concentration. The general conclusion which may be drawn from peroxidase specific activities is that Ca^{2+} promoted the copelletability of peroxidases with the 4 fractions. The most effective Ca^{2+} concentration seemed to be 10^{-5} M, except for B fraction (10^{-7} M). The Ca^{2+} effect is also visualized by the ratio between the pelletable activity and the activity remaining in the supernatant. On the other hand, it was shown that the Ca^{2+}-mediated

Table 1. Effect of Ca^{2+} concentration on reassociation of solubilized peroxidases to the 4 fractions isolated by density gradient centrifugation

Fraction	Ca^{2+} (M)	Pellet size μg protein	Pellet peroxidase specific activity*	Peroxidase activity† pellet/supern.	pellet + supern.
A	0	74	11.0	0.06	13.4
	10^{-7}	63	14.0	0.12	12.3
	10^{-5}	96	15.2	0.13	13.7
	10^{-3}	161	5.3	0.12	13.0
B	0	179	11.1	0.10	22.1
	10^{-7}	159	17.1	0.20	16.7
	10^{-5}	167	12.6	0.16	14.8
	10^{-3}	163	14.0	0.20	13.8
C	0	453	9.5	0.20	26.4
	10^{-7}	404	9.1	0.17	24.7
	10^{-5}	468	13.1	0.39	21.9
	10^{-3}	473	12.1	0.42	19.8
D	0	125	2.2	0.02	12.5
	10^{-7}	104	2.6	0.02	16.7
	10^{-5}	147	2.9	0.03	15.2
	10^{-3}	121	2.2	0.02	13.4

* ΔA_{470}/8 min. mg protein.
† ΔA_{470}/8 min.

binding of peroxidases to the B and C fractions induced a loss in the total amount of peroxidase activity which could be recovered (pellet + supernatant). This result, which may be interpreted as a partial inactivation of the bound enzymes was observed only in the fractions which mainly consisted of smooth or rough endoplasmic reticulum (B and C). This was an additional difficulty for studying the Ca^{2+} effect, since the binding was monitored through the measurement of the enzyme activity. Most likely, the amount of peroxidases bound to endoplasmic reticulum was much higher than indicated by the specific activities reported in Table 1.

It clearly appeared that the degraded ribosomes, which form the A fraction [11], could bind peroxidases when Ca^{2+} was present at low concentrations. As RNA is known to easily bind Ca^{2+} [12], the results of Table 1 could be explained by the formation of Ca^{2+} bridges between RNA and peroxidases. Some experiments performed with undissociated native ribosomes prepared from zucchini hypocotyl hooks showed that they had exactly the same binding properties as RNP. Thus, degradation of ribosomes was not the cause of the Ca^{2+}-mediated binding of peroxidases. On the other hand, one must point out that the 3 other fractions contained some contaminating RNA (Fig. 2). For this reason, it is difficult to attribute the effect of Ca^{2+} observed in these fractions to the RNA contamination or to membranes. However, considering the inactivation of peroxidases following the addition of Ca^{2+} to the B and C fractions or the level of specific activity bound through Ca^{2+}, one may imagine that endoplasmic reticulum is one of the cellular structures which bind peroxidases through Ca^{2+}.

Isoelectric focusing in thin layer polyacrylamide gel was used to separate the isoperoxidases of zucchini hypocotyl hooks (Fig. 4). A crude extract (1500 *g* supernatant) contained 10 isoperoxidases distributed among 2 very distinct groups: 4 in the alkaline part of the pH gradient and 6 in the acidic part. Nine of these bands were found associated to the 1500–20000 *g* pellet, but only isoperoxidases 3, 4 and 5 were completely pelletable at 20000 *g*. The effect of Ca^{2+} on the binding of the isoperoxidases was studied by comparing the intensity of each

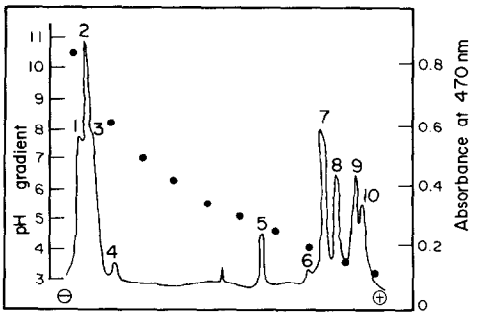

Fig. 4. Isoperoxidase profile of a crude extract resolved by isoelectric focusing in polyacrylamide gel.

