Analysis of the β -1,3-Glucanolytic System of the Biocontrol Agent *Trichoderma harzianum*

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The biocontrol agent *Trichoderma harzianum* IMI206040 secretes β -1,3-glucanases in the presence of different glucose polymers and fungal cell walls. The level of β -1,3-glucanase activity secreted was found to be proportional to the amount of glucan present in the inducer. The fungus produces at least seven extracellular β -1,3-glucanases upon induction with laminarin, a soluble β -1,3-glucan. The molecular weights of five of these enzymes fall in the range from 60,000 to 80,000, and their pIs are 5.0 to 6.8. In addition, a 35-kDa protein with a pI of 5.5 and a 39-kDa protein are also secreted. Glucose appears to inhibit the formation of all of the inducible β -1,3-glucanases detected. A 77-kDa glucanase was partially purified from the laminarin culture filtrate. This enzyme is glycosylated and belongs to the exo- β -1,3-glucanase group. The properties of this complex group of enzymes suggest that the enzymes might play different roles in host cell wall lysis during mycoparasitism.

Trichoderma harzianum is a mycoparasitic soil fungus which has been extensively used as a biocontrol agent because it attacks a large variety of phytopathogenic fungi responsible for major crop diseases (7). Several modes of action have been proposed to explain the suppression of plant pathogens by *Trichoderma*; these modes of action include production of antibiotics, competition for key nutrients, production of cell wall-degrading enzymes, stimulation of plant defense mechanisms, and a combination of these possibilities (24). The first detectable event during interaction with a host is directed hyphal branching (10); when the mycoparasite reaches the host, its hyphae coil around it and penetrate into the mycelium after partial degradation of the cell wall (2, 15).

Production of extracellular β -1,3-glucanases, chitinases, and a proteinase increases significantly when a *Trichoderma* species is grown in a medium supplemented with either autoclaved mycelium or host fungal cell walls (6, 14, 17). These observations, together with the fact that chitin, β -1,3-glucan, and protein are the main structural components of most fungal cell walls (30), are the basis for the suggestion that lytic enzymes produced by some *Trichoderma* species play an important role in the destruction of plant pathogens (8, 9).

β -1,3-Glucanases are enzymes which hydrolyze the O-glycosidic linkages of β -glucan chains by two mechanisms. Exo- β -1,3-glucanases (EC 3.2.1.58) hydrolyze a substrate by sequentially cleaving glucose residues from the nonreducing end, and endo- β -1,3-glucanases (EC 3.2.1.39) cleave β -linkages at random sites along the polysaccharide chain, releasing short oligosaccharides. Degradation of β -glucan by fungi is often accomplished by the synergistic action of both endo- and exo- β -glucanases (31); in fact, in most cases multiple β -glucanases rather than a single enzyme have been found (34, 37).

A number of fungal β -1,3-glucanases have been the subject

of basic and applied research, as they seem to have different functions during development and differentiation (30). It has been suggested that β -1,3-glucanases play a nutritional role in saprophytes and mycoparasites (7, 35), and these enzymes have also been implicated in autolysis (37). Furthermore, β -1,3-glucanases are among the plant defense responses to pathogen attack (34). Production of four β -1,3-glucanases by *T. harzianum* has been described, although different growth conditions and strains were used in the studies (14, 19, 22, 28). These enzymes are distinguishable on the basis of differences in molecular weight and isoelectric point. However, only one gene (*bgn13.1*) has been cloned. Expression of this gene might be repressed by glucose and induced by fungal cell walls, mycelia, or autoclaved yeast cells (13).

The present report describes the different components of the complex β -1,3-glucanolytic system observed in *T. harzianum* and the influence of culture conditions on enzyme expression. In addition, the most abundant β -1,3-glucanase produced under simulated mycoparasitism conditions was partially purified and characterized in this study.

