Polypeptides Associated with Gliotoxin Production in the Biocontrol Fungus *Gliocladium virens*

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ABSTRACT

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Two polypeptides (18.7- and 33.8-kDa) were purified from mycelial extracts of *Gliocladium virens* G20 with two-dimensional electrophoresis and used for the production of antiserum in the eggs of turkey hens. The resulting antisera were used to investigate the association of these polypeptides with gliotoxin production in *G. virens*. Mycelia from 20 strains of *G. virens* and three other gliotoxin-producing fungal genera were extracted into denaturing electrophoresis buffer and analyzed with immunoblotting procedures. Both polypeptides were detected in mycelial extracts from 15 strains that produced gliotoxin but were not detected in extracts from five strains that did not produce gliotoxin or in extracts from three other fungal genera. Antiserum against the 33.8-kDa polypeptide also reacted against mycelium of *G. virens* G20 extracted into nondenaturing Tris-HCl buffer. Gliotoxin was detected in the culture filtrate of *G. virens* grown in Weindling's medium after incubation of germinating chlamydospores for 24 h, whereas the 18.7-kDa polypeptide was detected in denatured mycelial extracts of *G. virens* after 16 h of growth. The 33.8-kDa polypeptide was detected after growth of germinating chlamydospores for 64 h. The chlamydospores contained two polypeptides (78- and 100-kDa) that cross-reacted with the antiserum raised against the 33.8-kDa polypeptide. These results may facilitate the identification and characterization of genes expressed during the production of gliotoxin by *G. virens*.

The biocontrol fungus *Gliocladium virens* J. H. Miller, J. E. Giddens, & A. A. Foster exerts its effect on plant pathogens primarily through production of a range of lytic enzymes and secondary metabolites (9,14,20,21,23,25,26). The importance of specific metabolites in biocontrol depends in part on the target pathogen, the strain of *G. virens*, and the substrate on which the antagonist is grown (8). The antibiotic gliotoxin is produced by *G. virens* and is particularly active against several important plant pathogens (2,23). Gliotoxin is the principal metabolite produced in soilless media by a formulation of *G. virens* that has been registered by the U.S. Environmental Protection Agency (14–16) for control of soilborne diseases of greenhouse-grown plants. Therefore, if the regulatory factors that enhance gliotoxin production can be determined, biocontrol performance might be improved by strain selection, nutrient amendment, or genetic transformation.

Gliotoxin belongs to the epipolythiodioxopiperazine group of fungal metabolites and contains a disulfide bridge that confers activity. The antibiotic is probably derived from phenylalanine via the intermediate *cyclo* (L-phenylalanyl L-seryl) (12). Although the molecule has been synthesized chemically (24), none of the biosynthetic events involved in the production of gliotoxin by fungi has been established. Indeed, even the biosyntheses of com-

mercially important antibiotics, such as tetracycline, rifampicin, and erythromycin, are poorly understood, probably because the biosynthetic enzymes and other important proteins are difficult to isolate and characterize (24).

Many enzymes and other proteins are likely to be involved in the production of secondary metabolites by fungi. They perform functions such as biosynthesis, regulation, conformation within the cell, export, and self-resistance to antibiotics (17). Some of the detailed physiology of gliotoxin production could therefore be determined if important cellular proteins could be identified and characterized. Such detailed understanding may establish the basis for differences in gliotoxin yield between strains and help explain how the final yield can be affected by nutritional and environmental factors. In fungi such as *Penicillium chrysogenum* and *Aspergillus nidulans,* the genes coding for biosynthetic enzymes are clustered in the same region of DNA (18). If this is also true for gliotoxin production, the DNA region encoding several genes could possibly be identified with a probe on the basis of the amino acid sequence of just one key protein. Several polypeptides have been tentatively correlated with gliotoxin production in two strains of *G. virens* (22).

The objective of this study was to confirm whether any of the polypeptides previously reported (22) are consistently associated with gliotoxin production and, if so, to determine some of their characteristics.

MATERIALS AND METHODS

Strains of *G. virens* and other gliotoxin-producing fungi. *G. virens* strains G1–G20 (13) were from the culture collection of the Biocontrol of Plant Diseases Laboratory; *G. virens* NRRL-2299, *Penicillium bilaii* NRRL-3625, *Aspergillus ruber* NRRL-75, and *Trichoderma viride* NRRL-1829 were obtained from the culture collection of the USDA NRRL (Northern Regional Research Laboratory, now the National Center for Agricultural Utilization Research, Peoria, IL). All cultures were maintained on V8 agar (13).

