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Characterization and Ultrastructural Localization of Chitinases from *Metarhizium anisopliae*, *M. flavoviride*, and *Beauveria bassiana* during Fungal Invasion of Host (*Manduca sexta*) Cuticle

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Extracellular chitinases have been suggested to be virulence factors in fungal entomopathogenicity. We employed isoelectric focusing and a set of three fluorescent substrates to investigate the numbers and types of chitinolytic enzymes produced by the entomopathogenic fungi *Metarhizium anisopliae*, *Metarhizium flavoviride*, and *Beauveria bassiana*. Each species produced a variety of *N*-acetyl-β-D-glucosaminidases and endochitinases during growth in media containing insect cuticle. *M. flavoviride* also produced 1,4-β-chitobiosidases. The endochitinases could be divided according to whether they had basic or acidic isoelectric points. In contrast to those of the other two species, the predominant endochitinases of *M. anisopliae* were acidic, with isoelectric points of about 4.8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis resolved the acidic chitinases of *M. anisopliae* into two major bands (43.5 and 45 kDa) with identical N-terminal sequences (AGGYVNAVYFY TNGLYLSNYQPA) similar to an endochitinase from the mycoparasite *Trichoderma harzianum*. Use of polyclonal antibodies to the 45-kDa isoform and ultrastructural immunocytochemistry enabled us to visualize chitinase production during penetration of the host (*Manduca sexta*) cuticle. Chitinase was produced at very low levels by infection structures on the cuticle surface and during the initial penetration of the cuticle, but much greater levels of chitinase accumulated in zones of proteolytic degradation, which suggests that the release of the chitinase is dependent on the accessibility of its substrate.

After cellulose, chitin is the most abundant polymer found in nature. It is a common structural component of the arthropod exoskeleton and fungal cell walls, so these organisms produce chitinases for growth regulation (7). Some other organisms which do not contain chitin also produce chitinases; the production of chitinases by plants as a protection against fungi provides an example which underlines the significance of chitinases in ecological interactions between organisms (7).

Since the use of extracellular enzymes to degrade the structural barriers of the host appears to be a commonly used strategy of fungal pathogens (14), chitinases could also be involved in the penetration of a host by mycoparasites or by entomopathogenic fungi. It has been frequently suggested that *Trichoderma* sp. chitinases have a role in mycoparasitism, and such suggestions have led to considerable efforts being directed towards elucidating the diversity and function of chitinases (9). Chitin microfibrils also constitute some 30% of the insect cuticle, the primary site of entry and infection by entomopathogenic fungi (23), and therefore represent a potential barrier to invasion. Consistent with this, ultrastructural studies have shown enhanced fungal penetration through the cuticle of insects treated with an inhibitor of chitin synthesis (11). Consequently, chitinolytic enzymes, together with proteases, are the enzymes most frequently considered critical in facilitating invasive mycosis of insects (3). In contrast to the abundant data that have accumulated on the regulation, amino acid sequences, and function of cuticle-degrading proteases (19, 22, 26, 27), there is very little information available on the molecular and functional properties of entomopathogenic chitinases. Some controversial experimental results have been reported with regard to the potential role of chitinases in entomopathogenicity. For example, several studies utilizing *Beauveria bassiana*, *Nomuraea rileyi*, and other fungi have suggested that virulence is correlated (at least in part) with chitinase activity (1, 4, 5, 33). By contrast, a chitinase-deficient mutant of *Verticillium lecanii* was still capable of infecting aphids (12). While it has been possible to demonstrate the extracellular presence of chitinases during growth in chitin or host cuticle under liquid culture conditions, demonstration of these enzymes in situ during penetration of host cuticles has proved more difficult. Chitin degradation products were not detected during the penetration of the cuticle of *Manduca sexta* by *Metarhizium anisopliae* (25). It was suggested that the late appearance of chitinase, as compared with that of protease, was the result of induction, as chitin eventually becomes available after degradation of the encasing cuticle proteins (21, 23, 24). These experiments were complicated by the difficulty of detecting low levels of chitin degradation products in infected cuticles. Nevertheless, several groups of workers have demonstrated in vitro activity of chitinases against insect cuticles (17, 18, 23), confirming the potential of these enzymes in facilitating invasion.

