Plant Physiol. (1991) 95, 450–455
0032-0889/91/95/0450/06/$1.00/0

Received for publication August 6, 1990
Accepted October 22, 1990

Purification and Characterization of an Antifungal Chitinase from *Arabidopsis thaliana*

John G. Verburg* and Q. Khai Huynh

Monsanto Corporate Research, Department of Protein Biochemistry, 700 Chesterfield Village Parkway, Chesterfield, Missouri, 63198

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, Yallaly 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 systemically 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 *A. 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 $\mu E\ m^{-2}\ s^{-1}$ 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 regenerated 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 $A_{280\,nm}$, 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[1]-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 chitooligosaccharides (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

[1] Abbreviation: TPCK-trypsin, *N*-tosyl-L-phenylalanine chloromethyl ketone-trypsin.

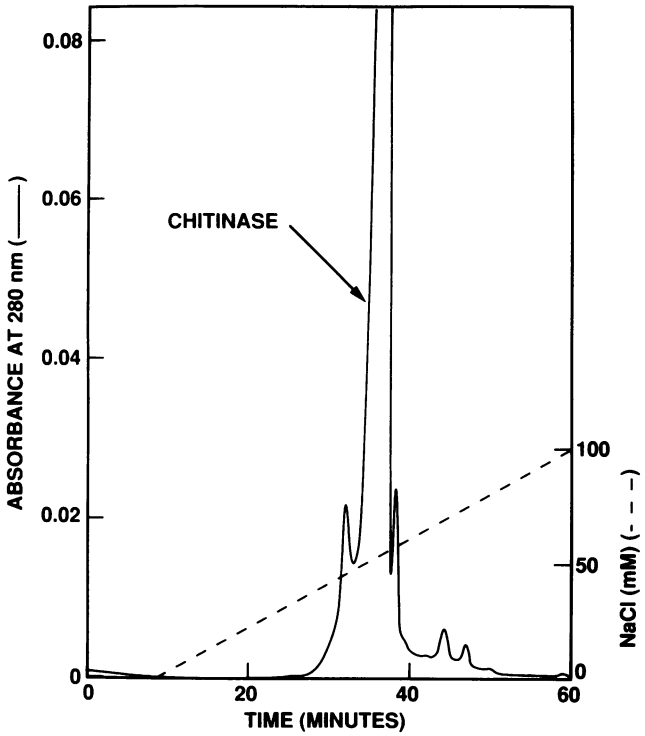

Figure 1. Purification of the *A. thaliana* chitinase by Mono S ion exchange chromatography. The pH 3.0 fraction of the chitin affinity chromatography, which contained the majority of the chitinase activity, was concentrated to approximately 10 mL and dialyzed against 10 mM sodium acetate (pH 5.0) at 4°C overnight. The sample was applied to a Mono S HR 5/5 column equilibrated in 10 mM sodium acetate (pH 5.0) and eluted with the gradient of increasing NaCl concentration in 10 mM sodium acetate (pH 5.0). Protein-containing fractions, which were detected at $A_{280\ nm}$, were collected manually and assayed for chitinase activity as described in "Materials and Methods."

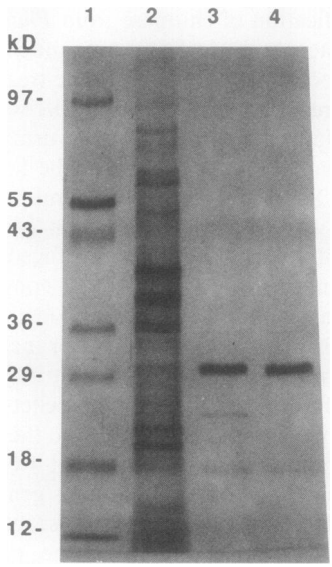

Figure 2. Purification of chitinase from *A. thaliana* monitored by SDS-PAGE. Lane 1, molecular mass standards; lane 2, 20 to 60% ammonium sulfate pellet; lane 3, chitin affinity chromatography, pH 3.0 wash; lane 4, Mono S, ion exchange chromatography. The molecular mass (kD) of the standards used is indicated in the margin.

