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Immunocytochemical localization of pectinesterases in hyphae of *Phytophthora infestans*

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Evidence for the localization of extracellular pectinesterases was obtained in hyphae of *Phytophthora infestans* with the antibody – protein A – gold technique. Hyphae were fixed in 1% formaldehyde and 0.5% glutaraldehyde, and the immunocytochemical localization was done on ultrathin sections of tissue embedded at low temperature in Lowicryl K4M. Gold particles were mainly present over three different types of vesicles and over dictyosomes. In older parts of the hyphae, the plasma membrane was heavily labeled. This might suggest that the hyphal cell wall in subapical regions is not as permeable as in the hyphal tips.

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L'on a obtenu une preuve de la localisation de pectine-estérases extracellulaires dans les hyphes de *Phytophthora infestans* à l'aide de la technique anticorps – protéine A – or. Les hyphes sont fixés dans la formaldéhyde 1% et la glutaraldéhyde 0,5%. La localisation immunocytochimique est réalisée sur des sections ultraminces de tissus enrobés à basse température dans du Lowicryl K4M. Les particules d'or sont surtout présentes sur trois types différents de vésicules et sur les dictyosomes. Dans les parties plus âgées des hyphes, la membrane plasmique est fortement marquée. Ceci pourrait suggérer que la paroi cellulaire de l'hyphe dans les régions sous-apicales est moins perméable qu'aux apex des hyphes.

[Traduit par la revue]

Introduction

Young hyphae of *Phytophthora* contain a highly organized "functional" cytoplasm. It consists of typical eucaryote organelles, including nuclei, mitochondria, dictyosomes, ribosomes, microbodies, and various vesicle types. It differs distinctly from the "reserve" cytoplasm of aged hyphae, sporangia, chlamydospores, and oospores (Hemmes 1983). The hyphal tip, being characterized by an accumulation of vesicles, is responsible for growth of the mycelium, and during pathogenesis it invades the tissue of the host plant.

The aim of our studies was to characterize the components of the "functional" cytoplasm of hyphae of *Phytophthora infestans* (Mont.) de Bary, the causative agent of late blight disease of potatoes and tomatoes, by localization of pectinesterases. Pectolytic enzymes of plant pathogenic fungi play an important role in the infection process, since pectin is a major component of the primary plant cell wall and the middle lamella. Demethylation of pectin by pectinesterases is a prerequisite for the action of chain-splitting enzymes like polygalacturonases and pectate lyases. Pectic enzymes also might induce host resistance by releasing elicitor-active carbohydrates from plant cell walls (West et al. 1985).

In a previous study (Förster and Rasched 1985) we purified extracellular pectinesterases from *P. infestans*. One of the enzymes (PE II) was purified to homogeneity. A second enzyme (PE I), which was more abundant, revealed two protein bands after sodium dodecyl sulfate electrophoresis, which were not separable by a number of different techniques and are further referred to as PE I complex.

With the advent of low-temperature tissue processing, which improves antigen preservation, and sensitive antigen detection methods using antibodies and gold-labeled protein A (Kellenberger et al. 1980; Roth 1982) more specific methods for enzyme localization are now available. We report here the localization of the PE I complex in hyphae of *P. infestans* by these techniques.

Material and methods

Fungus culture

Phytophthora infestans was grown on pea agar at 16°C. Pea particles, which settled on the bottom of the agar medium, later helped to localize the agar blocks with the fungus in the Lowicryl resin.

Enzyme purification

Extracellular pectinesterases from culture filtrates of *P. infestans* were purified as described previously (Förster and Rasched 1985). Pectinesterase complex PE I was used for antibody production.

Antibodies

Antibodies were raised in Chinchilla white rabbits. Before the first antigen injection, preimmune serum was obtained. Two milligrams of the purified antigen were injected in three portions as follows. The first injection with complete Freund's adjuvant was intracutaneous, the second (2 weeks later) and third injections (3 weeks later) were intramuscular without adjuvant. Animals were bled 10 days after the third injection. The specificity of the antiserum was tested by Ouchterlony diffusion tests (Garvey et al. 1977) and immunoblotting. For this latter test, the culture filtrate was prepurified with DEAE–cellulose (Förster and Rasched 1985) and separated by the same electrophoretic procedures as shown earlier (Förster and Rasched 1985). The proteins were transferred to nitrocellulose, and the sheet was incubated overnight in 200 μL γ-globulin antibodies and developed with protein A – peroxidase – chloronaphthol, following the method of Towbin et al. (1979). The result is shown in Fig. 1. The immunoglobulin G (IgG) fractions of the preimmune serum and the antiserum were obtained by repeated ammonium sulfate precipitation (Garvey et al. 1977). For immunocytochemistry the IgG fractions were diluted 1:150 with borate-buffered saline (BBS) (Garvey et al. 1977) containing 1% ovalbumin. This dilution was optimal for the labeling, while the background was negligible.