As all the experiments described above were performed in vitro, experimental data are needed to demonstrate the accumulation and localization of chitinases during the initial penetration of insect cuticle by fungi. A great deal of information has been obtained on the spatial and temporal coordination of proteases in *M. anisopliae*-insect interactions by immunocytochemical methods and electron microscopy (8, 29). Surprisingly, ultrastructural localization of chitinases with respect to mycoparasites and entomopathogens has not been documented. In this report, we describe the characterization of chitinases in three species of entomopathogenic fungi and

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show that an acidic chitinase is produced by *M. anisopliae* during penetration of the cuticle.

MATERIALS AND METHODS

Abbreviations. The abbreviations used in this paper are as follows: IEF, isoelectric focusing; SDB, Sabouraud dextrose broth; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MU, 4-methylumbelliferone; NAG, *N*-acetylglucosamine; and PBS, phosphate-buffered saline.

Organisms and growth. The fungal isolates (*M. anisopliae* ME-1, *Metarhizium flavoviride* ARSEF 324, and *B. bassiana* ARSEF 252) and culture media were described before (21, 30).

Preparation and analysis of culture filtrates. Standardized mycelial inocula (5 g wet weight) from 48-h SDB cultures (28) were incubated with shaking (100 rpm) at 27°C for up to 5 days in 100 ml of basal media (0.02% KH_2PO_4, 0.01% $MgSO_4$ [pH 6]) supplemented with 1% (wt/vol) cockroach cuticle prepared as described previously (19). Culture filtrates (100-ml aliquots) were concentrated for IEF by lyophilization. The residual solids were dissolved in 15 ml of distilled water and desalted with Amicon (Danvers, Mass.) Centriprep-10 ultrafiltration units.

Preparative IEF. Preparative broad-range IEF (pH 3 to 10) and narrow-range IEF (pH 3 to 5) were performed in a Rotofor (Bio-Rad) with 1% ampholytes (Bio-Lyte 3/10 or 3/5; Bio-Rad) by procedures described in the manufacturer's handbook. Enzyme activities and protein levels in the fractions were determined after the removal of the ampholytes with Amicon Centricon-10 ultrafiltration units.

Electrophoresis and enzyme characterization. Gel electrophoresis was performed in Bio-Rad minigel cells with SDS by the method of Laemmli (15). Protein was stained with Coomassie blue G-250 (Bio-Rad).

For glycol chitin-SDS-PAGE, electrophoresis was done in 9% (wt/vol) polyacrylamide gels containing 0.1% glycol chitin (31). After electrophoresis, the gels were incubated for 5 h (35°C) in renaturation buffer (0.1 M acetate buffer [pH 5.1] containing 2.5% [vol/vol] Triton X-100). Chitinase activity was detected by staining residual glycol chitin with Calcofluor White M2R.

Analytical IEF on ultrathin polyacrylamide gels was performed as previously described (19). Chitinase activity was visualized by overlaying the gels with a 0.03% solution of an MU monosaccharide, disaccharide, or trisaccharide derivative of NAG (the monomer of chitin) in 0.1 M sodium acetate buffer (pH 5.1) containing 1% low-melting-point agarose (32). The trisaccharide derivative was suspended in dimethyl sulfoxide and added to the acetate buffer as a fine suspension. This technique allowed activities to be detected either as clearing zones in the particulate substrate or by the release of MU. The appearance of fluorescent bands was monitored with a UV lamp. Alternatively, the overlay contained 0.01% glycol chitin in place of the MU glucosides, and the degradation of the glycol chitin was detected as dark zones after the chitin had been stained with Calcofluor White M2R.

