Purification and Characterization of an Antifungal Chitinase from Arabidopsis thaliana

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ABSTRACT

Plants exhibit an altered pattern of protein synthesis in response to pathogen invasion and abiotic stress. One of these 'pathogenesis-related' proteins has been identified as chitinase, which is capable of inhibiting fungal growth in vitro. This observation has led to the suggestion that the in vivo role of chitinases is to protect plants against fungal invasion. Here, we report the purification and characterization of a basic chitinase from Arabidopsis thaliana (L.) Heynh. Columbia wild type. The purified enzyme has a molecular mass of approximately 32 kilodaltons as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, and an apparent pI of approximately 8.7 as determined by isoelectric focusing. The purified protein is an effective inhibitor of the growth of Trichoderma reesei in vitro but does not affect the growth of several other fungi. Amino acid composition analysis of the intact protein as well as amino acid composition analysis and automatic Edman degradation of isolated tryptic fragments of the enzyme indicate that it may be identical to the product of a chitinase gene isolated from an Arabidopsis genomic library (Samac DA, Hironaka CM, Yallay PE, Shah DM [1990] Plant Physiol 93: 907–914).

Because plants do not contain an immune system, they have evolved other mechanisms to protect themselves from invasion by potential pathogens. Many plants respond to pathogenic infection with an altered pattern of protein synthesis in which enzymes whose apparent function is to inhibit the growth of parasitic invaders are produced. Many of these proteins are involved in the synthesis of low mol wt inhibitory molecules such as phytoalexins, phenols, lignins, tannins, and melanins (3). Others, such as the 'pathogenesis-related' proteins, include the fungal cell wall degrading enzymes, chitinases and β-1,3-glucanases, which appear to act directly on the invading pathogen.

Chitinases catalyze the hydrolysis of chitin, a β-1,4-linked homopolymer of N-acetyl glucosamine. Although chitin is not a component of plant cells, it is a major component of the cell walls of many fungi (2). It has been postulated that plants produce chitinases in order to protect themselves from chitin-containing parasites (1, 3, 4), but, until recently, direct evidence supporting that hypothesis was lacking (4). In 1986, however, Roberts and Selitrennikoff (22, 23) reported that an endochitinase purified from barley was capable of inhibiting the growth of Trichoderma reesei, Alternaria alternaria, Phycomyces blakesleeanus, and Neurospora crassa. In addition, Mauch et al. (16) have reported that, in combination, chitinases and β-1,3-glucanases act synergistically to inhibit fungal growth. Taken together, these results support the hypothesis that the in vivo role of these pathogenesis-related proteins is to protect the host from invasion by fungal pathogens, and that, as such, they are an integral component of a general disease resistance mechanism.

Synthesis of the pathogenesis-related proteins can be induced in a number of ways. For example, in Phaseolus vulgaris, the basic chitinase is systematically induced by the plant stress-related hormone ethylene (5, 8) and accumulates in the central vacuole (17). Although chitinases from a variety of sources have been purified and characterized, little is known about the enzyme from Arabidopsis thaliana, a plant whose small genome, simple genomic organization, and short life cycle have made its use in the study of plant molecular genetics common (18). In many cases, plants synthesize a number of closely related chitinases which are encoded by a gene family (12, 14, 15). These enzymes have been divided into two classes based upon whether they have basic or acidic isoelectric points. A. thaliana, on the other hand, produces a single basic chitinase, encoded by a single-copy gene (25). Here, we report purification and characterization of a basic chitinase from ethylene-treated Arabidopsis thaliana (L.) Heynh. Columbia wild type.

MATERIALS AND METHODS

Plant Culture and Treatment with Ethylene

Seeds of Arabidopsis thaliana (L.) Heynh. Columbia wild type were sown into Metromix Terralite 200 (Grace Horticultural Products, Cambridge, MA) and subirrigated daily with 14:15:16 (N:P:K) Peat-lite fertilizing solution. Plants were maintained at approximately 350 μE m⁻² s⁻¹ for 16 h/d at 21°C, 19°C at night, and 50% RH. Approximately 26 d after planting, plants initiated a flowering shoot. At this point, plants were sprayed to runoff with a 1 mg/mL solution of 2-chloroethylphosphonic acid (Sigma Chemical Company) which hydrolyzes to produce ethylene. The pots were then enclosed in plastic bags, and the plants were maintained in this condition for 72 h. Plants were then harvested and stored at -70°C until used.

