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Biochemical Characterization and Ultrastructural Localization of Two Extracellular Trypsins Produced by *Metarhizium anisopliae* in Infected Insect Cuticles

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Proteinase 2 (Pr2) is a fungal (*Metarhizium anisopliae*) serine proteinase which has a tryptic specificity for basic residues and which may be involved in entomopathogenicity. Analytical and preparative isoelectric focusing methods were used to separate two trypsin components, produced during growth on cockroach cuticle, with isoelectric points of 4.4 (molecular mass, 30 kDa) and 4.9 (27 kDa). The catalytic properties of the proteases were analyzed by their kinetic constants and by a combination of two-dimensional gelatin-sodium dodecyl sulfate-polyacrylamide gel electrophoresis and enzyme overlay membranes. Both Pr2 isoforms preferentially cleave at the carboxyl sides of positively charged amino acids, preferring arginine; the pI 4.4 Pr2 isoform also possessed significant activity against lysine. Compared with the pathogen's subtilisin-like enzyme (Pr1), the pI 4.4 Pr2 isoform shows low activity against insoluble proteins in a host (*Manduca sexta*) cuticle. However, it degrades most cuticle proteins when they are solubilized, with high-molecular-weight basic proteins being preferentially hydrolyzed. Polyclonal antibodies raised against each Pr2 isoform were isotype specific. This allowed us to use ultrastructural immunocytochemistry to independently visualize each isoform during penetration of the host (*M. sexta*) cuticle. Both isoforms were secreted by infection structures (appressoria) on the cuticle surface and by the penetrant hyphae within the cuticle. The extracellular sheath, which is commonly observed around fungal cells, often contained Pr2 molecules. Intracellular labelling was sparse.

Studies from a number of laboratories have highlighted the importance of proteolytic enzymes in the metabolism of fungi, as well as in the pathogenesis of diseases caused by parasitic fungi (6, 30). There is also accumulating evidence that the proteinases of protozoa and bacteria facilitate infestations of animal hosts (1). In fact, protease production seems to be one of the first biochemical responses by many parasitic organisms following host contact (5). This is also true of those entomopathogenic fungi that effect entry into insects by breaching the proteinaceous outer integument (26, 28). These are the only microbial means of controlling sap-sucking insects because these hosts cannot ingest other pathogens that infect through the gut wall. The possibility of using entomopathogenic fungi for pest control has led to considerable interest in the biological significance of proteinases in insect-pathogen interactions, with particular attention having been paid to their possible role in cuticle degradation (2, 6, 7). The cuticle-degrading subtilisin-like proteases (Pr1) produced by commercially important fungi such as *Metarhizium anisopliae* currently provide the best-understood model of a fungal determinant of entomopathogenicity (28).

The plant pathogen *Fusarium oxysporum* (17) and several species of entomopathogenic fungi, including *M. anisopliae*, produce serine proteases (Pr2) characterized by their trypsin-like activity towards peptides containing arginine and lysine residues (23, 24). Trypsin-related proteases perform a variety of physiological functions in animal systems (4, 16), but there is as yet no evidence for any particular role for the fungal enzymes. Aside from the proteolytic degradation of the cuticular barrier, other possible roles include the utilization of host proteins for nutrition, the destruction of antifungal proteins of the host, and the release of amino acids for amine production to elevate the pH (26).

Our earlier observations during studies using preparative IEF raised the possibility that there are different isoforms of Pr2 (23), and Cole et al. (3) have described a trypsin-like cysteine protease (Pr4) from *M. anisopliae*. Recently, we obtained N-terminal sequence data for 13 proteins produced during growth on insect cuticles. Two of these proteins, with isoelectric points of 4.4 (molecular mass, 30 kDa) and 4.9 (27 kDa), were 56% homologous and also showed similarities to bacterial and animal trypsins (29). The object of the present work was to unravel the complexity of the trypsin proteinase's composition by characterizing individual enzymes. As these experiments were performed in vitro, additional experimental data were needed to demonstrate the production, accumulation, and localization of individual isoforms in insects during infection processes. We previously obtained information on the role of Pr1 by the direct visualization of Pr1 within cuticle infected by *M. anisopliae* (7). In this article, we report the results of an immunocytochemical investigation using gold-labelled rabbit antisera to follow the fate of the two major Pr2 isoforms as they are synthesized by infection structures.

MATERIALS AND METHODS

Abbreviations. The abbreviations used in this paper are as follows: AFC, 7-amino-4-trifluoromethyl coumarin; Ben, benzoyl; BSA, bovine serum albumin; CH_2Cl, chloromethyl ketone; 2-D, two-dimensional; E-64, *trans*-epoxy-succinyl-L-leucylamido (4-guanidino) butane; EOMs, enzyme overlay membranes; IEF, isoelectric focusing; NA, 4-nitroanilide; PBS, phosphate-buffered saline; Pr1 (proteinase 1), subtilisin-related proteinase of *M. anisopliae*; Pr2 (proteinase 2), trypsin-related proteinase of *M. anisopliae*; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Suc, succinyl; Z, benzyloxycarbonyl.