Preparation of antibodies and immunoblotting. Chitinases partially purified by preparative IEF were separated by SDS-PAGE. After electrophoresis, the gel was washed briefly in water and stained for 10 min with 0.05% Coomassie blue prepared in water. The gel was washed repeatedly in water, and stained protein bands identified by N-terminal sequence analysis were excised with a scalpel. The gel slices were lyophilized, ground into a powder, and resuspended in a volume of water equal to one-half of the original volume. The suspension was divided into three aliquots, mixed with Freund's complete adjuvant, and injected at 14-day intervals into New Zealand White rabbits.

For Western blot (immunoblot) analysis, proteins were transferred from gels to nitrocellulose and immunoblotted as described previously (29). Blots were developed with the ProtoBlot Western Blot AP system (Promega).

Immunogold labeling. Cuticles from fifth-instar *M. sexta* larvae were excised, soaked in a saturated solution of phenylthiourea (to inhibit phenoloxidase) inoculated with conidia, and following incubations for up to 60 h, processed for electron microscopy as described previously (8). Ultrathin sections of LR White-embedded tissue were placed in blocking solution (8) for 30 min, treated with a 10^{-2} dilution of antiserum for 2 h, washed in PBS–0.05% Tween 20, and then treated with a 10^{-2} dilution of Protein A-gold (particle diameter, 10 nm; Sigma) in PBS–0.05% Tween 20–0.2% bovine serum albumin for 1.5 h. The sections were then washed in distilled water and stained for contrast in 4% (wt/vol) uranyl acetate in 50% (vol/vol) ethanol for 20 min. Observations were made with a Zeiss EM10 transmission electron microscope.

Specificity of the labeling was determined by: (i) incubation of the sections with serum obtained before the rabbits were immunized, (ii) omission of primary antisera, and (iii) treatment with Protein A prior to treatment with Protein A-gold.

Miscellaneous. Glycol chitin was synthesized from glycol chitosan as previously described (31). N-terminal sequence analysis followed previously used procedures (19). Unless otherwise stated, all chemicals were from Sigma and were the purest grade available.

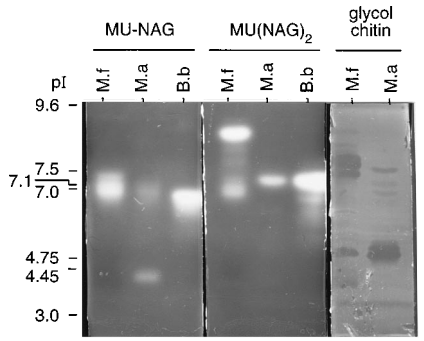

FIG. 1. Analytical (polyacrylamide) IEF (pH 3 to 10) of cultures of *M. flavoviride* (M.f), *M. anisopliae* (M.a), and *B. bassiana* (B.b) grown on 1% cockroach cuticle (4 days) and band development with MU-NAG, MU-$(NAG)_2$, and glycol chitin. The samples (2 μl) applied to the gels contained about 4 μg of protein. Isoelectric points are from IEF standards (Bio-Rad). The results are representative of at least three similar experiments using different enzyme preparations.

RESULTS

Catalytic properties of fungal chitinases. The complexity and diversity of the chitinolytic systems produced by *M. anisopliae*, *M. flavoviride*, and *B. bassiana* during their growth on insect cuticle were assessed on IEF gels with glycol chitin or a set of three fluorescent substrates (Fig. 1 and 2). The multiple chitinases produced by each species could be divided into acidic and basic isozymes, which allowed them to be designated according to their isoelectric points as well as substrate specificities. (i) Acidic activities produced by each of the three species against glycol chitin showed no activity against MU-NAG or MU-$(NAG)_2$ and little activity against MU-$(NAG)_3$, suggesting a specificity for NAG oligomers with more than four residues. (ii) *B. bassiana* Chit-7.5, *M. flavoviride* Chit-9, and *M. anisopliae* Chit-7.5 hydrolyzed glycol chitin and released MU from MU-$(NAG)_3$; *M. flavoviride* Chit-9 also released MU from MU-$(NAG)_2$ but not from MU-NAG, suggesting that they are endochitinases. (iii) *M. flavoviride* Chit-7.7 (three bands) hydrolyzed glycol chitin. MU-$(NAG)_3$ was also solubilized to produce only nonfluorescent products, presumably diacetylchitobiose and MU-NAG, suggesting that Chit-7.7