Tissue preparation for electron microscopy

Conventional embedding in Spurr's resin

Agar blocks (1.5 mm^3) removed from the edge of the fungus culture

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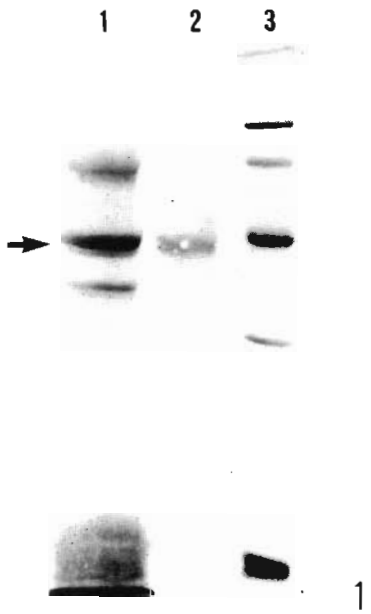


FIG. 1. Immunoblotting of extracellular proteins from *P. infestans* with pectinesterase complex PE I antibodies. Lane 1, 15 μg protein of the culture filtrate (arrow: pectinmethylesterase I). Lane 2, the proteins in lane 1 were transferred to nitrocellulose; the sheet was incubated overnight in 200 μL γ-globulin antibodies and developed with protein A – peroxidase – chloronaphthol. Lane 3, protein molecular mass standard with phosphorylase B (92.5 kDa), BSA (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa).

were fixed in 2% glutaraldehyde buffered with 0.05 M sodium phosphate buffer, pH 7.2, for 2 h at room temperature (20–22°C). After washing for 1.5 h in the same buffer, the agar blocks were postfixed in buffered 2% osmium textroxide at 4°C overnight. The samples were washed in buffer, then in distilled water, and dehydrated in a graded ethanol series. Specimens were infiltrated with Spurr's resin and cured at 70°C for 8 h.

Hyphal tips were selected in 20-μm plastic sections under the light microscope. By using a dissecting microscope, excess plastic around the hyphal tip was removed with a scalpel. The specimens were photographed to facilitate accurate trimming and were remounted on a sectioning block. Serial sections were made with glass knives, using a Reichert Om U3 ultramicrotome, and mounted on Pioloform-coated nickel grids. Good staining of the sections was obtained by the following procedures. After incubation for 15 min in 3% aqueous uranyl acetate, the grids were washed in distilled water and alkaline distilled water, incubated for 3 min in uranyl acetate, washed, and incubated for 3 min in Reynolds' lead citrate (Reynolds 1963).

Low-temperature embedding in Lowicryl K4M

Agar blocks from fungus cultures were fixed in 0.05 M sodium phosphate buffer (pH 7.2) containing 1% paraformaldehyde (w/v) and 0.5% glutaraldehyde (v/v) for 5 h at 4°C. The specimens were then washed in buffer for 1.5 h with several changes at 4°C and dehydrated in a graded series of ethanol at progressively lower temperatures: twice in 30% EtOH at 0°C for 30 min, once in 50% EtOH at −12°C for 1 h, once in 70% EtOH at −30°C overnight, once in 96% EtOH at −30°C for 1 h, and three times in 100% EtOH at −30°C for 0.5 h each. Infiltration with Lowicryl K4M (Chemische Werke Lowi, 8264 Waldkraiburg, Federal Republic of Germany) at −30°C was carried out as follows: EtOH – Lowicryl K4M (1:1) for 1 h; EtOH – Lowicryl K4M (1:2) for 1.5 h; 100% Lowicryl K4M twice for 1 h; 100% Lowicryl K4M overnight. The specimens were then transferred to fresh resin. Polymerization at −30°C was induced overnight, using indirect ultraviolet irradiation from a Sylvania F815/BLB fluorescent lamp (maximum wavelength, 366 nm). Then, the blocks were irradiated for another 2–3 days at room temperature to improve the sectioning properties. Hyphal tips were selected and sectioned as described in the previous section. Thin sections, mounted on nickel grids, were processed for immunocytochemistry.

