JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

# Proteomic Study of Biocontrol Mechanisms of *Trichoderma harzianum* ETS 323 in Response to *Rhizoctonia solani*

SHIH-CHI TSENG,[†] SHU-YING LIU,[§] HSUEH-HUI YANG,[#] CHAUR-TSUEN LO,[⊥] AND KOU-CHENG PENG*,[†]

Institute of Biotechnology, National Dong Hwa University, Hualien, 97401, Taiwan; Department of Molecular Biotechnology, Da-Yeh University, Changhua 51591, Taiwan; Department of Research, Buddhist Tzu Chi General Hospital, General Education Center, Tzu Chi College of Technology, 97002, Hualien, Taiwan; and Department of Biotechnology, National Formosa University, Yunlin 63208, Taiwan

To elucidate the entire range of proteins that are secreted by *Trichoderma harzianum* ETS 323 in its antagonism with *Rhizoctonia solani*, an in vivo interaction between them was mimicked and not only the secreted cell wall-degrading enzymes (CWDEs) but also all of the proteome were investigated. Seven CWDEs, chitinase, cellulase, xylanase, $\beta$-1,3-glucanase, $\beta$-1,6-glucanase, mannanase, and protease,were revealed by activity assay, in-gel activity stain, 2-DE, and LC-MS/MS analysis. Extracellular protein extracts from media that contained *R. solani* exhibited much higher CWDE activities than media that did not contain *R. solani*. Cellulase and mannanase activity, however, were insignificant. Activity stain also revealed that $\beta$-1,3-glucanase, $\beta$-1,6-glucanase, and xylanase activity occurred exclusively in media that contained *R. solani*. Furthermore, 35 of the 43 excised spots on the 2-DE gel were successfully analyzed by LC-MS/MS, and eight proteins were identified. They were two glycoside hydrolases, two proteases, two $\beta$-glucosidases, one endochitinase and, interestingly, one amino acid oxidase. Additionally, a possible mechanism was proposed to elucidate how the cell walls of *R. solani* are systematically enveloped and disintegrated.

**KEYWORDS: Proteome; biocontrol; *Trichoderma harzianum*; *Rhizoctonia solani*; cell wall-degrading enzymes**

## INTRODUCTION

Phytopathogenic fungi, such as *Rhizoctonia*, *Botrytis*, *Pythium*, and *Fusarium*, cause various plant diseases (*1*) that are responsible for serious economic and agricultural loss as they attack cash crops such as peppers, tomatoes, potatoes, cucumbers, and tobacco (*2*). To minimize the damage caused by these fungi, fungicides such as Benomyl, Carboxin, and Pyrimethanil (*3*) are adopted. Chemical treatment for controlling plant diseases not only pollutes the environment but also allows phytopathogenic fungi to evolve resistant strains. Alternatively, biocontrol agents (BCAs) that antagonize phytopathogenic fungi can be used to control plant disease. Biocontrol involves the introduction of particular microorganisms, either natural or genetically modified, into an agricultural ecosystem to reduce the effects of undesirable organisms and to favor the survival of organisms that are of benefit to humans (*4, 5*).

Various strategies of biocontrol have been proposed. They include the creation of competition for nutrients or space; the production of antibiotics and lytic enzymes; the inactivation of the enzymes of phytopathogenic fungi; and parasitism (*5*). The cell wall-degrading enzymes (CWDEs), mostly chitinases, glucanases, and proteases, are major lytic enzymes that are secreted by biocontrol agents (*2*). CWDEs attack the cell wall of phytopathogenic fungi to cause cell lysis and subsequent death. Although the mechanism of mycoparasitism is not completely understood, this process has been assumed to involve the expression of extracellular CWDEs (*1*). Recently, homologues of proteins encoded by avirulence genes have been identified in *Trichoderma* strains. These proteins can induce hypersensitive responses and other defense-related reactions in plant cultivars that contain the corresponding resistance genes (*2*). *Trichoderma harzianum*, a filamentous fungus that is commonly found in the rhizosphere, has been accepted as one of the most potent biocontrol agents against plant diseases and has been used as an antagonist against many soilborne phytopathogenic fungi over the past few years (*6*).

To the best of the authors' knowledge, biocontrol mechanisms have been investigated mostly on a single protein, but little work has been performed on the total protein expressed by a genome.

* Corresponding author (telephone +886 3 863 3635; fax +886 3 863 3630; e-mail kcpeng@mail.ndhu.edu.tw).
[†] National Dong Hwa University.
[§] Da-Yeh University.
[#] Tzu Chi College of Technology.
[⊥] National Formosa University.

10.1021/jf703626j CCC: $40.75 
Published on Web 07/19/2008

Proteomics is the study of the proteome, which constantly changes over various stages of the life cycle and under various environmental conditions. The combination of two-dimensional gel electrophoresis (2-DE) with protein identification by mass spectrometry constitutes the main method for identifying proteomics. 2-DE is a useful tool for separating thousands of proteins in complex mixtures and can resolve over 5000 proteins simultaneously; it has the ability to detect <1 ng of protein per spot (*7*). This approach has proven to be powerful in studying the change in protein expression in an organism under various environmental conditions (*8*). Advances in modern technology in the fields of mass spectrometry, along with a growing genome sequence database, enable protein identification by peptide mass fingerprinting. Liquid chromatography−tandem mass spectrometry (LC-MS/MS) can theoretically identify any protein when the genome sequence of the organism under study is available (*9*).

Proteomic studies of *Trichoderma* have previously addressed its cellular proteins (*10, 11*), the inducement of secreted proteins in phytopathogenic fungal cell walls (*12*), mitochondria proteins (*13*), and three-way interactions among *Trichoderma atroviride*, plant and fungal pathogens (*14*). The goal of the present investigation, however, is to demonstrate the range of proteins that are secreted by *T. harzianum* ETS 323 that is grown in the presence or absence of *R. solani* mycelia. It identifies the target proteins that are directly related to biocontrol mechanisms by elucidating the proteome and activity of the CWDEs.

## MATERIALS AND METHODS

**Fungal Material.** *R. solani*, isolated in Taiwan, was maintained on potato dextrose agar (PDA) and grown in a 250 mL potato dextrose broth (PDB) (Difco) at 28 °C. It was then incubated while being shaken at 150 rpm in a 500 mL Erlenmeyer flask for 7 days. Mycelia were collected by centrifugation at 3000*g* for 10 min at room temperature. The mycelia were washed twice using 50 mL of 18.2 MΩ sterile deionized water and then boiled for 20 min twice to obtain deactivated mycelia, which were then stored at −20 °C until use. An aliquot of mycelia was incubated on PDA and observed for 7 days to determine whether the deactivated mycelia were completely deactivated.

*T. harzianum* ETS 323 was also maintained on PDA (*15*). To mimic the biocontrol environment, four supplements were separately added to a basic broth medium. The ingredients of the basic medium were 1.4 g of $(NH_4)_2SO_4$, 0.2 g of $KH_2PO_4$, 6.9 g of $NaH_2PO_4 \cdot H_2O$, 0.3 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of peptone, and 0.3 g of urea. The supplements were 10 g of glucose (medium I), 5 g of glucose and 5 g of deactivated *R. solani* mycelia (medium II), 10 g of deactivated *R. solani* mycelia (medium III), and 10 g of deactivated *T. harzianum* mycelia (medium IV).

The conidia was grown in a 250 mL broth medium at 22 °C with shaking, and the culture supernatants were collected every 24 h for a total of 120 h by centrifugation at 3000*g* and 4 °C for 10 min.

**Antagonism between T. harzianum ETS 323 and R. solani.** *T. harzianum* ETS 323 and *R. solani* were incubated separately for 5 days due to *T. harzianum*'s much faster growth. Then, a 0.5 mm mycelium was incised from the edge of each, transferred to opposite sides of a single 90 mm PDA plate, and then further incubated for 6 days at 25 °C. The growths of both fungi were photographed every 24 h.