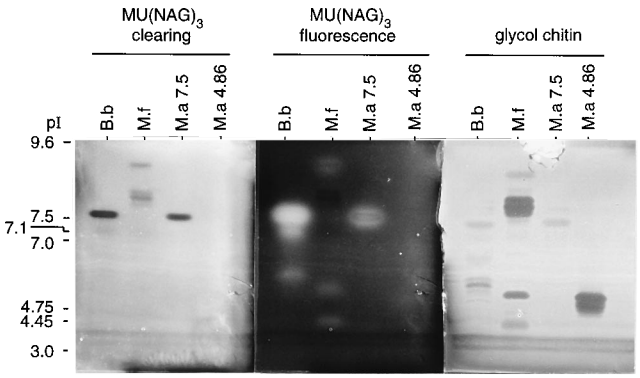

FIG. 2. Analytical IEF (pH 3 to 10) of *B. bassiana* (B.b) and *M. flavoviride* (M.f) chitinases and analytical IEF of Rotofor fractions of *M. anisopliae* (M.a) chitinases in 4-day cockroach cuticle medium. The preparative experiment was performed at pH 3 to 10 in a Rotofor column for 4.5 h at 630 to 940 V. The fractions were collected, the pHs were determined, and pH fractions 4.86 and 7.50 were analyzed by polyacrylamide IEF. Chitinolytic activity against MU-$(NAG)_3$ was detected either as clearing zones in the particulate substrate or by the release of fluorescent MU.

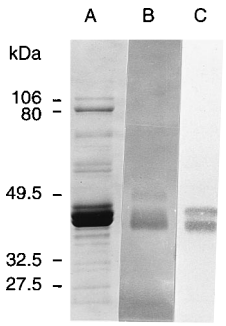

FIG. 3. SDS-PAGE analysis of the acidic chitinase activity of *M. anisopliae*. Narrow-range (pH 3 to 5) IEF of chitinases in filtrates from 4-day cuticle cultures was performed in a Rotofor column for 5 h at 640 to 1,040 V. The fractions were collected, and the pHs were determined. (A) SDS-PAGE analysis of the Rotofor fraction with a pH of 4.79. The protein is stained with Coomassie blue. (B) Glycol chitin-SDS-PAGE analysis of the 4.79 Rotofor fraction. Cleared regions in the gel represent chitinase activity. (C) A Western blot was prepared by probing 10 μg of lyophilized crude cuticle culture filtrate with rabbit antibodies raised against the 45-kDa band. Each lane contained about 10 μg of protein. The numbers on the left refer to the molecular mass markers (kilodaltons).

causes the progressive exotype release of diacetylchitobiose. A similar enzyme produced by *Trichoderma harzianum* has been designated a 1,4-β-chitobiosidase (10). (iv) *M. anisopliae* Chit-4.4 released MU from MU-NAG but not from the other substrates, indicating that it is a β-1,4 *N*-acetylglucosaminidase. *M. flavoviride* Chit-7, *M. anisopliae* Chit-7, and *B. bassiana* Chit-6.8 also show a preference for MU-NAG over other substrates.

Immunological characterization of acidic *M. anisopliae* chitinase. Having determined that acidic isozymes are the major endochitinase activities produced by *M. anisopliae* during growth in vitro on insect cuticle, we set about developing specific antibodies for these enzymes.

Enzymes produced after 4 days of growth in cuticle-containing media were subjected to narrow-range IEF (pH 3 to 5). Analysis of each of the Rotofor fractions by glycol chitin-SDS-PAGE identified chitinase activity bands corresponding to the positions of two closely spaced proteins with molecular masses of 43.5 and 45 kDa and pIs of about 4.8. N-terminal sequencing confirmed that each band consisted of a single protein. The N-terminal sequences of the two bands were identical (Ala-Gly-Gly-Tyr-Val-Asn-Ala-Val-Tyr-Phe-Tyr-Thr-Asn-Trp-Gly-Leu-Tyr-Leu-Ser-Asn-Tyr-Gln-Pro-Ala) and showed 66% identity with that of the corresponding region of the recently cloned (2), 42-kDa endochitinase of *T. harzianum* (Ala-Asn-Gly-Tyr-Ala-Asn-Ser-Val-Tyr-Phe-Thr-Asn-Trp-Gly-Iso-Tyr-Asp-Arg-Asn-Phe-Gln-Pro-Ala-Asp).

