Chitinolytic Enzymes of *Trichoderma harzianum*: Purification of Chitobiosidase and Endochitinase

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ABSTRACT

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Trichoderma harzianum strain P1 produces a variety of chitinolytic enzymes including *N*-acetyl-β-D-glucosaminidases, chitin 1,4-β-chitobiosidases, and an endochitinase. Chitobiosidases and an endochitinase were purified from dialyzed, concentrated culture filtrates using gel filtration, chromatofocusing, and isoelectric focusing. Three protein bands were evident in the purified chitobiosidase preparation, representing different levels of *N*-glycosylation of the same protein. The pI of all purified proteins was ~ 3.9. The molecular mass of the principal glycosylated chitobiosidase was 40 kDa, the deglycosylated form was 35 kDa, and the endochitinase was 41 kDa. The chitobiosidases did not react with a polyclonal antibody prepared against the endochitinase, nor did the endochitinase react with an antibody prepared against the chitobiosidase. The endochitinase and the chitobiosidase had broad pH optima. The larger glycosylated chitobiosidase had a 4.0–7.0 pH optimum. The endochitinase optimum activity was at ~ pH 4.0 and gradually declined as pH increased.

Additional keywords: biocontrol, biological control, mycoparasitism.

Trichoderma spp. are potent agents for the biocontrol of plant pathogens. Among the action mechanisms proposed for them is mycoparasitism with concomitant production of enzymes that degrade fungal cell walls (3). Chitinolytic enzymes, together with β-glucanases or cellulases, are the enzymes most frequently considered critical in biocontrol (3). In addition, chitinolytic enzymes may be important industrially for decomposing chitinous wastes from shellfish (4).

However, work done thus far with these fungi has not examined or considered in detail the kinds, numbers, or properties of chitinolytic enzymes produced. Ridout et al (14) have partially characterized an *N*-acetyl-β-D-glucosaminidase. Usui et al (21) have purified and characterized an endochitinase from these fungi. Uloha and Peberdy (19,20) have purified two chitinolytic enzymes from *T. harzianum* and identified them as a *N*-acetyl-β-D-chitobiase and a chitinase. However, based on the enzyme assays used, we consider their chitobiase to be an *N*-acetyl-β-D-glucosaminidase and their chitinase to be, perhaps, a chitobiosidase or an endochitinase (see Discussion). In addition, they did not provide full purification and characterization of the chitinolytic enzymes produced by these fungi.

This article describes the range of chitinolytic enzymes produced by *T. harzianum*, gives procedures for purification of an endochitinase and chitobiosidases, and partially characterizes the purified proteins.

MATERIALS AND METHODS

Enzyme production. Enzymes were produced by *T. harzianum* Rifai strain P1 (ATCC 74058), an effective biocontrol agent (16,17). This strain was grown on a medium containing 10 g of KNO_3, 5 g of KH_2PO_4, 2.5 g of $MgSO_4 \cdot 7H_2O$, 2 mg of $FeCl_3$, 10 g of crab shell chitin (Sigma Chemical Co., St. Louis, MO), 150 ml of V8 juice, 10 g of polyvinylpyrrolidone (Polyclar AT, GAF Corp., Wayne, NJ), and 1,000 ml of water. The medium was adjusted to pH 6.0 and sterilized by autoclaving in Erlenmeyer flasks (100 ml per 250-ml flask). The medium was inoculated with a spore suspension to give a final concentration of ~5 × 10^6 conidia per milliliter and placed on a rotary shaker at 150

rpm at 25 C for 4–5 days. The culture medium containing the enzymes of interest was separated from biomass by centrifugation at 8,000 *g* for 10 min. Residual particulates were removed by filtration through a glass fiber filter (type A/E, Gelman Sciences, Ann Arbor, MI).

Enzyme nomenclature and assays. Nomenclature of chitinolytic enzymes is confused. *Enzyme Nomenclature* (2) lists two chitinolytic enzymes: 1) chitinase 3.2.1.14, which randomly cleaves chitin polymers and 2) *N*-acetyl-β-glucosaminidase 3.2.1.30, which hydrolyzes "terminal, nonreducing *N*-acetyl-β-D-glucosamine residues from chitobiose and higher analogues." This terminology is insufficent for chitinolytic enzymes from *T. harzianum* because this fungus produces three distinct classes of enzymes. Further, the "chitinase" may refer to any enzyme that catalyzes the cleavage of chitin or only to endochitinase. Finally, enzymes that cleave chitobiose are described as chitobiase (19) even though *Enzyme Nomenclature* (2) no longer recognizes this terminology. Therefore, in this article, we refer to all enzymes with activity against chitin as chitinolytic enzymes and refer to those that cleave randomly as endochitinases. Exochitinases that release monomeric units are referred to as *N*-acetyl-β-glucosaminidase, hereafter called glucosaminidase, which is consistent with the terminology in *Enzyme Nomenclature* (2). Because our fungi also produced enzymes that release dimeric units, we needed an additional term. In the nomenclature for cellulolytic enzymes is EC 3.2.1.91, cellulose 1,4-β-cellobiosidase, which catalyzes "hydrolysis of 1,4-β-D-glucosidic linkages in cellulose . . . releasing cellobiose from the nonreducing ends of the chains." This enzyme is analogous to enzymes from *T. harzianum* that release chitobiose from chitin. Therefore, the enzymes with this activity are referred to as chitin 1,4-β-chitobiosidase, hereafter called chitobiosidase. Enzymes from *T. harzianum* endochitinases require at least the tetramer of chitin for activity, chitobiosidases require the trimer, and glucosaminidases require at least the dimer.

