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Electrophoretic Forms of Chitinase Activity in Xanthi-nc Tobacco, Healthy and Infected with Tobacco Mosaic Virus

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Tobacco leaf homogenates and intercellular fluid extracts were analyzed in native polyacrylamide gel systems for detection of chitinase activity. They were compared using clarified homogenates from several tobacco organs. In gels specific for acidic proteins, one major and five minor activities were found in diseased tissue, while only the major activity could be detected at a low level in healthy leaf tissue. Up to eight activities, including the six activities from infected leaf tissue, could be revealed in flower parts and apical leaves from healthy tobacco plants. In gels specific for basic proteins, two major and five minor activities were observed both in infected leaf tissue and in healthy flower parts. All tissue extracts contained one of the major basic chitinases. In both gel systems, the precise identification of pathogenesis-related (PR) proteins with chitinase activity was precluded because bands in one-dimensional native gels often contained more than one protein. Thus, chitinase activity was also studied in sodium dodecyl sulfate denaturing gels. Estimated molecular masses were 25–26 kDa and 30 kDa. The most prominent activity was the 30-kDa protein in all tissues, except for some flower parts. This activity was highly stimulated in infected tissue. The activities at 25–26 kDa were also stimulated in infected tissue and present in healthy flower parts. The major acidic chitinase stimulated in infected tissue hydrolyzed 4-methylumbelliferylchitotriose but not 4-methylumbelliferylchitobiose. The two major basic chitinases stimulated in infected tissue hydrolyzed the chitotriose derivative, but the chitobiose substrate was only hydrolyzed by one of the enzymes. Both basic chitinases had lysozyme activity. A two-dimensional gel system was devised to allow identification of individual proteins with chitinase activity. Four acidic proteins could be renatured in such gels, and three proteins corresponded to PR-P, PR-Q, and PR-b_{6c}. Chitinase activity of PR-b_{6c} was much higher than the three other activities combined. Four basic chitinases could be identified. One corresponded to PR-b_{13} and the others to unidentified proteins. Up to 13 (6 acidic, 7 basic) activities could be detected in Xanthi-nc leaf tissue infected with tobacco mosaic virus.

Additional keywords: chitinolysis, hydrolases, *Nicotiana*, two-dimensional polyacrylamide gel.

Plant chitinases (EC. 3.2.1.14) represent potential antifungal hydrolases (Schlumbaum *et al.* 1986; Roberts and Selitrennikoff 1988; Wargo 1975) that often act synergistically with β-1,3-glucanases on fungal cell walls (Mauch *et al.* 1988b). Chitinases have been characterized in various organs, including wheat germ, tomato stem, bean leaf, pea pod, yam roots, soybean seeds (Boller 1985, 1987), barley, maize, and wheat grains (Roberts and Selitrennikoff 1988), barley endosperm (Leah *et al.* 1987), thorn apple leaves (Broekaert *et al.* 1988), and oat leaves (Fink *et al.* 1988). Some chitinases also have lysozyme activity (EC. 3.2.1.17) (Boller 1987), and plant lysozymes display various levels of chitinase activity (Audy *et al.* 1988a; Bernasconi *et al.* 1987; Roberts and Selitrennikoff 1988).

Chitinase activity and its regulation have been studied in stressed plant tissues of cultured carrot cells (Kurosaki *et al.* 1987a, 1987b), melon leaves (Roby *et al.* 1986), and cultured parsley cells (Kombrink and Halbrock 1986), as well as in bean cell suspension cultures (Hedrick *et al.* 1988) and in ethylene-treated bean leaves (Mauch and Staehelin 1989). Chitinase activity has also been investigated in several host-pathogen interactions involving tobacco (Vögeli-Lange *et al.* 1988), melon (Roby and Esquerré-Tugayé 1987; Roby *et al.* 1988), pea (Mauch *et al.* 1988a), maize (Nasser *et al.* 1988), cucumber (Métraux and Boller 1986), and tomato (Pegg and Young 1982; Joosten and De Wit 1989).

Recently, several chitinases have been identified as extracellular pathogenesis-related (PR) proteins in potato (Kombrink *et al.* 1988), cucumber (Métraux *et al.* 1988), maize (Nasser *et al.* 1988), tomato (Joosten and De Wit 1989), and tobacco (Legrand *et al.* 1987). Up to now, three virus-plant combinations have been studied for stimulation of chitinases: tobacco mosaic virus (TMV) and Samsun NN tobacco (Legrand *et al.* 1987); tobacco necrosis virus (TNV) and hypersensitive cucumber (Boller and Métraux 1988); and brome mosaic virus (BMV) and systemic maize (Nasser *et al.* 1988). In the case of hypersensitive tobacco infected with TMV, four PR proteins were identified as chitinases: two acidic proteins (corresponding to PR-P [or PR-b_{7b}] and PR-Q [or PR-b_{8b}]) and two basic proteins not related to previously known PR proteins (Legrand *et al.* 1987). These four proteins are all endochitinases, are serologically related, and account for all chitinase activity in tobacco leaves infected with TMV. Acidic isoforms (PR-P and PR-Q) have relatively low specific activity when compared to basic chitinases and represent about one-third of all chitinase activity in leaves showing necrotic local lesions to TMV.

Despite all these results, there is still a lack of information about the complete set of molecular forms of chitinases in healthy versus infected tissue. Moreover in identifying PR proteins as chitinases, researchers often rely on the comparison of electrophoretic mobilities of chitinases with mobilities of PR proteins in one-dimensional polyacryl-

amide gels. The use of two-dimensional gel systems is necessary to separate and identify all tobacco PR proteins (Parent and Asselin 1984; Parent *et al.* 1985; Hogue and Asselin 1987).

Detection techniques for chitinase activity in native and denaturing gels were recently developed (Trudel and Asselin 1989). In this report, such techniques were used to study the various molecular forms of chitinase activity in healthy Xanthi-nc tobacco and in Xanthi-nc tobacco infected with TMV. A new technique was also used to allow detection of chitinase activity in two-dimensional gel systems. By using such techniques, the major acidic chitinase activity in tobacco leaves infected with TMV was found to correspond to PR protein b_{6c} and not to PR-P and PR-Q. Other molecular forms of chitinase activity were detected in several tissues of healthy tobacco and in tobacco leaf tissue infected with TMV.

MATERIALS AND METHODS

Chemicals. All chemicals for electrophoresis, analytical grade mixed bed resin AG 501-X8 (20–50 mesh), protein molecular mass markers, and Coomassie Brilliant Blue R 250 were from Bio-Rad (Mississauga, Ontario, Canada). Protein concentration was determined with Bio-Rad protein assay reagent. Lyophilized *Micrococcus lysodeikticus* (syn. *luteus* (Schroeter) Conn) (American Type Culture Collection [ATCC] 4698) cells and all other chemicals were from Sigma Chemical Co. (St. Louis, MO), and dialysis membranes were from Bethesda Research Laboratories (Burlington, Ontario, Canada). Glycol chitin was synthesized as previously described (Trudel and Asselin 1989).