Purification of Chitinase

Chitinase was purified from A. thaliana with minor modification of a purification protocol described by Boller et al.
(5) for the purification of chitinase from *Phaseolus vulgaris*. The following steps were performed, as much as possible, at 4°C. Leaves, shoots, and roots of ethylene-treated *A. thaliana* plants (2 kg) were homogenized in 4 L of 0.1 M sodium citrate, pH 5.0, in a Janke & Kunkel Ultra-Turrax homogenizer (Tekmar Company, Cincinnati, OH). The homogenate was centrifuged in a Sorvall GS3 rotor for 45 min at 13,800 g. The resulting pellet was rehomogenized in an additional 3 L of 0.1 M sodium citrate (pH 5.0) and centrifuged as before. The supernatant from this centrifugation was combined with the first, and solid ammonium sulfate was added to a final concentration of 20% saturation at 4°C. After incubation for 2 h with gentle stirring, this solution was centrifuged in a Sorvall GS3 rotor for 45 min at 13,800 g. The pellet was discarded, and solid ammonium sulfate was added to the supernatant to a final concentration of 60% saturation at 4°C. This solution was incubated overnight with continuous gentle stirring and centrifuged as before. The pellet was dissolved in approximately 125 mL of 10 mM sodium acetate, pH 5.0. Sodium bicarbonate was added to a final concentration of 20 mM, and the pH was adjusted to pH 8.4 with 0.1 N NaOH. This solution was dialyzed against two changes of 20 mM sodium bicarbonate (pH 8.4). Approximately 3.5 g of regeneranted chitin which had been preequilibrated in 20 mM sodium bicarbonate (pH 8.4), were suspended in the enzyme solution, and the chitin was washed successively with 200 mL of 20 mM sodium bicarbonate (pH 8.4), 200 mL of 20 mM sodium acetate (pH 5.5), and finally, 100 mL of 20 mM acetic acid (pH 3.0). Between washes, the chitin was collected by centrifugation in a Sorvall GSA rotor for 45 min at 27,000 g.

Chitinase, which was bound to the chitin at pH 8.4, and which was eluted from the chitin at pH 3.0, was purified to near homogeneity as follows. The pH 3.0 wash was concentrated to approximately 10 mL with an Amicon stirred cell concentrator with a YM-10 membrane (Amicon Division, W. R. Grace & Co., Danvers, MA). This solution was dialyzed against two changes of 10 mM sodium acetate (pH 5.0) and subjected to cation exchange chromatography as follows. The sample was applied to a Mono S HR 5/5 column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) equilibrated in 10 mM sodium acetate (pH 5.0). Adsorbed proteins were eluted with a linear gradient between 10 mM sodium acetate (pH 5.0) and 10 mM sodium acetate (pH 5.0), containing 200 mM NaCl, over 100 min. Fractions containing protein were detected by monitoring at A$_{380}$ nm, collected manually, and assayed for chitinase activity as described below.

**Protein Determinations and Other Analytical Methods**

Protein concentrations were determined by reaction with bicinchoninic acid (Pierce, Rockford, IL) according to the method described by Smith et al. (27) with BSA as a standard. Absorbance was determined with a Beckman model DU-64 spectrophotometer (Beckman Instruments, Fullerton, CA). Liquid chromatography was preformed with a Waters Associates model 510 Liquid Chromatograph (Waters Chromatography Division, Millipore Corporation, Milford, MA) equipped with a Waters Associates Model 440 Absorbance Detector and Extended Wavelength Module. Ion exchange fast protein liquid chromatography was performed on a Pharmacia Mono S HR 5/5 column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). For amino acid composition analysis, samples were hydrolyzed at 115°C for 24 h in constant boiling 6 N HCl and analyzed on a Beckman model 6300 high performance amino acid analyzer (Beckman Instruments, Fullerton, CA). Tryptic fragments were generated after reduction and carboxamidomethylation of cysteine residues by digesting the enzyme with TPCK$_{5}$-trypsin (Cooper Biomedical), at a concentration of 1:50 (w/w) at 37°C for 4 h. The tryptic fragments were resolved by reversed phase HPLC on a Vydac C-18 reversed phase column (4.6 × 250 mm, 5 μm particle size) (Alltech Associates, Inc., Deerfield, IL). Automatic Edman degradation of purified tryptic fragments was performed with an Applied Biosystems model 470A sequenator.