Preparation of the protein A – gold complex

Colloidal gold was prepared by the tannic acid – citrate reduction method of Slot and Geuze (1985).

Gold particles were coupled to protein A according to Bendayan (1984); however, the protein A – gold pellet after centrifugation was suspended in BBS containing 0.02% polyethylene glycol (molecular weight 20 000). It was stored up to 4 weeks at 4°C. For the incubation of thin sections a 5000-fold dilution of this stock solution in BBS was used. The protein A – gold particles obtained by these techniques were uniform in size and had a diameter of about 15 nm.

Immunocytochemistry

Ultrathin sections of cells embedded in Lowicryl K4M were labeled as follows at room temperature: (1) floating on a droplet of 1% ovalbumin in borate buffered saline (BBS) for 10 min; (2) washing with distilled water for 1 h; (3) incubation (1 h) in the diluted antibody solution; (4) washing with BBS for 1 h; (5) incubation (30 min) in protein A – gold; (6) washing with BBS for 30 min; (7) washing with distilled water for 30 min.

The sections were then stained with 3% aqueous uranyl acetate for 20 min.

In control experiments the antibody solution was replaced by the preimmune serum or the specimens were treated with the protein A – gold alone.

Electron microscopy

Grids were examined and photographed in a Siemens Elmiskop 1A microscope.

Statistical analysis

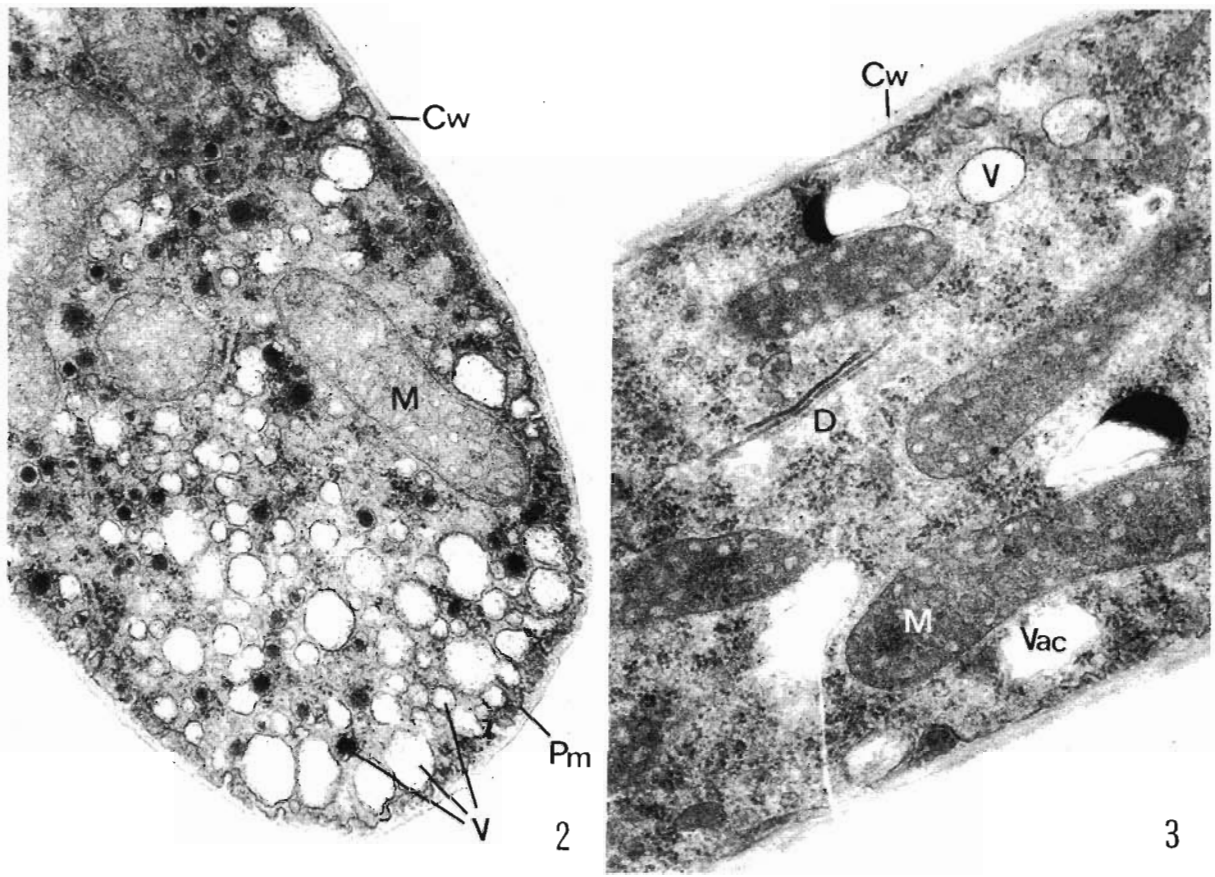
To evaluate the statistical significance of our data, gold particles distributed over vesicle types *b* and *c*, over the cell wall and the plasma membrane, over dictyosomes, over the remaining cytoplasm, and over the resin were counted and compared. The relative area was determined by cutting out the different structures from micrographs with fine scissors and weighing. The density of labeling is expressed as the number of gold particles per unit area; one unit area corresponds to 1 mg paper weight. Statistical analysis was done by the Student *t*-test ($p = 0.05$). The densities of labeling of the cellular structures were compared with the labeling of the remaining cytoplasm.