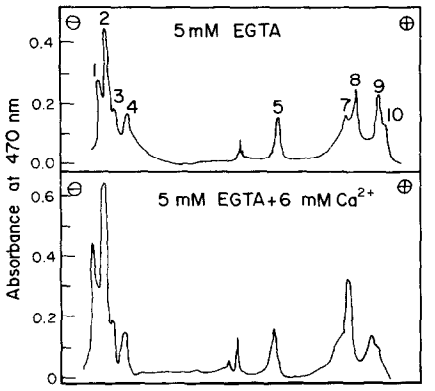

Fig. 5. Effect of Ca^{2+} on isoperoxidase profile of 1500–20000 *g* pellets resolved by isoelectric focusing in polyacrylamide gel. Pellets were sedimented in the presence of 5 mM EGTA or 5 mM EGTA + 6 mM Ca^{2+}.

band in 1500–20000 *g* pellets obtained from a crude extract containing 5 mM EGTA or 5 mM EGTA and 6 mM Ca^{2+} (Fig. 5). These 2 isoperoxidase profiles show that Ca^{2+} enhanced the pelletability of isoperoxidases 1, 2 and 8, whereas the pelletability of isoperoxidases 9 and 10 was reduced. Thus, only five among the 10 isoperoxidases were affected by Ca^{2+}. This cation appeared to have a differential effect: it induced the binding of 3 isozymes and detached 2 other ones. This latter effect can be explained by the enhancement of the ionic strength. It is interesting to note that 2 of the isoperoxidases (1 and 2) bound through Ca^{2+} were strongly cationic (isoelectric point $\geqslant$ 10).

DISCUSSION

Calcium induced the attachment of peroxidases to ribosomes and, probably, to endoplasmic reticulum or other cell membranes in extracts prepared from zucchini hypocotyl hooks. It is known that glycoproteins, such as peroxidases [13], bind Ca^{2+} particularly well [14]. Most likely, the cation can act as cross-linking agent between peroxidases and cellular components. The fact that the peroxidases bound through Ca^{2+} were mostly cationic, as well as the independence of the binding towards pH, indicate that Ca^{2+} probably did not interact with negative groups of the proteins such as carboxyl

groups. It was already reported [15] that RNP particles extracted from pea seedlings or rabbit liver contained a peroxidase activity which was released by EDTA. It was then concluded that the enzyme was associated with the particles through metals. This conclusion was confirmed by the present work which indicated that the metal involved is calcium. Besides the association to ribosomes, we had some indications that endoplasmic reticulum also bound peroxidases through Ca^{2+}. As Lipetz and Garro [16] and Parish [17] previously showed that Ca^{2+} released peroxidases from cell wall, we may conclude that this cation plays a role in the intracellular localization of peroxidases.

An effect of Ca^{2+} on peroxidases was already found in durum wheat semolina, where it acted as an activator of these enzymes, changing their kinetic properties [18]. On the other hand, Ca^{2+}, given to etiolated pea seedlings, decreased the activity of 2 isoperoxidases [19]. In our own experiments, increasing the Ca^{2+} concentration often induced a reduction of the total amount of recoverable peroxidase activity. All these observations indicated that Ca^{2+} is able to modify the activity of peroxidases. This effect is not restricted to peroxidases and was shown for other plant enzymes [20].

Much work is still required to make sure that the effect of Ca^{2+} is not an artifact and to precisely localize the binding sites for peroxidases within the cell. This localization could be difficult, because peroxidases appeared to be differently distributed in each plant cell type [1]. Finally, it can be noted that Ca^{2+} interacts with several aspects of auxin physiology including IAA transport [21], auxin binding [22] and auxin effect on plasma membrane [23]. Since peroxidases are involved in IAA degradation [24], a relation between IAA, peroxidases and Ca^{2+} might be physiologically significant.