Plant material: Tobacco leaf extracts infected with TMV and mock inoculated. Tobacco plants (*Nicotiana tabacum* L. cv. Xanthi-nc.) were grown to 10–12 leaves (counting only leaves that were at least 5-cm long) under greenhouse conditions. Inoculation was made on leaves 6 to 8 from the bottom of the plant. The inoculum was TMV (U_2 strain) at 2.5 μg/ml in 100 mM sodium phosphate buffer, pH 7.0. Plants rubbed with buffer were used as control healthy plants. Top leaves were removed, and plants were kept in growth chambers for 7 days with a 14-hr photoperiod (175 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 22° C and 80% relative humidity. This treatment yielded about 100 to 200 lesions per infected leaf.

Homogenates were made in 100 mM sodium phosphate buffer, pH 7.0, (1:1 [w/v]) at 4° C for 1 min at maximum speed in a Sorvall Omnimixer followed by centrifugation at 10,000 × *g* for 15 min at 4° C. To extract intercellular fluid (IF), three successive infiltrations (Hogue and Asselin 1987) were made with the same buffer, and extracts were recovered (1,000 × *g*, 10 min at 4° C). All IF extracts and homogenates were dialyzed overnight (cutoff 6,000–8,000) against distilled water at 4° C.

Extracts from healthy plant tissue. Tissue homogenates were made as described above in 50 mM Tris-HCl, pH 7.5, containing 50 mM $CaCl_2$ (1:1 [w/v]) except for the dilution of seed extracts (1:5 [w/v]). Tissues were taken from plants with 15 leaves, except for cotyledons (15-day-old plantlets) and floral tissues (plants with more than 30 leaves). Tissue extracts from young leaves were from leaves less than 8-cm long and included apical buds. Senescent and fully expanded leaves were the second and third leaves and the eighth leaf from the bottom of the plant, respectively. Roots were washed in running tap water before extraction.

Native and sodium dodecyl sulfate (SDS)-PAGE in one-dimensional gels and chitinolytic activity. Sample preparation and staining for chitinase activity after PAGE under native conditions for acidic and basic proteins and under denaturing conditions (SDS-PAGE) were performed as previously described (Trudel and Asselin 1989). Renaturation of enzyme activity after SDS-PAGE was in 25 mM sodium acetate, pH 5.0, containing 1% (v/v) purified Triton X-100 (Potvin *et al.* 1988; Audy *et al.* 1989) for 18 hr at 37° C. Optimal tobacco chitinase activity in gels was found at pH 5.0 at low ionic strength (10–100 mM sodium acetate). Controls not previously described were used. Overlay gels (Trudel and Asselin 1989) without glycol chitin were processed in the same manner as gels containing glycol chitin and stained with Calcofluor White M2R (Trudel and Asselin 1989). These controls were run because some plant crude extracts can give rise to nonfluorescent bands (dark bands normally interpreted as bands with chitinase activity) in gels without glycol chitin as the substrate (unpublished results). However, no such false positive band was detected in any of the tobacco extracts.

Two-dimensional gels and chitinolytic activity. First-dimensional native PAGE was performed in the Davis system for acidic proteins and in the Reisfeld system for basic proteins as previously described (Hogue and Asselin 1987; Parent *et al.* 1988). Vertical sections 5-mm wide were cut from native gels with a sharp blade, and proteins were denatured by heating for 5 min at 100° C in 4 ml (per gel slice) of 2.5% SDS in 125 mM Tris-HCl, pH 6.7, containing 0.01% (w/v) bromophenol blue as the tracking dye. Sections of gels were then transferred on top of a 1-mm thick 10–15% linear gradient polyacrylamide SDS preparative gel containing 0.01% glycol chitin (Trudel and Asselin 1989). Electrophoresis was run for 65 min at 25 mA at room temperature. Gels were then incubated with gentle shaking in renaturing buffer (25 mM sodium acetate, pH 5.0, 1% purified Triton X-100) for 2, 6, and 18 hr at 37° C and stained with Calcofluor White M2R (Trudel and Asselin 1989) followed by Coomassie blue and silver staining (Hogue and Asselin 1987) with the omission of steps 1 and 2 (Morrissey 1981). Protein molecular mass markers were lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), bovine serum albumin (66.2 kDa), and phosphorylase b (92.5 kDa).

Activity toward 4-methylumbelliferyl (4-MU) derivatives. After PAGE under native conditions, gels were incubated in 100 mM sodium acetate, pH 5.0, for 5 min at 37° C. Vertical sections of gels were put on clean glass plates and covered with Whatman 3 MM paper impregnated with 0.02% (w/v) of 4-MU derivatives in 25 mM sodium acetate buffer at pH 5.0. After 30 min at 37° C in a closed box under moist conditions, the papers were removed and the gels immediately photographed with a UV transilluminator (TM-40, UV Products, San Gabriel,

CA) using Polaroid (Eastman Kodak, Rochester, NY) type 55 black-and-white film with UV-Haze and Y3 yellow filters. The exposure time was 2 to 3 min at f-stop 4.7 with a 127-mm lens.

Lysozyme activity. After PAGE under native conditions, the gels were overlayered with a 0.75-mm thick 10% acrylamide gel containing 0.15% (w/v) lyophilized *M. lysodeikticus* cells (Audy *et al.* 1988a) and placed between two Whatman 3 MM papers soaked in distilled water. This sandwich was inserted in a Bio-Rad transblot cell containing 3 liters of 25 mM sodium acetate, pH 5.0, and electrotransferred for 4 hr at 37° C at constant 20 mA current. (The overlay gel should face the cathode side in the Reisfeld system and the anode side in the Davis system.) Lysozyme activity was then visualized on the overlay gel and photographed as previously described (Audy *et al.* 1988a). Bands with lysozyme activity appear as clear bands against the opaque *Micrococcus* cell background (Audy *et al.* 1988a, 1989).

RESULTS

Acidic electrophoretic forms of chitinase activity in tobacco leaf tissue, healthy and infected with TMV. Homogenates of healthy Xanthi-nc tobacco leaf tissue and leaf tissue infected with TMV before (Fig. 1A, HB) and after (Fig. 1A, HA) three successive IF extractions (Fig. 1A; IF1, IF2, and IF3) were analyzed for chitinase activity with glycol chitin as the substrate in the overlay gel after separation in a 15% polyacrylamide native gel system for acidic proteins (Davis system) (Trudel and Asselin 1989). An IF extract enriched in Xanthi-nc tobacco PR proteins was included as the reference for silver-stained PR proteins. Chitinase activity was detected as dark (nonfluorescent)