Glucosaminidase and chitobiosidase were assayed by modifications (18) of procedures described by Ohtakara (13) and Roberts and Seltrinnikoff (15). Briefly, assays measured the release of nitrophenol from *p*-nitrophenyl-β-D-*N*-acetylglucosaminide or from *p*-nitrophenyl-β-D-*N,N'*-diacetylchitobiose (both from Sigma), respectively, at pH 6.7 in microtiter plates. These substrates function as dimeric and trimeric substrates, respectively, with the *p*-nitrophenyl group serving as one monomer (15). Plates were read at 410 nm in a microtiter plate reader. Activity was expressed as nkatals (nmoles of nitrophenol released per second per milliliter of enzyme). Endochitinase activity was measured by the reduction of turbidity of a suspension of colloidal chitin (2,18). A suspension containing 1% (w/v) of moist colloidal chitin (about 88% moisture content), purified as described by Vessey and Pegg (22), was prepared in 50 mM potassium phosphate buffer, pH 6.7. A mixture consisting of 0.5 ml each of the chitin suspension and the enzyme solution to be tested was prepared and incubated for 24 h at 30 C. Subsequently, the mixture was diluted with 5 ml of water and the optical density was read at 510 nm. Activity was calculated as the percentage of reduction in turbidity relative to that of a similar suspension that contained water rather than enzyme solution; one enzyme unit was defined as the amount of enzyme required to reduce the turbidity of a chitin suspension by 5%. Alternatively, endochitinase activity could be quantitated using a microtiter plate assay as described for glucosaminidase and chitobiosidase but using *p*-nitrophenyl-β-D-*N,N',N''*-acetylchitotriose as the substrate (18).

Enzyme purification. Culture filtrates were first dialyzed against 50 mM potassium phosphate buffer (6 L of buffer per liter of culture filtrate), pH 6.7, overnight at 4 C with stirring. The dialysis tubes were then placed in polyethylene glycol (35,000 molecular weight; Fluka Chemika-Biochemika, Buchs, Switzerland) at room temperature until the volume was reduced 15- to 25-fold. If the resulting dialyzed, concentrated culture filtrate was stored for more than 1 wk, 0.02% NaN_3 was added as a preservative and the material was kept at 4 C until used. Subsequently, it was injected into a chromatography column (5 × 60 cm) packed with Sephacryl S-300 (Pharmacia LKB Biotechnology, Uppsala, Sweden). The column was equilibrated and eluted with 50 mM potassium phosphate buffer, pH 6.7, containing 200 mM NaCl and 0.02% NaN_3. Samples and elution buffer were pumped from the bottom of the column at a rate of 2.5 ml/min, and fractions were collected every 5 min.

Fractions exhibiting activity of various enzymes were pooled (see Results and Fig. 1A). The pooled sets were concentrated in dialysis tubing immersed in polyethylene glycol and dialyzed overnight against approximately a 10-fold volume of 25 mM imidazole-HCl buffer, pH 7.0, and then applied to a chromatofocusing column. The chromatofocusing column (1 × 30 cm) was packed with PBE 94 (Pharmacia) equilibrated with the 25 mM imidazole buffer. The column was eluted with Polybuffer (Pharmacia), pH 7.0–4.0, according to the manufacturer's directions.

Peak fractions containing chitobiosidase activity were further purified by electrofocusing using a Rotofor apparatus (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's directions and Bio-Lyte 3/5 ampholyte (10% v/v of the total sample). Peak fractions were collected and separated again on the Rotofor apparatus.

Numerous similar purifications were conducted for chitobiosidase and endochitinase, and the elution profiles presented in Figure 1 are typical examples of results obtained.