Antigen purification and primary antibody production. The five antigens selected for purification from *G. virens* strain G20, a subisolate of GL-21, were those with approximate molecular weights and isoelectric points (pIs) of 33.8, 7.35; 33.8, 7.10; 18.7, 6.75; 27.2, 7.25; and 15.1, 4.6, respectively (22). The two 33.8-kDa polypeptides were pooled. Aqueous conidial suspensions (1 ml containing 10^6 spores) were transferred to rectangular polyethylene containers (280 × 170 × 130 mm) that each contained 250 ml of modified Weindling's medium (11). After growth for 4 days without agitation at 25 C in the dark, the mycelium was harvested by filtration through Nitex nylon mesh (25-μm pore size) (Tetko Inc., Briarcliff Manor, NY). The mycelium was washed in distilled water (250 ml), filtered on Nitex nylon mesh, and blotted to remove excess moisture. It was weighed, transferred to a mortar chilled to −20 C, mixed with glass powder (0.3 g) (25-μm diameter) (Heat Systems Company, Melville, NY) and liquid nitrogen, and ground to a fine powder. Extraction buffer (22) was added in a volume (μl) equal to the weight (mg) of the mycelium, and the mixture was centrifuged at 200,000 *g* at 10 C for 1 h to remove cell debris. The supernatant had a concentration of approximately 3.5 μg of crude polypeptide per microliter of buffer (22). Eight replicate two-dimensional gels were used to purify the selected antigens. The first-dimension tube gels were each loaded with 30 μl (approximately 100 μg) of crude polypeptide. All two-dimensional electrophoresis procedures have been described previously (22).

Replicate gels were stained with aqueous Coomassie Blue R250 (0.05%, w/v) (7) in order to locate selected antigens on gels. A single gel piece that contained both of the 33.8-kDa polypeptides was excised with a scalpel and used directly for the production of antisera. Single gel pieces that contained the single 18.7-, 27.2-, and 15.1-kDa polypeptides were excised in the same way.

Duplicate polyacrylamide gel pieces that contained the selected polypeptides were pooled and used for the primary injection. The amounts of polypeptides used for injection were estimated from the intensity and size of the spot to be 24, 10, 18, and 8 μg for the pooled 33.8-, 18.7-, 27.2-, and the 15.1-kDa polypeptides, respectively. The gel pieces were macerated in Freund's complete adjuvant, and the emulsion was injected intradermally on the backs between the wings of large White Breeder hen turkeys (*Meleagris gallopavo*) (British United Turkeys of America, Louisburg, WV). Two replicate hens were used for each of the four polypeptides. After 28 days, each turkey was given a booster injection with the same amount of polypeptide in gel slices macerated in Freund's incomplete adjuvant.

Eggs were collected before the primary injection and for about 40 days after the secondary injection and were stored at 4 C until required. To extract antibodies, yolks of selected eggs were separated, mixed with an equal volume of phosphate buffered saline (PBS) (Sigma Chemical Co., St. Louis, MO), pH 7.8, and centrifuged at 30,000 *g* for 1 h. The supernatant was precipitated with 60% ammonium sulfate and shaken (100 cycles per minute) at 4 C for 16 h. The preparation was centrifuged at 10,400 *g* for 20 min, and the floating pellet was dissolved in a small volume of PBS, pH 7.8. The resulting solution was dialyzed against two changes of PBS for a total of 27 h. Antibody reactions against the selected antigens were determined with crude extracts of *G. virens* G-20 in electrophoresis buffer (6). Aliquots (25 μl containing approximately 65 μg of protein) were subjected to one-dimensional electrophoresis, electroblotting, and development with the procedures described below. Egg yolk preparations with strong antibody reactions were pooled and stored at −80 C until they were used.

Electrophoresis and immunoblotting procedures. One-dimensional electrophoresis was performed with a Ready Gel (12%) (BioRad, Richmond, CA) in a Mini Protean II electrophoresis cell (BioRad). All protein samples were heated at 90 C for 5 min in electrophoresis buffer (5), and the gels were either stained with Coomassie Blue R250 (6) or used for immunoblotting. For immunoblotting, prestained molecular weight standards (BioRad) were included in one or more lanes to give an approximation of molecular weights and to allow comparison between gels. Two-dimensional electrophoresis was performed with a 10% polyacrylamide gel. Samples were dissolved in electrophoresis buffer as described previously (22).

