The Ethylene Biosynthesis-Inducing Xylanase: Its Induction in *Trichoderma viride* and Certain Plant Pathogens

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ABSTRACT

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A protein component of Cellulysin is known to induce ethylene biosynthesis in a variety of plant tissues and harbors an endo-β-1,4-xylanase activity. Antiserum to the native ethylene biosynthesis-inducing xylanase immunoprecipitates both the enzymatic and biological activities. However, antisera raised against either the denatured 22- or 14-kilodalton (kDa) polypeptides comprising the native protein were ineffective in precipitating either activity. All three antibodies recognized the 14- and 22-kDa antigens on immunoblots. Synthesis of a single 22-kDa extracellular polypeptide detectable on immunoblots with the three antisera against the Cellulysin polypeptides was inducible in *Trichoderma viride* during growth on D-xylose, xylan, or crude cell-wall preparations. Induction was not observed when the fungus was grown on L-xylose, β-methyl-D-xylose, glucose, or several purified cell-wall polymers, including pectin, polygalacturonate, arabinogalactan, and cellulose. Production of this protein was influenced by substrate concentration and culture pH. When grown in the induction medium, several other species of *Trichoderma* and *Gliocladium* also synthesize a 22-kDa xylanase that could be detected on immunoblots and was capable of inducing ethylene biosynthesis. Isoelectric focusing demonstrated that the cross-reactive polypeptides from these fungi exist as isoforms. The primary form had a pI of 9.4, and less abundant forms focused at pI 8.4 and lower. Culture filtrates of two plant pathogens, *Fusarium oxysporum* f. sp. *pisi* and *Macrophomina phaseolina*, also contained ethylene biosynthesis-inducing and xylanase activities, as well as a 22-kDa cross-reactive polypeptide. *M. phaseolina* filtrates also contained substantial amounts of a 14-kDa polypeptide similar to that found in Cellulysin.

Additional keywords: cell-wall hydrolases, pathogenesis.

Ethylene production is one of the earliest responses by plant tissues to attack from pathogenic organisms (23,30). The rapid induction of ethylene biosynthesis can be mimicked by treatment of plant tissue or cells with elicitors derived from extracellular material from either fungi or plants (1,12,36,37,39). Treatment with these elicitors also induces the rapid synthesis of proteins such as L-phenyalanine ammonia-lyase (13), chitinase (9,31), proteinase inhibitors (6), and hydroxyproline-rich glycoprotein (38). Some researchers think that these proteins comprise an inducible defense mechanism against plant pathogens (8,32,40). However, other evidence suggests that such pathogen-related

ethylene is merely a symptom of infection with little or no direct involvement in pathogen resistance (25,29).

Ethylene biosynthesis in plant tissues also may be induced by commercial preparations of cell-wall hydrolyzing enzymes used primarily to prepare plant protoplasts (1–4,26). We have been working to elucidate how ethylene biosynthesis is induced by Cellulysin, a product of the nonpathogenic fungus *Trichoderma viride* Persoon ex Fries. The ethylene biosynthesis-inducing factor from Cellulysin was purified to a fraction containing three polypeptides of molecular masses 18, 12, and 10 kilodaltons (kDa) (19). This protein fraction contains an endo-β-1,4-xylanase (EC 3.2.1.8) activity, which is consequently referred to as the ethylene biosynthesis-inducing xylanase (EIX) (20). Antibodies raised against this protein pool immunoprecipitate both the ethylene biosynthesis-inducing and xylanase activities and immunodecorate the 18- and 12-kDa polypeptides of the antigen preparation on blots.

In the course of the present work, we found that the molecular masses of the 18- and 12-kDa polypeptides in the Cellulysin EIX fraction (20) migrate as 22- and 14-kDa polypeptides in a modified electrophoretic system. This is primarily a result of altering the acrylamide-to-bisacrylamide ratio of the gels and the differing mobilities of biotinylated versus prestained molecular weight markers. Hereafter, the primary polypeptides will be referred to as measuring 22 and 14 kDa.

Although ethylene induction is inseparable from the xylanase activity for EIX, ethylene biosynthesis-inducing activity is not a trait common to all xylanases. Of the limited number of commercial enzyme preparations tested, only those from *T. viride* contained proteins cross-reactive with the antibodies. In this report we demonstrate that synthesis of the ethylene biosynthesis-inducing xylanase by *T. viride* is induced by growth on appropriate substrates, that both xylanase and ethylene biosynthesis-inducing activites reside on the same protein(s), and that similar proteins are produced by several species of fungi when grown in media containing xylan as the carbon source. Preliminary reports of these studies have been made (2,14).

MATERIALS AND METHODS

Organisms and reagents. Fungal strains were kindly provided by G. C. Papavizas of the Biocontrol of Plant Diseases Laboratory, Beltsville Agricultural Research Center, Beltsville, MD. Pansorbin and Cellulysin were obtained from Calbiochem Corp., La Jolla, CA; Cellulase "Onozuka" RS was from Yakult Pharmaceutical Industry Co., Ltd., Nishinomiyo, Japan; *T. viride* cellulases Types V and VI were from Sigma Chemical Co., St. Louis, MO; and *T. viride* xylanase was from Fluka Chemical Corp., Ronkonkoma, NY. Birchwood β-D-xylan was from Atomergic Chemetals Corp., Farmingdale, NY; citrus pectin and polygalacturonic acid were from ICN Pharmaceuticals, Inc., Covina, CA; carboxymethyl cellulose (Type 7 MP) was from Hercules, Inc., Wilmington, DE; potato starch was from Fisher Scientific Co., Pittsburgh, PA; ground corn cobs (#30 mesh) were from The Andersons, Maumee, OH; peanut hulls were from Birdsong Peanuts, Franklin, VA; and NutriSoy was from Archer-Daniels-Midland Co., Decatur, IL. Tobacco cell walls were prepared from *Nicotiana tabacum* L. 'Xanthi' leaves by the method of Tong et al (36). All other carbon sources were from Sigma. Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and biotinylated molecular weight markers were obtained from Bio-Rad Laboratories, Richmond, CA; polyvinylidene difluoride (PVDF) membrane (Immobilon-P) was from Millipore, Bedford, MA; ampholytes (pH 3–10) were from Pharmacia LKB Biotechnology, Inc., Piscataway, NJ; and isoelectric focusing (IEF)-grade agarose, GelBond film, and pI markers were from FMC BioProducts, Rockland, ME. All other reagents were obtained at the highest purity available and used without further purification.