**Sample Preparation.** Ammonium sulfate (Riedel-de Haen, Seelze, Germany) was added to the *T. harzianum* ETS 323 culture supernatant and stirred at 4 °C for 12 h to give up to 80% saturation. Following centrifugation at 17000*g* for 30 min at 4 °C, the precipitated protein pellets were resuspended in 3 mL of deionized water. A 0.1% protease inhibitor cocktail (P8215, Sigma, St. Louis, MO) was added, and samples were dialyzed [CelluSep dialysis membrane molecular weight cut off (MWCO) = 6000−8000, Seguin) against a 100-fold volume of deionized water at 4 °C for 12 h and then stored at −20 °C until use. The protease activity assay was conducted from the sample without the addition of protease inhibitors.

**SDS-PAGE.** The prepared extracellular proteins in media I, II, and III were loaded onto 12.5% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) (*16*) at 20 mA, for 1.5 h.

**Enzyme Activity Assay.** Individual enzyme activities were determined by measuring the reduction of sugar from their respective substrates using a dinitrosalicylic acid (DNS) reagent (*17*). The substrates used were carboxylmethylcellulose (CMC) (Fluka, Buchs, Switzerland), xylan, laminarin, galactomannans of locust bean gum (Sigma), and pustulan (Calbiochem, Darmstadt, Germany), which corresponded to the enzymes cellulase, xylanase, $\beta$-1,3- glucanase, mannanase, and $\beta$-1,6-glucanase, respectively. Forty micrograms of extracellular protein extract was mixed with 120 $\mu$L of 1% substrate for 20 min at 50 °C. Then, DNS reagent was added, and the system was then incubated for 30 min at 90 °C to terminate the reaction and develop color. The reaction mixture was chilled, and $OD_{570}$ readings were recorded. Prespecified concentrations of glucose or xylose solutions were used to determine the extinction coefficient. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 $\mu$mol of product per minute under the assay conditions.

To quantify chitinase activity, 10 $\mu$L of 0.5% artificial substrate 4-nitrophenyl *N*,*N*′-diacetyl- $\beta$-D-chitobioside (Sigma) and 120 $\mu$g of extracellular protein samples were mixed in 200 $\mu$L of 100 mM acetate buffer (pH 5.0). After 30 min, 50 $\mu$L of 0.4 M $Na_2CO_3$ was added to terminate the reaction, and $OD_{415}$ readings were recorded (4-nitrophenyl $\varepsilon = 18300\ cm^{-1}\ M^{-1}$). One unit of enzyme activity was defined as the amount of enzyme required to produce 1 $\mu$mol of product per minute under the assay conditions.

Protease activity was determined following the manufacturer's protocol using the QuantiCleave Protease Assay Kit (Pierce, Rockford, IL). Briefly, in the presence of protease and succinylated casein as substrate, the peptide bonds of succinylated casein were cleaved, exposing primary amines, which then reacted with 2,4,6-trinitrobenzene sulfonic acid (TNBSA). $OD_{450}$ was then determined. One benzoyl-L-arginine ethyl ester (BAEE) unit was defined as the amount that caused a change in absorbance at 253 nm of 0.001 per minute in 3.2 mL of reaction mixture (pH 7.6, 25 °C). Two hundred and seventy BAEE units correspond to 1 international unit.

**In-Gel Activity Stain.** The extracellular protein samples were loaded onto 15% SDS−polyacrylamide gels, which contained 1% of chitin, CMC, birch wood xylan, laminarin, pustulan, or locust bean gum. SDS-PAGE was performed three times at 20 mA until the dye reached the bottom of the gel, which was then soaked in 1% Triton X-100 (Sigma) for 20 min. The gel was washed twice in 10 mM acetate buffer (pH 5.0) at 4 °C for 1 h and then incubated at 50 °C for 24 h. It was stained with 0.1% Congo red (Sigma) for 1 h and washed in 1 M sodium chloride (Riedel-de Haen) until bands appeared. If the enzyme reacted with the substrate contained in the gel, the substrate would decompose into oligosaccharides or monosaccharides, which would not take the Congo red dye and would exhibit translucent zones following washing.

**Two-Dimensional Electrophoresis for Extracellular Protein Extracts.** Extracellular protein samples were concentrated to 5 mg $mL^{-1}$ using an Amicon Ultra MWCO 5000 centrifugal filter (Millipore, Bedford, MA). Urea (USB, Cleveland, OH) and 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate (CHAPS) (J. T. Baker, Phillipsburg, NJ) were added to 200 $\mu$L of concentrated extracellular protein samples to final concentrations of 8 M and 2% (w/v), respectively. Three milligrams of freshly prepared DTT (Sigma), 2 $\mu$L of carrier ampholytes (Bio-Rad, Hercules, CA), and a trace amount of bromophenol blue (Sigma) were mixed with the samples. IPG strips (pH 4−7, 17.1 cm, Bio-Rad) were actively rehydrated at 50 V for 12 h and then exposed to a total of 90000 V-h in steps using a Bio-Rad Protean IEF cell at 20 °C. The voltage was programmed as follows; a rapid ramp to 300 V for 4 h and a linear ramp to 10000 V for 8 h, which was maintained until the total 90000 V-h was reached. Then, the strips were stored at −20 °C until use. Before the second dimension, the IPG strips were equilibrated for 15 min in an equilibration buffer [50 mM Tris-HCl (pH 8.8); 2% (w/v) SDS, 1% (w/v) DTT, 6 M urea, and 30% (w/v) glycerol] and then for another 15 min in the same equilibration buffer of 4% (w/v) iodoacetamide (Sigma) instead of DTT. The second-

dimensional electrophoresis was conducted in 12.5% SDS-PAGE at 25 mA for 6.5 h, and proteins were stained with Coomassie Blue R-250 (J. T. Baker).

**Analysis of Images.** The 2-DE gel images were analyzed using Imagemaster 2D platinum software (Amersham Biosciences, New Territories, Hong Kong).

**In-Gel Digestion.** Protein spots were excised into 1 mm cubes, placed in a 0.5 mL microfuge tube, and then washed in 50 mM ammonium bicarbonate and acetonitrile (1:1, v/v) (J. T. Baker) for 15 min. After the liquid was removed, sufficient acetonitrile was added to cover the gel cubes. This step was repeated until the gel cubes became colorless, and then the solution was dried in a vacuum centrifuge. For reduction and alkylation, the gel pieces were swollen in 10 mM dithiothreitol/25 mM ammonium bicarbonate and incubated for 45 min at 56 °C. After it had been cooled, the solution was drained and replaced by 55 mM iodoacetamide in 25 mM ammonium bicarbonate (Riedel-de Haen) and incubated for 30 min at room temperature in the dark. The gel pieces were washed twice in 50 mM ammonium bicarbonate and acetonitrile (1:1) for 15 min. Acetonitrile was then added to shrink the gel pieces, and this solution was further dried in a vacuum centrifuge. Then, sequencing-grade modified trypsin (10 ng/$\mu$L) (Promega, Madison, WI) in 25 mM ammonium bicarbonate was added to each gel piece to cover it, and then the gel was incubated at 37 °C for 30 min. Twenty-five millimolar ammonium bicarbonate was added to keep the gel wet while it was incubated at 37 °C for 16 h. Following incubation, the gel was sonified for 20 min, and the supernatant was recovered. Three microliters of 50% acetonitrile in 1% trifluoroacetic acid was added to recover the rest of the peptides.