Enzyme characterization. Polyacrylamide gel electrophoresis (PAGE) was used to assess purity of enzyme preparations and to characterize purified proteins. All electrophoresis utilized a PhastSystem (Pharmacia). Native and sodium dodecyl sulfate (SDS)-PAGE was conducted using 20% homogeneous, discontinuous gels; isoelectric focusing (IEF) was done on gels with a range of pH 3.0–9.0. All were run according to the manufacturer's directions. Proteins were prepared for SDS-PAGE according to the procedure of Laemmli (10). Proteins were visualized with Coomassie blue stain as provided by Pharmacia for the PhastSystem. Protein standards were provided by Sigma and Pharmacia for IEF and SDS-PAGE, respectively. For SDS, the molecular mass range of the six standard proteins was 14.4–94 kDa; for IEF, the pI range of the 12 standard proteins was pH 3.5–9.3. Molecular weights of *Trichoderma* enzymes were estimated from a regression equation of the log of molecular weight of the standard proteins versus distance migrated. The pIs were determined from a regression of pI of standard proteins versus distance migrated. Both cases used a linear regression with an r^2 value range of 0.94–0.99. On native or IEF gels, the position of bands determined by staining was compared with that of activity of the electrophoresed enzyme. Fluorescent bands produced by the release of 4-methylumbelliferone from 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide, 4-methylumbelliferyl-β-D-*N,N'*-diacetylchitobioside, or 4-methylumbelliferyl-β-D-*N,N',N''*-triacetylchitotriose (all substrates from Sigma) were utilized to detect activity of glucosaminidase, chitobiosidase, and endochitinase, respectively (18). The methylumbelliferyl substrates were dissolved at 300 μg/ml in 100 mM acetate buffer (pH 5.1), and 1% (w/v) low-melting-point agarose was added. The substrates and agarose were dissolved by heating, and the mixture was equilibrated to 37 C. Agarose mixtures were poured over the surface of the Phast gels immediately after the run was complete, and the presence of enzymes was visualized by the occurrence of fluorescent bands under ultraviolet illumination (Fig. 2).

Protein in samples was quantified using the Micro BCA protein assay according to the manufacturer's directions (Pierce, Rockford, IL) with soybean trypsin inhibitor (Sigma) as the standard. For determination of pH optima, mixtures containing 50 mM citric acid and 50 mM of either K_2HPO_4 (endochitinase) or K_3PO_4 (chitobiosidase) were prepared. These two solutions were mixed in various ratios to give the pH values indicated in Figure 3, and microtiter assays were run in triplicate for each pH value and for each enzyme. Nitrophenyl-β-D-*N,N'*-diacetylchitobioside or nitrophenyl-β-D-*N,N',N''*-triacetylchitotriose was used as substrate for chitobiosidase or endochitinase, respectively. Amino acid analyses were performed at the analytical and synthesis facility of the biotechnology program, Cornell University. Amino

acid composition was determined using the Picotag amino acid analysis procedure of Heinrikson and Meredith (8). Amino acid sequences were determined on an ABI gas phase protein sequencer as described by Hunkapiller et al (9).

Antibodies were prepared in separate rabbits against the purified endochitinase and chitobiosidase using standard techniques (7). These antibodies were used to determine cross-reactivity of enzymes using enzyme-linked immunosorbent assay (ELISA)(6).

The possibility that purified proteins may contain *N*-linked glycosidic groups was tested by treating proteins with *N*-glycosidase F (Boehringer Mannheim Biochemicals, Indianapolis, IN). Samples were prepared as suggested by the manufacturer. Reaction mixtures consisted of 2 μg of the chitinolytic enzyme tested in 2 μl of water, 1 μl of a buffer consisting of 500 mM potassium phosphate (pH 6.7), 100 mM sodium ethylenediaminetetraacetic acid and 3 μl of water, and 3 μl of *N*-glycosidase F containing 0.6 units of activity. In some experiments, the chitinolytic enzymes were denatured by boiling for 1 min in a mixture containing 50 mM potassium phosphate buffer (pH 6.7), 0.5% Nonidet P-40 (Sigma), 0.5% β-mercaptoethanol, and 0.1% SDS before adding *N*-glycosidase F. The chitinolytic enzyme-glycosidase mixture was incubated for 16–40 h at 37 C and then SDS-PAGE was done as described above.

RESULTS

Fractionation of concentrated, dialyzed culture filtrate using gel filtration on Sephacryl S-300 separated several enzymes. The first large peak of activity occurred between fractions 80 and 110 and consisted of glucosaminidase activity and a smaller quan-