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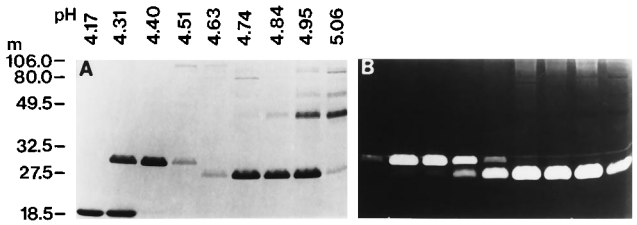


FIG. 1. SDS-PAGE analysis of Rotofor fractions exhibiting Pr2 activity. Narrow-range (pH 3 to 5) IEF of proteases in filtrates from 30-h cuticle cultures was performed in a Rotofor column for 5 h at 12 W, with the voltage increased from 635 to 1,130 V during this period. (A) SDS-PAGE analysis of each Rotofor fraction. Protein is stained with Coomassie blue. (B) Gelatin-SDS-PAGE analysis of Rotofor fractions. Cleared regions of the gel represent proteinase digestion of gelatin copolymerized with acrylamide. The molecular mass (m) markers, in kilodaltons, are on the left. The pI values of the IEF fractions are given at the top of panel A.

Organisms and growth. The fungal isolate (*M. anisopliae* ME-1) and culture media were described before (22).

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 48 h in 100 ml of basal medium (0.02% KH_2PO_4, 0.01% $MgSO_4$; pH 6) supplemented with cockroach cuticle at 1% (wt/vol). Culture filtrates (100-ml aliquots) were concentrated for IEF by lyophilization. The residual solids were dissolved in 15 ml of distilled water and desalted by using Centriprep-10 ultrafiltration units (Amicon, Danvers, Mass.).

IEF. Preparative narrow-range IEF (pH 3 to 5) of proteins (25 to 40 mg per run) was performed in a large Rotofor chamber (Bio-Rad) with 1% ampholytes (Bio-Lyte 3/5; Bio-Rad) following procedures described in the manufacturer's handbook. The ampholytes were removed from each fraction with Amicon Centricon-10 ultrafiltration units, and enzyme activities were determined.

Electrophoresis. Protein samples for electrophoresis were dissolved in SDS-PAGE buffer (15% [wt/vol] sucrose, 0.01% bromophenol blue, and 2.5% [wt/vol] SDS in 0.125 mM Tris-HCl [pH 6.7]) or IEF buffer (9.5 M urea, 2.0% Triton X-100, 1.6% ampholytes [pI 5 to 7], 0.4% ampholines [pI 3.5 to 10]). Samples in SDS-PAGE buffer were boiled (5 min) immediately before being loaded onto gels.

Gel electrophoresis was performed in Bio-Rad miniature gel cells with SDS by the method of Laemmli (13). Protein was stained with Coomassie blue G-250 (Bio-Rad).

For gelatin-SDS-PAGE, electrophoresis was done in 12% (wt/vol) polyacrylamide gels containing 0.2% copolymerized gelatin (14). After electrophoresis, the gels were incubated for 60 min (35°C) in renaturation buffer (0.05 M Tris [pH 8] containing 2.5% [vol/vol] Triton X-100). Protease activity against gelatin was detected by staining residual gelatin with Coomassie blue G-250.

The samples were analyzed by 2-D gel electrophoresis according to the method of O'Farrell (15). IEF was done in the first dimension in a 4% acrylamide gel containing 9.2 M urea, 2.0% (vol/vol) Triton X-100, 1.6 and 0.4% ampholines (pI 5 to 7 and pI 3 to 10, respectively), 0.01% ammonium persulfate, and 0.1% *N,N,N′,N′*-tetramethyethylenediamine. Electrophoresis was carried out in the second dimension in SDS-polyacrylamide (12%) slab gels with or without 0.15% gelatin. The proteins were silver stained with a kit from Sigma. Protease activity in gelatin-containing gels was detected by staining residual gelatin with Coomassie blue G-250 after renaturation. The isoelectric points of the separated proteases were determined from a graph of relative mobility (R_f) versus pI values of standard proteins (Bio-Rad). Inhibitors were tested after electrophoresis by including them in renaturation buffer at various concentrations.

To characterize isoforms, gels were overlaid with EOMs. They were nitrocellulose membranes previously impregnated with the fluorogenic substrates Suc-$(Ala)_2$-Pro-Phe-AFC, D-Val-Leu-Arg-AFC, D-Val-Leu-Lys-AFC, Z-Gly-Gly-Arg-AFC, Z-Arg-Arg-AFC, and Z-Phe-Arg-AFC (Enzyme Systems Products) by procedures described by Smith (18). Gels were incubated at 37°C, and the appearance of fluorescent bands was monitored with a UV lamp. The contact side of the membrane was photographed.

To further classify proteases, some gels were preincubated for 20 min in one of the following inhibitor preparations: leupeptin and antipain (20 μg ml^{-1} each), cystatin and E-64 (30 μg ml^{-1} each), 1,10-phenanthrolene (2 mM), (4-amidinophenyl)-methanesulfonyl fluoride and diisopropylfluorophosphate (3 mM each), or Suc-$(Ala)_2$-Pro-Phe-CH_2Cl (10 μmol). All the inhibitors were dissolved in water except the chloromethyl ketone, which was added from a stock solution in dimethyl sulfoxide (5% [vol/vol] final concentration).

Preparation of antibodies and immunoblotting. Proteins, 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 the stained protein bands 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 subdivided into three aliquots, each containing 30 to 60 μg of protein; the aliquots were each mixed with Freund's complete adjuvant and were injected 14 days apart into New Zealand White rabbits.

For Western blot (immunoblot) analysis, proteins were transferred from gels to nitrocellulose and immunoblotted as described previously (28). The blots were developed with the ProtoBlot Western Blot AP system (Promega).

Immunogold labelling. Cuticles from fifth-instar *Manduca sexta* larvae were excised, inoculated with conidia, and, following incubation for up to 60 h, processed for electron microscopy as described previously (7). Ultrathin sections of LR White-embedded tissue were placed in blocking solution (7) 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 (Sigma; 10-nm-diameter particle size) in PBS–0.05% Tween 20–0.2% BSA 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.