Polypeptides in the gels were electroblotted onto Transblot nitrocellulose or polyvinylpyrrolidone membranes (BioRad) with a Mini Protean II gel apparatus. To detect specific antigens, the membranes were incubated for 2 h in a suspension prepared with powdered nonfat milk in PBS (5%, w/v), pH 7.4, washed twice in PBS (5 min each), and then incubated for 18 h in primary antibody diluted 100-fold in PBS containing Tween 20 (ICN Pharmaceuticals, Cleveland, OH) (0.2%, v/v). The membranes were washed with four changes of PBS, pH 7.4 (5 min each), incubated for 2 h in phosphatase-labeled goat antiturkey immunoglobin G (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD), and diluted 750-fold in PBS, pH 7.4, containing Tween 20 (0.2%, v/v). The membranes were washed in four changes of PBS and developed with the 5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium phosphatase substrate system (Kirkegaard and Perry Laboratories, Inc.). Membranes were stained for total protein with India ink (Pelikan AG, Hannover, Germany) diluted 1,000-fold in PBS, pH 7.4, containing Tween 20 (0.2%, v/v).

Occurrence of 33.8- and 18.7-kDa antigens. The presence of the 18.7- and 33.8-kDa antigens in mycelium of *G. virens* strains G1–G20 was determined. An aqueous conidial suspension (1 ml containing 10^6 spores) of each strain was transferred to two replicate Erlenmeyer flasks (250-ml) each containing 30 ml of modified Weindling's medium. After 4 days' growth without agitation at 25 C in the dark, the mycelium was removed by filtration, weighed, and ground in liquid nitrogen as described above. Electrophoresis buffer (6) was added in a volume (μl) equivalent to the fresh weight (mg) of mycelium, which yielded a constant protein concentration of 65 μg/μl (22). The samples were centrifuged at 16,400 *g* for 10 min. Aliquots (30 μl) of the supernatant were electrophoresed in a 12% Ready Gel (BioRad), electroblotted, and developed as described above. The experiment was repeated.

In other experiments, mycelium of G20 was also grown from conidial suspensions and extracted into Tris HCl-buffer (0.1 M), pH 6.8. After they were centrifuged, the samples were mixed with an equal volume of double-strength electrophoresis buffer, heated, centrifuged, electrophoresed, and blotted as described. Blots were developed from both antibodies. The presence of the two antigens in the mycelium of *G. virens* NRRL-2299, *P. bilaii* NRRL-3625, *A. ruber* NRRL-75, and *T. viride* NRRL-1829 was investigated as described, except that *A. ruber* and *T. viride* were grown for 18 and 4 days, respectively, in Erlenmeyer flasks (1-L) that contained 60 ml of modified Weindling's medium (three flasks per replicate). The mycelia were extracted into electrophoresis buffer.

To determine whether the antiserum reacted to the 33.8-kDa antigen when extracted into Tris-HCl buffer, the mycelium was extracted into the buffer with the procedures described. The protein concentration was determined by the method of Bradford (1) with bovine serum albumen as the standard. Serial dilutions were prepared and the extracts adsorbed onto a Transblot nitrocellulose membrane with a HYBRI.DOT manifold (Bethesda Research Laboratories, Gaithersburg, MD) with 3.0-mm-diameter transblot wells. The membrane was developed as described.

The presence of metabolites in all the culture filtrates was determined with thin-layer chromatography (TLC). The filtrate (30 ml) was mixed with 30 ml of chloroform (Sigma) and shaken vigorously for 30 s. The chloroform was separated and then evaporated to dryness with a rotary evaporator. The residue was resuspended in chloroform (100 μl) and analyzed with TLC as described previously (16). Pure standards of gliotoxin (Sigma) or viridin (ICI, Jealott's Hill, UK) were run on the same TLC plate, and the metabolites were detected with a mineralight UV GL-58 ultraviolet light (UVP-Inc., San Gabriel, CA) at 254 nm.

Quantitative estimates of the production of antigen, gliotoxin, and viridin were made for *G. virens* strains G2, G6, G7, G12, G15, and G20. Cultures were grown, and the polypeptides were electrophoresed and blotted as above with three replicates per strain. The intensities of the immunological reactions were determined as specified below. Gliotoxin and viridin were extracted into chloroform as above, developed on TLC plates, and quantified with video image analysis as described previously (16).