Results

Ultrastructural preservation and fungal ultrastructure

Fixation in 1% formaldehyde and 0.5% glutaraldehyde and embedment in Lowicryl K4M (Figs. 4–12) revealed poorer ultrastructural preservation of hyphae of *P. infestans* compared with conventional fixation and embedment in Spurr's resin (Figs. 2 and 3). However, all cellular constituents could be distinguished easily. The ultrastructural organization of the hyphae was similar as described for *Pythium* (Grove et al. 1970). Following Lowicryl embedment, the hyphal tip was characterized by an accumulation of three morphologically different types of vesicles: (*a*) electron-translucent vesicles of various sizes (0.10 to 0.45 μm in diameter); (*b*) large vesicles with electron-dense contents (0.22 ± 0.05 μm in diameter); and (*c*) small vesicles with electron-dense contents (0.08 ± 0.02 μm in diameter) (Figs. 4, 5, 6). Following Spurr embedment the hyphal tip contained small and large electron-translucent vesicles and small electron-dense vesicles (Fig. 2).

The first organelles that appeared in the subapical zone in addition to vesicles were mitochondria; they were followed by



ABBREVIATIONS USED: Cw, cell wall; D, dictyosome; ER, endoplasmic reticulum; M, mitochondrion; Pm, plasma membrane; V, vesicle; Va, vesicle type *a*; Vb, vesicle type *b*; Vc, vesicle type *c*; Vac, vacuole.

FIGS. 2 and 3. Transmission electron micrographs of *P. infestans* after 2% glutaraldehyde fixation and Spurr's embedment. Fig. 2. Hyphal tip region. Numerous vesicles are present at the apex. × 30 000. Fig. 3. Subapical zone of a hypha. × 33 000.

dictyosomes, endoplasmic reticulum, small vacuoles, and nuclei. Dictyosome vesicles were surrounded by a membrane and their contents appeared relatively electron translucent after embedment in Lowicryl resin; they represent a fourth type of vesicle (arrow, Figs. 7, 8). There were usually more ribosomes in the subapical zone than in the apex. The subapical zone merged into a zone of vacuolation. Here the size of the vacuoles increased with increasing distance from the tip.

Immunocytochemistry

Labeling for pectinesterases with the antibody – protein A – gold technique demonstrated gold particles over the following cellular constituents. In hyphal tips (Fig. 4) they were generally located over the electron-dense large (type *b*) and small (type *c*) vesicles, but not over the electron-translucent vesicles (type *a*). In the subapical zone, dictyosome cisternae and their vesicles were labeled (Figs. 7 and 8). All three vesicle types of the hyphal tip were also found here; gold particles were located only over those with dark staining, similar to what was observed in the tip (Figs. 4–6). Some labeling was found on the cytoplasm. Quite obvious labeling was also observed on the cytoplasm along the wall of more distant parts of the subapical zone, presumably the plasmalemma (Fig. 9). This labeling was especially obvious in older parts of the hyphae (Fig. 10) and in the highly vacuolated hyphae (Fig. 11). The subapical zone contained a well-developed rough endoplasmic reticulum, but no labeling occurred here (Figs. 7, 8, 9).