The 45-kDa band was used to raise polyclonal antibodies. These were analyzed by immunoblotting extracellular proteins secreted by the pathogen in cuticle-containing media (Fig. 3). Two major activities corresponding to the isoelectric points and molecular masses of the acidic chitinase isoforms were visible following glycol chitin-SDS-PAGE. The antibody recognized both of these activities.

Immunogold localization of the acidic chitinases of *M. anisopliae*. Conidia of *M. anisopliae* germinated to form appressoria on the surface of *M. sexta* and penetrated cuticles within 40 h of inoculation as described previously (20, 26). The labeling of sections from cuticles at 24, 36, 44, and 60 h postinoculation with antibodies to chitinase revealed very little labeling in or over the appressorial wall, suggesting the presence of low levels of chitinase on prepenetration fungal structures, even after 36 h, when penetration of the cuticle was well advanced (Fig. 4a).

Variation in chitinase distribution made difficult the quantitation of gold particles according to the time course of cuticle degradation. Nevertheless, it was apparent that overall labeling increased around fungal hyphae between 36 and 60 h postinfection. Labeling around most hyphae at 36 h varied from slight to moderate, with an average of 9 ± 7 gold particles within 1 μm^2 of the circumference of penetrant hyphae. Nineteen out of 45 hyphae showed no labeling (Fig. 4a), while two hyphae were intensely labeled (>20 gold particles) (Fig. 4b). At 44 and 60 h postinoculation, 86% ($n = 44$) of hyphae showed moderate to intense labeling (Fig. 4c and d).

Initially, the enzyme was sometimes confined to the hyphal cell wall (Fig. 4b). Normally, the enzyme diffused into the attacked cuticle itself (Fig. 4b to d), suggesting easy passage of the enzyme through the walls and/or the development of a porous cuticle structure. Very few gold particles were found intracellularly.

Control sections (see Materials and Methods) showed only a very low level of nonspecific binding of gold particles (0.13 ± 0.12 gold particles per μm^2), with the results for all controls being negative (Fig. 4e), confirming that use of antisera to the chitinase in conjunction with the Protein A-gold complex is a valid labeling method for detecting the enzyme in insect cuticle.

DISCUSSION

We previously reported that isolates of *M. anisopliae* and *B. bassiana* produce multiple activities against glycol chitin which are not hydrolyzed by the proteases produced by the same organisms (31). Since it is not a substrate for exochitinase, glycol chitin should detect only endochitinases (13, 16). As a preliminary to the immunolocalization of a chitinase of *M. anisopliae*, it was necessary to investigate the diversity of these enzymes and determine which of the multiple activities is likely to be of most relevance to pathogenicity. Using IEF and fluorescent substrates, we elucidated a complex mixture of endo- and exoacting chitinases produced by entomopathogenic fungi during growth on insect cuticle. Although the role of this complex mixture in cuticle penetration is unknown, the endoacting enzymes are likely to be the most effective at solubilizing cuticle polymers, assisting penetration. The exochitinases may function to further degrade oligomers released by endochitinases, resulting in chitobiose and NAG, which can be taken up for nutrition.

Many plants secrete multiple isozymes of endochitinases, which can be divided into basic and acidic chitinases (6). A similar situation exists with entomopathogenic fungi. When *M. anisopliae* was grown on cuticle, the acidic isozymes were the major ones secreted. This result contrasts with results with other fungal species, in which basic isozymes predominate. Judging from the identical N-terminal sequences, it seems possible that the multiple forms within the acidic endochitinase of *M. anisopliae* may not all represent separate gene products. Likewise, multiple chitinases of *T. harzianum* may result from posttranslational modification, particularly glycosylation (10). Sequencing data revealed a close relationship between the *M. anisopliae* acidic chitinase and the *T. harzianum* enzyme previously implicated in mycoparasitism (10), indicating adaptation of similar systems to attack disparate hosts.