Mean intensities of the antigen reactions were compared with the mean amounts of gliotoxin and viridin produced. In addition, since all the cultures were grown independently, the intensities of the antigens were correlated with the amounts of gliotoxin and viridin by use of a regression analysis program (SAS, Cary, NC).

Changes in profiles of the 33.8- and 18.7-kDa antigens during germination of chlamydospores. Chlamydospores of *G. virens* strain G20 were supplied by W. R. Grace & Co., Columbia, MD. The chlamydospores (20 g) were aseptically washed twice by suspension in 500 ml of sterile distilled water and filtration on Nitex nylon mesh (10-μm). The washed chlamydospores were resuspended in 50 ml of distilled water. Aliquots (1.0 ml) of the suspension were transferred to standard petri dishes that contained 15 ml of modified Weindling's medium buffered to pH 6.5 with 2-[N-morpholino]ethanesulfonic acid (0.1 M) (Sigma). Nonagitated cultures were incubated at 25 C in the dark, and the mycelium was harvested by filtration through 25-μm Nitex nylon mesh at 0, 16, 24, 40, 64, 76, 160 (about 7 days), and 232 h (about 10 days) with two replicate cultures for each sampling time. The chlamydospores and mycelia were blotted dry, weighed, and ground in liquid nitrogen as described above. Electrophoresis buffer (6) (μl) was added to mycelium or chlamydospores (mg) at the ratio of 3:1. The preparations were centrifuged at 16,400 *g* for 10 min, and 25-μl aliquots of the supernatants were used for electrophoresis.

Quantification of metabolites and immunological reactions. The amounts of gliotoxin and viridin in culture filtrates were quantified with video image analysis as described previously (16) and were compared to known standards. The intensities of bands on immunoblots that corresponded to 33.8- and 18.7-kDa polypeptides were quantified with a model LKB 2222-010 scanning densitometer (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) at 633 nm.

RESULTS

Antibody production and immunoblotting. Antibodies were successfully raised against both the 33.8- and 18.7-kDa antigens, but no activity against the 15.1- or 27.2-kDa antigens could be detected. Antibodies raised against the 33.8-kDa antigen detected a single band on one-dimensional immunoblots with no background staining from nonspecific reactions. Two-dimensional immunoblots revealed two prominent spots that corresponded to the two injected antigens. In addition, a faint spot was detected with the molecular weight-pI coordinates of 33.8 and 6.9 (Fig. 1A). Antibodies raised against the 18.7-kDa antigen detected a single prominent band in one-dimensional immunoblots and a single prominent spot in two-dimensional immunoblots (Fig. 1B). However, nonspecific background reactions in the 80–100-kDa molecular weight region were detected with the 18.7-kDa antibody preparation. Both antigens could be detected when the mycelium was first extracted into Tris-HCl buffer, followed by mixing with electrophoresis buffer, electrophoresis, and immunoblotting. Antiserum against the 33.8-kDa polypeptide also reacted against the total protein (2.5–160 μg) extracted into Tris-HCl buffer and adsorbed onto a nitrocellulose membrane with the HYBRI.DOT manifold. There was no reaction against either antigen when the preinjection egg serum was used. Antigenic reactions against both antigens could be detected at 14 days after the second injection and could be detected in serum from eggs laid for at least the next 5 wk.

Detection of 33.8- and 18.7-kDa antigens in *G. virens* in and other fungal genera. Mycelial extracts of strains G1, G3, G4, G8, and G9 did not react against either antigen, whereas all other strains reacted against both antigens (Fig. 2, Table 1). Intensities