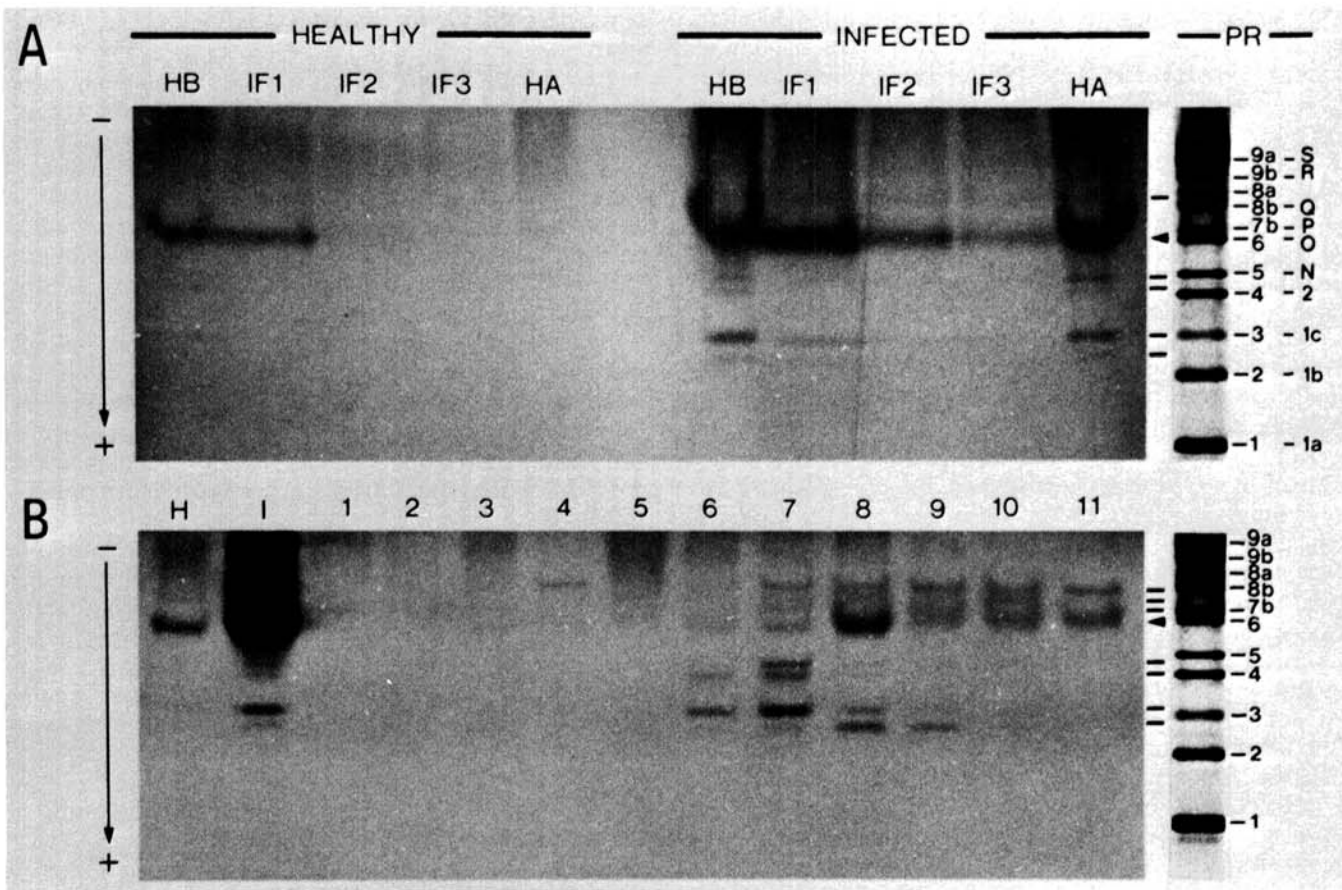

Fig. 1. Chitinase activity after electrophoresis in a 15% (w/v) polyacrylamide gel at pH 8.9. Three successive intercellular fluid (IF1, IF2, and IF3) extracts and homogenates before (HB) and after (HA) infiltrations from healthy Xanthi-nc tobacco leaf tissue and leaf tissue infected with tobacco mosaic virus (TMV) (panel **A**) were subjected to PAGE under native conditions for acidic proteins (Davis system). In panel **B**, homogenates of healthy leaf tissue (H) and of leaf tissue infected with TMV (I) were subjected to PAGE with clarified homogenates of healthy tissue: seed (1), cotyledon (2), root (3), stem (4), senescent leaf (5), fully expanded leaf (6), apical leaf (7), sepal (8), petal (9), pistil (10), and stamen (11). An IF extract containing pathogenesis-related (PR) proteins from leaf tissue infected with TMV was subjected to PAGE, and proteins were stained with aqueous silver nitrate (PR). Nomenclature for PR proteins is shown in two systems (Hogue and Asselin 1987; Kauffmann *et al.* 1987). Samples (20 μl) contained 25 μg of protein for tissue homogenates (panel **B**, 1 to 11). The IF extracts from infected leaf tissue contained 20 μg (IF1), 1 μg (IF2), and 0.5 μg (IF3), and the IF extracts from healthy leaf tissue had 0.5 μg (IF1) and 0.1 μg of protein (IF2 and IF3). Homogenates contained 50 μg of protein (HB, HA, and H) and 70 μg of protein (I). After electrophoresis, an overlay polyacrylamide gel containing 0.01% (w/v) glycol chitin was incubated for 1 hr on top of the separating gel. After staining with Calcofluor White M2R, bands with lytic activity appeared as dark zones after UV illumination. Arrows on the left indicate the direction of migration. Arrowheads on the right indicate the migration of PR-O (PR-b_6). Bars on the right indicate the various bands with chitinase activity.

bands against a UV fluorescent background of intact glycol chitin stained with Calcofluor White M2R (Trudel and Asselin 1989). Results in Figure 1A show a low level of chitinase activity in one band in the homogenate of healthy leaf tissue (Fig. 1A, HB) and in the first IF extract (Fig. 1A, IF1). The band with chitinase activity in healthy Xanthi-nc tobacco leaf tissue migrated at the position of the PR-O (or PR-b_6) protein (Fig. 1A). The electrophoretic pattern of chitinase activity in tissue infected with TMV was different in two ways. First, the band with chitinase activity migrating at the position of PR-O was highly stimulated in all extracts of infected tissue (Fig. 1A, arrowhead on the right). Second, up to five other minor bands with chitinase activity could be found in some extracts of infected tissue. A doublet of bands migrated near the PR-1c protein, another doublet near PR-N, and one band could be observed near PR-Q (Fig. 1A, bars on the right).

Results thus indicate that one major activity and five minor acidic chitinase activities are found in leaves after localized TMV infection. The major band and some minor ones (like the doublet near PR-1c) seem to accumulate (at least partially) in IF extracts like the PR proteins. This is in agreement with reports showing the presence of chitinases in intercellular washing fluids (Kombrink *et al.* 1988). However, bean chitinase was not detected in IF extracts (Mauch and Staehelin 1989).

Our results with the gel assay indicate that some amounts of chitinases accumulate extracellularly. From such results, we cannot determine if they are actively secreted or simply released outside of the altered cells. However, a substantial amount of chitinase activity is still present in the homogenate after the three successive IF extracts (Fig. 1A; HA, infected). This indicates that significant amounts of these chitinases can be found intracellularly. The same observation was recently reported for oat chitinases where extracellular and intracellular chitinases could be found. With oat chitinases, six infiltration steps released up to 5% of soluble chitinase (Fink *et al.* 1988). It could well be that large amounts of tobacco leaf chitinases accumulate with vacuolar protein aggregates as occurred in stressed bean leaves (Mauch and Staehelin 1989).