**Identification of Protein.** In-gel and solution digests of proteins were analyzed by a nanoscale capillary LC-MS/MS using an UltiMateTM capillary LC system, Switchos valve switching unit, and Famos autosampler (LC Packings, San Francisco, CA), coupled to a quadrupole time-of-flight mass spectrometer (QSTAR XL, Applied Biosystem/MDS Sciex, Foster City, CA) that was equipped with a nanospray ionization source. Two and a half microliters of sample was injected through an autosampler into the LC system at a flow rate of 30 $\mu$L min$^{-1}$ and preconcentrated on a 300 $\mu$m × 5 mm PepMap C18 precolumn (LC Packings). The peptides were then eluted onto a 75 $\mu$m × 150 mm NanoEase C18 analytical column (Waters Corp., Milford, MA), which had been pre-equilibrated with solution A (5% ACN, 95% water, 0.1% formic acid), and the peptide was separated using a linear gradient of 5−60% in solution B (80% ACN, 20% water, 0.1% formic acid) over 47 min at a flow rate of 200 nL min$^{-1}$. This flow rate through the column was reduced from 200 $\mu$L min$^{-1}$ from pumps A and B by flow splitting. The LC eluent was directed to the electrospray source with a PicoTip emitter (New Objectives, Woburn, MA). The mass spectrometer was operated in positive ion mode. A TOF analyzer was calibrated with multipoint calibration using selected fragment ions from the collision-induced dissociation (CID) of Glu-fibrinopeptide B. MS/MS spectra were obtained in a data-dependent acquisition method (DDA). Subsequently, MS/MS data were subjected to search algorithms against the Swiss-Prot protein sequence database using Mascot software (Matrix Science Ltd., London, U.K.) with the following parameters: variable modifications, oxidation (M) and carbamidomethyl (C); peptide mass tolerance, ±2 Da; fragment mass tolerance, ±0.8 Da; maximum missed cleavages, 1; instrument type, ESI-QUAD-TOF.

## RESULTS

**Antagonism between R. solani and T. harzianum ETS 323.** *T. harzianum* ETS 323 and *R. solani* were simultaneously inoculated on opposite sides of a 90 mm PDA plate. The mycelia of *T. harzianum* ETS 323 spread more rapidly than those of *R. solani*. After 96 h of incubation, the mycelia of the two species were located and their boundary was observed. After a brief standoff between the two, white mycelia of *T. harzianum* ETS 323 were mounted on top of *R. solani*'s mycelia. After 120 h of incubation, the white mycelia of *T. harzianum* ETS 323 covered the entire plate. After 148 h of incubation, green conidia also covered the plate.

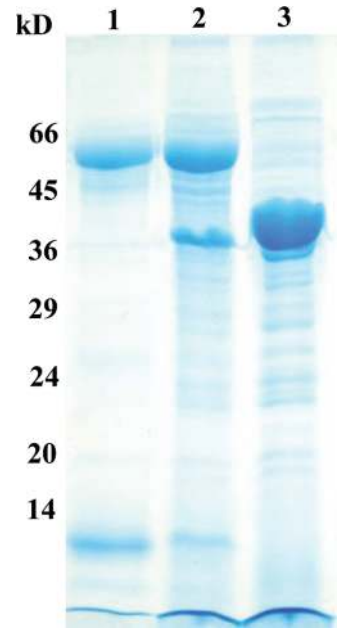



**Figure 1.** Extracellular protein extracts of *T. harzianum* ETS 323 resolved by 12.5% SDS-PAGE. Lanes: 1, extracts from *T. harzianum* ETS 323 grown in media I (glucose only); 2, extracts from *T. harzianum* ETS 323 grown in media II (glucose plus deactivated *R. solani* mycelia); 3, extracts from *T. harzianum* ETS 323 grown in media III (deactivated *R. solani* mycelia only). Molecular mass markers: bovine serum albumin, 66 kDa; chicken egg ovalbumin, 45 kDa; rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; bovine erythrocytes carbonic anhydrase, 29 kDa; bovine pancreas trypsinogen, 24 kDa; soybean trypsin inhibitor, 20 kDa; bovine milk α-lactalbumin, 14 kDa.

**Extracellular Proteins of T. harzianum in Various Media.** The extracellular proteins that were obtained from cultures grown in media I (glucose only), II (glucose plus deactivated *R. solani* mycelia), and III (deactivated *R. solani* mycelia only) were harvested and saturated with 80% ammonium sulfate. Each protein profile was then demonstrated using SDS-PAGE. Extracts from media II and III exhibited much higher protein varieties compared to the extract from medium I and had similar banding patterns, although the predominant bands are a bit different (**Figure 1**). 2-DE gels were expected to produce the same result.

**Analysis of Enzyme Activities of Extracellular Proteins.** The enzyme activities of the extracellular proteins were determined. These were chitinase, cellulase, xylanase, $\beta$-1,3-glucanase, $\beta$-1,6-glucanase, mannanase, and protease. Extracts from media II and III had much higher activities of chitinase, xylanase, $\beta$-1,3-glucanase and $\beta$-1,6-glucanase than those from medium I after 48 or 72 h of incubation, whereas the enzyme activities of extracts from medium IV were all extremely low (**Figure 2**). Cellulase and mannanase activities, however, were insignificant. Protease activity was high on day 4 in medium III and on day 5 in medium II (**Figure 3**). However, no activity of protease from the extracts of medium I (glucose only) or IV (deactivated *T. harzianum* mycelia only) was detected.

**Enzyme Activity Stain.** $\beta$-1,3-Glucanase, $\beta$-1,6-glucanase, and xylanase activities were evident on SDS gel that had been stained to reveal such activity (**Figure 4**, AII, BII, CII). Extracellular proteins in each preparation were resolved in SDS-PAGE that contained a substrate that was specific to each enzyme. The translucent areas indicated activity and were thus distinguished from the surrounding redness. Bands of $\beta$-1,3-glucanase and xylanase activity were observed in the extracel-

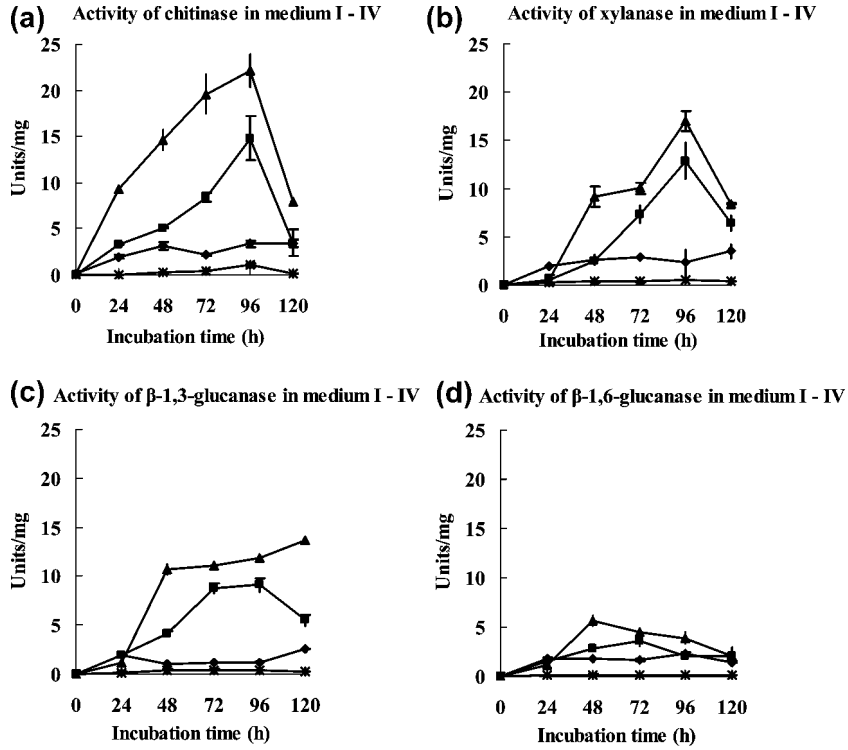



**Figure 2.** Enzyme activities of the extracellular protein extracts from *T. harzianum* ETS 323 grown in different media over a 120 h incubation period: (**a**) chitinase activity; (**b**) xylanase activity; (**c**) $\beta$-1,3-glucanase activity; (**d**) $\beta$-1,6-glucanase activity; (◆) enzyme activities detected in medium I; (■) enzyme activities detected in medium II; (▲) enzyme activities detected in medium III; (∗) enzyme activities detected in medium IV. Basic medium with supplement of 10 g of glucose (medium I); 5 g of glucose and 5 g of deactivated *R. solani* mycelia (medium II); 10 g of deactivated *R. solani* mycelia (medium III); and 10 g of deactivated *T. harzianum* mycelia (medium IV).