Studies of the timing of the production of proteases, chitinases, and other factors in insects could provide important information about the role of the accumulated hydrolytic enzymes during pathogenesis. In the same system studied here, i.e., *M. anisopliae* infecting *M. sexta* larvae, we found high levels of subtilisin-like and trypsin-like proteases associated with

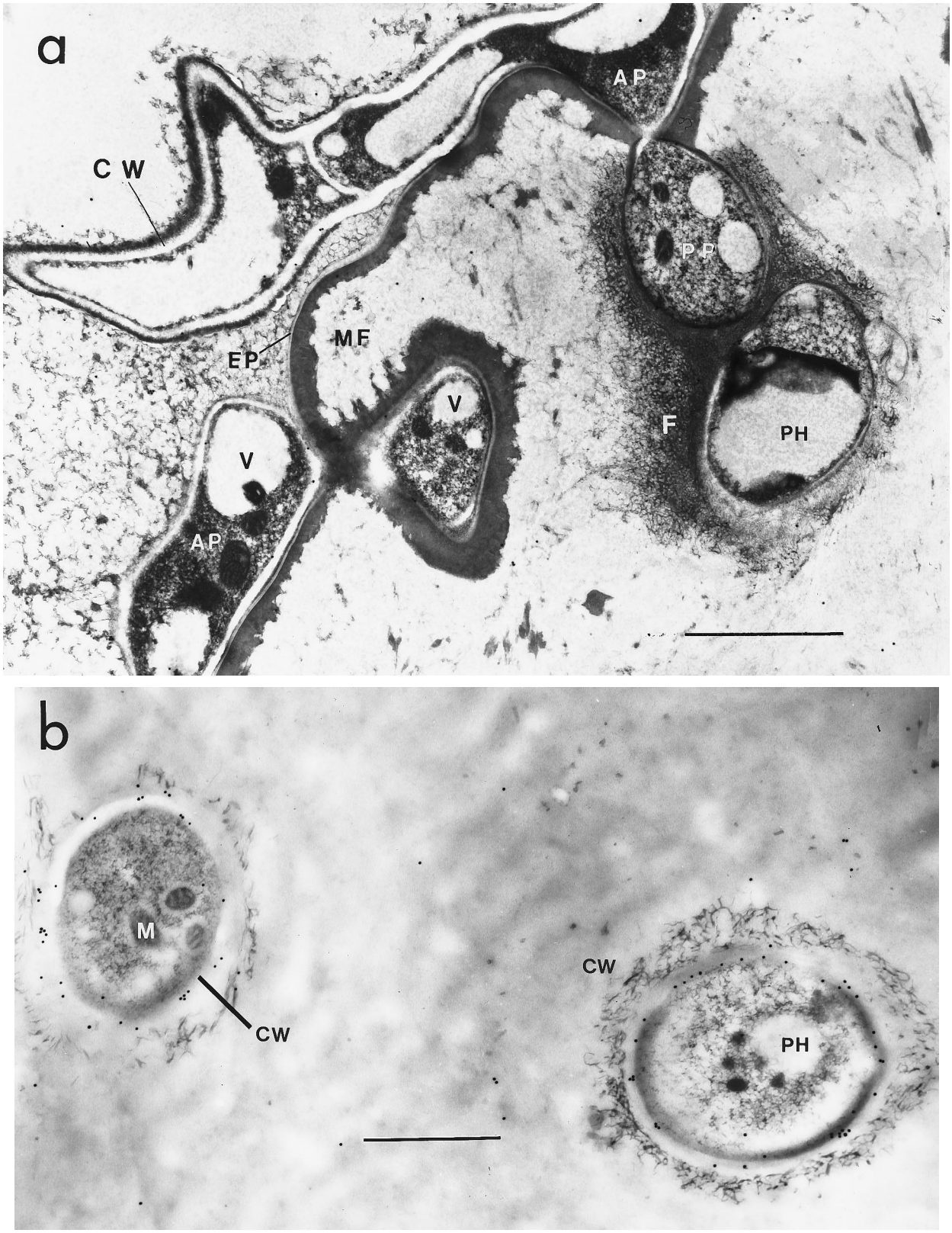

FIG. 4. Immunocytochemical localization of acidic chitinase secreted by *M. anisopliae* in host (*M. sexta*) cuticle. Sections were incubated with rabbit antibodies raised against the 45-kDa band shown in Fig. 2, and this procedure was followed by incubation with Protein A-gold. Absence of labeling with gold particles around appressoria and penetrant hyphae at 36 h after inoculation was observed (a). Gold particles in the cuticle were considered background, since they were comparable to labeling observed in some negative controls. The distribution and concentrations of gold particles were very variable from cell to cell during the early stages of infection. The hyphal cross-sections (b) possess some of the most intense labeling observed at 36 h postinoculation. By contrast, most hyphae were intensely labeled at 44 (c) and 60 (d) h after inoculation. These studies clearly show a largely extracellular distribution of chitinase and the ability of the enzyme to diffuse into the area surrounding the hyphae. A negative control (e) in which preimmune serum was used for the primary incubation shows only a few gold particles. AP, appressorium; CW, cell wall; EP, epicuticle; F, fibrous or matrix-like residues which surround some infection structures and which may be fungal mucilage; M, mitochondria; MF, microfold; PH, penetrant hyphal body; PP, penetration peg; V, vacuole. Bars, 1 μm.