The specificity of the labelling was determined as follows: (i) by incubation of the sections with serum obtained before the rabbits were immunized, (ii) by omission of primary antisera, and (iii) by treatment with protein A prior to treatment with protein A-gold.

Preparation of cuticle substrates. Cuticle was obtained from the giant cockroach (*Blaberus giganteus*) by extracting soft tissue from homogenized insects with sodium tetraborate and added to culture media as previously described (20). Clean samples of cuticle from 3-day-old fifth-instar *M. sexta* larvae were prepared as described previously (25). Proteins bound by electrostatic and hydrophobic forces were extracted by washing cuticles of *M. sexta* in 10 mM Tris-HCl, pH 7.5, containing 0.5 M KCl (20). Extracted proteins were precipitated by incubating them overnight at −20°C with 3 volumes of 0.1 M ammonium acetate in methanol. Proteins were collected by centrifugation (10,000 × *g*, 10 min) and redissolved in water for use as an enzyme substrate. Following extraction, the residual cuticle was washed by repeated centrifugation (2,000 × *g*, 5 min) with water and dried in vacuo.

Enzyme assays. The subtilisin-like protease Pr1 of *M. anisopliae* was purified as described previously (23). Protease activity against *M. sexta* cuticle and solubilized cuticle proteins and anilidase activity (against blocked peptide nitroanilides) were determined as described previously (23). Assays were performed in duplicate. All results are representative of at least two similar experiments using different enzyme and cuticle preparations.

Materials. Reagents were procured as follows: Suc-$(Ala)_2$-Pro-Phe-CH_2Cl and EOM components were from Enzyme Systems Products, and other enzyme substrates and inhibitors were from Sigma.

RESULTS

Separation of Pr2 isoforms. Pr2 isoforms were separated from each other by preparative narrow-range IEF (pH 3 to 5). Analysis of each of the Rotofor fractions by gelatin-SDS-PAGE (Fig. 1) identified the two main components, with molecular masses of 30 kDa (mean pI ± standard deviation, 4.36 ± 0.08; $n = 7$ runs on the Rotofor) (designated 4.4-Pr2) and 27 kDa (4.86 ± 0.15; $n = 7$) (designated 4.9-Pr2), previously identified as trypsin-like enzymes from their N-terminal sequences (29).

A kinetic analysis was performed with IEF fractions (pH 4.31 and 4.95) which contained only one of the isoforms (Fig. 1; Table 1). The isoforms showed similar preferences, for Ben-Phe-Val-Arg-NA over Ben-Pro-Phe-Arg-NA, reflected in both the K_m and V_{max} values. 4.9-Pr2 differed from 4.4-Pr2 in that it

TABLE 1. Kinetic constants of substrates cleaved by Pr2 isoforms[a]

Substrate	Substrate concn range (mM)	4.4-Pr2		4.9-Pr2	
		K_m (mM)	V_{max} (mmol ml^{-1} min^{-1})	K_m (mM)	V_{max} (mmol ml^{-1} min^{-1})
Ben-Phe-Val-Arg-NA	0.05–2.0	0.30	2.73	0.14	2.45
Ben-Pro-Phe-Arg-NA	0.05–3.0	3.48	0.22	2.24	0.24
D-Val-Leu-Arg-NA	0.05–2.0	0.22	2.46	0.20	2.83
D-Val-Leu-Lys-NA	0.05–2.0	1.34	1.25	2.61	0.20

[a] The two protease preparations were IEF fractions 4.31 and 4.95 as represented in Fig. 2.

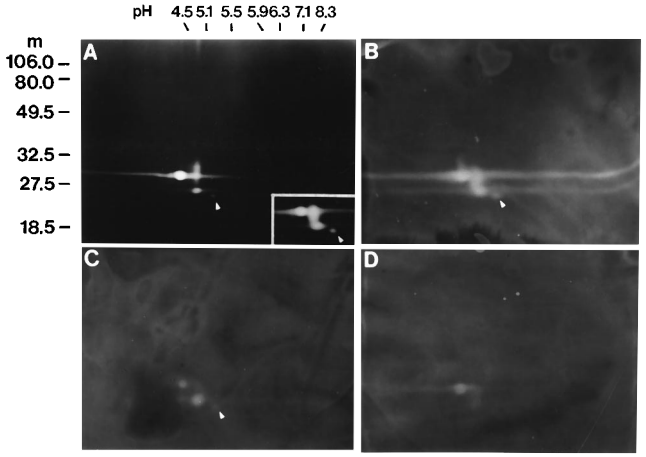


FIG. 2. 2-D SDS-PAGE analysis of substrate preferences of Pr2 activities in 30-h cuticle culture filtrates. The total proteinase pattern was determined by gelatin-SDS-PAGE. Following electrophoresis, the gel was incubated in renaturation buffer for 1 h (A) or 2 h (inset) prior to staining with Coomassie blue. Zymograms were prepared with EOMs containing peptidyl-AFC substrates incubated for 5 min (D-Val-Leu-Arg-AFC [B]) or 12 min (Gly-Gly-Arg-AFC [C] or Val-Leu-Lys-AFC [D]). Only trace activity against Z-Phe-Arg-AFC or Z-Arg-Arg-AFC was detected (data not shown). Arrowheads point to the 5.3-Pr2 isoform. The molecular mass (m) markers, in kilodaltons, are on the left. The pI values are given at the top of panel A.

possessed a more stringent specificity for arginine over lysine at P_1, as evidenced by the 14-fold and 2-fold increases in V_{max}s achieved with D-Val-Leu-Arg-NA compared with those attained with D-Val-Leu-Lys-NA for 4.9-Pr2 and 4.4-Pr2, respectively.