MATERIALS AND METHODS

Microorganisms. The following strains were used in this work: *T. harzianum* IMI206040, *Mucor rouxii* IM80 (= ATCC 24905), *Neurospora crassa* 74-OR8-1a (= FGSC 4200), *Saccharomyces cerevisiae* S 288c, and *Rhizoctonia solani* AG1.

Preparation of fungal cell walls. *S. cerevisiae* was grown in YPD medium (1% yeast extract, 1% peptone, 2% D-glucose), *N. crassa* was grown in Vogel medium (38), *M. rouxii* was grown in YPG (0.3% yeast extract, 1% peptone, 2% D-glucose), and *R. solani* was grown in potato dextrose broth (PDB) (Difco). *S. cerevisiae* yeast cells and the different fungal mycelia were collected by filtration through Whatman 3MM filter paper, washed with sterile water, and resuspended in 20 mM sodium phosphate buffer (pH 7.0). Cells were disrupted ballistically with a homogenizer (Braun, Melsungen, Germany), and cell walls were separated from other cell debris by centrifugation at 2,500 $\times$ g and washed with buffer until they appeared to be free of cytosol, as judged by microscopic observation after cotton blue staining. Cell walls were lyophilized and added to mineral medium for induction of lytic enzymes as described below.

β -1,3-Glucanase induction. Briefly, *T. harzianum* mycelia were obtained by inoculating half-strength PDB with 10^6 conidia/ml and were incubated for 14 h at 28°C to synchronize cultures. The mycelia were collected by filtration through Millipore filter paper (pore size, 5 μ m), transferred to mineral medium (11), and incubated for an additional 12 h at 28°C. The mycelia were then filtered, trans-

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ferred to fresh mineral medium containing either 0.2% cell walls, 0.2% commercial polysaccharide (laminarin [95% pure; Sigma], pustulan [Calbiochem], or pullulan [Sigma]), or 2% glucose as a sole carbon source, and grown with agitation. Aliquots were removed from each flask at different times, and mycelia were immediately removed by filtration. The culture filtrates were either precipitated with 80% acetone and recovered by centrifugation at $27,000 \times g$ for 45 min at 4°C or extensively dialyzed against distilled water at 4°C and lyophilized. The concentrated samples were resuspended in 50 mM sodium acetate buffer (pH 5.0) and used as sources of β -1,3-glucanase. Protein concentration was measured as described previously (4).

β -1,3-Glucanase activity assays. The standard assay mixture (volume, 500 μ l) contained 250 μ l of protein concentrate, 5 mg of laminarin per ml, and 50 mM sodium acetate buffer (pH 5.0). Each reaction mixture was incubated for 1 h at 50°C, and the production of reducing sugars was determined by the procedure described by Somogyi (36) and Nelson (25). One unit of β -1,3-glucanase activity was defined as the amount of enzyme that catalyzed the release of 1 mmol of glucose equivalents per min.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by using the system of Laemmli (21) with 4% acrylamide stacking and 10% acrylamide separating gels. The gels were stained with silver as described by Nielson (26) or with Coomassie brilliant blue to visualize proteins. Isoelectric focusing (IEF) was performed with Ampholine PAG plates (pH 3.5 to 9.5) and a multiphor system (Pharmacia) according to the manufacturer's instructions. The plates were stained with Coomassie brilliant blue.

Activity staining of gels. Following electrophoresis, SDS-PAGE gels were incubated in 1% Triton X-100 in 50 mM sodium acetate buffer (pH 5.0) for 30 min to remove the SDS and equilibrated with fresh buffer for 15 min, and β -1,3-glucanase activity was determined as previously described (29). For IEF gels, β -1,3-glucanase activity was detected as described above, except that no pretreatment with Triton X-100 was required.

Glycoprotein detection. Following SDS-PAGE, proteins were transferred from gels to Immobilon-P membranes as described by Burnette (5). The blots were used for glycoprotein staining with the concanavalin A-biotin-streptavidin-alkaline phosphatase system (Boehringer Mannheim) according to the recommendations of the supplier.