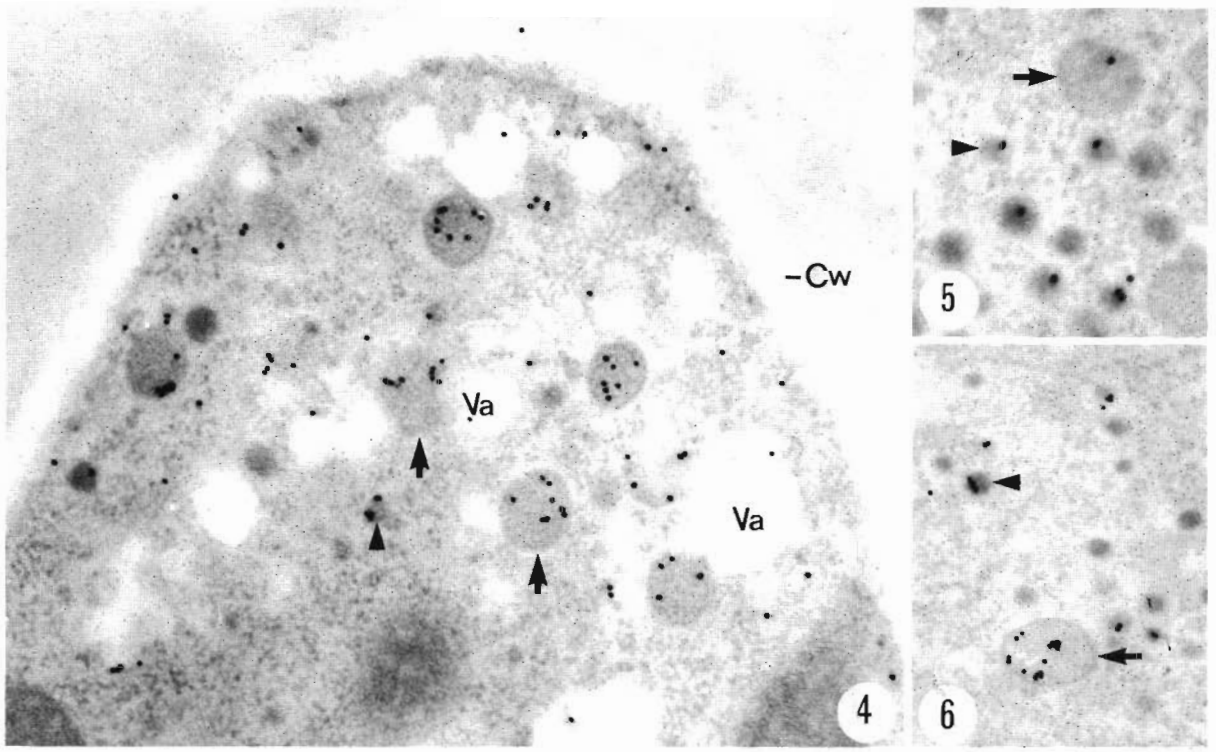
In control experiments, where the sections were treated with a preimmune serum instead of the antibody, only a few gold particles were found (Fig. 12). When the sections were incubated in the protein A – gold complex alone, similar results were obtained.

Quantification and statistical analysis of our data are shown in Table 1. Because of poor ultrastructural preservation, especially with the type *c* vesicles, it often was difficult to delineate the cell structure while determining the area. Thus, these data are subject to error. The high standard deviation can be explained by a varying overall labeling between different experiments.

Discussion

Fixatives that preserve ultrastructural details well may cause conformational changes of antigens (Hayat 1981). Therefore, we fixed the specimens to be used for immunocytochemistry with a very low concentration of glutaraldehyde. Since osmium fixation interferes with the Lowicryl embedding technique (Armbruster et al. 1982) and also inhibits immunological reactions (Roth 1982), it was omitted. Ultrastructural preservation after embedment in Lowicryl K4M was less satisfactory in comparison with conventional embedment, but it is generally assumed that protein extraction and denaturation processes are quite reduced during low-temperature embedment.