2-D gel analysis. To supplement the preparative analysis presented in Fig. 1, we also performed analytical 2-D gelatin-SDS-PAGE. As can be seen in Fig. 2, two major activities are visible, and the isoelectric points and molecular masses correspond exactly to the 4.4-Pr2 and 4.9-Pr2 isoforms. This technique also identified a third minor activity (25 kDa; pI 5.3). A diffuse spot (pI 4.9; 30 kDa) appeared on some gels. This spot did not occur when samples were boiled before IEF, and it may result from 4.9-Pr2 activity on gelatin during electrophoresis. We performed additional studies on enzyme specificity with EOMs by 2-D gelatin-SDS-PAGE, as this technique resolves the most proteinase activities and does not require purified enzyme (Fig. 3). Among the peptides tested, the best substrate for all activities was Val-Leu-Arg-AFC. Replacing the arginine at the P_1 position with lysine reduced activities, particularly those of the 4.9-Pr2 and 5.3-Pr2 isoforms. Replacing the bulky residues at P_2 and P_3 with glycine also reduced activities, particularly that of 4.4-Pr2. Little activity against cathepsin substrates containing paired arginine residues or Phe-Arg occurred.

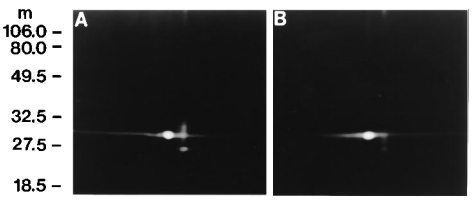


FIG. 3. 2-D SDS-PAGE analysis of 30-h cuticle culture filtrates, demonstrating selective inhibition of 4.4-Pr2 by E-64. The total proteinase patterns in controls (A) and in the presence of E-64 (B) were determined by gelatin-SDS-PAGE. The inhibitor was tested after electrophoresis by including E-64 (30 μg ml^{-1}) in renaturation buffer (50 mM Tris [pH 8] containing 2.5% [vol/vol] Triton X-100). The molecular mass (m) markers, in kilodaltons, are on the left. See Materials and Methods for details.

TABLE 2. Relative cuticle-degrading and anilidolytic activities of Pr1 and Pr2 in cultures grown on cockroach cuticle for 2 days

Substrate and inhibitor[a]	Pr1 and Pr2 activity[b]
Pr1 substrate: Suc-$(Ala)_2$-Pro-Phe-NA[c]	0.50 ± 0.02
Leupeptin	0.50 ± 0.02
Suc-$(Ala)_2$-Pro-Phe-CH_2Cl	0
Pr2 substrate: Ben-Phe-Val-Arg-NA[c]	0.18 ± 0.01
Leupeptin	0.005 ± 0
Suc-$(Ala)_2$-Pro-Phe-CH_2Cl	0.19 ± 0.005
Salt-extracted *M. sexta* cuticle[d]	27 ± 2.4
Leupeptin	25 ± 1.8
Suc-$(Ala)_2$-Pro-Phe-CH_2Cl	2 ± 0

[a] Culture filtrates were incubated with leupeptin (25 μg ml^{-1}) or Suc-$(Ala)_2$-Pro-Phe-CH_2Cl (0.1 mM) for 30 min before assaying with *M. sexta* cuticle or specific anilide substrates for Pr1 [Suc-$(Ala)_2$-Pro-Phe-NA] or Pr2 (Ben-Phe-Val-Arg-NA). Controls contained autoclaved enzyme.

[b] Activities were determined in crude extracts. Units are micromoles of NA per minute per milliliter (for Pr1 and Pr2 substrates) or micrograms of tyrosine equivalents per minute per milliliter (for cuticle substrate).

[c] Assays against NA substrates were performed at 23°C in 10 mM Tris-HCl, pH 8, 4% (by volume) dimethyl sulfoxide, and 0.6 mM substrate.

[d] Activity against salt-extracted *M. sexta* cuticle was determined at 23°C in 10 mM Tris-HCl, pH 8, containing 2.5 mg of cuticle ml^{-1}. Following incubation (1 h), the A_{280} of trichloroacetic acid-soluble products was read (28).

All of the enzymes were completely inhibited by incubating 2-D gelatin-SDS-PAGE gels with specific serine proteinase inhibitors [diisopropylfluorophosphate and (4-amidinophenyl)-methanesulfonyl fluoride at 3 mM each] or with leupeptin (20 μg ml^{-1}) and antipain (20 μg ml^{-1}), which are inhibitors of both cysteine proteinases and trypsin-like serine enzymes. The enzymes were not sensitive to the cysteine proteinase inhibitor cystatin (30 μg ml^{-1}) or the metalloprotease inhibitor 1,10-phenanthroline (2 mM). Selective inhibition of 4.9-Pr2 and 5.3-Pr2 was produced by the cysteine proteinase inhibitor E-64 (30 μg ml^{-1}) (Fig. 3).