Enzyme purification. Filtrates (1 liter) from 48-h laminarin-induced cultures were obtained and processed as described above. Each lyophilized powder was resuspended in 5 ml of 50 mM sodium acetate buffer (pH 5.0) containing 1 mM phenylmethylsulfonyl fluoride and E-64. All subsequent steps were carried out in the same acetate buffer at 4°C. The sample was applied to a Mono Q fast-performance liquid chromatography column (type HR 10/10; Pharmacia) and eluted with a linear NaCl gradient (0 to 0.5 M) with monitoring for total protein (A_{280}) and β -1,3-glucanase activity. Most active fractions were pooled, dialyzed, lyophilized, resuspended in 0.5 ml of the acetate buffer, and applied to a Bio-Gel P-200 column.

RESULTS

Induction of β -1,3-glucanase by various carbon sources. It has been reported that production of β -1,3-glucanases by *T. harzianum* is dependent on the carbon source available (14). In order to determine the best conditions for production of β -1,3-glucanases, a variety of polysaccharides and fungal cell walls were used as sole carbon sources, and the activity in each extracellular medium was determined. Several experiments indicated that the maximum activity occurred after 48 h of incubation with all of the inducers tested. The highest activity was obtained with laminarin, although induction with purified cell walls from *S. cerevisiae* and *R. solani* also resulted in high specific activities. A basal level of activity was detected when 2% glucose was used as the sole carbon source (Fig. 1).

To determine whether an extractable fraction of *S. cerevisiae* could induce β -1,3-glucanase activity in *Trichoderma* preparations, mineral medium supplemented with cell walls was autoclaved (15 min, 115°C) and filtered to eliminate insoluble material. The filtrate was used in a β -1,3-glucanase induction experiment. Figure 1 shows that the level of activity induced by this filtrate was about 70% of the level observed with whole cell walls (19 and 27 U/mg, respectively).

As mentioned above, in the presence of 2% glucose β -1,3-glucanase activity is very low (Fig. 1). It has been proposed that several of the genes coding for cell wall-degrading enzymes in *T. harzianum* are repressed by glucose. To examine this possibility, the effect of glucose on β -1,3-glucanase production was

FIG. 1. Effect of carbon source on the production of β -1,3-glucanase by *T. harzianum*. Culture filtrates were obtained by using mineral medium supplemented with cell walls from *M. rouxii* (bar 1), *N. crassa* (bar 2), *R. solani* (bar 3), or *S. cerevisiae* (bar 4), pustulan (bar 5), pullulan (bar 6), laminarin (bar 7), filtrate of autoclaved *S. cerevisiae* cell walls (bar 8), or glucose (bar 9). β -1,3-Glucanase activity was determined as described in the text.

tested by using mycelia that were pregrown in half-strength PDB, starved for 12 h, and transferred to mineral medium supplemented with *S. cerevisiae* cell walls in the absence of glucose. β -1,3-Glucanase activity was determined after 24 h of incubation. At this time, 2% glucose was added to a parallel culture, and both cultures were incubated for an additional 24 h. Figure 2 shows that the production of β -1,3-glucanase activity was inhibited; the level of activity obtained was only 51% of the level observed without the addition of glucose (14 and 27 U/mg, respectively).

***T. harzianum* has a complex glucanolytic system.** Concentrated culture filtrates obtained with the best β -1,3-glucanase inducers (Fig. 1) were subjected to SDS-PAGE to determine whether the observed differences in activity correlated with a specific protein pattern. Complex protein patterns were observed with the two inducers tested (Fig. 3). When samples obtained with *R. solani* cell walls (Fig. 3, lane 3), laminarin (Fig. 3, lane 1), and 2% glucose (Fig. 3, lane 2) were compared, six major protein bands which were not present when glucose was the sole carbon source were observed in the cell wall samples, and four major protein bands which were not present when glucose was the sole carbon source were observed in the laminarin samples.