Assays. Protein was estimated versus a lysozyme standard by the method of Bradford (10). Induction of ethylene biosynthesis in tobacco leaf disks was measured as described previously (19). Xylanase activity was measured as nanomoles of reducing sugar released from birchwood β-D-xylan per minute using a modification of the bicinchonic acid (BCA) assay (41). The xylan composition was 85% by weight neutral sugars, of which 100% was D-xylose (K. Gross, *personal communication*). Appropriately diluted enzyme in 100 μl of 20 mM sodium acetate (pH 5.0) was mixed with 100 μl of 0.01% xylan in the same buffer on ice. After a 10-min incubation at 50 C, reactions were stopped by boiling for 1 min. BCA reagent (500 μl) was added and the tubes were boiled for an additional 15 min. After cooling to room temperature, the absorbance at 560 nm was determined and compared to a standard curve for D-xylose. Reaction blanks were made by adding xylan substrate to the tube after the enzyme had been boiled.

Preparation of antibodies. Cellulysin EIX purified by adsorption to Sephacryl S-200 was electrophoresed in SDS-PAGE gels and stained with Coomassie Blue R-250 as described previously (19). Stained bands corresponding to the 22- and 14-kDa polypeptides were excised from the gels, and protein was electroeluted from the gel slices. Polyclonal antibodies were raised against each of the electroeluted polypeptides in New Zealand White rabbits (20) and purified from sera by three rounds of $(NH_4)_2SO_4$ precipitation (33% saturation).

Culturing conditions. All fungi were grown in a minimal salts medium (5) containing 0.1% (w/v) carbon substrates as described. Cultures were inoculated with 2-day-old mycelia growing on potato-dextrose agar and incubated at 25 C with 100 cycles/min orbital agitation. Cultures other than those used for time course experiments were harvested after 4 days of growth. For the induction experiments with different fungal species, the culture medium was to 0.1% birchwood xylan and pH 7.0.

Culture filtrates were prepared by vacuum filtration through glass-fiber filters (GF/C, Whatman Chemical Separation, Inc., Clifton, NJ) to remove the bulk mycelia before centrifugation at 10,000 *g* for 15 min to remove the remaining debris. Filtrates were stored at −20 C without further treatment. For culture filtrates requiring concentration, total protein was precipitated by the addition of solid $(NH_4)_2SO_4$ to 80% saturation. Precipitated protein, collected by centrifugation (45 min, 14,000 *g*), was resuspended in and dialyzed against 20 mM sodium acetate (pH 5.0). After a final centrifugation (20 min, 40,000 *g*), concentrates were stored at −20 C.

SDS-PAGE. Proteins were fractionated in 20% polyacrylamide minigels (5 × 8 × 0.075 cm) (7). Biotinylated molecular weight markers were run on each gel. Protein bands were visualized by silver staining (43); however, the 14-kDa EIX polypeptide was negatively stained by this procedure. The 14-kDa EIX band was visualized by first destaining the gel with sequential addition of 0.075% potassium ferricyanide and 2% sodium thiosulfate. Destaining did not need to be complete. The gel then was washed thoroughly in H_2O, and the staining procedure was repeated.

Immunoblotting. Proteins from unstained SDS-PAGE gels were electroblotted to PVDF membranes by a 1-hr transfer at 100 V. The blots were then probed with antibodies as described by Johnson et al (24). Incubation with the primary antibody (that raised against the 22-kDa polypeptide, unless otherwise noted) at 1:10,000 dilution was carried out for at least 3 hr. After extensive washing in phosphate-buffered saline, blots were incubated for 1 hr with goat anti-rabbit IgG-alkaline phosphatase conjugate (Bio-Rad) and ExtrAvidin-alkaline phosphatase conjugate (Sigma) to detect immunodecorated bands and biotinylated molecular weight markers, respectively. Bands were visualized by the nitroblue tetrazolium/5-bromo-4-choro-3-indolyl-phosphate staining protocol provided by Bio-Rad. Blots were photographed after air drying.

Analytical isoelectric focusing. Isoelectric focusing was performed in agarose gels consisting of 1% IEF-grade agarose, 12% sorbitol, and 6.33% ampholytes (pH 3–10) cast on GelBond polyester sheets. The anode and cathode buffers were 40 mM aspartic acid and 1.0 M NaOH, respectively. Gels were run for a total of 1,000–1,200 volt hours in 90 min at a temperature of 5 C. Gels to be stained were fixed in 10% trichloroacetic acid

plus 5% sulfosalicylic acid for 1 hr, washed thoroughly in 30% methanol containing 10% acetic acid, and air dried. Dried gels were stained with 0.05% Blue W (Serva Biochemicals, Westbury, NY) in water (w/v) for 30 min and destained in water. For immunoblotting, a sheet of PVDF membrane was wetted in methanol and rinsed in water before being placed on the surface of the gel. The gel and membrane then were inverted onto filter paper in a gel drier and vacuum was applied for 10 min. Blots were probed with the antibody and photographed as described for SDS-PAGE immunoblots.

RESULTS

Antibody characterization. The antibodies raised against the native Cellulysin EIX immunoprecipitate the endoxylanase and ethylene biosynthesis-inducing activities in parallel (20) and immunodecorate the 22- and 14-kDa polypeptides on immunoblots (Fig. 1A and B). Antibodies raised against either the 22- or 14-kDa polypeptide (Fig. 2C and D) immunodecorate the same polypeptides as antibodies against the native enzyme (Fig. 2B) on immunoblots. All three antibodies also cross-react with 14- and 22-kDa polypeptides in other commercial preparations of hydrolytic enzymes of *T. viride*. Because the antibodies raised against the denatured 22- and 14-kDa polypeptides immunodecorate the antigenic bands on blots more strongly than the antibodies against native enzyme, the antibody against the 22-kDa polypeptide was selected for routine analyses of further blots. The antiserum against the 14-kDa polypeptide was not selected for routine use because it immunodecorated a second pair of polypeptides found only in Cellulysin preparations (Fig. 2D, lanes 3 and 6).