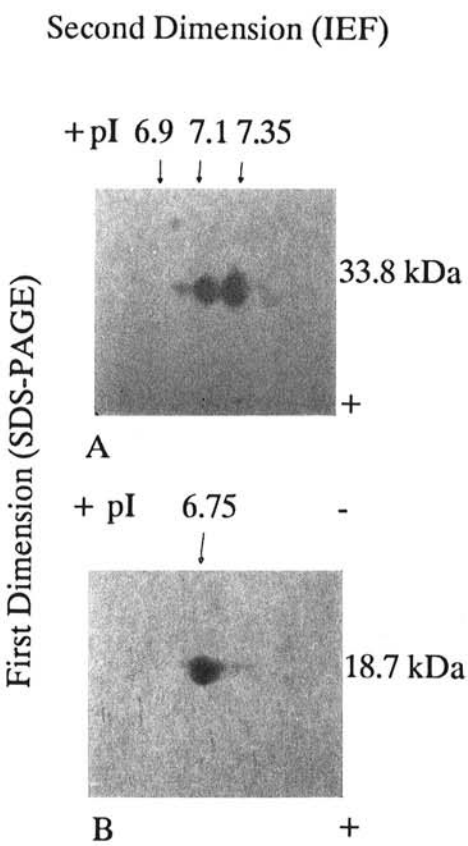

Fig. 1. Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focus (IEF) immunoblot showing reactions with **A,** 33.8-kDa and **B,** 18.7-kDa polypeptides from the mycelium of *Gliocladium virens* (G20). pI = Isoelectric point; kDa = molecular weight in kilodaltons.

of the reactions varied among the strains, and a double band was detected in the 33-kDa region for G18. All strains produced viridin, but gliotoxin was produced only by those strains that reacted with antibodies against the 18.7- and 33.8-kDa antigens. *P. bilaii, A. ruber*, and *T. viride* all produced gliotoxin, but their mycelial extracts did not react against either antibody. *G. virens* NRRL-2299 produced gliotoxin and reacted against both antibodies.

Correlation of antigenic reactions with gliotoxin and viridin production. Mycelial fresh weights were similar for all strains of *G. virens*. However, the amounts of gliotoxin produced varied among strains and was greatest for strain G7 and least for G2. The production of gliotoxin by G20, G15, G12, and G6 ranged between these extremes (Table 1). The intensities of the antigenic reaction also varied among strains, even though the same amount of protein (65 μg) was loaded.

Total amounts of gliotoxin and viridin present in the medium were correlated with the intensities of the antigenic reactions, with each replicate treated as an independent sample (18 observations) (Fig. 3). The observations were normally distributed about a mean value, $P = 0.05$. There was no significant correlation between gliotoxin and the 33.8-kDa polypeptide nor between viridin and the 33.8- and the 18.7-kDa polypeptides. The *F*-ratio/probability values were 1.69/0.21, 0.10/0.92, and 2.10/0.17, respectively. There was a significant correlation between the 18.7-kDa polypeptide and gliotoxin ($F = 7.84$, $P = 0.012$). The slope parameters for this comparison were determined to be $G = 0.35 + 2.6$ (I), where G = amount of gliotoxin produced, and I = the intensity of the 18.7-kDa antigenic reaction. The correlation coefficient was determined to be $r^2 = 0.33$.

Production of gliotoxin and antigens during germination of chlamydospores. Gliotoxin was detected in the culture medium after 24 h of growth, and high concentrations were maintained after 48 h (Fig. 4). The 33.8-kDa antigen was not detected in the initial chlamydospore extract ($T = 0$ h) but was first detected after 64 h of growth (Fig. 5A). This antigen reached a peak concentration at 96 h and then declined. Two additional polypeptides with approximate molecular weights of 78 and 100 kDa were detected when this antibody was used in protein extracts from nongerminated chlamydospores and during the first 64 h after germination. These polypeptides reached a peak intensity of reaction between 16 and 24 h. Polypeptides with approximate molecular weights of 78 and 104 kDa reacted with this antibody after 96 h. Mycelium that had been extracted after 230 h contained numerous polypeptides that reacted against this antibody.

The 18.7-kDa polypeptide was not detected in the initial chlamydospore extract but was first detected after 16 h (Fig. 5B). The intensity of this band increased after 96 h. Changes in other polypeptides that reacted to this antibody could not be monitored accurately because of the high nonspecific reaction of the egg serum in the 80–100-kDa region.