By using the antibody – protein A – gold technique with



FIGS. 4–6. Transmission electron micrographs of hyphae of *P. infestans* after fixation in 1% formaldehyde and 0.5% glutaraldehyde and embedment at low temperature in Lowicryl K4M. Sections were incubated in antipectinesterase IgG followed by protein A – gold. Fig. 4. Section through a hyphal tip. Gold particles are located mainly over vesicle types *b* (arrows) and also *c* (arrowhead). × 45 000. Figs. 5 and 6. Section through the subapical zone of a hypha. The vesicle types *b* (arrow) and *c* (arrowhead) are labeled. Fig. 5. × 55 000. Fig. 6. × 42 000.

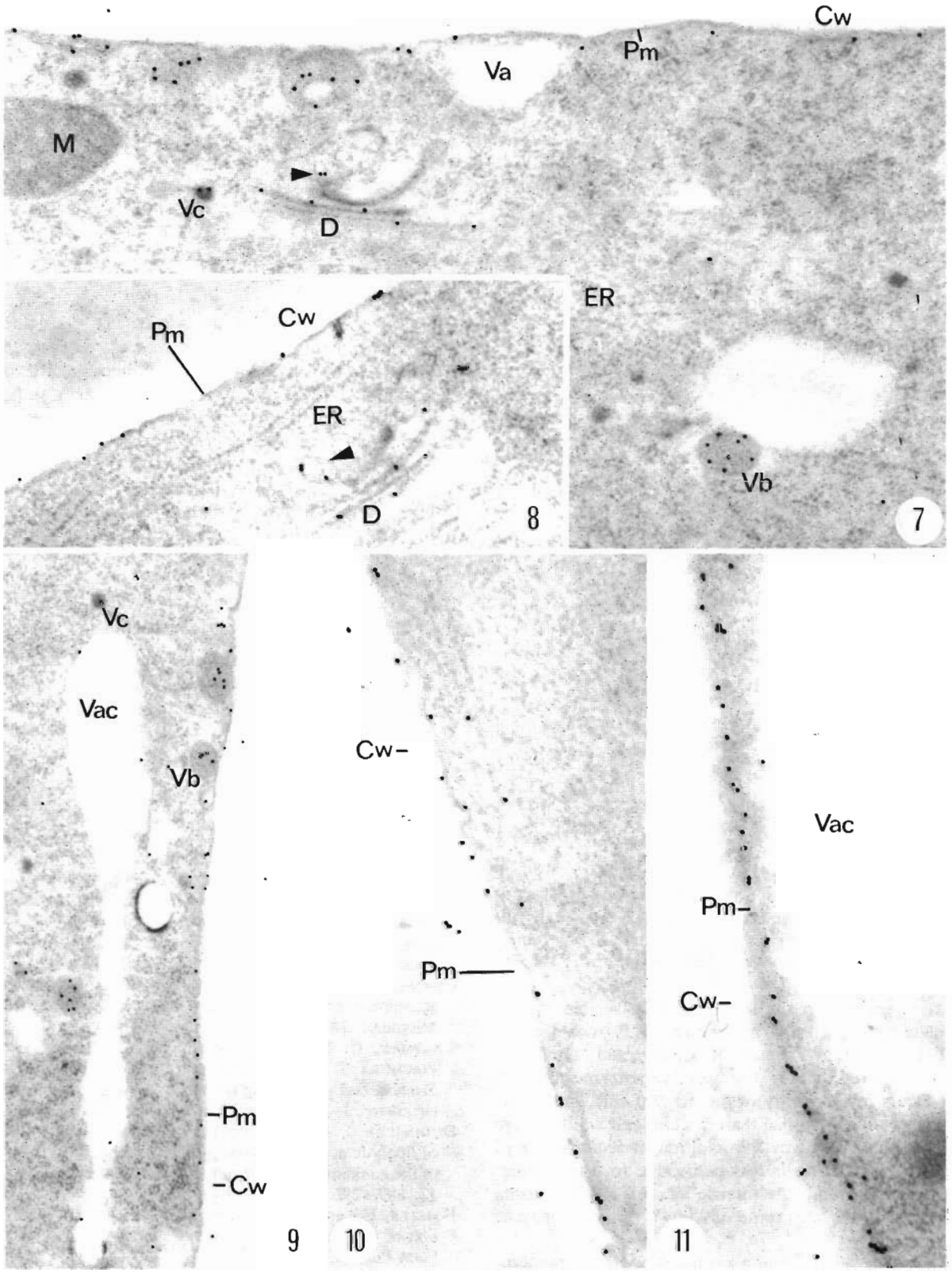
ultrathin Lowicryl K4M embedded tissue sections, we obtained evidence for the localization of the pectinesterase complex I in distinct hyphal compartments of *P. infestans*. Gold particles were found to be located on three of the four different vesicle types that looked different morphologically: a population of larger and smaller vesicles with electron-dense contents (types *b* and *c*) and the dictyosome vesicles with electron-translucent contents. The former types were distributed over the entire hypha but were most numerous in the tip region. We do not know the physiological significance of the vesicle types. We speculate that either the two pectinesterases of the PE I complex are located in different vesicles or that the morphologically distinct vesicles contain different forms of the enzymes, for example, glycosylated and non-glycosylated forms.