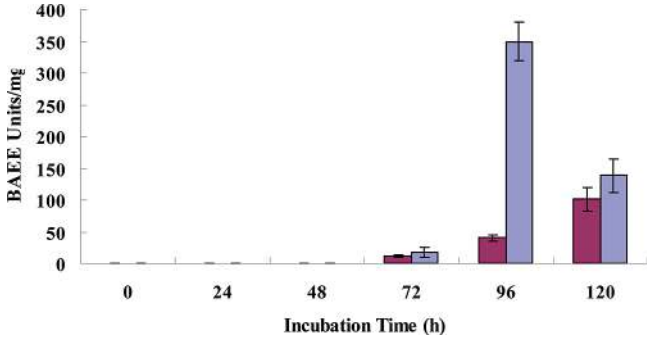



**Figure 3.** Protease activities of the extracellular protein extracts from *T. harzianum* ETS 323 grown in media II (purple bars) and III (blue bars) over a 120 h incubation period. There was no detectable activity in extracts from media I and IV. At least three replicates were performed for each experiment.

lular extracts from medium I on day 2 of incubation. $\beta$-1,3-Glucanase, $\beta$-1,6-glucanase, and xylanase activities were observed in the extracelluar extracts from media II (days 3, 4, and 5) and III (days 2, 3, and 4). Xylanase activity was observed in medium III on days 2−4 and in medium III on days 3−5. $\beta$-1,3-Glucanase and $\beta$-1,6-glucanase activities was observed in medium III on days 2−5 and in medium II on days 3−5. Additionally, four isozymes of $\beta$-1,3-glucanase (45, 24, 20, and 18 kDa), three isozymes of $\beta$-1,6-glucanase (45, 20, and 18 kDa), and four isozymes of xylanase (24, 20, 18, and 14 kDa) were identified (**Tables 1** and **2**).

In medium III, $\beta$-1,3-glucanase activity was first observed on day 2 (revealed as bands A3 and A5 in **Figure 4**, AIII). On days 3 and 4, activity bands A2, A3, A4, and A5 were all observed on the gel. On day 5, only A2 was observed. $\beta$-1,6-Glucanase activity was observed on days 2−5 (bands B3 and B4 in **Figure 4**, BIII). On days 3−5, activity band B2 was also present. Xylanase activity was first observed on day 2 (bands C2, C3, and C5 in **Figure 4**, CIII). On day 3, activity bands C2, C3, C4, and C5 were observed. On day 4, only C3 and C4 were observed. No activity band was observed on day 5 (**Tables 1** and **2**).

In medium II, $\beta$-1,3-glucanase activity was first observed on day 3 (bands A3′ and A5′ in **Figure 4**, AII). On days 4 and 5, activity bands A2′, A3′, A4′, and A5′ were all observed on the gel. $\beta$-1,6-Glucanase activity was first observed on days 3−5 (bands B3′ and B4′ in **Figure 4**, BII). On days 4−5, activity band B2′ was also observed. Xylanase activity was first observed on day 3 (bands C2′, C3′, and C5′ in **Figure 4**, CII). On day 4, activity bands C2′, C3′, C4′, and C5′ were observed. On day 5, only bands C3 and C4 were observed. Interestingly, in medium II, the activity bands of all three enzymes appeared 1 day after those in medium III (**Figure 4**; **Tables 1** and **2**).

**Proteomic Investigation.** The extracellular proteins from *T. harzianum* ETS 323 grown in media I, II, and III for 96 h underwent 2-DE analysis, which revealed significant proteomic differences (**Figure 5**). Through image analysis, spots 2-9 and 3-1 were identified as the same protein, as were spots 2-3 and 3-9 and spots 3-4 and 2-7. All other spots represented unique proteins. Protein spots further underwent in-gel digestion for LC-MS/MS identification.

**Protein Identification.** After the protein spots of interest had been incised and digested in gel, LC-MS/MS was adopted to determine the sequence of the protein fragments. Of the 43 selected spots, 35 exhibited LC-MS/MS signals. Every sequenced fragment was input to the Mascot search engine to determine the function of the selected proteins. Eventually, eight proteins were identified and are listed below (**Table 3**). Spot 1-5 was matched to a single peptide sequence of

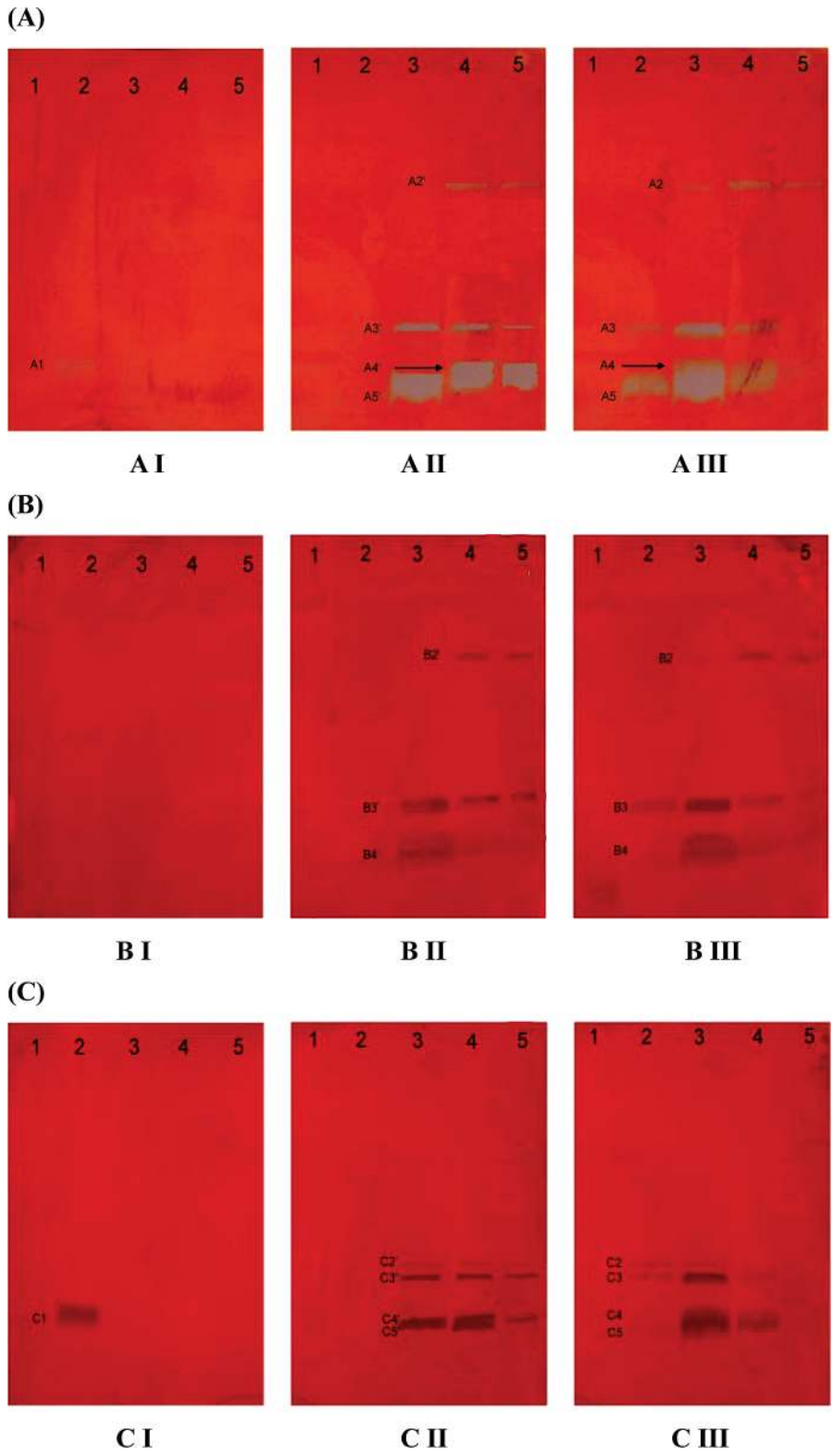



**Figure 4.** Activity stain of the extracellular protein extracts from *T. harzianum* ETS 323 grown in media I, II, and III: (**A**) $\beta$-1,3-glucanase activity on the gel containing laminarin; (**B**) $\beta$-1,6-glucanase activity on the gel containing pustulan; (**C**) xylanase activity on the gel containing birchwood xylan (band color difference due to different substrate breakdown byproducts). Lanes 1−5: days 1−5 on 15% SDS-PAGE contained 1% indicated substrate.