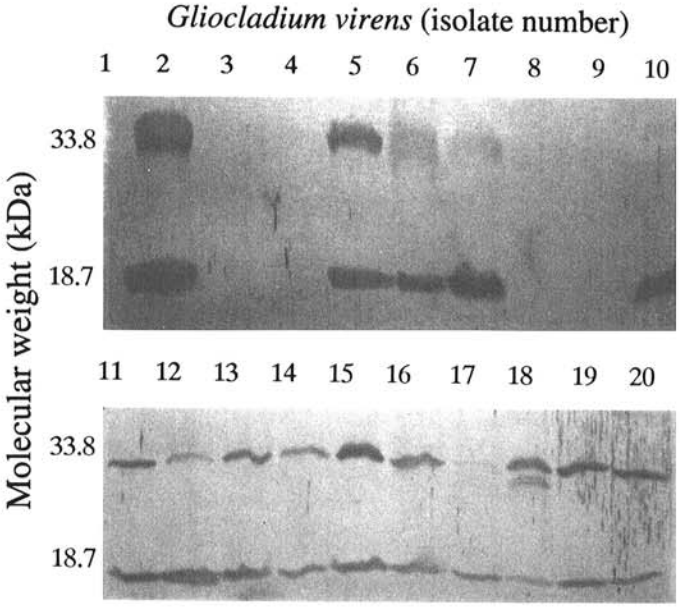

Fig. 2. Reaction of mycelial extracts from 20 strains of *Gliocladium virens* with antibodies against 33.8- and 18.7-kDa polypeptides. Upper panel, immunoblot of strains G1–G10 (lanes 1–10, respectively); lower panel, immunoblot of strains G11–G20 (lanes 11–20, respectively). Molecular weights are in kilodaltons.

TABLE 1. Production of gliotoxin and viridin and antigenic reactions with 33.8- and 18.7-kDa antigens of six selected strains of *Gliocladium virens*

Strain	Fresh weight (mg)	Metabolite concentration (μg/ml)[a]		Antigen intensity[b]	
		Gliotoxin	Viridin	33.8 kDa	18.7 kDa
G2	108.6 ± 49.0	1.47 ± 0.73	2.31 ± 1.00	0.93 ± 0.12	0.54 ± 0.10
G6	104.6 ± 16.0	3.77 ± 0.46	2.87 ± 0.67	2.10 ± 0.72	1.22 ± 0.33
G7	88.3 ± 13.6	6.26 ± 0.82	4.76 ± 0.62	2.18 ± 0.13	1.52 ± 0.19
G12	108.0 ± 26.8	3.36 ± 0.39	1.77 ± 0.22	1.91 ± 0.77	1.22 ± 0.29
G15	115.0 ± 15.7	2.90 ± 0.45	2.62 ± 0.14	1.96 ± 0.52	1.31 ± 0.06
G20	103.0 ± 21.0	2.58 ± 22.1	1.80 ± 0.42	2.27 ± 0.56	1.16 ± 0.18

[a] Gliotoxin and viridin were produced in culture filtrates (30 ml). Values are means of three replicate culture filtrates ± standard deviations.

[b] Peak area relative intensities were determined by scanning laser densitometry. Values are based on three replicate determinations ± standard deviations.

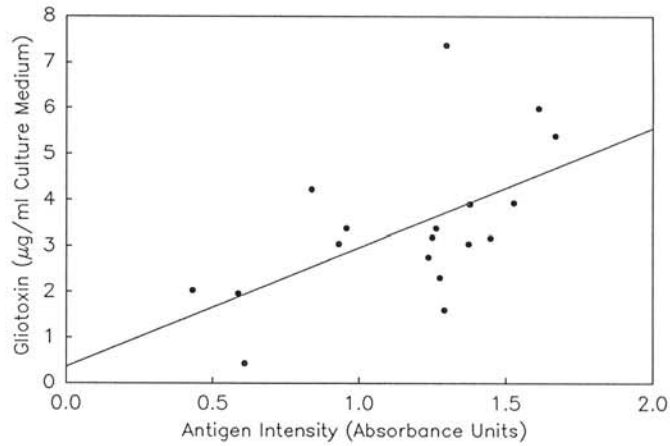

Fig. 3. Scatter plot showing the intensity of the antigenic reaction measured by scanning densitometry of the 18.7-kDa polypeptide correlated with the amount of gliotoxin produced per milliliter of culture filtrate of *Gliocladium virens* (G20).

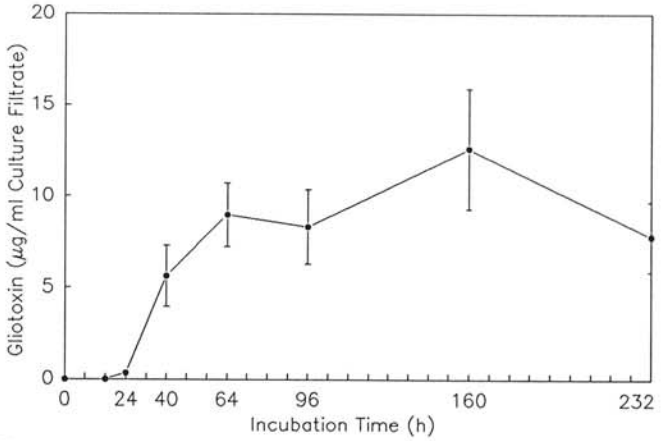

Fig. 4. Amounts of gliotoxin produced in cultures of *Gliocladium virens* (G20) derived from chlamydospores after they had germinated for various periods of time at 25 C. Amounts shown are the means of four replicates ± standard deviations.