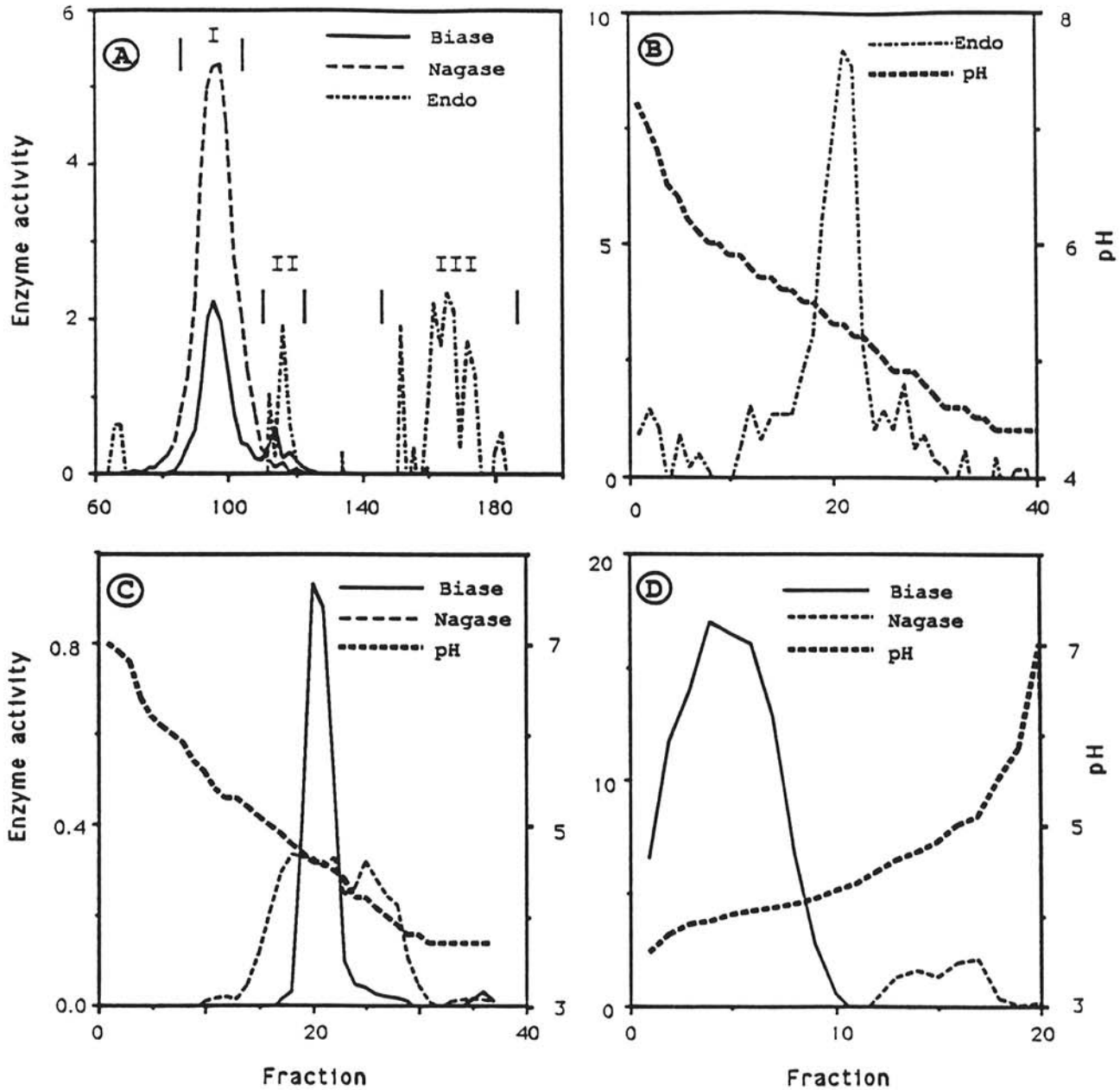

Fig. 1. Fractionation of the chitinolytic enzymes from *Trichoderma harzianum*. About 800 ml of culture filtrate was dialyzed and concentrated to about 40 ml. **A,** Approximately 20 ml of this filtrate was applied to a gel filtration column packed with Sephacryl S-300 (Pharmacia LKB Biotechnology, Uppsala, Sweden) and eluted with 200 mM NaCl dissolved in 50 mM potassium phosphate buffer, pH 6.7. **B,** Fractions were collected and pooled into sets I, II, and III. Set III was dialyzed against imidazole-HCl buffer, pH 7, and applied to a chromatofocusing column that was eluted with Polybuffer from pH 7 to 4.2. **C,** Set II was similarly dialyzed and run on a chromatofocusing column (**C**). **D,** The peak chitobiosidase fractions were pooled and these were subjected to isoelectric focusing. Nagase = *N*-acetyl-β-D-glucosaminidase activity, biase = chitobiosidase activity, and endo = endochitinase activity. Enzyme activity in all figures is in units of nkatals per second per milliliter of enzyme for glucosaminidase and chitobiosidase. One enzyme unit of colloidal chitin for endochitinase is the amount of enzyme required to reduce the turbidity of a chitin suspension by 5%. No activity was detected prior to fraction 60.

tity of chitobiosidase activity (Fig. 1A). Fractions in this peak were designated set I, pooled, and applied to a chromatofocusing column. Several peaks of activity were detected and this complex mixture was not fractionated further in this study. A smaller peak of chitobiosidase activity, together with some endochitinase activity was detected in fractions 110–120 (Fig. 1A). Fractions in this peak were designated set II, pooled, and saved for chromatofocusing. Fractions 150–190 contained most of the endochitinase activity. These fractions were designated set III, pooled, and also saved for further purification. Proteins in set III probably did not separate on the basis of molecular weight; apparently they were adsorbed to the gel matrix because they eluted at, or greater than, the total column volume.