Acidic electrophoretic forms of chitinase activity in tissues of healthy tobacco plants. The acidic electrophoretic forms of chitinase activity in homogenates from healthy leaf tissue (Fig. 1B, H) and leaf tissue infected with TMV (Fig. 1B, I) were compared with the ones in homogenates from healthy tissue: seed (Fig. 1B, 1), cotyledon (Fig. 1B, 2), root (Fig. 1B, 3), stem (Fig. 1B, 4), senescent leaf (Fig. 1B, 5), fully expanded leaf (Fig. 1B, 6), apical leaf (Fig. 1B, 7), sepal (Fig. 1B, 8), petal (Fig. 1B, 9), pistil (Fig. 1B, 10), and stamen (Fig. 1B, 11). Results indicate that all bands with chitinase activity in diseased tissue could be found in varying amounts in some healthy tissues.

Two extracts are especially interesting. The extract from sepals had the strongest band of activity, migrating at the position of PR-O, and also contained several minor bands of activity (Fig. 1B, 8). One minor band of activity (the one migrating near PR-P [PR-b_{7b}]) was detected in some extracts from healthy leaf tissue; this band could not be clearly detected in leaf tissue infected with TMV. The extract from apical leaves showed the strongest band of activity migrating near PR-1c. Some extracts showed very little or no activity (seeds, cotyledons, roots, and senescent leaves). Flower organs and apical leaves were the best sources of acidic electrophoretic forms of chitinases in healthy tissue.

Basic electrophoretic forms of chitinase activity in healthy tobacco leaf tissue and leaf tissue infected with TMV. An analysis with the same extracts as those for acidic proteins was also performed in an overlay gel containing glycol chitin after separation in a 15% polyacrylamide native gel for basic proteins (Reisfeld system) (Trudel and Asselin 1989). Chitinase activity was revealed after digestion of glycol chitin as it was for acidic proteins. Results in Figure 2A show four bands of chitinase activity in homogenates before IF extracts from healthy tissue (Fig. 2A, HB). The same four bands can be observed at a reduced level in homogenates after successive infiltrations (Fig. 2A, HA). This indicates that these chitinases can be found at least partially extracellularly (Fig. 2A; IF1, IF2, IF3, healthy).

Basic forms of chitinase activity are stimulated in infected leaf tissue (Fig. 2A, infected). Two major bands of stimulated activity (Fig. 2A, arrowheads on the right) correspond to the chitinases in extracts from healthy leaf tissue. Five other bands of activity can be observed (Fig. 2A, bars on the right). One major band of activity migrated at the position of PR-b_{13}-b_{14} (Hogue and Asselin 1987); the other upper major band migrated near a protein previously identified as x_2 (Hogue and Asselin 1987). One band of activity near the top of the gel was present in infected but not in healthy leaf tissue. The two lower minor bands seemed to be present only in both homogenates of infected leaf tissue and migrated below PR-b_{13}-b_{14} proteins. So, three bands (one with the lowest mobility and two with the highest mobilities) seemed dependent upon TMV infection.

Basic electrophoretic forms of chitinase activity in tissues of healthy tobacco plants. The basic electrophoretic forms of chitinase activity in homogenates from healthy leaf tissue and leaf tissue infected with TMV were compared to the ones in homogenates from healthy tissue as it was done for acidic proteins (Fig. 2B). The two major bands of activity (Fig. 2B, arrowheads) can be found in all extracts except for the lower one in cotyledons and stems. Results indicate that flower parts (except stamens) contain small amounts of chitinase activity, with bands migrating near the top of the gel (Fig. 2B; 8, 9, 10, upper bar). The two activities with the highest electrophoretic mobilities can be found in flower parts (Fig. 2B; 8, 9, 10, 11, lower bars) as well as in infected leaves (Fig. 2B, 1). The upper band of these same two bands can also be detected in large amounts in the seed homogenate (Fig. 2B, 1).

Analysis of chitinase activity in healthy tobacco leaf tissue and in leaf tissue infected with TMV after SDS-PAGE. Because using one-dimensional native gels does not allow precise identification of several tobacco PR proteins (Parent and Asselin 1984; Hogue and Asselin 1987), analysis of chitinases was also performed in denaturing gels to estimate the molecular masses of chitinases. The same leaf extracts as the ones used in Figure 1 and 2 were subjected

to SDS-PAGE in gels containing glycol chitin as the substrate and analyzed for chitinase activity (Trudel and Asselin 1989) after renaturation of enzymes in buffered Triton X-100 (Potvin *et al.* 1988; Audy *et al.* 1989; Leclerc and Asselin, in press).

Results in Figure 3A indicate that one band near 30 kDa was present in the homogenate of healthy leaf tissue before IF extracts (Fig. 3A, HB) and in the first two IF extracts (Fig. 3A; IF1, IF2). The same band was stimulated in infected tissue and became visible as a diffuse band. A large diffuse band near 25–26 kDa was present in infected tissue, especially in the extract from the homogenate before IF extracts (Fig. 3A, HB). Estimated molecular masses of some PR proteins (such as PR-b_{6c}) are different from previous estimations (Hogue and Asselin 1987) because the molecular mass of carbonic anhydrase was used as 31 kDa in this work instead of 29 kDa.

Analysis of chitinase activity in tissues of healthy tobacco plants after SDS-PAGE. Denatured proteins from several tissues of healthy tobacco plants were analyzed after SDS-PAGE for chitinase activity. The major band migrating near 30 kDa was detected in varying amounts in all tissues, except for petal (Fig. 3B, 9) and pistil (Fig. 3B, 10) extracts. However, these extracts showed activity in smears. The lower level migrating near 25–26 kDa, previously detected in infected leaf tissue, could be found in flower parts (Fig. 3B; 8, 9, 10, 11).

Hydrolysis of 4-MU chitobiose, 4-MU chitotriose, and *M. lysodeikticus* cells in native gels with extracts from healthy tobacco leaves and from leaves infected with TMV. Chitinases display various activities toward oligomers of *N*-acetyl-D-glucosamine (Robbins *et al.* 1988). Leaf proteins in homogenates from healthy tissue (Fig. 4, H) and tissue infected with TMV (Fig. 4, I) were separated in native gels for acidic (Fig. 4, A) or basic (Fig. 4, B) proteins and assayed for hydrolysis of 4-MU chitobiose (Fig. 4, 4-MUCB) or 4-MU chitotriose (Fig. 4, 4-MUCT). Lytic activity was assessed by the presence of UV fluorescent bands generated by the release of 4-methylumbelliferone from the dimer or trimer of *N*-acetyl-D-glucosamine. In native gels for acidic proteins, only the fluorescent band at the position of PR-O (PR-b_6) (Fig. 4, bar on the left) was detected in infected tissue when 4-MU chitotriose was the substrate. Fluorescent diffuse bands near the top of