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 I 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 pI 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 I. *Purification of Chitinase from A. thaliana*

Step	Protein	Activity	Specific Activity	Yield	Purification
	mg	*units*	*units/mg*	%	*-fold*
1. Crude extract	6203	81	0.013	100	1
2. 20–60% $(NH_4)_2SO_4$	1182	59	0.050	73	4
3. Chitin affinity chromatography	6	9	1.5	11	115
4. Mono S chromatography	0.35	0.7	1.9	1	146

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).

Amino Acid	Chitinase	Theoretical	Percent of Theoretical
Asx	34.68	31	111.9
Thr	18.05	17	106.2
Ser	16.95	16	106.0
Glx	23.62	24	98.4
Pro	19.07	22	86.7
Gly	39.78	41	97.0
Ala	26.49	31	85.5
Cys	16.43	17	96.6
Val	9.37	9	104.2
Met	2.81	3	93.7
Ile	8.30	13	63.8
Leu	12.64	14	90.3
Tyr	14.70	14	105.0
Phe	13.06	9	145.1
His	3.27	3	108.9
Lys	8.95	9	99.4
Arg	14.77	14	105.5

acid composition of a gene encoding a basic *Arabidopsis* chitinase (25) are shown in Table II. These results indicate that the amino acid composition of the purified protein is similar to that deduced from the cloned *Arabidopsis* chitinase. In addition, following reduction of disulfide bonds and carboxamidomethylation of cysteine residues, a 150-μg portion of the purified chitinase was digested with TPCK-trypsin under conditions which resulted in partial digestion of the enzyme, and the tryptic fragments were resolved by reversed phase HPLC. Automatic Edman degradation of one of the tryptic fragments for 20 cycles, revealed the following single sequence: Ala-Ala-Ile-Trp-Phe-Trp-Met-Thr-Ala-Gln-Pro-Pro-Lys-Pro-Ser-Cys-His-Ala-Val-Ile. Cys was detected as carboxamidomethyl-Cys. Comparison with the deduced sequence of the cloned *Arabidopsis* chitinase (25) indicated that this peptide is identical in sequence to a region of the cloned enzyme. Further, several additional tryptic fragments, designated T1 through T5, were subjected to automatic amino acid composition analysis, and the results are shown in Table III. These results are consistent with the composition of tryptic fragments predicted from the deduced amino acid sequence of the cloned *Arabidopsis* chitinase (25). Taken together, these data indicate that the purified *Arabidopsis* chitinase may be identical to the cloned chitinase. Finally, within a 20 amino acid overlap, a computer search of the NBRF/SWISSPROT protein and the translated GenBank databases revealed a high degree of sequence identity between the sequence of the tryptic

Table III. *Amino Acid Composition of Tryptic Fragments of the A. thaliana Chitinase*