glycosyl hydrolase from *Gibberella zeae*, which is of the same order, *Hypocreales*, as *T. harzianum* ETS 323. Spot 1-11 was matched to a single peptide sequence of glycosyl hydrolase from *Aspergillus fumigatus*, a filamentous fungus that is similar to *T. harzianum* ETS 323. Spot 2-1 was matched to four peptide sequences of putative L-amino acid oxidase (LAAO) from *T. harzianum*. Spot 2-6 was matched to five peptide sequences of putative aspartic protease from *T. harzianum*. Spot 2-8 was matched to one peptide sequence of $\beta$-glucosidase from *Talaromyces emersonii*, a filamentous fungus that is similar to *T. harzianum* ETS 323. Spot 3-2 was matched to one peptide sequence of endochitinase from *Hypocrea* (*Trichoderma*) sp. Spot 3-8 was matched to one peptide sequence of $\beta$-glucosidase from *Cryptococcus neoformans*, which belongs to the Basidiomycota family. Spot 3-10 was matched to two peptide sequences of serine protease

**Table 1.** Summary of Types of Enzyme Activities from Activity Stained Gels

| band | molecular mass (kDa) | type of enzyme activities |
|---|---|---|
| A1 | 18 | $\beta$-1,3-glucanase |
| C1 | | xylanase |
| A2 A2′ | 45 | $\beta$-1,3-glucanase |
| B2 B2′ | | $\beta$-1,6-glucanase |
| C2 C2′ | 24 | xylanase |
| A3 A3′ | | $\beta$-1,3-glucanase |
| B3 B3′ | 20 | $\beta$-1,6-glucanase |
| C3 C3′ | | xylanase |
| A4 A4′ | | $\beta$-1,3-glucanase |
| B4 B4′ | 18 | $\beta$-1,6-glucanase |
| C4 C4′ | | xylanase |
| A5 A5′ | 14 | $\beta$-1,3-glucanase |
| C5 C5′ | | xylanase |

**Table 2.** Summary of the Activity Bands Shown on Activity Stained Gels

| | molecular mass[a] | | | | |
|---|---|---|---|---|---|
| medium−day | 45 kDa | 24 kDa | 20 kDa | 18 kDa | 14 kDa |
| I−1 | | | | | |
| I−2 | | | | 3, X | |
| I−3 | | | | | |
| I−4 | | | | | |
| I−5 | | | | | |
| II−1 | | | | | |
| II−2 | | | | | |
| II−3 | | X | 3, 6, X | 6 | 3, X |
| II−4 | 3, 6 | X | 3, 6, X | 3, 6, X | X |
| II−5 | 3, 6 | | 3, 6, X | 3, 6, X | |
| III−1 | | | | | |
| III−2 | | X | 3, 6, X | | 3, X |
| III−3 | 3, 6 | X | 3, 6, X | 3, 6, X | 3, X |
| III−4 | 3, 6 | | 3, 6, X | 3, 6, X | |
| III−5 | 3, 6 | | | | |

[a] 3, $\beta$-1,3-glucanase activity; 6, $\beta$-1,6-glucanase activity; X, xylanase activity.

from two fungal species, *Hypocrea virens* and *Porphyromonas gingival*.

## DISCUSSION

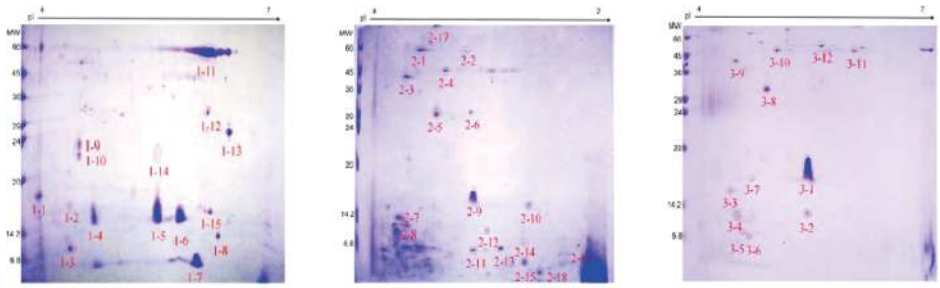
When both *T. harzianum* ETS 323 and *R. solani* were coinoculated on a single PDA plate, *T. harzianum* ETS 323 outgrew *R. solani*, satisfying the requirements for one of the mechanisms of biocontrol (*18*)—that a faster-growing agent maintains competitive advantages over a slower-growing one. After 5−6 days of incubation, not only did the conidia of *T. harzianum* ETS 323 form on the area that had been previously occupied by *R. solani* but also the mycelia of *R. solani* were eventually not detectable. Therefore, *T. harzianum* ETS 323 is strongly suggested to be able to outgrow and eventually inhibit the growth of *R. solani*. Furthermore, yellow pigments were observed on the plate; these were most likely anthraquinone derivatives, which have been claimed to participate in partial roles in inhibiting *R. solani* (*15*).

Chitinase, $\beta$-1,3-glucanase, $\beta$-1,6-glucanase, and protease activities were significant in the extracellular extracts of *T. harzianum* ETS 323 grown in the presence of deactivated mycelia of *R. solani*. The higher activity of CWDEs from *T. harzianum* ETS 323 grown in media II and III than in CWDEs grown in medium I demonstrates that deactivated mycelia of *R. solani* stimulated *T. harzianum* ETS 323 to generate these enzymes. This phenomenon was observed in the daily reduction of the mycelia of *R. solani* when cocultured with *T. harzianum* ETS 323 on a single PDA plate.

Because cell wall-degrading isozyme profiles of some *Trichoderma* have been presented elsewhere (*19*), possible isozyme activities were also analyzed by activity stain in the present study. The activities of three enzymes were detected on polyacrylamide gel that contained specific substrates; they were $\beta$-1,3-glucanases, $\beta$-1,6-glucanases, and xylanase. Because some of the activity bands had a particular molecular mass, either each enzyme may cross-react with various substrates or different enzymes have a single molecular mass.

The broad spectrum of $\beta$-glucosidase with no specific substrate was also identified (*20*). Bands A1 and C1 had the same molecular mass (18 kDa) and appeared in the same incubation period. Bands A1 and C1 represented a single enzyme that exhibited the activity of both $\beta$-1,3-glucanase and xylanase or two enzymes of equal molecular mass. A 17 kDa $\beta$-1,3-glucanase (*21*) and xylanase (*22*) with a similar molecular mass (18 kDa) have been previously identified elsewhere. These two enzymes are supposed to be either nonspecific, and both exhibit such functions on polysaccharides as those of xylan, laminarin, and pustulan, or specific, as described above. Bands A2, A2′, B2, and B2′ (45 kDa) also appeared at a single size position on day 3. A2, A2′, B2, and B2′ may be the same enzyme that exhibits the activity of both $\beta$-1,3-glucanase and $\beta$-1,6-glucanase. A 46 kDa $\beta$-1,6-glucanase has been identified (*23*), but no $\beta$-1,3-glucanase of equivalent size has been described. Bands C2 and C2′ (24 kDa) exhibited exclusively xylanase activities. To date, no xylanase of similar size has been identified. Bands A3, A3′, B3, B3′, C3, and C3′ had the same size (20 kDa) and exhibited the activities of all three enzymes. A 20 kDa xylanase (gil627018) has been submitted to the NCBI protein database, but no $\beta$-1,3-glucanase or $\beta$-1,6-glucanase of similar size has been identified. Bands A4, A4′, B4, B4′, C4, and C4′ had the same molecular mass (18 kDa) and also exhibited the activities of all three enzymes. Bands A5, A5′, C5, and C5′ were of equal size and exhibited the activities of xylanase and $\beta$-1,3-glucanase. Again, no similar molecular masses of these two enzymes have been described. The late appearance of CWDE activities in medium II is attributable to *T. harzianum*'s consumption of the mycelia of *R. solani* once the glucose was gone. In contrast, the weak CWDE activity and the absence of activity band in medium IV may follow from the lack of a nutrient source for growth and secreting protein.