Chromatofocusing of set III, primarily endochitinase, gave the elution profile shown in Figure 1B. Electrophoresis of this purified enzyme on native, SDS (Fig. 2A), or IEF gels (not shown) showed that it consisted of a single protein. Activity, as determined by florescence of the methylumbelliferyl substrate, corresponded to the protein band on IEF and native gels. This 41-kDa protein banded at the same level as the 36-kDa protein standard, but regression of the log of molecular weight versus distance migrated gave the values indicated. The discrepancy occurred because the putative 36-kDa standard fell below the regression line generated when all proteins were considered. Throughout, all molecular weights are accurate to within 1 to 2 kDa; because regression is to the log of molecular weight, this difference represents less than 1 mm on gels. Endochitinase was not affected by *N*-glycosidase F.

Chromatofocusing of set II gave the elution profile shown in Figure 1C. In addition to chitobiosidase and glucosaminidase, a small overlapping peak of glucanase activity was detected by hydrolysis of nitrophenyl-β-D-glucopyranoside in some column runs (data not shown). This enzyme eluted in fractions equivalent to 22 and 23, so in most cases only the fractions prior to 22 were pooled for further purification. In most purification runs, endochitinase was not detected in the eluant from set II. Some endochitinase activity was adsorbed to the chromatofocusing medium and was not eluted by the Polybuffer gradient, but it could be eluted with 1 M NaCl.

The chitobiosidase from the chromatofocusing step was further purified by separation according to pI on a Rotofor apparatus (Fig. 1D). The peak of chitobiosidase activity occurred at pH 4.0; *N*-acetylglucosaminidase focused at a higher pH. Glucanase was not separated from chitobiosidase on the Rotofor. Therefore, if fractions from chromatofocusing columns contained chitobiosidase and glucanase activity, then a second chromatofocusing run was required to remove glucanase. After purification, the chitobiosidase consisted of three closely spaced protein bands on SDS gels (Fig. 2B). The largest of the proteins (40 kDa) stained most intensely, the intermediate protein (38 kDa) was faint, and the smallest protein (35 kDa) stained at an intermediate intensity (Fig. 2A). The proteins giving rise to the three bands of chitobiosidase differed only in *N*-glycosylation. After the sample was dried in a SpeedVac apparatus (Savant Instruments, Farmingdale, NY), only the 40-kDa protein could be detected on SDS-PAGE (Fig. 2C). This was also the case if purification was not completed within a few weeks. When this protein was subjected to *N*-glycosidase F, two bands were detected that corresponded to the 40-kDa and the 38-kDa proteins. When the denatured 40-kDa protein was subjected to the same enzyme, bands corresponding to the 38- and 35-kDa proteins were obtained (Fig. 2D). Protein bands (visualized with Coomassie blue stain) corresponded to activity bands (determined with methylumbelliferyl overlay) on IEF and native gels (data not shown).

The specific activity of the purified endochitinase was calculated to be 0.86 units per microgram of protein with the turbidity reducing assay and 2.2 nkat/mg of protein with nitrophenyl-β-D-*N,N',N''*-triacetylchitotriose as the substrate. The original culture filtrate contained an efficient inhibitor of endochitinase activity; in crude dialyzed, concentrated culture filtrates no endochitinase activity was detected. This inhibitor prevented measurement in nonpurified fractions; only specific activity of the purified enzyme could be calculated. The specific activity of the purified chitobiosidase was 127 nkat/mg of protein, and the purification provided about a 200-fold increase in this value (Table 1). However, the apparent level of purification and percentage of recovery should be viewed with caution because the inhibitor present in the crude culture filtrate may also inhibit chitobiosidase activity. If so, the values for percentage of recovery would be larger than if no inhibitor were present. The large difference in specific activity of the endochitinase on nitrophenyl-β-D-*N,N',N''*-triacetylchitotriose versus chitobiosidase on *p*-nitophenyl-β-D-*N,N'*-diacetylchitobiose was probably due to the fact that nitrophenyl-β-D-*N,N',N''*-triacetylchitotriose is a poor substrate for endochitinase.