Unlike the antiserum against the native EIX polypeptides (Fig. 1), the antisera against the denatured polypeptides showed little or no ability to immunoprecipitate either xylanase or ethylene biosynthesis-inducing activities from solution (Figs. 3 and 4). There was some enhancement of activity in the bioassay for enzyme samples treated with increasing amounts of the nonprecipitating antibodies, but this could be mimicked with preimmune serum or bovine serum albumin and therefore was ascribed to a protein stabilization effect.

Production of EIX by *T. viride* in culture. *T. viride* T-1 grown in a minimal salts medium containing 0.1% birchwood β-D-xylan produced significant levels of both xylanase and ethylene biosynthesis-inducing activities (Table 1). These culture filtrates may be fractionated to a pool containing > 90% of the total ethylene biosynthesis-inducing activity by ion-exchange chromatography on carboxymethyl-Sepharose (J. F. D. Dean and J. D. Anderson, *unpublished*). This pool contained two polypeptides of 22 and 27 kDa; the 22-kDa polypeptide is strongly cross-reactive with all three antisera on immunoblots (Fig. 2, lane 2). This cross-reactive polypeptide, as well as both enzyme activities, was produced by *T. viride* grown on oat-spelt β-D-xylan, D-xylose, and crude cell-wall preparations but not by fungus grown on D-glucose, L-xylose, β-methyl-D-xylose, α-cellulose, CM-cellulose, polygalacturonate, or arabinogalactan (Table 1). The ethylene biosynthesis-inducing and xylanase activities partially purified from xylan-grown cultures were both immunoprecipitated in parallel by antiserum against the native EIX (Fig. 1D) but not by the antisera against the 22- and 14-kDa polypeptides (Figs. 3D and 4D). And only the 22-kDa polypeptide

TABLE 1. Induction of *Trichoderma viride* ethylene biosynthesis-inducing xylanase by various carbon sources

Carbon source	Specific activity xylanase (μmoles red. equiv./min/mg protein)	Specific activity ethylene induction (μl C_2H_4/g fresh wt./hr/mg protein)
D-glucose	0.0	1
D-xylose	3.5	143
L-xylose	0.2	0
Methyl-D-xylose	0.0	0
Xylan, birchwood	6.8	286
Xylan, oat-spelt	4.6	211
α-cellulose	0.0	0
CM-cellulose	0.0	0
Polygalacturonate	0.0	0
Arabinogalactan	3.0	0
Pectin, citrus	0.0	25
Tobacco cell wall	0.8	66
Corn cob	3.2	144
Peanut hull	0.6	82
NutriSoy	18.0	2,860

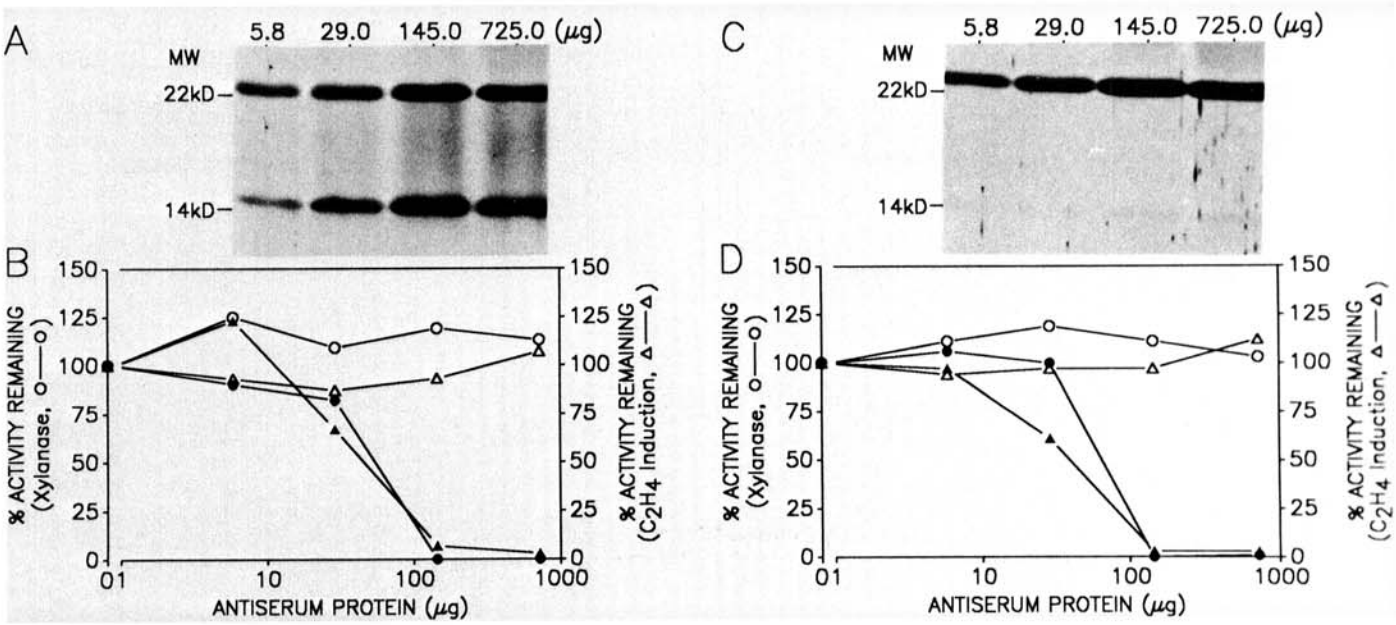

Fig. 1. Immunoprecipitation of Cellulysin and *Trichoderma viride* ethylene biosynthesis-inducing xylanases (EIXs) by antiserum against native EIX. Equivalent amounts (100 ng) of **A and B,** purified Cellulysin, or **C and D,** *T. viride* EIX were incubated with increasing amounts (0, 5.8, 29, 145, and 725 μg) of antiserum overnight at 4 C, and antibody-EIX complexes were removed by adsorption to Pansorbin (5 μl for 3 hr at 25 C) followed by centrifugation. Panels A and C are immunoblots of material adsorbed to Pansorbin detected with antibody against the 22-kDa polypeptide. Panels B and D depict the relative enzymatic activities remaining after immunoprecipitation with antiserum against native enzyme (closed symbols) versus control antiserum (open symbols).