Although Lowicryl-embedded hyphal tips contained three vesicle types, only two types could be distinguished following Spurr embedment. We assume that the light vesicles (type *a*) detected in Lowicryl-embedded hyphae correspond to the light vesicles of various sizes in Spurr-embedded tissue and that the small dark-staining vesicles (type *c*) correspond to the dark vesicles observed following Spurr embedment. The electron-dense vesicles (type *b*) that were intensely labeled for pectinesterases after Lowicryl embedment seemed to be missing in Spurr-embedded tissue. This might be due to extraction during tissue processing, and consequently these vesicles would be indistinguishable from the electron-translucent vesicle type. On the other hand, the translucent large vesicles following Lowicryl embedding (type *a*) might represent an artifact due to rupturing or extraction of vesicle types *b* and *c*.

Meyer et al. (1976) investigated hyphal tip growth in germinating cysts of *P. palmivora* by density-gradient distribution of enzymes and other techniques. They suspect that wall-lytic and wall-synthesizing enzymes are located separately in the two vesicle types observed after Spurr embedment. While the larger vesicles are supposed to be dictyosome derived, the origin of the smaller ones is suspected to be endoplasmic reticulum derived. It remains speculative, considering the entire enzymatical capacity of the cell, which enzymes might be present in the same vesicles and which are localized in a different type.

According to the hypothesis on production and secretion of fungal enzymes, proteins are synthesized at the rough endoplasmic reticulum, vesicles are produced, and the protein is transported to the Golgi apparatus. Vesicles budding from the Golgi apparatus are transported to the plasmalemma at the site of secretion where the vesicle membrane fuses with the plasmalemma (Byrde 1982). The evidence for this hypothesis is supported by a few studies using secretary mutants (Novick et al. 1981) and histochemical (Hislop et al. 1974; Duddridge and Sargent 1978) and immunohistochemical methods (Chapman et al. 1983). However, in none of these studies was the whole sequence of enzyme production and secretion demonstrated.

Our studies on pectinesterase localization in hyphae of *P. infestans* provide evidence for this hypothesis: dictyosomes are involved and vesicles containing the enzymes seem to be transported to the site of secretion, mainly the hyphal tips. Here the vesicles are likely to fuse with the plasmalemma, allowing the protein to pass through the cell wall. Some vesicles are also found on the edge of older parts of the hypha. Here the plasmalemma is labeled by numerous gold particles. We speculate that the cell wall of hyphal tips is not a barrier for enzyme passage. However, in older hyphal parts, the wall seems to be



FIGS. 7–11. Transmission electron micrographs of hyphae of *P. infestans* after fixation in 1% formaldehyde and 0.5% glutaraldehyde and embedment at low temperature in Lowicryl K4M. Sections were incubated in antipectinesterase IgG followed by protein A – gold. Figs. 7 and 8. Sections through the subapical zone of a hypha. Gold particles are mainly located over dictyosomes, dictyosome vesicles (arrowhead) over vesicle types *b* and *c*, and on the plasma membrane. There is no labeling of the endoplasmic reticulum. Fig. 7. × 42 000. Fig. 8. × 42 000. Fig. 9. Labeling of the plasmalemma in the subapical zone. × 32 000. Fig. 10. Labeling in the zone of beginning vacuolation. × 40 000. Fig. 11. Labeling in the fully vacuolated zone of the hypha. × 48 000.