DISCUSSION

Our results demonstrate that the presence of the 18.7- and 33.8-kDa polypeptides is consistently associated with the production of gliotoxin by certain strains of *G. virens*. These polypeptides appear to be so consistently associated with the ability of *G. virens* strains to produce gliotoxin that antisera against them can differentiate, with high confidence, the mycelial protein of gliotoxin-producing strains from that of non-gliotoxin-producing strains of the fungus. This characteristic may prove useful in the study of specific interactions between gliotoxin-producing and non-gliotoxin-producing strains and for the development of rapid enzyme-linked immunosorbent assay (ELISA) procedures for screening wild-type strains or for the identification of transformed or mutated strains that have lost the ability to produce gliotoxin. Antisera were not successfully raised against the 15.1- and 27.2-kDa polypeptides, which suggests lower antigenicity compared with the 18.7- and 33.8-kDa polypeptides. A positive reaction against the 15.1- and 27.7-kDa polypeptides could probably be achieved if larger amounts were used for production of antisera.

The 18.7-kDa antigen was not detected in mycelium of strains that did not produce gliotoxin, even though such strains appear to possess low concentrations of this polypeptide (22). If the polypeptides were the same in gliotoxin-producing and non-gliotoxin-producing strains, then some reaction would have been detected with mycelial extracts of non-gliotoxin-producing strains. This result suggests that the polypeptide in non-gliotoxin-producing strains is antigenically different but has a similar molecular weight and pI. Antibodies against the 18.7- and 33.8-kDa polypeptides also did not react against mycelial extracts from other gliotoxin-producing fungal genera, which indicates that the epitopes recognized by the polyclonal antisera were not present in their mycelia.

Both the 18.7- and 33.8-kDa polypeptides could be detected in immunoblots when first extracted into Tris-HCl buffer followed by mixture with electrophoresis buffer, which indicates that β-mercaptoethanol and sodium dodecyl sulfate are not required for their initial solubilization. This result will permit the study and further purification of native forms of both polypeptides under nondenaturing conditions. The membrane ELISA test also gave a positive reaction against the mycelial protein extracted into Tris-HCl buffer. Since the immunoblots demonstrated that there was no background reaction, the positive reaction is likely due to the presence of the 33.8-kDa antigen. The membrane ELISA test was not used for the 18.7-kDa polypeptide because of the high nonspecific reaction against this antigen.

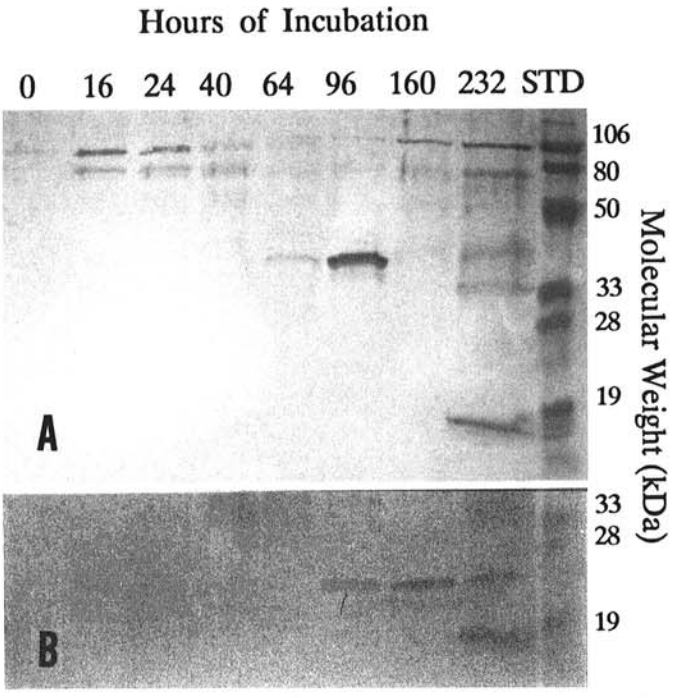

Fig. 5. Immunoblot showing the appearance of polypeptides of *Gliocladium virens* after cultures were grown for various periods of time at 25 C. The initial culture was from germinating chlamydospores of *G. virens* (G20). **A,** Immunoreaction against the 33.8-kDa polypeptide; **B,** immunoreaction against the 18.7-kDa polypeptide. Lanes 1–8 show reaction after incubation for 0, 16, 24, 40, 64, 96, 160, and 232 h, respectively. Lane 9 shows prestained standards to provide an approximate indication of molecular weights (kDa).