**Enzyme Assays**

Regenerated chitin was prepared by acetylation of chitosan (BioShell Inc., Albany, OR) in aqueous methanolic acetic acid essentially as described by Hirano et al. (11) and Molano et al. (19).

Chitinase activity was determined spectrophotometrically essentially as described by Boller et al. (5) and Boller and Mauch (6). Briefly, reaction mixtures contained chitinase and 1 to 2 mg of regenerated chitin in 300 μL of 10 mM sodium acetate (pH 4.5). After incubation at 37°C for 2 h, the reaction was terminated by centrifugation in an Eppendorf model 5415 microfuge for 10 min at 10,000 g. A 150-μL aliquot of the supernatant was transferred to a second tube which contained 15 μL of 1.0 M potassium phosphate (pH 7.1), and 10 μL of a 30 mg/mL solution of protoplast-forming enzyme from snail gut (Boehringer Mannheim Biochemicals, Indianapolis, IN) in 10 mM KCl, 1 mM EDTA (pH 6.8). This solution was incubated at room temperature for 1 h to hydrolyze the chitosaccharides (9). The resulting monomeric N-acetyl glucosamine was determined spectrophotometrically after reaction with p-dimethylaminobenzaldehyde as described by Reissig et al. (21).

Lysozyme activity was determined by monitoring the hydrolysis of a suspension of *Micrococcus lysodeikticus* (Sigma). Reaction mixtures contained chitinase and 0.33 mg of *M. lysodeikticus* in 1.0 mL of 50 mM sodium acetate (pH 6.24). The decrease at A$_{450}$ nm was determined at intervals.

**Antifungal Activity of Chitinase**

Antifungal activity of *A. thaliana* chitinase was estimated by a hyphal extension-inhibition assay essentially as described by Roberts and Selittrennikoff (22). Briefly, a disc, removed from an actively growing fungal culture, was placed in the center of a fresh agar medium. After incubation for a period of time sufficient to establish growth on the new plate, protein solutions to be tested were placed on sterile paper discs (Difco Laboratories, Detroit, MI) around the perimeter of the fungal culture. Hyphal extension results in outward growth from the central disc. If the protein being tested is an effective inhibitor

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1 Abbreviation: TPCK-trypsin, N-tosyl-L-phenylalanine chloromethyl ketone-trypsin.
of hyphal extension, a crescent shaped zone of inhibition is observed around the disc. In this manner, the effect of the chitinase purified from A. thaliana on the growth of Trichoderma reesei, Alternaria solani, Fusarium oxysporum, Sclerotinia sclerotiorum, Gaeumannomyces graminis, and Phytophthora megasperma was examined. The fungal strains used in this study were isolated and characterized by the Crop Protection Group, Monsanto Agricultural Company.

RESULTS AND DISCUSSION

As has been reported for the purification of chitinases from many sources, the chitin affinity chromatography employed here resulted in purification of chitinase to near homogeneity in a single step, since the vast majority of Arabidopsis proteins are not selectively adsorbed onto chitin. Once the nonbound proteins were removed, proteins that were selectively adsorbed were eluted at low pH. Subsequent ion exchange chromatography on Mono S removed minor contaminating proteins as shown in Figure 1. Purification of chitinase during each of these steps is summarized in Table 1 and was monitored by SDS-PAGE as illustrated in Figure 2. From the gel illustrated in Figure 2, an apparent mol wt of approximately 32,000 was estimated for the Arabidopsis chitinase. A portion of the purified Arabidopsis chitinase was also subjected to isoelectric focusing. An apparent pl of approximately 8.7 was estimated for the Arabidopsis chitinase (results not shown). It has been reported that chitinases purified from bean (P. vulgaris) (5) and pea (Pisum sativum) (15) exhibit lysozyme activity. Similarly, the purified Arabidopsis chitinase exhibited lysozyme activity, assessed by monitoring the hydrolysis of a suspension of M. lysodeikticus as described in “Materials and Methods” (results not shown).