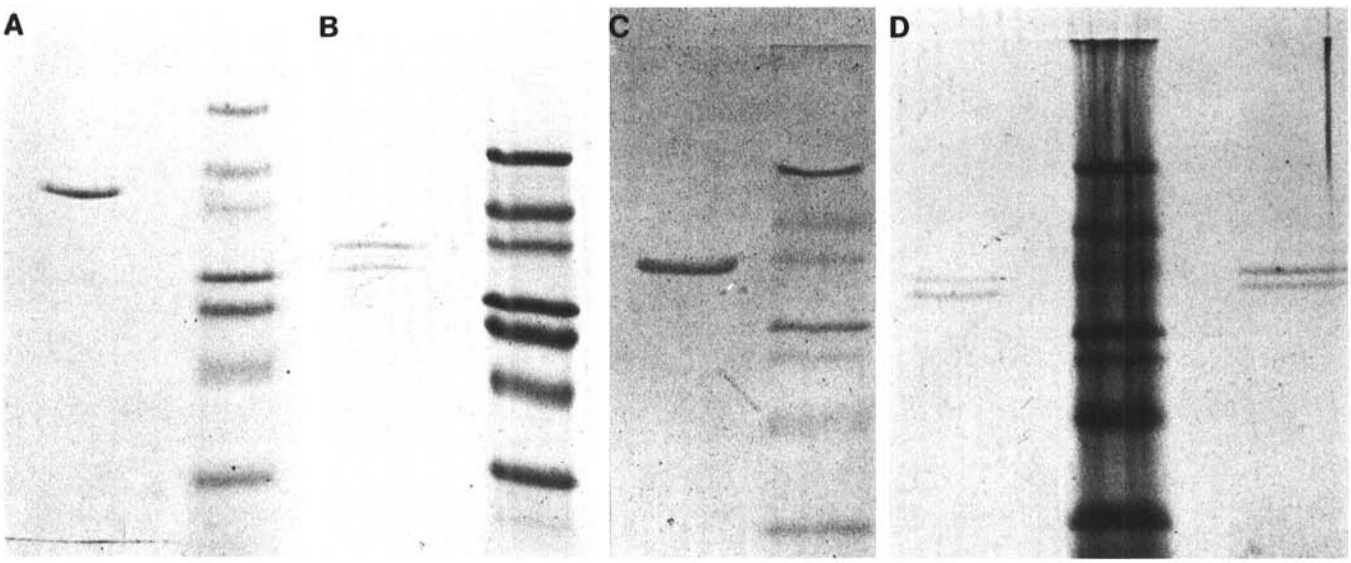

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified endochitinase and chitobiosidase. **A,** Left lane contains purified endochitinase (41 kDa), right lane contains molecular weight markers 66, 45, 36, 29, 24, 20.1 and 14.2 kDa (markers obtained from Sigma Chemical Co., St. Louis, MO). **B,** Left lane contains the purified chitobiosidase (40, 38, and 35 kDa), right lane contains the molecular weight markers. **C,** Left lane shows the chitobiosidase purified as in B after drying, right lane contains the molecular weight markers. **D,** Left lane contains denatured 40-kDa chitobiosidase after treatment for 40 h with *N*-glycosidase F, the center lane contains the molecular weight markers, and the the right lane contains the native 40-kDa chitobiosidase after similar treatment with *N*-glycosidase F. All gels were stained with Coomassie blue stain. Molecular weights of enzymes were estimated from a linear ression of the log of molecular weight of the standards versus distance from the bottom of the gels.

The pIs were determined for the purified endochitinase and the chitobiosidases. The pIs of the proteins were very similar, with values of 3.8–3.9. The enzymes could be resolved on IEF gels; in one typical run, the pIs were determined to be 3.82, 3.94, and 3.95 for a minor chitobiosidase (presumably the deglycosylated 35-kDa protein), the major chitobiosidase, and the endochitinase, respectively. The two chitobiosidases produced bands separated by less than 1 mm on native gels, but the endochitinase was separated from the chitobiosidases by about 5 mm (the total gel is 50 mm long).

The first 20 *N*-terminal amino acids of the endochitinase were sequenced and found to be alanine, serine, glycine, tyrosine, alanine, asparagine, alanine, valine, tyrosine, serine, threonine, asparagine, tryptophan (or aspartic acid), lysine, isoleucine, tyrosine, glycine, arginine, asparagine, and serine. The amino acid composition of the enzyme was aspartic acid (16%), leucine (6%), glutamic acid (5.8%), serine (10.4%), glycine (9.7%), histidine (1.3%), arginine (1.5%), threonine (4.4%), alanine (8.5%), proline (6%), tyrosine (8.5%), valine (4.2%), methionine (1.7%), cysteine (0.8%), isoleucine (4.3%), phenylalanine (5.5%), and lysine (5.3%). Similarly the first 10 amino acids of the 40-kDa chitobiosidase were alanine, leucine, aspartic acid, an unresolved amino acid, histidine, threonine, proline, arginine, valine, and aspartic acid. A polyclonal antibody prepared against the purified endochitinase did not cross-react with the chitobiosidases; similarly, an antibody prepared against the 40-kDa chitobiosidase did not react with the endochitinase, as determined using ELISA.