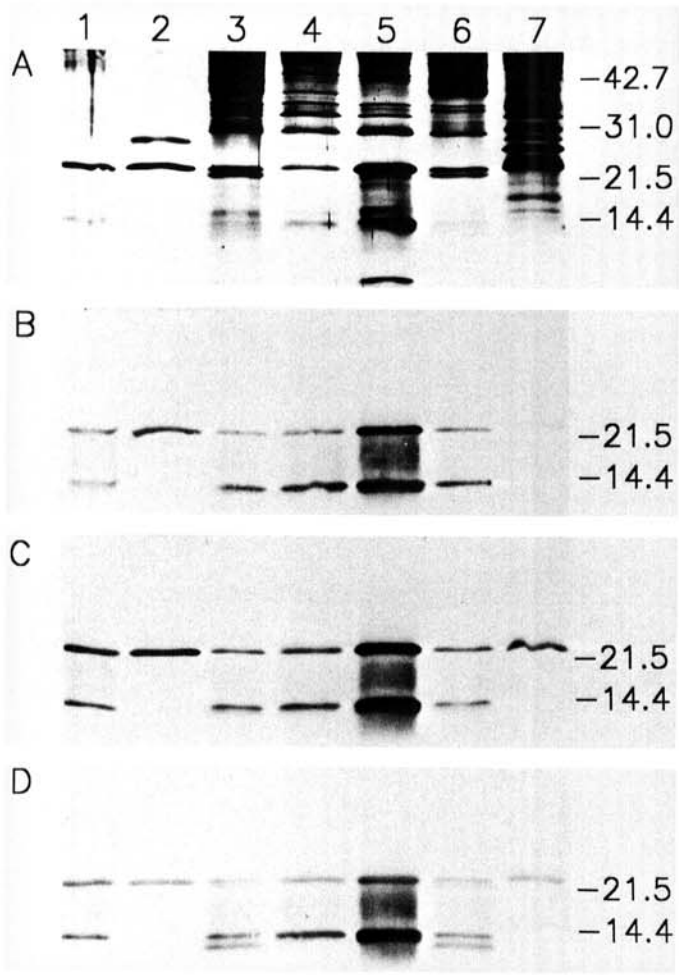

Fig. 2. Immunoblots of purified ethylene biosynthesis-inducing xylanase (EIX) and of commercial preparations of *Trichoderma viride* hydrolytic enzymes. Proteins (2.5 μg) were silver stained **(A),** and cross-reactive polypeptides were detected with antibodies against: **B,** native EIX, **C,** 22-kDa polypeptides, or **D,** 14-kDa polypeptides. Lane 1, purified Cellulysin EIX (200 ng); lane 2, purified *T. viride* (T-1) EIX (100 ng); lane 3, Cellulysin; lane 4, Cellulase Onozuka RS; lane 5, xylanase from Fluka Chemical Corp.; lane 6, cellulase (type V) from Sigma Chemical Co.; lane 7, cellulase (type VI) from Sigma.

was detected on immunoblots of resuspended immunoprecipitates of the *T. viride* culture material (Fig. 1C).

Previous work showed that the greatest yield of *T. viride* EIX is obtained during growth on 0.1% xylan (2). The initial culture pH also affects the production of EIX by *T. viride*. Changing the pH of the medium from 4.0 to 8.3 before culturing results in a 3.5-fold increase in both specific activity and total EIX production at the higher pH (Fig. 5). The bulk of this increase occurs between pH 7.8 and 8.3.

EIX is one of the first proteins secreted by *T. viride* in response to growth on xylan (Fig. 6). When a culture of *T. viride* grown 3 days in a noninducing medium of 0.1% D-glucose is shifted to 0.1% xylan, significant levels of xylanase and ethylene biosynthesis-inducing activities are apparent by 4 hr after xylan addition, total activity levels off at 14 hr, and maximal levels are attained by 48 hr. The increases in both enzyme activities are paralleled by increased production of the 22-kDa cross-reactive polypeptide.

Production of EIX enzymes by other fungi. Several fungal species were grown on medium containing 0.1% xylan and culture filtrates were screened for ethylene biosynthesis-inducing and xylanase activities (Fig. 7). All of the *Trichoderma* and *Gliocladium* strains produced significant levels of both activities. The best producer in terms of both total and specific ethylene biosynthesis-inducing activity was *T. hamatum* (Bonorden) Bainier Tri-4. An immunoblot of the culture supernatants shows that all these fungi produced a nearly identical pattern of cross-reactive polypeptides with the primary product from each migrating at an apparent molecular weight of 22 kDa (Fig. 7B). However, minor component cross-reactive bands, primarily of lower apparent molecular weights, were detectable on blots from all these cultures.

Culture filtrates from all eight *Trichoderma* and *Gliocladium* strains were subjected to isoelectric focusing from pH 3 to pH 10 and then either stained or blotted to PVDF membrane for visualization with antiserum against the 22-kDa polypeptide. Figure 8 shows that the primary band in each sample, corresponding to the bulk of the fungal EIX protein, has an apparent pI of 9.4, whereas minor bands appear to focus at lower pIs. In contrast, the major isoform of the Cellulysin EIX focuses at pI 8.4, but other isoforms also appear at lower pI values (lane 10), resulting in a pattern that resembles the elution profile of this enzyme from an isoelectric focusing column (20).