The two-dimensional immunoblot demonstrated that antibodies were raised against both of the prominent 33.8-kDa polypeptides with pIs of 7.1 and 7.35 and against an additional 33.8-kDa antigen with a pI of 6.9. If the two prominent polypeptides are different, the antiserum contains a mixture of antibodies. The reaction against the third polypeptide may result from antibodies raised against a minor contaminant polypeptide injected with the two prominent polypeptides. However, since the two prominent polypeptides have the same molecular weight and similar pIs and are both characteristic of gliotoxin producers, they may be isoforms of the same polypeptide. If so, both isoforms may react against a single population of polyclonal antibodies. An additional isoform may account for the detection of a third polypeptide by use of this antiserum. Use of specific monoclonal antibodies or N terminal sequence analysis may indicate whether the three polypeptides are all isoforms. Strains of *G. virens* that do not produce gliotoxin are known to possess a 33.8-kDa polypeptide with a pI of 5.8 (22). However, the present study demonstrates that this polypeptide is so different from the 33.8-kDa polypeptide possessed by gliotoxin producers that it does not react against the antiserum.

The 18.7-kDa polypeptide can be detected, although in small quantities, after 16 h and before gliotoxin is detectable in the culture filtrate, which suggests its possible involvement in the initial synthesis or export of gliotoxin. The amount of this polypeptide is also significantly correlated with the amount of gliotoxin produced, which again suggests a close association. By contrast, the 33.8-kDa polypeptide appears after the initial production of gliotoxin, and the amount of polypeptide detected does not correlate with the amount of gliotoxin produced. The delayed production of this polypeptide may indicate its involvement in the self-resistance of *G. virens* to gliotoxin; the production of this polypeptide may be induced by the accumulation of gliotoxin to critical (toxic) concentrations. Alternatively, this polypeptide may be induced when the exogenous nutrient supply becomes depleted in some critical component.

Although the 33.8-kDa polypeptide is not present in chlamydospores, there are two polypeptides with approximate molecular weights of 78 and 100 kDa that react against the antibody. These polypeptides decline in intensity as the 33.8-kDa polypeptide increases. Although cross-reaction is not proof of a functional relationship, it is possible that the polypeptides with high molecular weights may be alternative forms of the 33.8-kDa polypeptide with the same antigenic site. Since they are prominent in the chlamydospores, they may be storage proteins that yield the active 33.8-kDa polypeptide after proteolysis during germination. Activation of enzymes after proteolytic cleavage of forms with high molecular weights has been reported in a range of fungi (3,5,19).

In a study of the metabolites produced by *G. virens,* Howell and Stipanovic (10) described two groups of *G. virens* strains on the basis of their ability to produce gliotoxin (Q group) or gliovirin (P group). Our results indicate the 18.7- and 33.8-kDa polypeptides are produced only by the Q group and provide further evidence of a difference in physiology between the two groups. Although the polypeptides are characteristic of gliotoxin-producing strains, our results do not enable the establishment of their exact nature or functions. The polypeptides might, for example, be involved directly in gliotoxin biosynthesis or self-resistance to the antibiotic. Alternatively, they may perform functions that are unrelated to gliotoxin metabolism but that are features of the physiology of strains that do metabolize gliotoxin.

Since the polypeptides are characteristic of gliotoxin-producing strains, establishing their function might enable a more detailed

understanding of the physiology of such strains and allow more effective selection procedures or formulations to be developed.

Indications of function can be obtained by the comparison of amino acid or corresponding DNA sequence homologies with known sequences in established databases. We are currently using microsequencing techniques to obtain amino acid sequences from blots of the polypeptides separated by two-dimensional electrophoresis. The sequences can then be compared directly with those in the database or used to prepare primers so that portions of DNA sequence coding for the polypeptides can be amplified by the polymerase chain reaction. Alternatively, an expression library of *G. virens* genes can be screened with the antibodies to isolate the corresponding gene. In this case, the background reaction against the 18.7-kDa polypeptide would have to be removed by using, for example, an affinity procedure based on the method described by Calza et al (4).

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