The pH optimum of the chitobiosidase was broad, with maximum activity extending from about pH 4.0 to 7.0. The optimum of the endochitinase peaked at about pH 4.0 and gradually declined to about pH 7.0 (Fig. 3).

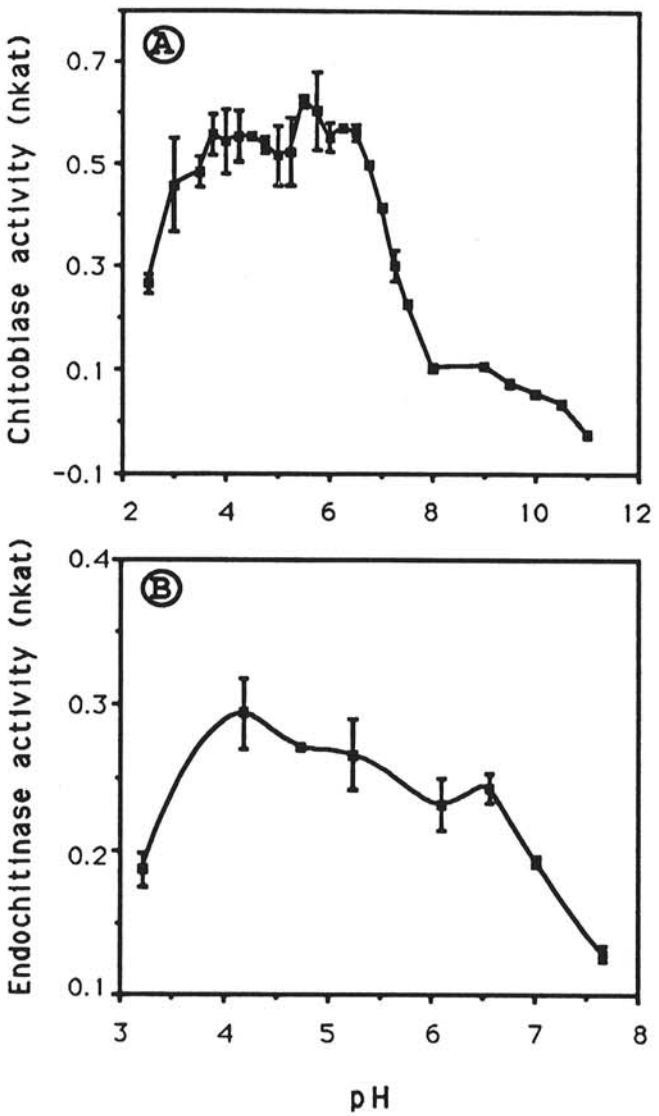

Fig. 3. A, pH optima of chitobiosidase. **B,** pH optima of endochitinase. Samples of purified enzyme (the chitobiosidase sample contained only the major 36-kDa protein) were suspended in 50 mM potassium phosphate-sodium citrate buffer at the pH indicated and microtiter plate assays performed using *p*-nitrophenyl-β-D-*N,N'*-diacetylchitobiose or *p*-nitrophenyl-β-D-N,N',N''-triacetylchitotriose as the substrate. Assays at each pH were performed in triplicate and averaged, and the entire experiment repeated. Values presented are averages of the two experiments and the error bars represent standard deviations of the means of the two experiments.

DISCUSSION

The data in this work clearly show that there are a number of chitinolytic enzymes produced by *T. harzianum* strain P1, and that there are multiple forms of glucosaminidase and chitobiosidase. The role of this complex mixture in biocontrol is unknown. However, the endochitinase and the chitobiosidase purified in this work are potent inhibitors of a range of chitin-containing fungi, including species of *Fusarium, Botrytis, Ustilago, Uncinula,* and other *T. harzianum* strains (11). Mixtures of enzymes are not required for fungal inhibition, but the chitobiosidase and endochitinase act synergistically to inhibit fungi (11). It should be emphasized that only two enzymes were examined (11). *T. harzianum* produces many chitinolytic enzymes. For example, chromatofocusing of set I indicates the presence of several other glucosaminidases and chitobiosidases in addition to the ones purified in this work. It is likely that several enzymes are required for maximum activity. *Pythium* spp., which do not contain chitin in their cell walls, are not inhibited by the chitinolytic enzymes of *T. harzianum* (11). A number of different cellulolytic enzymes from *T. reesei* and other organisms are synergistic in the degradation of cellulose (12). Similar interactions, including enzymes with other than chitinolytic function, may be necessary for maximum activity against fungal cell walls (11). The chitinolytic enzymes from *T. harzianum* are substantially more effective, or control a wider range of fungi, than chitinolytic enzymes from higher plants or bacteria (11).