Proteomics have been extensively adopted to study the protein expression of cells under various conditions. When deactivated *R. solani* was used as the sole carbon source, *T. harzianum* ETS 323 secreted various extracellular proteins, as presented in proteomic analysis. CWDEs such as proteases, chitinases, and glucanases were present in significant quantities in the extracellular extracts. To make sure *Rhizoctonia* proteins would not interfere with the protein identification following LC-MS/MS analysis, *Rhizoctonia* mycelia were disrupted by a cell disruptor and the supernatant was collected after centrifugation to eliminate debris. Two aliquots of the supernatant, 50 $\mu$g each, were treated with 350 BAEE units of TPCK trypsin (Pierce 20233) for 12 and 24 h, which is equivalent to the same amount of protease activity and duration as in the medium III at day 4. The same experiment was done but with 700 BAEE units of TPCK trypsin. SDS-PAGE analysis showed no significant



(A) (B) (C)

**Figure 5.** 2-DE analysis of extracellular protein extracts from *T. harzianum* ETS 323 grown in media I (**A**), media II (**B**), and media III (**C**).

**Table 3.** LC-MS/MS Identification of the 2-DE Protein Spots

| spot | protein | GenBank accession no. | theor MW and p*I* | amino acid sequence, coverage, and score | species |
|---|---|---|---|---|---|
| 1-5 | glycosyl hydrolase | AAR02399 | 37054, 8.92 | YWDCCKPSCSWSGKAK, 4%, 32 | *Gibberella zeae* |
| 1-11 | glycosyl hydrolase | XP753202 | 118156, 5.66 | NGNIT VNGVPVMFR, 1%, 36 | *Aspergillus fumigatus* |
| 2-1 | putative L-amino acid oxidase | CAC81784 | 50169, 4.94 | LQQAFGYYK<br>RVDAISIDLDAPDDGNMSVR<br>GLNLHPTQADAIR<br>FSYPWWIK, 11%, 96 | *Hypocrea lixii* (*T. harzianum*) |
| 2-6 | putative aspartic protease | CAI91181 | 39540, 5.61 | DTVSFGGLTVTSQSVGAAR<br>YTGSLTYFSTLK<br>AYNAFLSAAGGK<br>TDSSSGLAVFSK<br>YYAWINDGGSSGVNTIIGQK, 19%, 206 | *Hypocrea lixii* |
| 2-8 | $\beta$-glucosidase | AAL69548 | 92328, 4.87 | CVGQVGSIPR, 1%, 27 | *Talaromyces emersonii* |
| 3-2 | 42 kDa endochitinase | AAQ79795 | 22677, 4.94 | AYISGGVVASKIVLGMPIYGR,10%, 24 | *Hypocrea* sp. |
| 3-8 | $\beta$-glucosidase | AAW44922 | 94059, 5.60 | VAGGVLRWVQK, 1%, 23 | *Cryptococcus neoformans* |
| 3-10 | alkaline serine protease | XP749017 | 41050, 5.77 | FVDILAPGVDILSCWTGGPTSTK, 5%, 34 | *Aspergillus fumigatus* |

proteins present from samples following TPCK trypsin treatment. Therefore, there were no significant quantities of *Rhizoctonia* protein contaminants present in media II and III due to the cleavage action of *Trichoderma* protease activities. Further LC-MS/MS sequence analysis also revealed that none of the peptide sequence belongs to *Rhizoctonia*.

The first proteomic study of *T. harzianum* was performed by Grinyer, who identified intracellular proteins (*10*). The same group also studied extracellular proteins of *T. harzianum* that were induced by the cell walls of *R. solani* (*24*). Two proteins, 1,3-$\beta$-glucosidase and 42 kDa endochitinase, were identified in the culture supernatant of *T. atrovirid* using cell walls of *R. solani* as the sole carbon source. Rather than cell walls that were isolated from *R. solani*, deactivated *R. solani* was used herein to simulate the natural production of *T. harzianum*. Under such circumstances, numerous extracellular enzymes were identified, including CWDEs such as chitinase, glucanases, proteases, and xylanases (**Tables 1**–**3**). Although most of the corresponding proteins in the databases had different p*I* values and MWs from the spots in the 2-DE gels (**Table 3**; **Figure 5**), the differences may follow from such secondary modifications as glycosylation.

Chitin is one of the most naturally abundant polymers, and it is present as a structural polymer in fungi (*5*). Chitinase catalyzes the cleavage of the $\beta$-1,4-linkage between two units of *N*-acetyl-$\beta$-D-glucosamine (GlcNAc). Chitinases are of three types (*25*)—$\beta$-1,4-*N*-acetylglucosaminidases, endochitinases, and exochitinases (chitobiosidases). An endochitinase of about 42 kDa has been isolated from various strains of *Trichoderma* (*26, 27*). The 42 kDa endochitinase is thought to be a major enzyme that participates in mycoparasitic interaction. The expression of 42 kDa endochitinase is not induced by chitin or chito-oligosaccharides, but is triggered by carbon depletion and metabolic stress (*28*). The gene of 42 kDa endochitinase is up-regulated during the stage before contact between *T. atroviride* and *R. solani* (*29*). One endochitinase-like peptide sequence AYISGGVVASKIVLGMPIYGR (**Table 3**) was identified from a 14 kDa protein spot on the 2-DE gel. It may have been a novel endochitinase that is similar to the 42 kDa endochitinase that was described above.

Glucanases are also critical enzymes that participate in the biocontrol mechanisms of *T. harzianum* ETS 323. Most fungal cell walls comprise $\beta$-1,3-glucan, chitin, and a small amount of $\beta$-1,6-glucans (*5*). The major components of the cell wall of *R. solani* have been identified as $\beta$-glucan and chitin (*30*). Many $\beta$-1,3-glucanases from *T. harzianum* have been identified as exoglucanases (*31, 32*) and endoglucanases (*21, 33, 34*)—consistent with previous investigations that found that these enzymes are associated with the antagonistic function of *T. harzianum* (*18, 35*). $\beta$-1,3-Glucanases cleave the $\beta$-1,3 linkages between pairs of glucoses. The activities of $\beta$-1,3-glucanase and $\beta$-1,6-glucanase were detected experimentally herein when *T. harzianum* ETS 323 was grown in media II and III.

Glucose typically inhibits the secretion of $\beta$-1,3-glucanase (*32*), but in this work, the activity of $\beta$-1,3-glucanase was uninhibited for a short period when *T. harzianum* ETS 323 was grown with glucose for 24−48 h. Apparently, *T. harzianum* ETS 323 secreted a low level of $\beta$-1,3-glucanase to detect long-chain $\beta$-1,3-glucans. In a glucose-rich medium, the sentry enzymes are secreted for a short period and then degrade if no oligosaccharide with $\beta$-1,3-linkage is present. In a $\beta$-1,3-glucan-rich medium, however, oligosaccharides with $\beta$-1,3-linkages are generated and induce more $\beta$-1,3-glucanases to cleave the long-chain $\beta$-1,3-glucans to form glucose, thereby eventually disintegrating the cell wall. In the enzyme assay, $\beta$-1,3-glucanase exhibited the highest level of activity and corresponding protein

bands in the activity and stained gel when *T. harzianum* ETS 323 was grown in media II and III (**Figure 4**, AIII, A2, and A3).