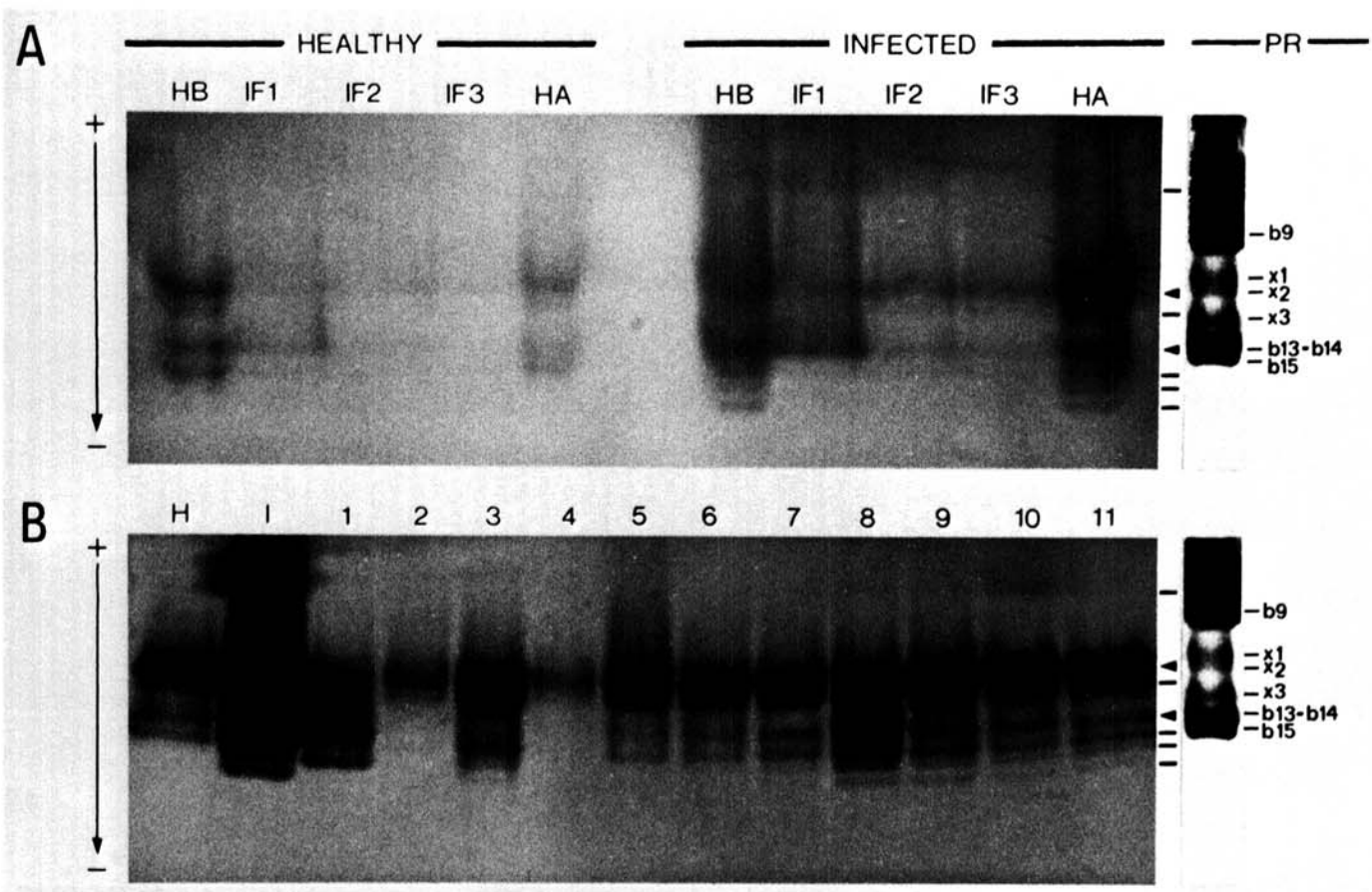

Fig. 2. Chitinase activity after electrophoresis in a 15% (w/v) polyacrylamide gel at pH 4.3. The same intercellular fluid (IF) extracts and homogenates given in Figure 1 were subjected to PAGE under native conditions for basic proteins (Reisfeld system). An IF extract containing pathogenesis-related (PR) proteins from leaf tissue infected with tobacco mosaic virus was subjected to PAGE, and proteins were stained as described in Figure 1. Nomenclature of PR proteins has been previously described (Hogue and Asselin 1987). Samples (20 μl) contained the same amounts of protein as given in Figure 1. Detection of chitinase activity was with an overlay gel system as given in Figure 1. Arrows on the left indicate the direction of migration. Arrowheads (lower one at the position of PR-b_{13}-b_{14}, upper one near x_2 protein) on the right indicate the migration of the two major bands of activity. Bars on the right indicate the various bands with chitinase activity.

the gels represented autofluorescent spots and not products of hydrolysis. In native gels for basic proteins, bands were only observed in infected tissue (Fig. 4, B) as was the case for acidic proteins. One fluorescent band was present with 4-MU chitobiose as the substrate and migrated near the x_2 protein as previously depicted in Figure 2. Two bands were found with 4-MU chitotriose as the substrate. The upper band had the same mobility as the band present with 4-MU chitobiose as the substrate; the lower band had the electrophoretic mobility of PR-b_{13}-b_{14} (Fig. 2). Extracts were tested for lysozyme activity (Fig. 4, L) in healthy leaf tissue (Fig. 4, H) and tissue infected with TMV (Fig. 4, I). In the native gel system for basic proteins (Fig. 4, B), two bands with lysozyme activity were observed. They were the same bands as the ones hydrolyzing either 4-MU chitobiose or 4-MU chitotriose. No sharp band of lysozyme activity could be seen with native gels for acidic proteins, except for smears from the top of the gel down to the level of migration of the PR-O protein (not shown).

Analysis of chitinase activity in two-dimensional gel systems with extracts from healthy tobacco leaves and from leaves infected with TMV. A new approach for detecting chitinase activity after separation of proteins in two-dimensional systems was devised to identify individual proteins with chitinase activity. Proteins were first separated in native PAGE for acidic (Fig. 5, a) or basic (Fig. 5, b) proteins and then subjected to SDS-PAGE in gels containing glycol chitin as the substrate. However, when the usual detection of chitinase activity based on fluorescence of glycol chitin stained with Calcofluor White M2R (Fig. 3 and previous results [Trudel and Asselin 1989]) was followed by Coomassie blue and silver staining, we found that glycol chitin could be differentially stained with aqueous silver nitrate. Intact glycol chitin embedded in gels was efficiently stained with silver nitrate to give a uniform dark background in the gel. If digested, glycol chitin was not stained with silver nitrate and a clear white zone of lysis could be detected. Moreover, prolonged staining allowed proteins to be visualized if proteins did not elute from the gel during the renaturation process. This technique thus allows direct visualization of proteins concomitant with lysis zones generated by chitinase activity.