FIG. 2. Effect of glucose on β -1,3-glucanase induction. Two parallel *T. harzianum* cultures were incubated with *S. cerevisiae* cell walls. At the time indicated by the arrow, 2% glucose was added to one of the cultures (○), whereas the second culture was used as a control (●). Incubation was continued for 24 h, and enzyme activities were determined at the time points indicated.

FIG. 3. SDS-PAGE analysis of the proteins secreted by *T. harzianum*. Culture filtrates were obtained by using mineral medium supplemented with either laminarin (lane 1), 2% glucose (lane 2), or *R. solani* cell walls (lane 3). Each filtrate was dialyzed and lyophilized, and 50 μ g of protein from each sample was subjected to SDS-PAGE. The gel was stained with Coomassie brilliant blue. Lane M contained molecular mass markers. The arrows indicate bands not present in lane 2.

To determine which of the polypeptides observed corresponded to β -1,3-glucanase, we assayed for enzyme activity by performing SDS-PAGE. The results indicated that in the presence of laminarin *T. harzianum* produced at least three bands with β -1,3-glucanase activity; two of these bands were at apparent molecular weights of 35,000 and 39,000, and the third band was a wide band at 60 to 80 kDa (Fig. 4, lane 1). In contrast, when glucose was used as the sole carbon source, only two β -1,3-glucanase bands (39 and 60 kDa) were found (Fig. 4, lane 2). The 39-kDa band and the band of activity at 60 to 80 kDa apparently corresponded to the 39- and 77-kDa protein bands observed after Coomassie brilliant blue staining when

FIG. 4. Detection of β -1,3-glucanase activity after SDS-PAGE of culture filtrates precipitated with acetone. Culture filtrates were obtained by using mineral medium supplemented with either laminarin (lane 1) or glucose (lane 2) and were precipitated with acetone, the concentrated samples were subjected to SDS-PAGE, and the gel was stained for β -1,3-glucanase activity. All lanes were loaded with 15 μ g of protein. Lane M contained prestained molecular mass markers.

FIG. 5. IEF of β -1,3-glucanase from *T. harzianum* filtrates. Culture filtrates were obtained by using mineral medium supplemented with different commercial polysaccharides or fungal cell walls as sole carbon sources. (A) Culture filtrates precipitated with acetone. Lane 1, glucose; lane 2, *M. rouxii* cell walls; lane 3, *N. crassa* cell walls; lane 4, *R. solani* cell walls; lane 5, *S. cerevisiae* cell walls; lane 6, *S. cerevisiae* cell wall filtrate; lane 7, *S. cerevisiae* residual cell walls; lane 8, pustulan; lane 9, laminarin; lane 10, pullulan. Lanes were loaded with 1 U of enzyme. (B) Culture filtrates that were dialyzed and lyophilized. Lane 1, laminarin; lane 2, *R. solani*; lane 3, glucose. All lanes were loaded with 15 μ g of protein.

laminarin and *R. solani* cell walls were used as carbon sources (Fig. 3).

To determine whether the 60- to 80-kDa activity band and the 39-kDa activity band present in the laminarin sample corresponded to the bands produced in the presence of glucose, enzymatic detection on IEF gels was carried out with acetone-precipitated culture filtrates. As Fig. 5A shows, *T. harzianum* produced two isoforms (pI 6.6 and 6.8) with all of the inducers and three different isoforms (pI 4.8, 5.7, and 5.9) with 2% glucose. Three additional β -1,3-glucanases (pI 5.0, 5.5, and 6.04) were detected in the culture medium when laminarin was used as the sole carbon source and the culture medium was lyophilized (Fig. 5B).

Due to the apparent complexity of the glucanolytic system of *T. harzianum*, a lyophilized sample of the laminarin-induced culture filtrate was subjected to two-dimensional gel electrophoresis and stained for glucanase activity. A complex pattern consisting of at least six glucanases was obtained (Fig. 6). Two of these enzymes had an apparent molecular weight of 77,000 and pI values of 6.8 and 6.6, and three of them appeared to be 60-kDa proteins with pI values of 6.0, 5.5, and 5.0. A sixth

FIG. 6. Detection of β -1,3-glucanase activity on a two-dimensional gel. Lyophilized laminarin culture filtrate was subjected to two-dimensional gel electrophoresis. (A) First-dimension activity pattern. (B) β -1,3-Glucanase activity pattern on the two-dimensional gel. The gel was loaded with 5 U of enzyme.