However, not all chitinolytic enzymes from biocontrol fungi are equally effective. Di Pietro et al (5) purified an endochitinase from *Gliocladium virens*. Although *G. virens* is closely related to *T. harzianum*, its endochitinase was substantially less active against fungi than was the endochitinase from *T. harzianum* (5,11). This occurred in spite of the fact that the *G. virens* enzyme was of similar size, had similar specific activity against colloidal chitin, and reacted to a polyclonal antibody prepared against the *T. harzianum* endochitinase (5). The native *G. virens* enzyme was about one-half as reactive serologically as was the *T. harzianum* enzyme. After denaturation with urea, both enzymes were equally reactive upon ELISA (5).

The pH at which each enzyme eluted from the chromatofocusing columns was dissimilar to the pI as determined by electrophoresis. This result was surprising because both procedures separate proteins on the basis of pI. The endochitinase eluted at a pH of about 5.2 from chromatofocusing columns; the pI as determined by electrophoresis was about 3.9. The chitobiosidases eluted at a pH of 4.3; the pIs as determined by electrophoresis were 3.9. The literature supplied by the manufacturer indicates that salt accumulates as chromatofocusing proceeds, so some enzymes may

TABLE 1. Specific activity and recovery of chitobiosidase

Purification step	Specific activity (nkat/mg protein)	Recovery (%)
Culture filtrate	0.54	100
After dialysis and concentration	12.3	39
Set II after chromatography	26.2	29
After chromatofocusing	51.7	13
After isoelectric focusing	127	12

be eluted by salt effects that increase as the column runs. Thus, the endochitinase may have eluted from the chromatofocusing column by a salt gradient rather than by pI.

It seems possible that the multiple forms within each of the three kinds of chitinolytic enzymes detected may not all represent separate gene products. Instead, posttranslation modification, particularly glycosylation, may be responsible for a substantial amount of the variation within each enzyme class. Clearly, the chitobiosidases purified in this work differ only in *N*-glycosylation. Preliminary evidence suggests that the larger chitobiosidases in set I are serologically related to the purified proteins and may, in fact, be concatamers of the enzymes described in this research (data not shown). Glycosylation may stabilize proteins (12); the less-glycosylated forms of chitobiosidase identified in this study were more labile than the fully glycosylated 40-kDa protein. The cellulolytic enzymes of *T. reesei* are a complex mixture, but the presence of tunicamycin in the growth media, which inhibits *N*-glycosylation, resulted in a less complex enzyme mixture (12).

Similar enzymes have been purified from other strains of *Trichoderma*. Usui (21) purified an endochitinase (identified as such by its ability to reduce turbidity of chitin solutions), but this enzyme was substantially larger (58 kDa) than the enzyme we purified from strain P1. Uloha and Peberdy (19) have purified a chitinolytic enzyme that they have designated as a chitobiase. This enzyme, however, releases nitrophenol from *p*-nitrophenyl-β-D-*N*-acetylglucosaminide and cleaves chitobiose. Therefore, it would be considered as a glucosaminidase by the nomenclature system used in this article and as defined in *Enzyme Nomenclature* (2). In general, glucosaminidases are considered to cleave a monomer from substrates and so can act on chitobiose or nitrophenyl-β-D-*N*-acetylglucosaminide. (As noted in Materials and Methods, *p*-nitrophenyl-β-D-*N*-acetylglucosaminide acts as a dimeric substrate with the nitrophenyl group serving as a monomer.) The 118-kDa enzyme purified by Uloha and Peberdy (19) was larger than the enzymes purified in this study. Uloha and Peberdy (20) also purified another chitinolytic enzyme they consider a chitinase (EC 3.2.1.14) that randomly cleaves chitin (2). However, they assayed their enzyme primarily by release of reducing groups and showed that the purified protein does not cleave chitobiose. Therefore, according to the nomenclature employed in this article, it could be either a chitobiosidase or an endochitinase. It had a molecular mass of about 40 kDa, which is similar to that of the enzymes described in this article.

T. harzianum is a rich source of chitinolytic enzymes. These should provide a number of new tools for the control of plant pathogenic fungi and, perhaps, for other uses, such as the degradation of shellfish wastes. Consequently, we will isolate genetic sequences coding for these enzymes and purify and characterize other enzymes in the chitinolytic complex of *T. harzianum*. The enzymes themselves may be useful for the control of plant pests or for other industrially important processes. In addition, the genes may be used to transform plants, increasing their resistance to plant pests or to transform microorganisms, increasing their biocontrol capabilities. The enzymes purified in this work are particularly attractive for these purposes because they are substantially more active and effective against a wider range of fungi than chitinolytic enzymes from plants or other microorganisms. They are especially active when used synergistically (11). Isolating these genetic sequences will permit deletion or addition of these enzymes to *T. harzianum* itself, and in this way the role of these enzymes in biocontrol of plant pathogens can be quantitatively determined.

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