Eight fungal pathogens also were incubated in the inducing medium and were tested for the production of EIX cross-reactive

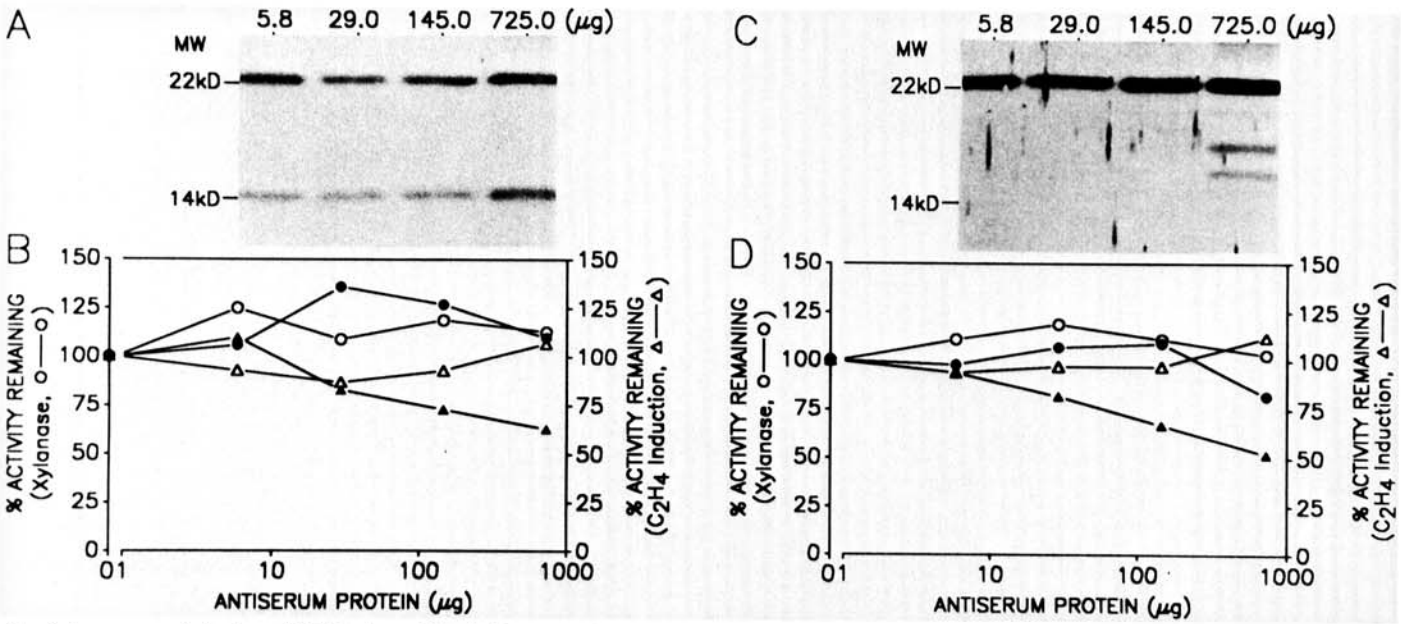

Fig. 3. Immunoprecipitation of Cellulysin and *Trichoderma viride* ethylene biosynthesis-inducing xylanases by antiserum against the 22-kDa polypeptide. Enzymatic activities and immunoprecipitated polypeptides were measured and depicted as detailed in Figure 1 after substitution of equal amounts of antiserum specific for the 22-kDa polypeptide in place of antiserum against the native enzyme.

polypeptides. Four of these, *Verticillium dahliae* Klebahn 18704, *Sclerotium cepivorum* Berkeley N23, *S. hydrophilum* Saccardo 32151, and *Phytophthora megasperma* Drechsler 16705, grew little or not at all in this medium. *Penicillium digitatum* Saccardo and *Rhizoctonia solani* Kuhn R23 grew well, but their culture media contained no cross-reactive polypeptides and no ethylene biosynthesis-inducing activity. However, filtrates from both *Fusarium oxysporum* f. sp. *pisi* (Hartig) Snyder et Hansen (F-52) and *Macrophomina phaseolina* (Maublanc) Ashby (MP-2) were able to induce ethylene biosynthesis in tobacco and contained polypeptides detectable on immunoblots (Fig. 7). After a 50-fold concentration of these culture filtrates by ion exchange chromatography, immunoblots of the *Fusarium* filtrate showed a single band of 22 kDa, whereas the *Macrophomina* filtrate contained cross-reactive polypeptides of 14 and 22 kDa (Fig. 7B, inset).

DISCUSSION

Initial experiments comparing immunoblots of several commercially available enzyme preparations of *T. viride* showed that nearly all contained the same 22- and 14-kDa polypeptides previously purified as EIX from Cellulysin. One commercial preparation (Fig. 2, lane 7) contained only the 22-kDa cross-reactive polypeptide, yet retained ethylene biosynthesis-inducing and xylanase activities comparable to the other preparations. Separate polyclonal antibodies against the SDS-PAGE-resolved 22- and 14-kDa polypeptides purified from Cellulysin recognized both polypeptides on immunoblots. The sharing of antigenic determinants by the 14- and 22-kDa polypeptides suggests that the 14-kDa polypeptide might be derived from the 22-kDa polypeptide.

Although the antibodies against the denatured 22- and 14-kDa polypeptides recognized both antigens on immunoblots, they were not very effective in immunoprecipitating either ethylene biosynthesis-inducing or xylanase activities at antibody concentrations equivalent to those used with the antibody against the native enzyme. Yet these antibodies can immunoprecipitate a small amount of either the 22- and 14-kDa polypeptides from Cellulysin EIX or the 22-kDa polypeptide from *T. viride* EIX (Figs. 1 and 3). Evidently, the primary epitopes recognized by the antibodies raised against the denatured forms of the enzyme are sequestered when the active protein is free in solution and