Effect of inhibitors on cuticle degradation. The activities of purified Pr1 (1 μg ml^{-1}) and 4.4-Pr2 (5 μg ml^{-1}), partially purified by IEF (Rotofor fraction, pI 4.4) (Fig. 1), against insoluble *M. sexta* cuticle (in 10 mM Tris-HCl, pH 8, containing 2.5 mg of cuticle ml^{-1}) were 54 and 7 μg of tyrosine equivalents min^{-1} ml^{-1}, respectively. The relative contributions of Pr1- and Pr2-type enzymes to host cuticle degradation were also assessed with specific inhibitors for each enzyme. The Pr1 inhibitor Suc-$(Ala)_2$-Pro-Phe-CH_2Cl (20) inhibited cuticle degradation by 92% in culture filtrates containing both Pr1 and Pr2 (Table 2). Leupeptin did not significantly inhibit cuticle degradation. The cuticle used in these experiments was the insoluble residue remaining after extraction of electrostatically bound proteins. A time course experiment involving 2-D gel analysis was used to assay the susceptibility of the various extracted proteins to 4.4-Pr2 activity (Fig. 4). The protein patterns indicate that high-molecular-weight basic proteins are preferentially hydrolyzed, but most of the solubilized cuticle proteins were eventually degraded by Pr2 action.

The immunological relationship between 4.4-Pr2 and 4.9-Pr2. Antibodies were tested for monospecificity by immunoblotting proteins secreted by *M. anisopliae* in cuticle-containing medium with polyclonal antibodies raised against each isoform in separate rabbits (Fig. 5). The antibodies to 4.4-Pr2 and 4.9-Pr2 usually gave rise to a single major spot on a 2-D gel,

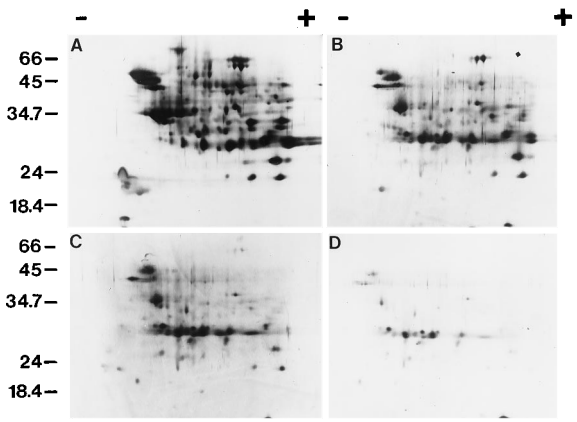


FIG. 4. Pr2 activity against proteins extracted from *M. sexta* cuticles. Activity against the salt-solubilized protein fraction from *M. sexta* cuticles was determined at 23°C in 10 mM Tris-HCl, pH 8, containing 1 mg of protein ml^{-1} and 0.1 μg of 4.4-Pr2 ml^{-1}. Following incubation for 0 min (A), 45 min (B), 90 min (C), and 180 min (D), proteins were precipitated from reaction mixtures with trichloroacetic acid (5% final concentration) and run on 2-D gels. Proteins were silver stained.

corresponding to the position of fluorescent activity on EOMs, but antibodies showed no cross-immunoreactivity, indicating that they are isotype specific. In some replicates the 4.9-Pr2 spot could be seen to be subdivided, forming two closely spaced activities (Fig. 5).

Immunogold localization of Pr2 isoforms. Application of antibodies to 4.4-Pr2 (Fig. 6B) and 4.9-Pr2 (data not shown) resulted in extensive gold labelling in the cell walls and peripheral cytoplasms of mycelia from liquid culture, while control sections incubated with preimmune serum showed only a very low level of nonspecific binding of gold particles (Fig. 6A), confirming that the use of antisera to the Pr2 isoforms in conjunction with the protein A-gold complex is a highly specific labelling method for detecting each isoform in insect cuticles.

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 (7, 21, 25). Labelling of sections from cuticles inoculated for 24, 40, and 60 h with antibodies to 4.4-Pr2 or 4.9-Pr2 indicated that both enzymes accumulated within the cell walls and on the surfaces of appressoria as well as on areas that contacted the cuticle. Production continued during penetration, with the formation of the penetrant peg and hyphal bodies. Different cells exhibited different labelling intensities (Fig. 7C to E); this may have been a reflection of cell type or cell age, factors which are difficult to distinguish in a section. Many penetrant structures had intense labelling within and around the circumference of the fungal cell wall and, to a lesser extent, within the peripheral cytoplasm-cell membrane regions, indicating that the Pr2 isoforms are rapidly secreted. No labelling of other fungal organelles, including mitochondria and the nucleus, was recognized. Both isoforms were confined to the immediate vicinity of penetrant fungal structures (Fig. 7B and E), suggesting that diffusion through the cuticle is hindered as described before for Pr1 (7). No labelling was observed over undegraded cuticles. A characteristic of the penetration process was the large amount of electron-dense, matrix-like or fibrous material observed around fungal hyphae and sites of cuticle degradation. These materials presumably derive from fungal mucilage rather than host melanization, as the cuticles were pretreated with phenylthiourea before inoculation.

DISCUSSION

In previous studies of *M. anisopliae*, we demonstrated that the extracellular production of a protease (Pr2) specific for basic amino acids is favored by growth on insect cuticle or the soluble protein BSA (23, 27).

The present study distinguishes between two major trypsin activities (4.4-Pr2 and 4.9-Pr2) and a minor activity (5.3-Pr2) and defines functional features of these enzymes. The use of EOMs allowed the substrate preferences of the individual proteinases to be studied without pure enzyme preparations. The three Pr2 isoforms showed maximum activity against D-Val-Leu-Arg-AFC, a specific substrate for mammalian glandular kallikrein (EC 3.4.21.34). Like kallikrein (11), the fungal enzymes preferentially cleaved bonds with Arg in position P_1 and a bulky residue in P_2. Cole et al. (3) have described Pr4, a *Metarhizium* enzyme (pI, 4.6; 26.7 kDa) with a specificity for basic residues which was classified as a cysteine proteinase because of its susceptibility to sulfhydryl reagents. In this study, 4.9-Pr2 was inhibited by E-64, which is a specific inhibitor of cysteine proteinases, and was found to resemble Pr4 (3) in that it has stringent preference for arginine over lysine at the P_1 position. However, the N-terminal sequence of 4.9-Pr2 (IVGGSPAAAGEFPFIVSTLL) clearly establishes that it is related to 4.4-Pr2 (IVGGEAAAQGEFPYIVALL) and resembles other serine proteinases with trypsin specificity (29). Most likely, 4.9-Pr2 is a serine proteinase with a free sulfhydryl group close to the active site. Our results do not indicate whether each Pr2 isoform has unique, specific functions. The fact that the isoforms exhibit similar expression patterns during growth on cuticle suggests that any specific biological roles arise from differences in substrate specificities.