EXPERIMENTAL

Plant material. Zucchini seedlings (*Cucurbita pepo*, cv Black Beauty) were grown in the dark on moist paper, at 26° during 4 days. Hypocotyl hook segments were cut just below the cotyledons. Harvesting and all the subsequent manipulations were performed under green safelight at 0–4°.

Extraction procedure. Hook segments were minced with a razor blade and ground in a mortar and pestle in 25 mM *N*-morpholino-3-propanesulfonic acid (MOPS) containing 250 mM sucrose and adjusted to pH 7.2. The resulting homogenate was squeezed through nylon cloth and centrifuged for 5 min at 1500 *g*. The supernatant was centrifuged again during 30 min at 20000 *g* and the resulting pellet (1500–20000 *g* pellet) gently resuspended in extraction medium. Ethyleneglycol-bis (*β*-aminoethylether) *N,N'*-tetraacetic acid (EGTA) was added in a concn of 5 mM either in the supernatant of the 1500 *g* centrifugation or during the resuspension of the 1500–20000 *g* pellet. This point is specified in the text.

Sucrose density gradients. Discontinuous sucrose density gradients were constructed with 3 ml of 50% (w/w) and 4.4 each of 38.5, 30, 21 and 10% (w/w) sucrose in 25 mM MOPS (pH 7.2). In some cases, 50 mM KCl and 3 mM EGTA were added in all sucrose solns. Resuspended pellets were layered (1.5 ml for 4 *g* tissue) onto gradients which were spun for 3 hr at 27000 rpm in a MSE SS50 ultracentrifuge. The gradients were fractionated into 1-ml fractions and each fraction was assayed for protein, RNA and enzyme activities. In other cases, the bands were directly collected with a L-shaped needle fitted to a syringe at the 4 interfaces of the gradient.

Reassociation experiment. The resulting 4 fractions were desalted by group separation through Sephadex G25 equilibrated in 25 mM Tris–HCl buffer (pH 7.2) and a 24-hr dialysis against several l. of the same buffer. In the same way peroxidases solubilized from 1500–20000 *g* pellets were collected from the top of the gradients, centrifuged for 60 min at 30000 *g*, strained through a column of DEAE Sephadex A50 equilibrated with Tris–HCl buffer and dialysed. The reassociation of the solubilized peroxidases to the 4 fractions was performed as follows: the peroxidases were separately mixed to each fraction in the presence of various Ca^{2+} concns and then centrifuged for 30 min at 20000 *g* (fractions B–D) or for 60 min at 135000 *g* (fraction A). The resulting pellets were resuspended in 25 mM MOPS. Peroxidase activity was assayed in both the pellets and the supernatants and protein in the pellets.

Assays. Peroxidase activity was determined at pH 6.1 with guaiacol as H-donor (8.10^{-3} M) and 2.10^{-3} M H_2O_2. Change in absorbance at 470 nm was read after a time precised in each case. In the fractions eluted from the gradients, antimycin-insensitive NADH cytochrome *c* reductase [25], cytochrome *c* oxidase [26] and RNA [27] were determined. Protein was assayed according to Lowry *et al.* [28].

Isoelectric focusing. Isoelectric focusing in polyacrylamide gel was performed in a LKB Multiphor apparatus. Gel preparation and all further operations were conducted according to the LKB instruction manual. A 1-mm thick sheet of plastic with 5 × 10 mm holes was used to apply samples directly on the gel. Sodium deoxycholate (0.2%) was added to the samples prior to their application. The gel was stained for peroxidase in acetate buffer (pH 4.5) with 2.10^{-3} M benzidine and 10^{-3} M H_2O_2. The isoperoxidase profiles were monitored with an ISCO gel scanner.

Replication. All experiments were repeated at least twice.

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