Results in Figure 5 indicate that IF extracts from healthy tobacco leaf tissue had four spots after a two-dimensional

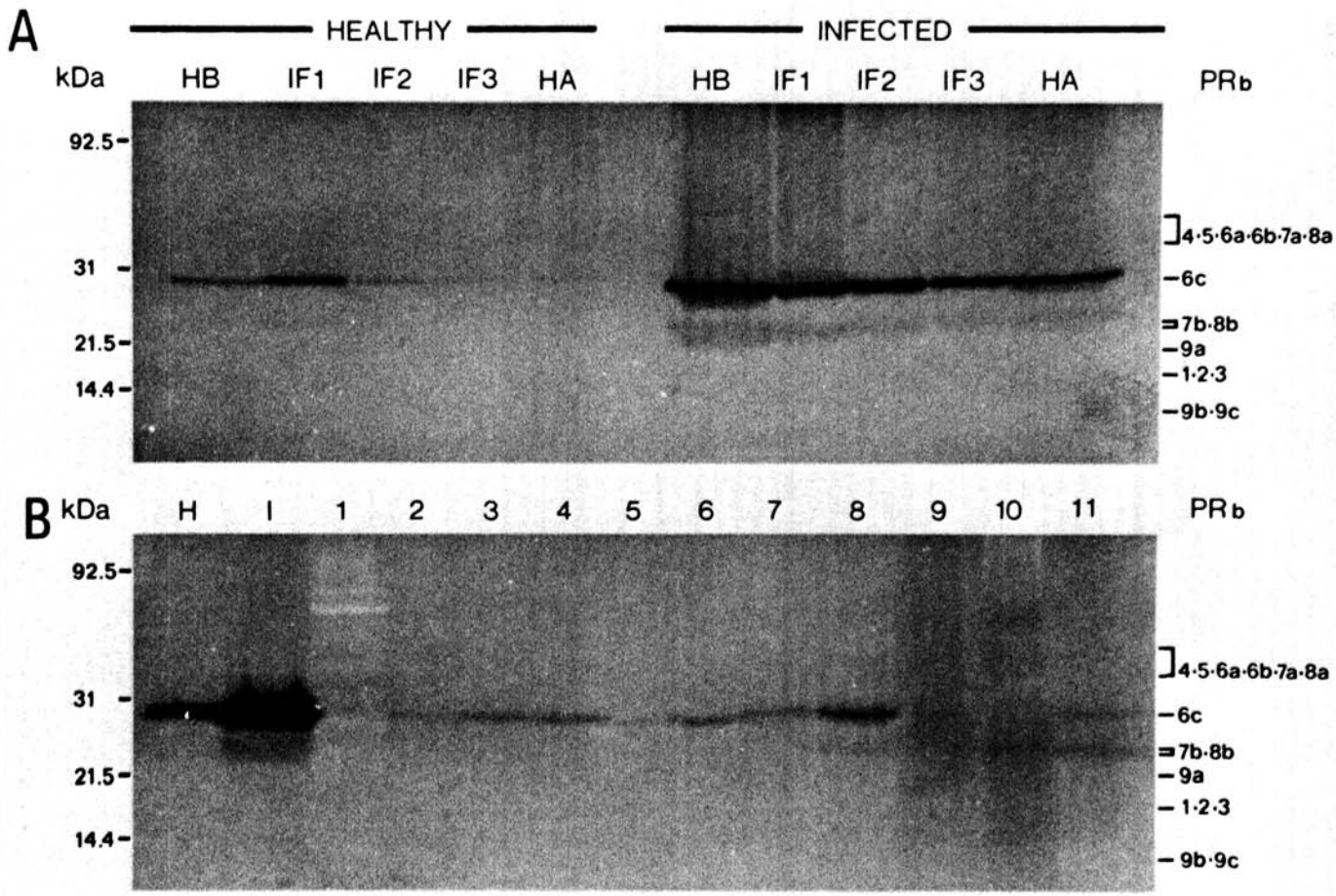

Fig. 3. Chitinase activity after sodium dodecyl sulfate (SDS)-PAGE in a linear gradient (10–15%) polyacrylamide gel containing 0.01% (w/v) glycol chitin as substrate. The same intercellular fluid (IF) extracts and homogenates given in Figure 1 were subjected to SDS-PAGE as previously described (Trudel and Asselin 1989). Molecular mass markers are indicated on the left. Samples (20 μl) contained the same amounts of protein as given in Figure 1. Detection of chitinase activity after SDS-PAGE was previously described (Trudel and Asselin 1989) with changes indicated in the text. The migration of most acidic PR proteins (PRb) is indicated by the bars on the right.

gel separation for acidic proteins (Fig. 5; healthy, a). These spots are indicated by numbered white arrowheads (Fig. 5; panel C, healthy, a). This technique is highly sensitive because it can reveal lysis zones within which no protein can be detected by silver staining (Fig. 5A; panels B and C, a, healthy, for example). One trailing spot could be detected in two-dimensional gels for basic proteins (Fig. 5; healthy, b). Three periods of renaturation were used to indicate the relative importance of active proteins. Analysis of IF extracts from tobacco leaf tissue infected with TMV showed increased activity of the four major acidic spots already detected in healthy tissue (Fig. 5; infected, a). Two of the spots corresponded to PR-P and PR-Q proteins (Fig. 5; panel C, infected, a, arrowheads 2 and 3). The major spot (arrowhead 1) corresponded to PR-b_{6c} and the fourth spot (arrowhead 4) to an unidentified protein. The relative intensity of lysis showed that PR-b_{6c} was by far the most active acidic chitinase. Analysis of silver-stained acidic PR proteins after (Fig. 5; panels A, B, and C, infected) and before renaturation (Fig. 5; panel D, a) revealed that PR-proteins b_1, b_2, b_3, b_{9b}, and b_{9c} were lost by elution during renaturation and incubation of proteins. The other PR proteins could be detected at various levels. Comparisons between the relative intensity of chitinases should take into account that chitinases can differ in specific activity and that the assay for chitinase in gels is not known to be linear under all conditions.

For basic proteins in infected leaf tissue (Fig. 5; infected, b), prolonged incubation (Fig. 5, panel C) revealed two trailing spots (arrowheads 6 and 7) and two definite spots (arrowheads 5 and 8). By comparing with a silver-stained two-dimensional pattern of the same PR proteins (Fig. 5; panel D, b), one basic protein corresponded to PR protein b_{13} (Hogue and Asselin 1987).

The sensitivity of detection of chitinase activity in the two-dimensional system is obviously much better than in one-dimensional gels. This increased sensitivity is partly due to the nature of the detection method (Coomassie blue and silver nitrate versus fluorescent brightener only). Samples with larger amounts of protein were also used because of some protein loss during the incubation of gels in buffer before electrophoresis in the second dimension. Detection of chitinase activity based on staining of glycol chitin with silver nitrate was not very useful in one-dimensional SDS-gels (as shown in Fig. 3) when compared to two-dimensional gels because lysis zones were masked by silver staining of multiple proteins comigrating as one band (like PR-O [PR-b_6] and PR-b_{13}-b_{14}, for example) (not shown). Staining of glycol chitin with silver nitrate is thus restricted to well-separated proteins such as those generated in two-dimensional gels.