FIG. 7. Size and staining characteristics of the purified β -1,3-glucanase as determined by SDS-PAGE. (A) Silver-stained gel. Lane M, molecular mass markers; lane 1, purified enzyme. (B) Laminarin zymogram. (C) Glycoprotein staining of the purified protein.

activity spot with an apparent molecular weight of 35,000 and a pI of 5.5 was detected. In contrast to the SDS-PAGE analysis, no activity was detected at 39 kDa.

Purification of a major component of the β -1,3-glucanolytic system. As shown in Fig. 6, *T. harzianum* secretes multiple β -1,3-glucanase isoforms into the culture medium. To study the properties of the most abundant species (Fig. 4), we decided to purify it from the culture filtrate. The procedure used consisted of three steps, lyophilization, anionic exchange, and size exclusion, and resulted in 108-fold purification and a 43% yield. At the end of the procedure, activity eluted as a single peak (data not shown). The estimated molecular size of the protein fraction with the highest activity obtained after the size exclusion step was 80 kDa. SDS-PAGE analysis of this fraction revealed a major protein band at a molecular mass of approximately 77 kDa, a molecular mass slightly smaller than the molecular mass estimated by column filtration, and two faint bands at lower molecular masses after silver staining of the gel (Fig. 7A). A single active band corresponding to the 77-kDa polypeptide was detected following activity staining, as shown in Fig. 7B. A blot of an equivalent sample was stained for glycoprotein detection with concanavalin binding. Three bands were revealed, one at 77 kDa, one at 70 kDa, and one at 58 kDa (Fig. 7C), indicating that the enzyme polypeptide is glycosylated. Although the 70- and 58-kDa protein bands were only faintly visible after silver staining, concanavalin binding indicated that they were highly glycosylated. Analysis of the purified fraction with an IEF gel revealed a major band with a pI of 6.8 after Coomassie brilliant blue staining. However, two minor bands with pI values of 6.6 and 6.0 were also observed after activity staining (data not shown). The purified β -1,3-glucanase efficiently hydrolyzed laminarin (2,804 U/mg) but was completely inactive on pustulan and pullulan. To determine whether the purified β -1,3-glucanase is an endoenzyme or an exoenzyme, the enzyme was incubated with oxidized laminarin (3). The absence of detectable hydrolysis suggested that the activity is an exoenzyme activity.

DISCUSSION

Our results show that *T. harzianum* produced β -1,3-glucanase when it was grown with all of the carbon sources examined. The level of production of β -1,3-glucanase varied depending on the carbohydrate source. The specific activity increased in the presence of cell walls of *M. rouxii*, *N. crassa*, *S.*

cerevisiae, and *R. solani* (in ascending order of efficacy) and appeared to be dependent on the amount of β -1,3-glucan present in the cell walls of these organisms. In this regard, the mycelium of *M. rouxii* contains no detectable β -1,3-glucan, whereas *N. crassa* and *S. cerevisiae* cell walls contain 20.2 and 55% β -1,3-glucan, respectively (20, 23). The β -1,3-glucan content of *R. solani* cell walls has not been determined. In addition, β -1,3-glucanase activity was higher with laminarin (β -1,3-glucan) than with pustulan (β -1,6-glucan) or pullulan (α -1,6-glucan), suggesting that the induction patterns of the enzymes may vary in response to the glucan structure and that β -1,3-glucanase induction depends on the type of linkage. These data do not support the proposal that induction of β -1,3-glucanases in *T. harzianum* does not require β -1,3-glucan (22). In addition, results obtained with the filtrate of autoclaved *S. cerevisiae* cell walls suggest that the induction observed with cell walls may be triggered by two components, one extractable and one that remains cell wall bound. When all of the carbon sources tested were compared, the highest enzyme production was observed in laminarin-induced filtrates, in contrast to the surprisingly low levels detected by de la Cruz and coworkers with the same carbon source (13). Similar variations in different strains have been observed for various lytic enzymes in bacteria (16).