The great differences in specificity between different pancreatic serine enzymes are frequently produced by only one or two amino acid changes (4, 10, 12). In animal systems, trypsin isoforms have undergone multiple switches of specificity to produce the family of chymotrypsins and elastases (9). Nevertheless, the tryptic specificities of both Pr2 isoforms have been maintained in spite of their considerable divergence, which is reflected in the N-terminal amino acid sequences (29), electrophoretic differences, antigenic unrelatedness, and specificities for lysine residues. It may be that after the evolution of an effective broad-spectrum subtilisin-related protease such as Pr1, there was little selective pressure to diverge specificities and create chymotrypsins from one of the ancestral Pr2 genes. Conversely, as there are no reports of subtilisins with specificities for basic residues, it seems possible that a tryptic specificity imposes structural requirements which are not readily met by subtilisins.

The Pr1 protease is the major protein produced by *M. aniso-*

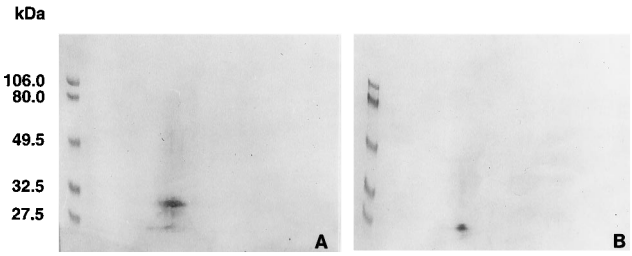


FIG. 5. Western blot showing the lack of cross-reactivity between 4.4-Pr2 and 4.9-Pr2. Proteins in 30-h cuticle culture filtrates were separated by 2-D SDS-PAGE, transferred to nitrocellulose, and tested with antibodies against 4.4-Pr2 (A) or 4.9-Pr2 (B). The bands on the left side of each panel are prestained molecular mass markers.