DISCUSSION

Identical patterns of chitinase activity were found in gels containing colloidal chitin instead of glycol chitin. Gels with colloidal chitin did not yield patterns as clear as the ones with glycol chitin. Because of its solubility in water, glycol chitin can be embedded uniformly in gels. That is not the case for colloidal chitin. So, the pattern of electrophoretic forms of tobacco chitinases with glycol chitin can be compared to assays with colloidal chitin. However, there could be cases in which chitinases would digest colloidal chitin but not glycol chitin. We know of only one plant

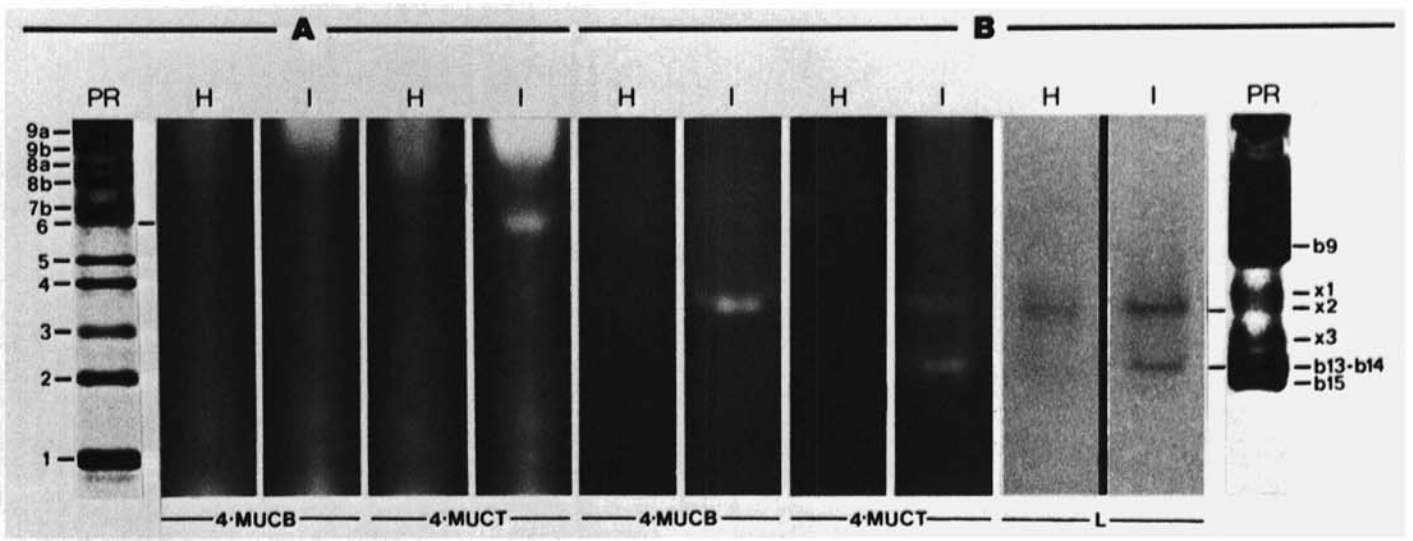

Fig. 4. Lytic activity toward 4-methylumbelliferylchitobiose, 4-methylumbelliferylchitotriose, and *Micrococcus lysodeikticus* (syn. *luteus*) cells after native PAGE for acidic or basic proteins. Homogenates from healthy leaf tissue (H) and from leaf tissue infected with tobacco mosaic virus (I) (50 μg of protein) were subjected to native PAGE for acidic proteins (**A**) as given in Figure 1 or to native PAGE for basic proteins (**B**) as given in Figure 2. After electrophoresis, gels were exposed to an overlay filter impregnated with 4-methylumbelliferylchitobiose (4-MUCB) or 4-methylumbelliferylchitotriose (4-MUCT). A gel for basic proteins was also used to detect lysozyme activity (L) against *M. lysodeikticus* cells. The bar on the left indicates the activity hydrolyzing 4-MUCT and the band of activity migrating at the position of PR-O (PR-b_6). The two bars on the right indicate the activities hydrolyzing 4-MUCB, 4-MUCT, and *Micrococcus* cells (L) with bands of activity migrating at the position of PRb_{13}-b_{14} and x_2. These two bands of activity represent the same major activities as shown by arrowheads in Figure 2. PR proteins (PR) are identified as given in Figure 1 (A, PR) or Figure 2 (B, PR).

chitinase (wheat germ) reported to digest colloidal chitin but not glycol chitin (Molano *et al.* 1979). It is also important to note that repeated freezing and thawing of samples did not change the number of chitinases. This held true when protease inhibitors were used (not shown).

It was noteworthy that PR-P and PR-Q did not have high levels of chitinase activity in the native gel system for acidic proteins. This result with Xanthi-nc tobacco is different from the one reported with Samsun NN tobacco (Legrand *et al.* 1987). We do not know whether PR-P and PR-Q from Samsun NN tobacco were contaminated by chitinase-active bands migrating at the position of PR-O (PR-b_6). In our gel assays, PR-P and PR-Q had rather low (minor) levels of activity. They were best revealed in