Approximately 15 μg of the purified chitinase were subjected to automatic amino acid composition analysis. The results and a comparison of the results to the deduced amino

Table 1. Purification of Chitinase from A. thaliana

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Activity (units)</th>
<th>Specific Activity (units/mg)</th>
<th>Yield (%)</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
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<td>1. Crude extract</td>
<td>6203</td>
<td>81</td>
<td>0.013</td>
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<tr>
<td>2. 20–60% (NH₄)₂SO₄</td>
<td>1182</td>
<td>59</td>
<td>0.050</td>
<td>73</td>
<td>4</td>
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<tr>
<td>3. Chitin affinity chromatography</td>
<td>6</td>
<td>9</td>
<td>1.5</td>
<td>11</td>
<td>115</td>
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<td>4. Mono S chromatography</td>
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<td>0.7</td>
<td>1.9</td>
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Table II. Amino Acid Composition of A. thaliana Chitinase

The theoretical data are from the deduced amino acid composition of the A. thaliana basic chitinase gene (25).

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Chitinase</th>
<th>Theoretical Percent of Theoretical</th>
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<tr>
<td>Asx</td>
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<tr>
<td>Thr</td>
<td>18.05</td>
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<tr>
<td>Ser</td>
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<tr>
<td>Glx</td>
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<td>Pro</td>
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<td>Cys</td>
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<td>17</td>
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<tr>
<td>Val</td>
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<td>Tyr</td>
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<tr>
<td>Phe</td>
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<tr>
<td>His</td>
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<td>3</td>
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<tr>
<td>Lys</td>
<td>8.95</td>
<td>9</td>
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<tr>
<td>Arg</td>
<td>14.77</td>
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Table III. Amino Acid Composition of Tryptic Fragments of the A. thaliana Chitinase

<table>
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<tr>
<th>Amino Acid</th>
<th>T1</th>
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<th>T3</th>
<th>T4</th>
<th>T5</th>
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</thead>
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<td>2</td>
<td>6</td>
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</tr>
<tr>
<td>Thr</td>
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<td>2</td>
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<tr>
<td>Ser</td>
<td>3</td>
<td>1</td>
<td></td>
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<tr>
<td>Gln</td>
<td>1</td>
<td>4</td>
<td>1</td>
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<tr>
<td>Pro</td>
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<td></td>
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</tr>
<tr>
<td>Gly</td>
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<td>1</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Ala</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td></td>
<td></td>
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<tr>
<td>Cys</td>
<td>2</td>
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<tr>
<td>Val</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
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<tr>
<td>Met</td>
<td>1</td>
<td>1</td>
<td></td>
<td>2</td>
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<tr>
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<td>1</td>
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<tr>
<td>Leu</td>
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<td>Tyr</td>
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<tr>
<td>Arg</td>
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The theoretical data are from the deduced amino acid composition of the A. thaliana basic chitinase gene (25).

The deduced amino acid sequence of the basic chitinase from A. thaliana (At) (25) is aligned with the amino acid sequences of tobacco (Nt) (26), potato (St) (10), and bean (Pv) (8) basic chitinas. Identity is indicated by (+), and gaps are indicated by (−). In addition, the amino acid sequence (upper case) of a tryptic fragment (residues 237–256) of the purified Arabidopsis chitinase is shown, as are the amino acid sequences (lower case) deduced from the amino acid composition of several additional tryptic fragments, designated T1 through T5 in Table III (T1, residues 300–305; T2, residues 172–193; T3, residues 111–123; T4, residues 199–214; and T5, residues 215–236). Numbering is from the deduced amino acid sequence of the cloned enzyme, including a signal peptidase. Glu-34 is presumed to be the N-terminal residue of the mature protein (25).

Figure 3. Sequence alignment of the A. thaliana, N. tabacum (tobacco) S. tuberosum (tattoo), and P. vulgaris (bean) chitinas. The deduced amino acid sequence of the basic chitinase from A. thaliana (At) (25) is aligned with the amino acid sequences of tobacco (Nt) (26), potato (St) (10), and bean (Pv) (8) basic chitinas. Identity is indicated by (+), and gaps are indicated by (−). In addition, the amino acid sequence (upper case) of a tryptic fragment (residues 237–256) of the purified Arabidopsis chitinase is shown, as are the amino acid sequences (lower case) deduced from the amino acid composition of several additional tryptic fragments, designated T1 through T5 in Table III (T1, residues 300–305; T2, residues 172–193; T3, residues 111–123; T4, residues 199–214; and T5, residues 215–236). Numbering is from the deduced amino acid sequence of the cloned enzyme, including a signal peptidase. Glu-34 is presumed to be the N-terminal residue of the mature protein (25).
fragment of the purified Arabidopsis chitinase and several other chitinases whose sequences are known, including tobacco (Nicotiana tabacum) (26), potato (Solanum tuberosum) (10) and bean (P. vulgaris) (8). These results are summarized in Figure 3.