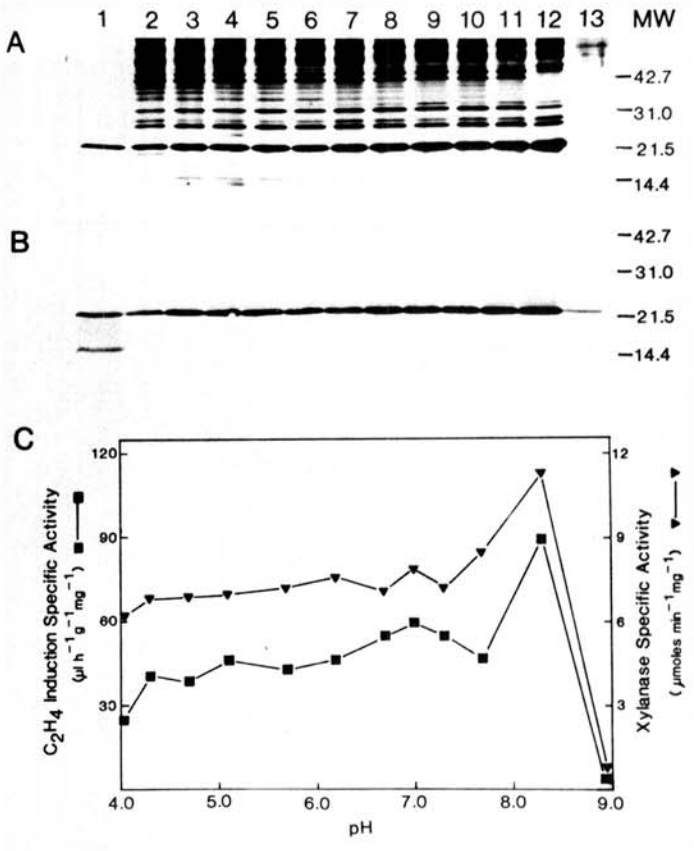

Fig. 5. The effect of initial culture pH on induction of *Trichoderma viride* ethylene biosynthesis-inducing xylanase (EIX). Trichloroacetic acid-precipitable protein in aliquots (50 μl) from 0.1% xylan cultures of increasing initial pH was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and either **A,** silver stained, or **B,** immunoblotted. Lane 1 contains a standard of purified Cellulysin EIX (100 ng). Samples corresponding to lanes 2–13 also were assayed for specific ethylene biosynthesis-inducing and xylanase activities as shown plotted against the initial culture pH **(C).** Units for ethylene biosynthesis are μl C_2H_4/hr/g fresh weight tobacco leaf tissue/mg EIX protein.

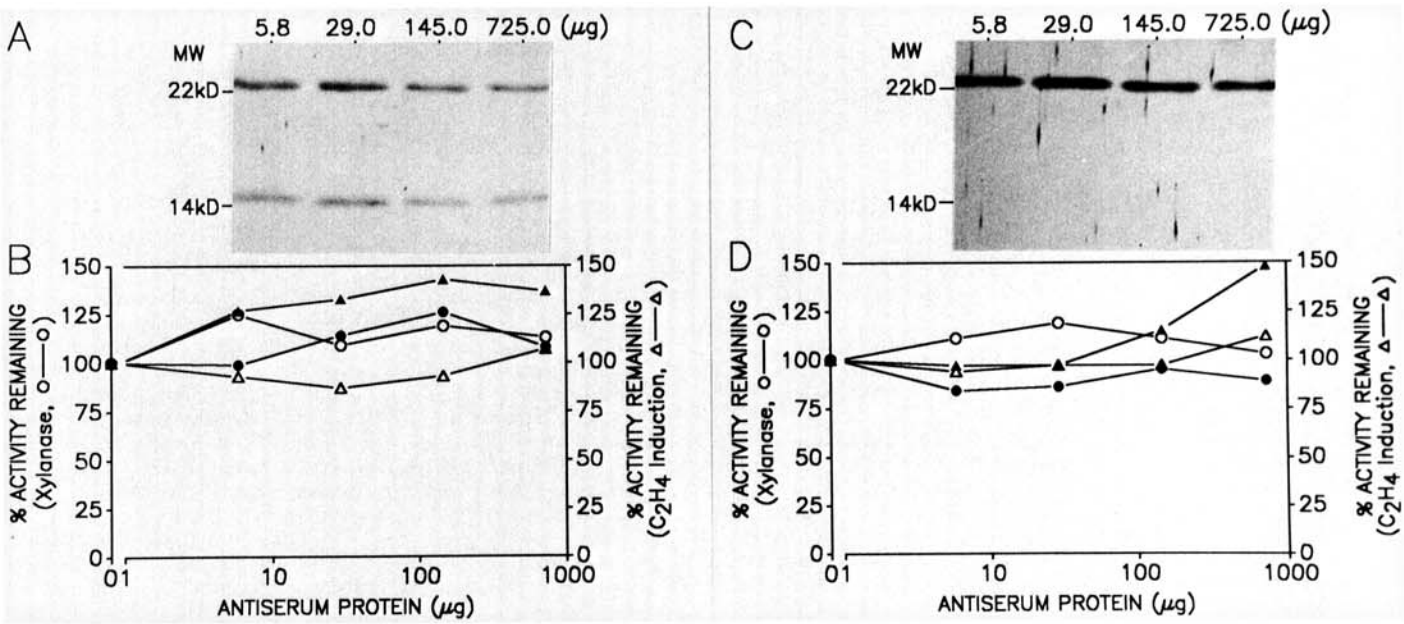

Fig 4. Immunoprecipitation of Cellulysin and *Trichoderma viride* ethylene biosynthesis-inducing xylanases by antiserum against the 14-kDa polypeptide. Enzymatic activities and immunoprecipitated polypeptides were measured and depicted as detailed in Figure 1 after substitution of equal amounts of antiserum specific for the 14-kDa polypeptide in place of antiserum against the native enzyme.

therefore are unavailable to participate during immunoprecipitation. The small amounts of polypeptides precipitated may represent either denatured and inactive enzyme or a very small clonal population of antibodies recognizing the native enzyme.

Different size polypeptides of EIX sharing the same epitopes could be generated by proteolysis such as that demonstrated for fungal endoglucanases in liquid culture (18,21,28), as well as in a commercial preparation of enzymes of *T. viride* (27). Alternatively, the two EIX proteins might result from a post-translational modification, such as glycosylation, which confers anomalous electrophoretic properties to the recipient, as has been demonstrated for a xylanase from *Stereum sanguinolentum* Albertini et Schweinitz ex Fries (17). At least one xylanase from *T. viride* previously has been shown to be glycosylated (35).

To understand better how the two polypeptides are related, *T. viride* T-1 was cultured in a minimal salt medium containing birchwood xylan as the sole carbon source. Under these conditions, substantial amounts of the 22-kDa EIX were secreted into the culture medium, but only low levels of the 14-kDa polypeptide were produced. A few other cross-reactive polypeptides also were faintly visible at molecular weights intermediate to the 14- and 22-kDa forms of EIX on heavily loaded immunoblots. This evidence would indicate that the 22-kDa EIX is the original form of the enzyme produced by *T. viride*. The identity of the minor cross-reactive forms is still uncertain but might also represent glycosylations.