β-1,6-Glucans are minor structural components of the fungal cell walls. *T. harzianum* has been demonstrated to secrete β-1,6-glucanase to hydrolyze the fungal cell walls, and three β-1,6-glucanases have been purified from extracellular proteins of *T. harzianum* (*23, 36, 37*). Although these enzymes in filamentous fungi are well-known, few of them have been purified (*23*). In this study, activity stain and activity assay were adopted, and the activity of β-1,6-glucanase was detected, but LC-MS/MS analysis did not reveal such an enzyme, possibly because insufficient information on this enzyme is available in the sequence database.

Xylanase catalyzes the hydrolysis of xylan to xylooligosaccharides and xylose and has participated in xylan degradation as a nutrient source, but not in biocontrol. However, in this work, *T. harzianum* ETS 323 grown in media II and III secreted xylanases. The role of xylanase in biocontrol has not been studied. The components of the cell wall of *R. solani* have been identified as chitin, β-1,3-glucan, and β-1,6-glucan (*30*) but not xylan. β-1,3-Glucanase or β-1,6-glucanase secreted by *T. harzianum* ETS 323 is assumed possibly to exhibit the activity of xylanase. Alternatively, xylan may be a component of the cell wall of *R. solani*. To prove this assumption, both β-1,3-glucanase and β-1,6-glucanase should be purified to test their activity against xylan, or the cell wall structure of *R. solani* should be analyzed more accurately.

L-Amino acid oxidase was identified from the extracellular proteins of *T. harzianum* ETS 323 that was grown in medium II. This enzyme cleaves amino acids and generates ammonia, hydrogen peroxide, and α-keto- aminocaproic acid. Hydrogen peroxide may have an active role in disrupting the integrity of the cell membrane. L-Lysine α-oxidase was first isolated from *T. viride* and may exhibit cytotoxic, antitumor, and antibacterial effects (*38*).

Some *Trichoderma* proteases have been described elsewhere (*36*). Also, an aspartic protease (33 kDa, p*I* 4.3) of *T. harzianum* was identified while incubated with the cell walls of *Pythium ultimum*, *Botrytis cinerea*, and *R. solani* (*12*). These proteases have been implicated in the degradation of the cell walls of *R. solani*. The high-level expression of protease during fungus−fungus interaction has been identified when *T. harzianum* and *R. solani* were involved in in vitro confrontation (*35*). In biocontrol, protease may be involved in lysing of the host by attacking structural proteins that are also part of the cell-wall skeleton (*5*). In this work, proteases' activities were identified in the extracellular extracts of *T. harzianum* ETS 323 that were grown in media II and III (peaking on day 4) but later than glucanases' activities (peaking on day 3).

Corresponding to the various amounts of CWDE activities detected during different incubation periods, this work proposes that the cell wall has various compositions toward the cell membrane. According to **Figure 2**, chitin is the main constituent throughout the cell wall and increased dramatically inward toward the cell membrane. Xylan presents little in the top of the cell wall and then distributes evenly, increasing to the cell membrane, although no solid evidence currently indicates that xylan is a component of fungal cell walls. The β-1,3-glucan content is little in the top of the cell wall and distributes evenly toward the cell membrane. Much less β-1,6-glucan than other glucans is found in the cell wall and is distributed with a slight decreasing inwardly. Each glucan comes into contact with or is inserted into the protein that is right next to the cell membrane (**Figure 3**). *R. solani* maintains its protoplast form after these CWDEs and protease activities, but the LAAO and some antibiotics that produce hydrogen peroxide finally eliminate the integrity of cell membrane of the *R. solani*.

In conclusion, proteins that participate in the biocontrol of *T. harzianum* ETS 323 in response to *R. solani* are studied, and a possible mechanism by which the cell walls of *R. solani* are enveloped and disintegrated is proposed. With respect to the proteins, 35 of the 43 excised spots on 2-DE gel were successfully analyzed using LC-MS/MS, and 8 proteins were identified herein. On the basis of these proteins, CWDEs, such as chitinase, β-1,3-glucanase, β-1,6-glucanase, protease, and xylanase, were revealed by activity assay, in-gel activity staining, 2-DE, and LC-MS/MS analysis. Chitinase, protease, and β-glucanases are known to have critical roles in the antagonism by *T. harzianum* ETS 323 with *R. solani*. The number of identified proteins was low, perhaps because the NCBI protein databank has insufficient sequence information on *T. harzianum*, covering only 241 proteins and the variety of the *Trichoderma* species. If the databank contained sufficient information, then more CWDEs and proteins associated with the biocontrol mechanism of *T. harzianum* would be identifiable. Intensive basic research, such as on protein purification and sequence identification, is strongly recommended to realize this goal. 2-DE analysis reveals the p*I* and molecular mass of the individual protein spots on gel, facilitating protein purification. With a complete databank, the proteomic method would be more powerful.

## ABBREVIATIONS USED

CWDEs, cell wall-degrading enzymes; MWCO, molecular weight cutoff; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; BAEE, benzoyl-L-arginine ethyl ester; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; PDA, potato dextrose agar; CID, collision-induced dissociation; DDA, data-dependent acquisition; LAAO, L-amino acid oxidase.

## ACKNOWLEDGMENT

We thank Ted Knoy for his editorial assistance.

## LITERATURE CITED

(1) Chet, I. *Trichoderma*: application, mode of action and potential as a biocontrol agent of soilborne plant pathogenic fungi. In *Innovative Approaches to Plant Disease Control*; Chet, I., Ed.; Wiley: New York, 1987; pp 137−160.

(2) Harman, G. E.; Howell, C. R.; Viterbo, A.; Chet, I.; Lorito, M. *Trichoderma* species-opportunistic, avirulent plant symbionts. *Nat. Rev.* **2004**, *2*, 43–56.

(3) Steffens, J. J.; Pell, E. J.; Tien, M. Mechanisms of fungicide resistance in phytopathogenic fungi. *Curr. Opin. Biotechnol.* **1996**, *7*, 348–55.

(4) Deng, S.; Lorito, M.; Penttilä, M.; Harman, G. E. Overexpression of an endochitinase gene (ThEn-42) in *Trichoderma atroviride* for increased production of antifungal enzymes and enhanced antagonist action against pathogenic fungi. *Appl. Biochem. Biotechnol.* **2007**, *142*, 81–94.

(5) Viterbo, A.; Ramot, O.; Chernin, L.; Chet, I. Significance of lytic enzymes from *Trichoderma* spp. in the biocontrol of fungal plant pathogens. *Antonie van Leeuwenhoek* **2002**, *81*, 549–556.

(6) Margolles-Clark, E.; Hayes, C. K.; Harman, G. E.; Penttila, M. Improved production of *Trichoderma harzianum* endochitinase