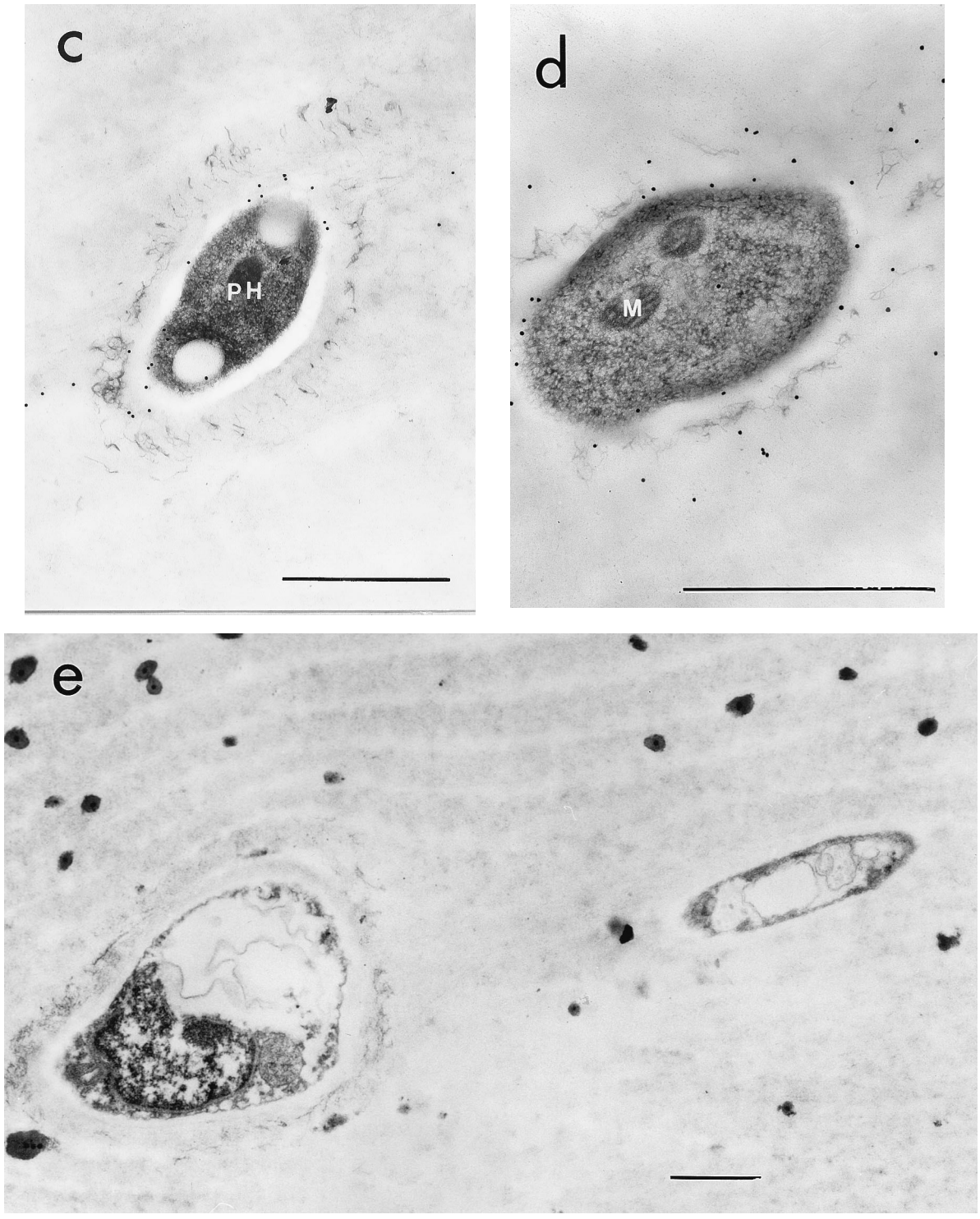

FIG. 4—*Continued.*

outer wall layers of appressoria and with regions of the cuticle undergoing initial attack (8, 29). The absence of chitinase at this time could be due to a failure to detect chitinase if it is more loosely bound to the epicuticle than proteases or is not fixed during preparation of the sample rather than to the absence of chitinase per se. However, we previously detected proteases and exochitinase but not endochitinase activity by enzyme assays and by looking for chitin degradation products as penetration commenced (26). In view of this result and the current data showing chitinase accumulation only after penetration has been achieved, it appears that proteases are more likely than chitinases to initiate cuticle degradation. The Pr1 protease remains associated with the fungal hyphae until about 40 h postinoculation, at which time it begins to permeate the

partially degraded cuticle (8). Removal of proteins by proteolytic hydrolysis of the cuticle before the chitin is exposed to chitinases at ca. 40 h postinoculation probably provides avenues for the chitinase to penetrate the procuticle, which suggests that release of the chitinase is dependent on the accessibility of its substrate. This hypothesis is consistent with the known protective action of protein associated with chitin microfibrils in cuticle (23), and the induction of chitinases by chitin degradation products that was shown in vitro (24) would be facilitated by the unmasking of cuticular chitin. The low concentration of gold particles over appressoria may reflect basal levels of chitinase synthesis, which occurs at about 3% of fully induced levels in the absence of chitin (24). Basal levels of chitinase may function to release inducers of chitinase from chitin when chitin is accessible.

The results of this study show that entomopathogenic fungi produce a complex mixture of chitinolytic enzymes during growth on insect cuticle. We also show that the major endochitinase of *M. anisopliae* is secreted into the cuticle, which presumably would allow close contact with the chitin substrate during cuticle degradation. Although it seems likely that proteases initiated cuticle degradation, allowing the chitinase to permeate the cuticle, there is substantial in vitro evidence that proteases and chitinases act synergistically in the solubilization of cuticle (23) so that the chitinase is probably associated with fungal modification of the procuticle while this barrier is being breached and after. The latter may be of little importance to infection processes, unless it facilitates the reemergence of the fungus through the cuticle to sporulate. We have evidence that there are families of genes that encode the cuticle-degrading proteases of *M. anisopliae* (19), and a similar multiplicity of genes may encode the various families of chitinases described here. Thus, single-gene disruption experiments are unlikely to reveal the role of these proteins in pathogenicity. The various hydrolases produced during growth on cuticle will need to be identified, characterized, and localized during the invasion process to provide an accurate representation of cuticle degradation.

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