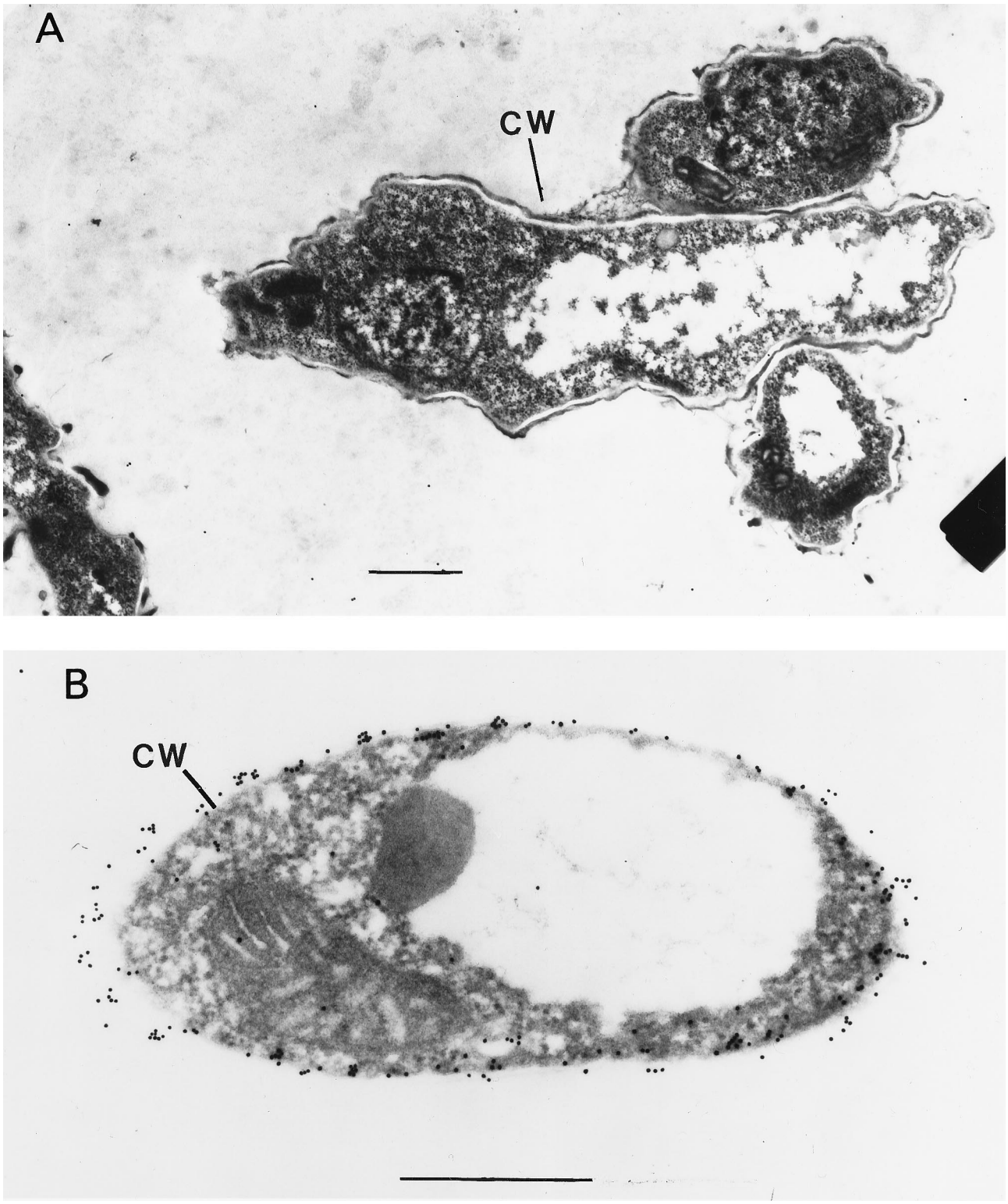
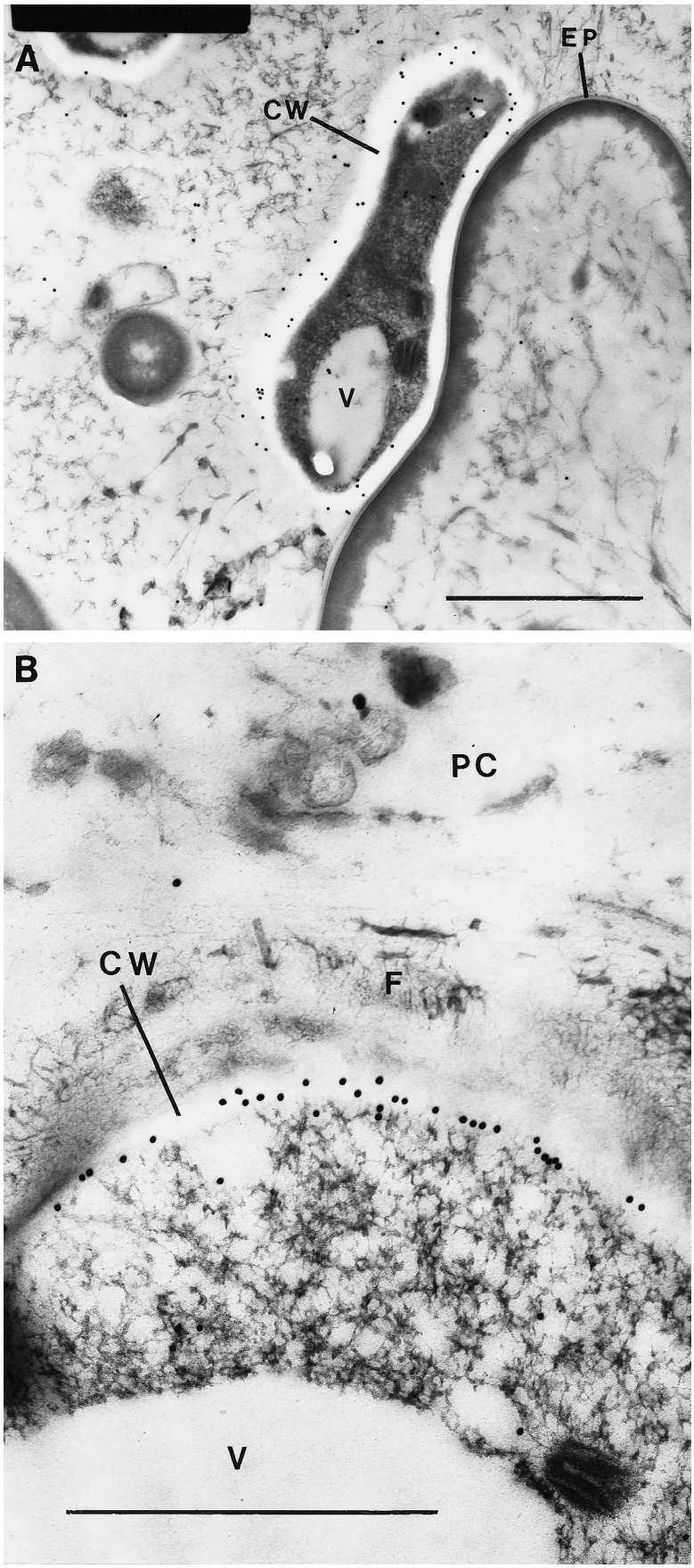


FIG. 6. Transmission electron micrographs of *M. anisopliae* cells from 72-h cuticle cultures, immunolabelled with 10-nm-diameter gold particles. (A) The negative control, in which preimmune serum was used for the primary incubation, shows only a few gold particles. (B) Gold labelling with antibodies against 4.4-Pr2 shows that Pr2 is principally located in the cell wall (CW). Bars, 1 μm.