by expression in *Trichoderma reesei*. *Appl. Environ. Microbiol.* **1996**, *62*, 2145–2151.
(7) Gorg, A.; Weiss, W.; Dunn, M. J. Current two-dimensional electrophoresis technology for proteomics. *Proteomics* **2004**, *4*, 3665–3685.
(8) Chu, P. W.; Yap, M. N.; Wu, C. Y.; Huang, C. M.; Pan, F. M.; Tseng, M. J.; Chen, S. T. A proteomic analysis of secreted proteins from xylan-induced *Bacillus* sp. strain K-1. *Electrophoresis* **2000**, *21*, 1740–1745.
(9) Medina, M. L.; Haynes, P. A.; Breci, L.; Francisco, W. A. Analysis of secreted proteins from *Aspergillus flavus*. *Proteomics* **2005**, *5*, 3153–3161.
(10) Grinyer, J.; McKay, M.; Nevalainen, H.; Herbert, B. R. Fungal proteomics: initial mapping of biological control strain *Trichoderma harzianum*. *Curr. Genet.* **2004**, *45*, 163–169.
(11) Grinyer, J.; Kautto, L.; Traini, M.; Willows, R. D.; Te'o, J.; Bergquist, P.; Nevalainen, H. Proteome mapping of the Trichoderma reesei 20S proteasome. *Curr. Genet.* **2007**, *51*, 79–88.
(12) Suarez, M. B.; Sanz, L.; Chamorro, M. I.; Rey, M.; Gonzalez, F. J.; Llobell, A.; Monte, E. Proteomic analysis of secreted proteins from *Trichoderma harzianum* identification of a fungal cell wall-induced aspartic protease. *Fungal Genet. Biol.* **2005**, *42*, 924–934.
(13) Grinyer, J.; McKay, M.; Herbert, B.; Nevalainen, H. Fungal proteomics: mapping the mitochondrial proteins of a *Trichoderma harzianum* strain applied for biological control. *Curr. Genet.* **2004**, *45*, 170–175.
(14) Marra, R.; Ambrosino, P.; Carbone, V.; Vinale, F.; Woo, S. L.; Ruocco, M.; Ciliento, R.; Lanzuise, S.; Ferraioli, S.; Soriente, I.; Gigante, S.; Turra, D.; Fogliano, V.; Scala, F.; Lorito, M. Study of the three-way interaction between *Trichoderma atroviride*, plant and fungal pathogens by using a proteomic approach. *Curr. Genet.* **2006**, *50*, 307–21.
(15) Liu, S. Y.; Lo, C. T.; Chen, C.; Liu, M. Y.; Chen, J. H.; Peng, K. C. Efficient isolation of anthraquinone-derivatives from *Trichoderma harzianum* ETS 323. *J. Biochem. Biophys. Methods* **2007**, *70*, 391–395.
(16) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.
(17) Sengupta, S.; Jana, M. L.; Sengupta, D.; Naskar, A. K. A note on the estimation of microbial glycosidase activities by dinitrosalicylic acid reagent. *Appl. Microbiol. Biotechnol.* **2000**, *53*, 732–735.
(18) Benitez, T.; Rincon, A. M.; Limon, M. C.; Codon, A. C. Biocontrol mechanisms of *Trichoderma* strains. *Int. Microbiol.* **2004**, *7*, 249–260.
(19) Sanz, L.; Montero, M.; Grondona, I.; Vizcaíno, J. A.; Llobell, A.; Hermosa, R.; Monte, E. Cell wall-degrading isoenzyme profiles of *Trichoderma* biocontrol strains show correlation with rDNA taxonomic species. *Curr. Genet.* **2004**, *46*, 277–286.
(20) Nierman, W. C.; Pain, A.; Anderson, M. J. Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. *Nature* **2005**, *438*, 1151–1156.
(21) Lorito, M.; Hayes, C. K.; Di Pietro, A.; Woo, S. L.; Harman, G. E. Purification, characterization and synergistic activity of a glucan 1,3-$\beta$-glucosidase and an *N*-acetylglucosaminidase from *T. harzianum*. *Phytopathology* **1994**, *84*, 398–405.
(22) de Paula Silveira, F. Q.; Sousa, M. V.; Ricart, C. A.; Milagres, A. M.; de Medeiros, C. L.; Filho, E. X. A new xylanase from a *Trichoderma harzianum* strain. *J. Ind. Microbiol. Biotechnol.* **1999**, *23*, 682–685.
(23) Montero, M.; Sanz, L.; Rey, M.; Monte, E.; Llobell, A. BGN16.3, a novel acidic $\beta$-1,6-glucanase from mycoparasitic fungus *Trichoderma harzianum* CECT 2413. *FEBS J.* **2005**, *272*, 3441–3448.
(24) Grinyer, J.; Hunt, S.; McKay, M.; Herbert, B. R.; Nevalainen, H. Proteomic response of the biological control fungus *Trichoderma atroviride* to growth on the cell walls of *Rhizoctonia solani*. *Curr. Genet.* **2005**, *47*, 381–388.
(25) Sahai, A.; Manocha, M. Chitinases of fungi and plants: their involvment in morphogenesis and host-parasite interaction. *FEMS Microbiol. Rev.* **1993**, *11*, 317–338.
(26) Carsolio, C.; Gutierrez, A.; Jimenez, B.; Van Montagu, M.; Herrera-Estrella, A. Characterization of ech-42, a *Trichoderma harzianum* endochitinase gene expressed during mycoparasitism. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 10903–10907.
(27) Garcia, I.; Lora, J. M.; de la Cruz, J.; Benitez, T.; Llobell, A.; Pintor-Toro, J. A. Cloning and characterization of a chitinase (chit42) cDNA from the mycoparasitic fungus *Trichoderma harzianum*. *Curr. Genet.* **1994**, *27*, 83–89.
(28) Mach, R. L.; Peterbauer, C. K.; Payer, K.; Jaksits, S.; Woo, S. L.; Zeilinger, S.; Kullnig, C. M.; Lorito, M.; Kubicek, C. P. Expression of two major chitinase genes of *Trichoderma atroviride* (*T. harzianum* P1) is triggered by different regulatory signals. *Appl. Environ. Microbiol.* **1999**, *65*, 1858–1863.
(29) Kullnig, C.; Mach, R. L.; Lorito, M.; Kubicek, C. P. Enzyme diffusion from *Trichoderma atroviride* (= *T. harzianum* P1) to *Rhizoctonia solani* is a prerequisite for triggering of *Trichoderma* ech42 gene expression before mycoparasitic contact. *Appl. Environ. Microbiol.* **2000**, *66*, 2232–2234.
(30) Ait-Lahsen, H.; Soler, A.; Rey, M.; de La Cruz, J.; Monte, E.; Llobell, A. An antifungal exo-$\alpha$-1,3-glucanase (AGN13.1) from the biocontrol fungus *Trichoderma harzianum*. *Appl. Environ. Microbiol.* **2001**, *67*, 5833–5839.
(31) Cohen-Kupiec, R.; Broglie, K. E.; Friesem, D.; Broglie, R. M.; Chet, I. Molecular characterization of a novel $\beta$-1,3-exoglucanase related to mycoparasitism of *Trichoderma harzianum*. *Gene* **1999**, *226*, 147–154.
(32) Ramot, O.; Cohen-Kupiec, R.; Chet, I. Regulation of $\beta$-1,3-glucanase by carbon starvation in the mycoparasite *Trichoderma harzianum*. *Mycol. Res.* **2000**, *104*, 415–420.
(33) de la Cruz, J.; Pintor-Toro, J. A.; Benitez, T.; Llobell, A.; Romero, L. C. A novel endo-$\beta$-1,3-glucanase, BGN13.1, involved in the mycoparasitism of *Trichoderma harzianum*. *J. Bacteriol.* **1995**, *177*, 6937–6945.
(34) El-Katatny, M. H.; Gudelj, M.; Robra, K. H.; Elnaghy, M. A.; Gubitz, G. M. Characterization of a chitinase and an endo-$\beta$-1,3-glucanase from *Trichoderma harzianum* Rifai T24 involved in control of the phytopathogen *Sclerotium rolfsii*. *Appl. Microbiol. Biotechnol.* **2001**, *56*, 137–143.
(35) Flores, A.; Chet, I.; Herrera-Estrella, A. Improved biocontrol activity of *Trichoderma harzianum* by over-expression of the proteinase-encoding gene prb1. *Curr. Genet.* **1997**, *31*, 30–37.
(36) de la Cruz, J.; Llobell, A. Purification and properties of a basic endo-$\beta$-1,6-glucanase (BGN16.1) from the antagonistic fungus *Trichoderma harzianum*. *Eur. J. Biochem* **1999**, *265*, 145–151.
(37) de la Cruz, J.; Pintor-Toro, J. A.; Benitez, T.; Llobell, A. Purification and characterization of an endo-$\beta$-1,6-glucanase from *Trichoderma harzianum* that is related to its mycoparasitism. *J. Bacteriol.* **1995**, *177*, 1864–1871.
(38) Lukasheva, E. V.; Berezov, T. T. L-Lysine $\alpha$-oxidase: physicochemical and biological properties. *Biochemistry (Moscow)* **2002**, *67*, 1152–1158.

**Received for review December 13, 2007. Revised manuscript received June 12, 2008. Accepted June 13, 2008. This work was supported by Grants NSC 96-2317-B-259-001 and NSC 96-2313-B-212-006 from the National Science Council in Taiwan.**

JF703626J