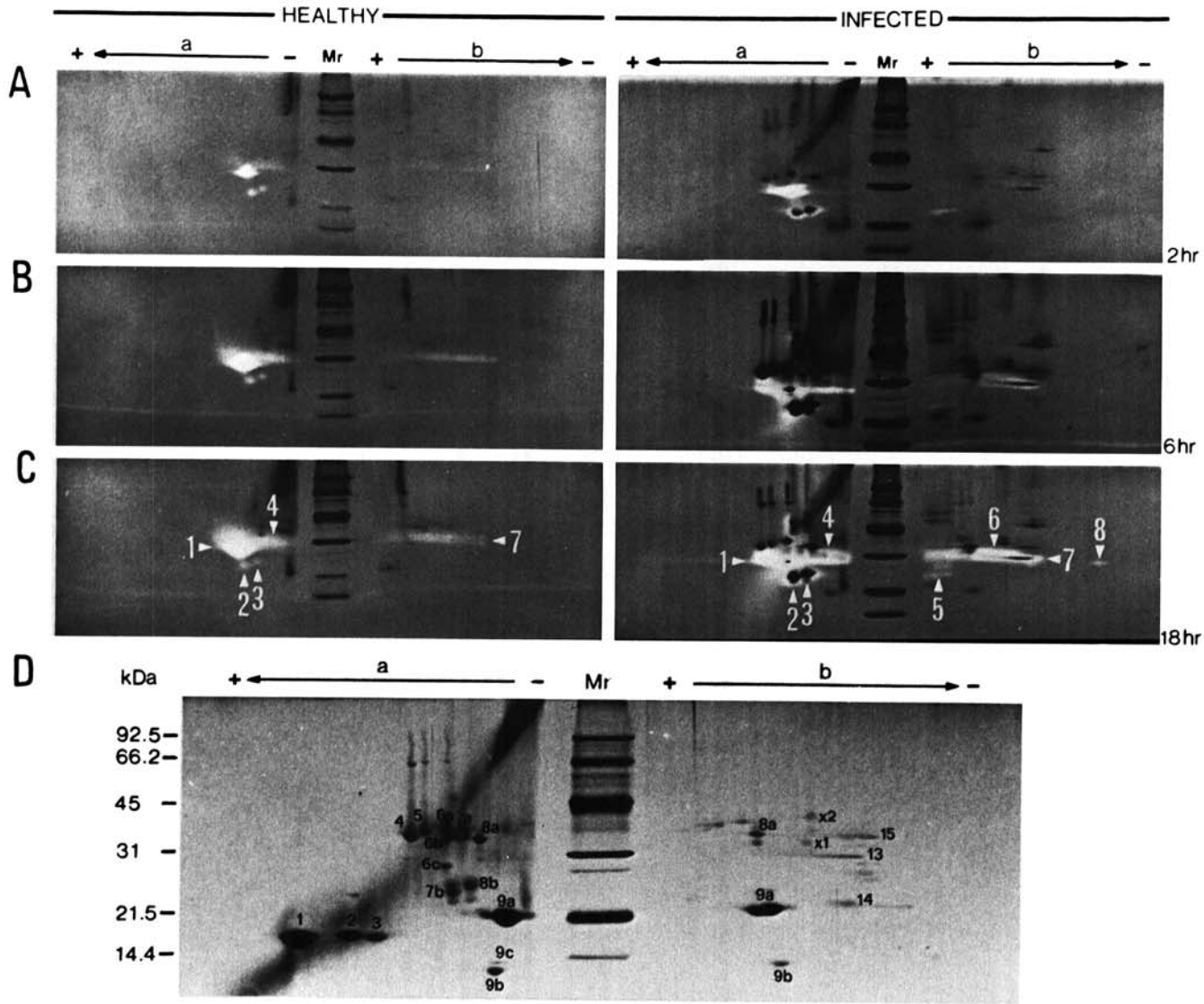

Fig. 5. Chitinase activity and silver staining after separation of proteins from intercellular fluid (IF) extracts in two-dimensional gel systems. IF extracts of healthy Xanthi-nc tobacco leaf tissue and leaf tissue infected with tobacco mosaic virus (TMV) were subjected to native PAGE for acidic proteins (a) (as given in Fig. 1) or basic proteins (b) (as given in Fig. 2) in the first dimension (horizontal arrows). After native PAGE, gel slices were incubated in sodium dodecyl sulfate (SDS) buffer and subjected to SDS-PAGE (as given in Fig. 3) (top to bottom of panels). After SDS-PAGE, renaturation of enzymes was allowed to proceed for 2 (panel **A**), 6 (panel **B**), and 18 hr (panel **C**). After renaturation, gels were stained with Calcofluor White M2R, Coomassie Brilliant Blue R 250, and aqueous silver nitrate. White zones correspond to lytic activity against glycol chitin embedded in the SDS gel. Samples of extracts from healthy leaf tissue and from leaf tissue infected with TMV contained 1.5 μg and 60 μg of protein, respectively (three times the amount used in Figs. 1 and 2). In panel **C** (18 hr) on the left, the four arrowheads in the gel for acidic proteins (a) indicate the position of pathogenesis-related (PR) proteins b_{6c} (arrowhead 1), PR-P (arrowhead 2), PR-Q (arrowhead 3), and an unidentified protein (arrowhead 4). In the same panel in the gel for basic proteins (b), arrowhead 7 indicates the position of PR-b_{13}. In panel **C** (18 hr) on the right, the arrowheads indicate the position of the same proteins with the addition of three arrowheads (arrowheads 5, 6, and 8) corresponding to the unidentified proteins. Molecular mass markers were denaturated with SDS and mercaptoethanol; intercellular fluid (IF) extracts were denatured only with boiling buffered SDS (Trudel and Asselin 1989). That is why hen egg white lysozyme (14.4 kDa) did not display chitinase activity (Trudel and Asselin 1989). In panel **D**, the same IF extracts were stained with Coomassie blue followed by aqueous silver nitrate. Protein nomenclature is according to Hogue and Asselin (1987). Horizontal arrows indicate the direction of migration in the first dimension.

two-dimensional gels and represented minor signals despite large amounts of these two proteins. Their specific activity must be very low unless different chitinases coincidentally comigrate with PR-P and PR-Q. The signals accompanying PR-P and PR-Q could not be enhanced by changing pH, ionic strength, temperature, buffer, or protein concentration (not shown).

The strongest acidic activity corresponded to PR-b_{6c} as previously identified in two-dimensional gels (Hogue and Asselin 1987). This is consistent with previous results indicating that three proteins comigrate at the level of PR-O (PR-b_6): one peroxidase (b_{6a}), one β-1,3-glucanase (b_{6b}) (Kauffmann *et al.* 1987; Côté *et al.*, 1989), and one chitinase (this work). The diversity of basic chitinases is as complex as the one for acidic proteins. Previously, only two activities differing in molecular mass were reported (Shinshi *et al.* 1987; Legrand *et al.* 1987; Hooft van Huijsduijnen *et al.* 1987). It could well be that the two major basic chitinases detected in gels are identical to the two basic chitinases previously described (Shinshi *et al.* 1987; Legrand *et al.* 1987). The estimation of the molecular masses of these two basic proteins differed slightly (about 30 kDa versus 34 and 32 kDa) from previous determinations (Shinshi *et al.* 1987).

Some isoforms of chitinases could not be detected in two-dimensional gels. For example, there is no lysis near PR-1c (PR-b_3) despite significant chitinase activity in native gels for acidic proteins. Some chitinases can only be renatured in the presence of SDS alone (without a reducing agent); others can resist denaturation by SDS and a reducing agent. Some of the factors affecting renaturation of chitinases or lysozymes have been previously discussed (Audy *et al.* 1989; Trudel and Asselin 1989). The absence of some chitinase activities in two-dimensional gels can reflect the sensitivity to denaturation, insufficient amounts of protein, or loss of protein during renaturation. Some chitinases (like the two chitinase-active bands migrating near PR-1c [Fig. 1A]) were eluted from preparative gels and concentrated (20×). Such concentrates yielded no signal, indicating that these enzymes could not be renatured (not shown).

The ratio of hydrolysis of 4-MU chitobiose and 4-MU chitotriose has been proposed as a new criterion for identifying endochitinases versus exochitinases (Robbins *et al.* 1988). According to this criterion, the main acidic activity (mobility of PR-O) in diseased tissue could be classified as an endochitinase. This would be the same for the basic chitinase at the position of PR-b_{13}-b_{14} (Hogue and Asselin 1987). However, the activity in healthy and diseased leaf tissue with bands migrating near the x_2 protein (Hogue and Asselin 1987) could be tentatively classified as an exochitinase (Robbins *et al.* 1988). To our knowledge, there are only two reports dealing with exochitinases in plants (Roby and Esquerré-Tugayé 1987; Fink *et al.* 1988).

Some chitinases, like wheat germ chitinase, yam root chitinases, and one cucumber chitinase do not display lysozyme activity (Molano *et al.* 1979; Tsukamoto *et al.* 1984; Métraux *et al.* 1989). However, the two main basic chitinases in tobacco tissue showed lysozyme activity in gels. Lysozyme activity for acidic chitinases was not well-resolved with respect to basic proteins but was significant at the position of PR-b_{6c}. It would be interesting to evaluate the capacity of such stimulated lysozyme to digest other microbial cells or cell walls (Potvin *et al.* 1988; Leclerc and Asselin, in press), or to interact with endogenous plant cell wall components (Audy *et al.* 1988b).

Identification of chitinases as PR proteins in one-dimensional gel systems should be interpreted with caution. Using two-dimensional gels should be favored in the identification of PR proteins with enzymatic activity. We now have gel systems allowing the visualization of electrophoretic forms of chitinase activity in plant tissue that can be complemented with assays of lysozyme activity and hydrolysis of chitin oligosaccharides. The diversity of chitinases in healthy and stressed tissue is more complex than previously estimated. The use of such assays will help to develop a better understanding of the array of hydrolases having potential antimicrobial activity.

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