A
EP
CW
V
B
PC
CW
F
V

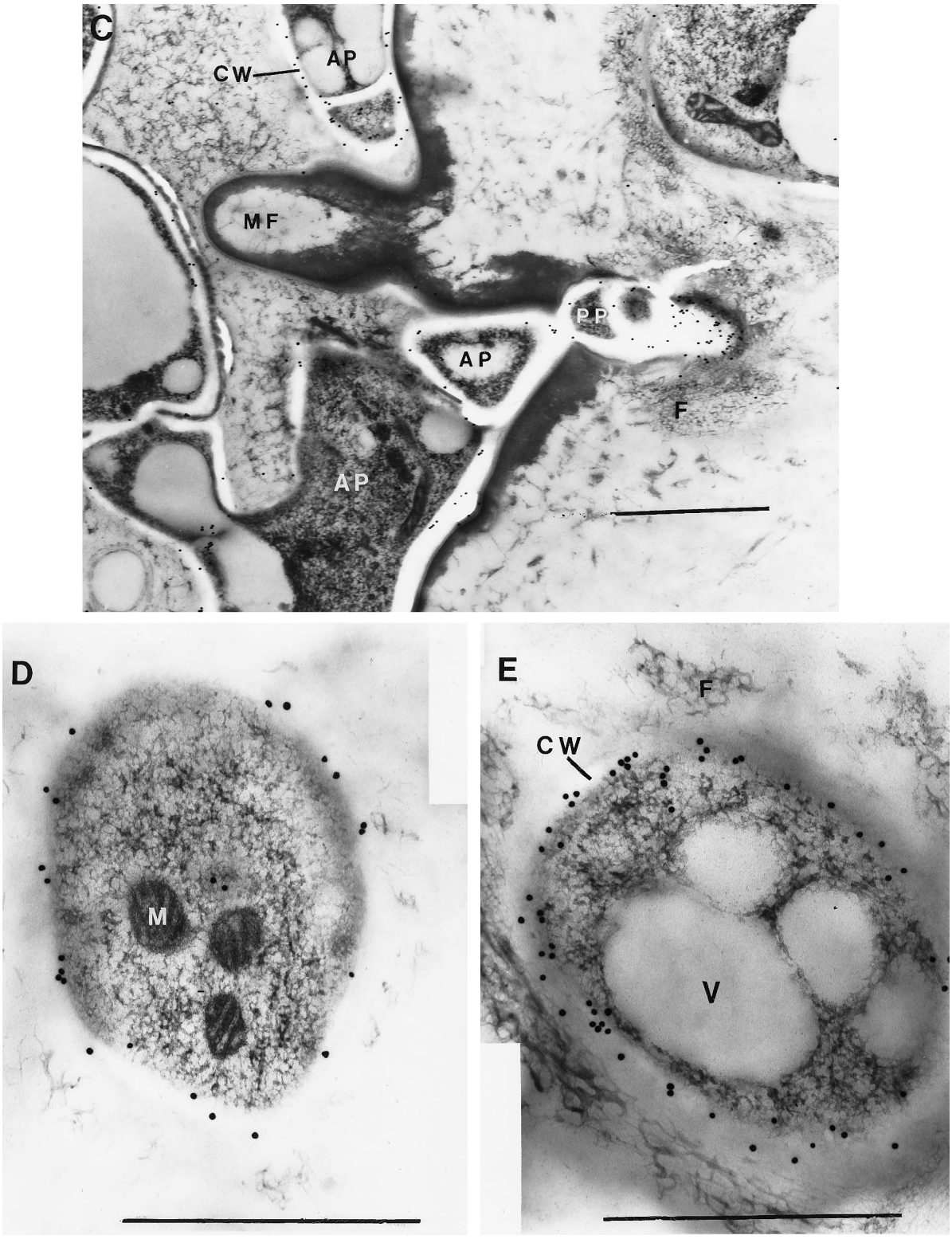


FIG. 7. Immunocytochemical localization of 4.4-Pr2 and 4.9-Pr2 in thin sections of *M. sexta* cuticles infected by *M. anisopliae*. Sections were incubated with antibodies raised against 4.4-Pr2 (A and B) or 4.9-Pr2 (C, D, and E) and then incubated with protein A-gold. (A and C) Deposition of gold particles around developing appressoria and penetration pegs, observed 24 h (A) and 40 h (C) after inoculation. Forty hours after inoculation, gold particles concentrate primarily in the fungal cell wall (B, D, and E). Electron-dense matrix-like material was also found in the invasion zone. AP, appressorium; CW, cell wall; EP, epicuticle; F, fibrous or matrix-like residue; M, mitochondrion; MF, microfold; PC, procuticle; PP, penetration peg; V, vacuole. Bars, 1 μm (A to D) and 0.5 μm (E).

pliae during infection processes (29), and a comparison of the overall rates of hydrolysis suggests that Pr1 has a much greater ability than Pr2 to degrade cuticle. Subtilisin-related proteases may therefore have a greater potential for pathogenicity and possibly also for scavenging extracellular proteins, their most likely biological role in saprophytic fungi (8), while trypsin-related enzymes have a more selective function. Pr2 isoforms were associated with appressoria, suggesting that these enzymes are available during the early stages of cuticle colonization. Immunogold labelling showed that both Pr2 isoforms are principally located in the fungal cell wall during growth through insect cuticles. The mechanisms which could either inactivate enzymes or prevent them from spreading from hyphae (i.e., molecular sieving, differential binding of enzymes to cuticle components, and resistance to enzymatic degradation and enzyme inhibitors) have been reviewed (19). The present ultrastructural study also revealed the occurrence of fibrillar extracellular sheaths surrounding the hyphae, apparently adhering to the cuticle. Extracellular mucilage could have a role in the support and transport of cuticle-degrading enzymes to their target, but the degree of mucilage diffusion into the cuticle exceeds that of the Pr2 molecules (Fig. 7B and E).

Production of the Pr2 isoforms by infection structures suggests that they have some role in degrading extracellular proteins complementary to that of Pr1. It would be expected that most of the hydrophilic Arg-Y or Lys-Y units susceptible to isoforms of Pr2 are on the periphery of globular proteins. However, for Pr1, which has a specificity for hydrophobic residues, a significant proportion of potentially susceptible units may be in the interior of the protein. A trypsin activity may complement Pr1, opening up proteins for further hydrolysis, assisting in penetration, and providing peptides which can be utilized as nutrients. These findings suggest that production of isoforms of Pr1 and Pr2 forms part of a cascade of pathogen reactions facilitating the penetration of host cuticles.

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