Because fungal xylanases may be either constitutive or induced (15), several other carbon sources were tested for the ability to induce EIX. The EIX, as determined by ethylene biosynthesis-inducing and xylanase activities, was best induced by β-D-xylan (birchwood and oat-spelts), D-xylose, and crude plant cell-wall preparations including ground corn cobs, soybean pressings (NutriSoy), and tobacco cell walls, with birchwood xylan giving the best overall yield (Table 1). The inducibility of EIX is demonstrated in Figure 6 as the appearance of xylanase and ethylene biosynthesis-inducing activities, as well as the 22-kDa cross-reactive band, within 4 hr of xylan addition. The 3.5-fold increase in EIX specific activity in response to an increase in culture pH from 4.0 to 8.3 (Fig. 5) is caused in part by an increase in the total amount of the 22-kDa polypeptide synthesized, as well as by an apparent qualitative and quantitative shift in the synthesis of the other secreted polypeptides.

Xylanases previously isolated from other *Trichoderma* species (5,22,33,34,42) share such physical characteristics as molecular

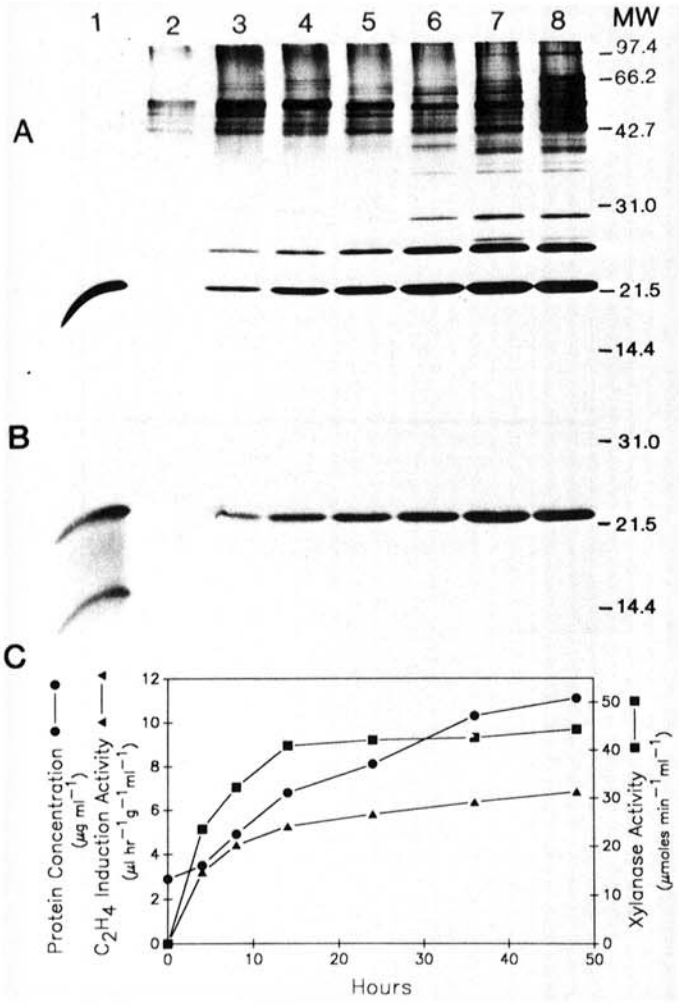

Fig. 6. Time course of *Trichoderma viride* ethylene biosynthesis-inducing xylanase (EIX) induction by xylan. A 3-day-old culture of *T. viride* grown on 0.1% D-glucose was made to 0.1% xylan, and samples were removed at subsequent times. Trichloroacetic acid-precipitable protein in aliquots (50 μl) was analyzed after sodium dodecyl sulfate-polyacrylamide gel electrophoresis by **A,** silver staining, or **B,** immunoblotting. Lanes 2–8 contain samples taken at 0, 4, 8, 14, 24, 36, and 48 hr after xylan addition, respectively. Lane 1 contains a standard of purified Cellulysin EIX (100 ng). Panel C depicts the protein concentration and the ethylene biosynthesis-inducing and xylanase activities in filtrates from each time point. Units for ethylene biosynthesis are μl C_2H_4/hr/g fresh weight tobacco leaf tissue/mg EIX protein.