TABLE 1. Quantification of immunocytochemical localization of pectinesterases in hyphae of *P. infestans*. The density of labeling is expressed as the number of gold particles per unit area

	Hyphal tip	Subapical zone	Zone of vacuolation
Vesicle type *b*	0.656±0.255 *a*	0.540±0.412 *a*	1.155±0.150 *a*
Vesicle type *c*	0.901±0.385 *a*	0.310±0.144 *a*	—
Cell wall and plasma membrane	0.138±0.079 *a*	0.122±0.063 *a*	0.184±0.116 *a*
Dictyosomes	—	0.218±0.180	—
Resin	0.002±0.001 *a*	0.007±0.005 *a*	0.003±0.002 *a*
Remaining cytoplasm	0.065±0.030	0.023±0.022	0.021±0.017
Total area analyzed (μm^2)	28.1	50.8	21.3

NOTE: Values followed by the letter *a* indicate that the data are significantly different against labeling of the remaining cytoplasm.

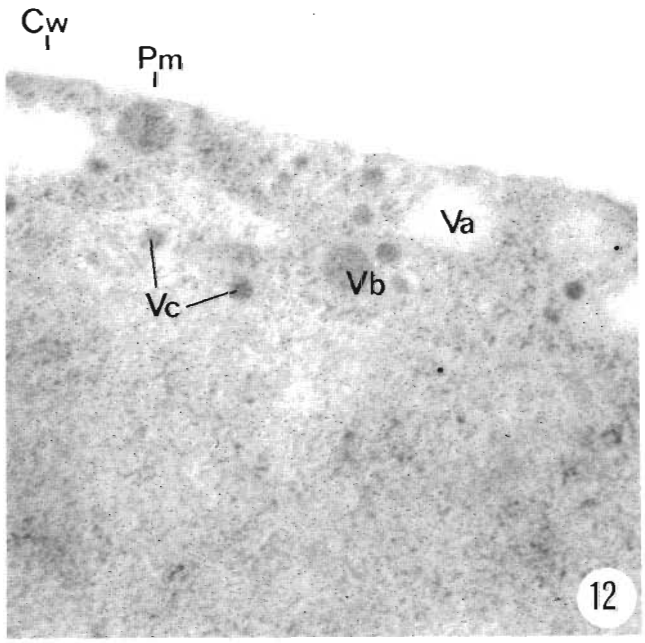


FIG. 12. Transmission electron micrograph of a hypha of *P. infestans* after fixation in 1% formaldehyde and 0.5% glutaraldehyde and embedment at low temperature in Lowicryl K4M. Section through the subapical region incubated in preimmune IgG followed by protein A – gold (control). Only a few gold particles are present. × 42 500.

less permeable, and enzymes released from the vesicles appear to accumulate at the plasma membrane. This would be in agreement with the hypothesis of Chang and Trevithick (1974), who proposed that the passage of exoenzymes through cell walls occurs more readily in the nascent cell wall of the apical region of fungal hyphae than in older cell walls, where the large pores are closed by the addition of secondary wall substances, making the walls less permeable for macromolecules. On the other hand, the intense labeling of the plasma membrane might indicate some unknown role in enzyme activity.

Our studies give no indication on the site of enzyme synthesis. We often found a well-developed rough endoplasmic reticulum, but no labeling was there. It might be argued that the methods used are not sensitive enough to detect the small protein quantities at the site of synthesis. On the other hand, even when very low antibody and gold concentrations were used, some gold particles were distributed over the cytoplasm, presumably on ribosomes. There is evidence that many proteins are synthesized on free polysomes, discharged into the cell sap and subsequently taken up into specific organelles (Sabatini et al. 1982). According to our results, such a mechanism of enzyme incorporation into the membrane system might exist with pectinesterases of *P. infestans*.

Further studies using monoclonal antibodies, which would bind to particular forms of the enzymes, might clarify the significance of the different vesicle types and also might rule out any unspecific binding, which might be present by using polyclonal antibodies and which are not detected by the controls used in our studies.

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