Only trace levels of β -1,3-glucanase activity were produced when the fungus was grown with glucose (Fig. 1). In addition, production of β -1,3-glucanase under otherwise inducing conditions was inhibited by addition of glucose (Fig. 2). The mechanism leading to the inhibition observed remains to be investigated. Furthermore, the analysis of the activity profiles on IEF zymograms indicated that the activity detected in the glucose samples correlated with a group of enzymes different from the enzymes produced with all other carbon sources (Fig. 5A). These data suggest that the latter results from enzyme induction. It could be that the β -1,3-glucanase species detected when glucose was used as the carbon source are required to sustain fungal growth.

IEF zymograms of the induced culture filtrates revealed two active polypeptides in acetone precipitates (Fig. 5A), in contrast to the five β -1,3-glucanase bands detected in the lyophilized preparations (Fig. 5B). There are two possible explanations for these results. First, treatment with acetone might not precipitate all of the β -1,3-glucanases produced by *Trichoderma* species. And second, some of the active bands observed in the lyophilized samples might be proteolytic products released from mature enzymes (1).

Two-dimensional gel electrophoresis revealed that the *T. harzianum* glucanolytic system was even more complex, encompassing at least six glucanases. In addition, a 39-kDa β -1,3-glucanase was observed in the SDS-PAGE analysis; this enzyme was not detected by the two-dimensional electrophoresis technique. Similar complex glucanolytic systems, including both endo- and exo- β -1,3-glucanases, have been described for other fungi (12, 18, 27, 32, 33). The molecular masses of the fungal β -1,3-glucanases characterized appear to vary considerably, not only between species but also within species (31). β -1,3-Glucanases with molecular masses of 31.5, 36.0, 66, and 78 kDa have been reported previously for different *T. harzianum* isolates (13, 19, 22, 28).

To gain insight into enzyme multiplicity, it is important to obtain specific information on each β -1,3-glucanase species secreted. Thus, one of the extracellular β -1,3-glucanases was partially purified. The β -1,3-glucanase purified in this study hydrolyzed laminarin but not pustulan or pullulan, indicating that it had a specific activity directed toward the β -1,3 linkage. A zymogram analysis showed that the major active band cor-

responded to a 77-kDa polypeptide with three isoforms (pI 6.8, 6.6, and 6.0) (data not shown). This is in contrast to the 66-kDa species (pI 7.7 and 8) and the 78-kDa species (pI 6.2) previously reported (13, 22). Since the difference in size is relatively small, the possibility that the different mobilities of the enzymes are due to different degrees of glycosylation cannot be ruled out. Clearly, the β -1,3-glucanase purified in this work differs in this regard from the nonglycosylated 66-kDa β -1,3-glucanase described by de la Cruz and coworkers (13). The failure to observe the 39-kDa β -1,3-glucanase in the two-dimensional electrophoresis analysis and throughout the purification procedure may have resulted from an increase in proteolytic activity after the purification procedure, particularly the lyophilization step.

In conclusion, *T. harzianum* produces a complex system consisting of at least seven β -1,3-glucanases under inducing conditions. The level of activity secreted is dependent on the proportion of β -1,3-glucan present in the inducer. The physiological role of each of the enzymes detected here remains to be investigated. Finally, based on the secretion of these enzymes during simulated mycoparasitism and considering that several other lytic enzymes secreted by *T. harzianum*, including a β -1,3-glucanase, have been shown to act synergistically (22), similar interactions that include enzymes with activities other than glucanolytic activities may be necessary for maximum activity against fungal cell walls.

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