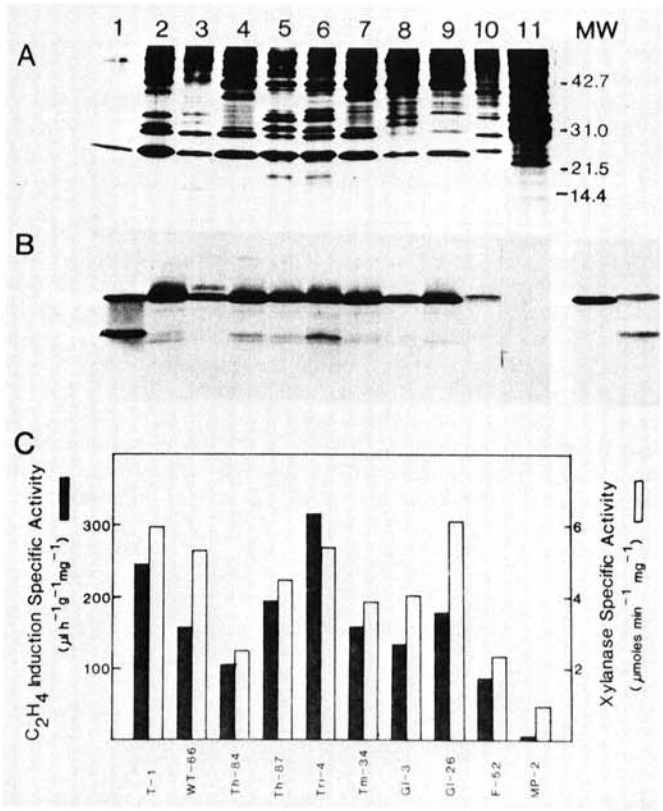

Fig. 7. Production of polypeptides cross-reactive with ethylene biosynthesis-inducing xylanase (EIX) antibodies by several fungal species. Trichloroacetic acid-precipitable protein (2.5 μg) from culture filtrates of several fungi grown on 0.1% xylan and concentrated by $(NH_4)_2SO_4$ precipitation was analyzed after sodium dodecyl sulfate-polyacrylamide gel electrophoresis by **A,** silver staining, or **B,** immunoblotting. Lanes contain: *Trichoderma viride*, T-1 (lane 2); *T. harzianum*, WT-66, Th-84, and Th-87 (lanes 3–5, respectively); *T. hamatum*, Tri-4 and Tm-34 (lanes 6 and 7, respectively); *Gliocladium virens*, Gl-3 and Gl-26 (lanes 8 and 9, respectively); *Fusarium oxysporum* f. sp. *pisi*, F-52 (lane 10); and *Macrophomina phaseolina*, MP-2 (lane 11). Lane 1 contains a standard of purified Cellulysin EIX (100 ng). The inset at the far right of panel B shows the cross-reactive polypeptides detected on an immunoblot after concentration of the pathogen proteins shown in lanes 10 and 11, respectively. Panel C depicts the specific ethylene biosynthesis-inducing and xylanase activities in $(NH_4)_2SO_4$ concentrates corresponding to lanes 2–11 in Panels A and B. Units for ethylene biosynthesis are μl C_2H_4/hr/g fresh weight tobacco leaf tissue/mg EIX protein.

weight, isoelectric point, and temperature stability with *T. viride* EIX. We tested several strains of *Trichoderma* and *Gliocladium* for the ability to produce an EIX-like protein in our induction medium. All *Trichoderma* and *Gliocladium* strains tested produced varying amounts of a 22-kDa polypeptide that could be detected on immunoblots (Fig. 7). It would thus appear that an endoxylanase of 22 kDa with a pI of 9.4 and the ability to induce ethylene biosynthesis in a variety of plant tissues is a common product of *Trichoderma* and *Gliocladium* grown on xylan. Filtrates of xylan-grown cultures of *F. oxysporum pisi* and *M. phaseolina*, the pathogens responsible for vascular wilt in pea and charcoal stem rot in soybean, respectively, likewise contain an ethylene biosynthesis-inducing activity and a 22-kDa band detectable on immunoblots. VanderMolen et al (39) have demonstrated that a partially purified endo-β-1,4-xylanase from cultures of *Fusarium oxysporum* f. sp. *cubense* (E. F. Smith) Snyder et Hansen, the organism responsible for vascular wilt in bananas, is capable of inducing formation of vascular gels in susceptible plants. Moreover, they found that formation of vascular gels also was induced by ethylene and that treatment of the plant tissue with the partially purified xylanase led to increased ethylene production.

Vascular occlusion in response to ethylene is illustrative of the dilemma faced by those wishing to relate ethylene and plant pathological responses. Vascular occlusion of a leaf petiole in response to ethylene produced by the leaf blade after pathogen attack might be considered a host defense if the leaf abscises and falls away from the plant. But ethylene-induced occlusion of the vascular system in response to fungal attack through the roots might be considered to enhance pathogenicity as the plant weakens and yields to further invasion. Each mode of ethylene induction and each form of ethylene action must be considered within a well-defined context. Thus, observations that an immediate effect of ethylene application to healthy tissues is an increase in steady-state levels of the mRNAs' encoding defense response proteins, such as chitinase (11,16), might suggest that one function of disease-induced ethylene is to stimulate host defenses in the diseased tissue and, perhaps, throughout the plant. But injection of purified *T. viride* EIX into tobacco leaves induces the synthesis of a pathogenesis-related chitinase even in the presence of inhibitors of ethylene production and action (Lotan and Fluhr, *personal communication*). The synthesis of similar ethylene biosynthesis-inducing xylanases by pathogenic and nonpathogenic fungi may have important implications for models relating ethylene biosynthesis and fungal pathogenesis, but more specific information concerning the mechanism of action of this fungal protein in plant systems is required.

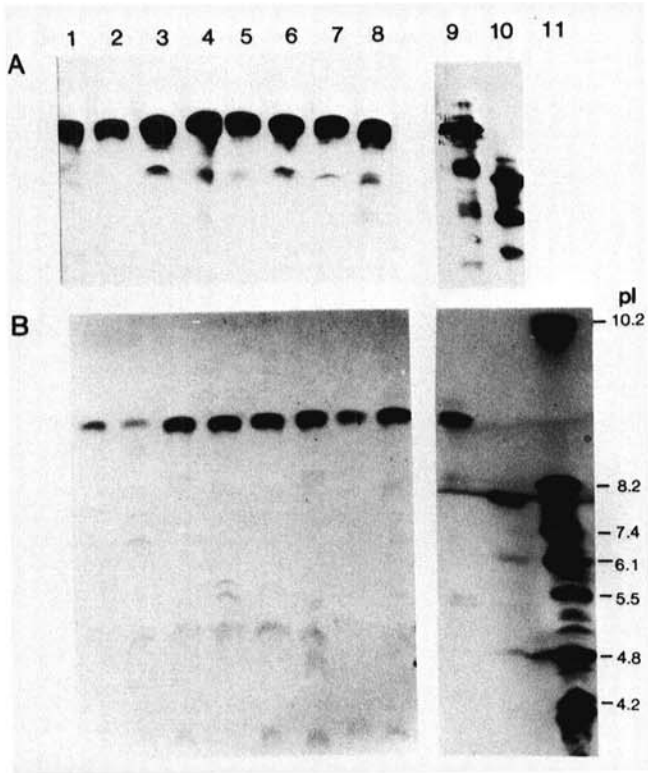

Fig. 8. Isoelectric points of the proteins from Cellulysin and several fungal species that cross-react with antibodies against ethylene biosynthesis-inducing xylanase (EIX). Samples (10 μg) of partially purified Cellulysin EIX or $(NH_4)_2SO_4$-concentrated *Trichoderma* and *Gliocladium* proteins were subjected to isoelectric focusing (pH 3–10) in agarose gels. Replicate gels were: **A,** blotted to PVDF membrane for immunodetection of cross-reactive polypeptides, or **B,** dried and stained. Lanes contain *G. virens*, Gl-26 and Gl-3 (lanes 1 and 2, respectively); *T. hamatum*, Tm-34 and Tri-34 (lanes 3 and 4, respectively); *T. harzianum*, Th-87, Th-84, and WT- 66 (lanes 5–7, respectively); *T. viride*, T-1 (lanes 8 and 9); Cellulysin EIX (lane 10); and pI markers (lane 11).

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