The results presented in Figure 4 indicate that the purified Arabidopsis chitinase is an effective inhibitor of growth of T. reesei, as assessed by the hyphal extension assay. Amounts of the purified protein as low as 0.5 μg/disc resulted in zones of inhibition. Interestingly, growth of several other fungi, A. solani, F. oxysporum, S. sclerotiorum, G. graminis, and P. megasperma, was not inhibited, even when as much as 8 μg of purified protein were applied per disc (results not shown). Similar results have been reported previously. For example, Broekhaert et al. (7) reported that chitinases purified from thorn-apple, tobacco, and wheat were effective inhibitors of spor germination and hyphal growth of Trichoderma hamatum and Phycomyces blakesleeanus, but that both spore germination and hyphal growth of Botrytis cinerea were not affected by these enzymes. These observations raise the intriguing question as to why some fungi are susceptible to treatment with chitinases, while others are apparently resistant. Although the molecular mechanism of the inhibition of fungal hyphal extension by chitinase is not known, it is reasonable to suggest that the enzyme hydrolyzes chitin in the rapidly growing extension zone of the hyphae, resulting, eventually, in a loss of integrity and localized necrosis. Although chitin is not a principal component of the cell walls of all fungal taxonomic groups, the vast majority of fungi, including all forms with septate mycelia, have chitin and glucan as the major polysaccharide components of their cell walls (2). Within the taxonomic group of fungi whose cell walls are composed primarily of chitin and glucan, however, the chitin content can vary widely (2), and it may be, at least in part, the amount of chitin in the cell wall that determines susceptibility to treatment with chitinase. Although there is not a great deal of quantitative data pertinent to this point, several authors have correlated the polysaccharide composition of fungal cell walls with susceptibility to hydrolysis catalyzed either by chitinase, glucanase, or combinations of chitinase and glucanase. For example, Rokem et al. (24) have estimated the ratio of N-acetyl glucosamine to glucose monomers following either acid hydrolysis or enzymatic hydrolysis of cell walls of several fungi. In their limited study, results ranging from a ratio of N-acetyl glucosamine to glucose of 2.5:1 for the cell walls of Morchella crassipes to a ratio of 1:5.5 for the cell walls of Candida utilis were presented (24). It is likely, however, that cell wall composition alone does not determine susceptibility to treatment with chitinase.

Although the majority of the available evidence has been interpreted in terms of a model in which the in vivo role of chitinases is to protect the host from fungal invasion directly by hydrolyzing chitin in the fungal cell wall, it has been suggested that the role of chitinases in the plant disease resistance mechanism is to release elicitors of defense-related lignification from fungal cell walls. Kurosaki et al. (13) reported that treatment of carrot cells with a partial hydrolysate of mycelial walls of Chaetomium globosum, obtained by treating mycelial walls with carrot chitinases, resulted in increased levels of phenylalanine ammonia lyase activity as well as the accumulation of phenolic acids. In addition, Pearce and Ride (20) reported that chitin and related substances serve as elicitors of the lignification response in wheat. It is possible, then, that chitinases play not only a direct role in the plant disease resistance mechanism by degrading cell walls of invading fungi, but an indirect role in that the products of chitinase activity on fungal cell walls stimulate the biosynthesis of phenolic compounds and lignification in plant cells.

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The authors would like to thank Drs. L. D. Bell and D. M. Shah for their strong encouragement and support of this project. In addition, we wish to thank J. F. Zobel for performing the automatic amino acid composition analyses, C. E. Smith for performing the automatic Edman degradation, and J. R. Borgmeyer for his assistance in performing the in vitro fungal growth inhibition assays described in this report. Finally, we would like to thank Dr. D. A. Samac for providing us with a copy of ref. 25 prior to its publication.

LITERATURE CITED

Comparison of some molecular, enzymatic and antifungal properties of chitinases from thorn-apple, tobacco and wheat.

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