Amino Acid	T1	T2	T3	T4	T5
Asx		2	1	2	6
Thr		1	2		
Ser		3		1	
Glx	1	4		1	
Pro		3		1	1
Gly	1	1	1	3	1
Ala		3	3		4
Cys		2		1	
Val					3
Met				1	
Ile	1		1		2
Leu				2	3
Tyr	1	1	2	2	
Phe	1		2		1
His					
Lys		1	1		1
Arg	1			1	

```
          10         20         30         40         50
At  MPPQKENHRT LNKMKTNLFL FLIFSLLLSL SSAEQCGRQA GGALCPNGLC
Nt                        SLLLLS ASA....S.. ...R.AS...
St        MRR HKEVNFVAYL LFSLLVLVSA ALAQN..S.G ..KA.AS.Q.
Pv        MKN RMMMMIWSVG VVWMLLLVGG SYG.......  ......G.N.

          60         70         80         90        100
At  CSFEGWCGNT EPYCKQPGCQ SQCTPGGTPP GPTGDLSGII SSSQFDDMLK
Nt  ..K.......  ND..GPGN..  ...P-..PT.  PGG...GS..  ...M..Q...
St  ..K.......  ND..GSGN..  ...PG..PG.  ..G...GSA.  .NSM..Q...
Pv  ..Q.....S.  TD..GPG---  C.SQC..PS.  A..-...AL.  .R.T..Q...

         110        120        130        140        150
                 gfytynafit aak
At  HRNDAACPAR GFYTYNAFIT AAKSFPGFGT TGDTATRKKE VAAFFGQTSH
Nt  ....N..QGK  ...S.....N  ..R.......  S...TA..R.  I....A....
St  ...ENS.QGK  N..S.....N  ..R.......  S..INA..R.  I....A....
Pv  ....G...AK  .....D...A  ...AY.S..N  ........R.  I...L.....

         160        170        180        190        200
                               qeqnpasdy cepsatwpca sgk    gp
At  ETTGGWATAP DGPYSWGYCF KQEQNPASDY CEPSATWPCA SGKRYYGRGP
Nt  ..........  ....A....W  LR..GSPG..  .T..GQ....  P.RK.F....
St  .......S..  ....A.....  LR.RGNPG..  .P..SQ....  P.RK.F....
Pv  ..........  ....A.....  VR.R....T.  .SATPQF...  P.QQ......

         210        220        230        240        250
    mqlswnynyg lcgraigvdl lnnpdlvand aviafkAAIW FWMTAQPPKP
At  MQLSWNYNYG LCGRAIGVDL LNNPDLVAND AVIAFKAAIW FWMTAQPPKP
Nt  I.I.H.....  P.........  ........T.  P..S..S.L.  ....P.S...
St  I.I.H.....  P.........  ........T.  P..S..T.L.  ....P.S...
Pv  I.I.......  Q.........  ..K.....T.  S..S..S.L.  ..........

         260        270        280        290        300
    SCHAVI                                              i
At  SCHAVIAGQW QPSDADRAAG RLPGYGVITN IINGGLECGR GQDGRVADRI
Nt  ...D..I.R.  ...S.....N  ....F.....  ..........  .T.S..Q...
St  ...D..I.R.  N..S.....N  ....F.....  ..........  .T.N..Q...
Pv  .S.D..TSR.  T..S..V..R  ......TV..  ..........  ...S......

         310        320        330
    gfyqr
At  GFYQRYCNIF GVNPGGNLDC YNQRSFVNGL LEAAI*
Nt  ...R...S.L  ..S..D....  G.....G...  .VDTM*
St  ...R...S.L  ..T..D....  V...W.G.A.  .VDTL*
Pv  ..FK...DLL  ..GY.N....  ...TP.G.S.  .LSDLVTSQ*
```

Figure 3. Sequence alignment of the *A. thaliana, N. tabacum* (tobacco) *S. tuberosum* (potato), and *P. vulgaris* (bean) chitinases. 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 chitinases. 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 peptide. Glu-34 is presumed to be the N-terminal residue of the mature protein (25).

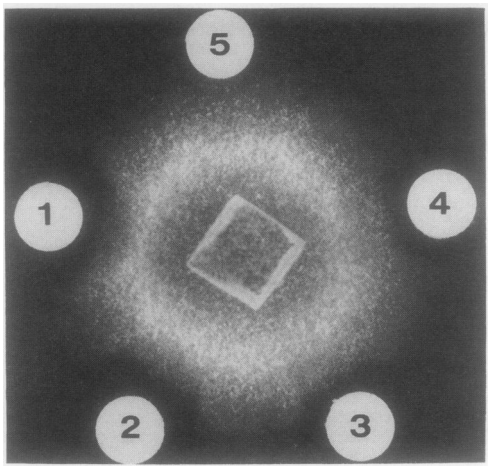

Figure 4. The antifungal activity of the *A. thaliana* chitinase against the growth of *T. reesei* assessed by the hyphal-extension inhibition assay (22) as described in "Materials and Methods." Serial dilutions of the purified protein in 40 μL of 10 mM sodium acetate (pH 5.0) were applied to discs 1 through 4 so that 4, 2, 1, and 0.5 μg of chitinase were applied, respectively. An equal volume of 10 mM sodium acetate (pH 5.0) was applied to disc 5 as a buffer blank.

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 spore 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.

ACKNOWLEDGMENTS

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

1. **Abeles FB, Bosshart RP, Forrence LE, Habig WH** (1970) Preparation and purification of glucanase and chitinase from bean leaves. Plant Physiol **47:** 129–134
2. **Bartnicki-Garcia S** (1968) Cell wall chemistry, morphogenesis and taxonomy of fungi. Annu Rev Microbiol **22:** 87–109
3. **Bell AA** (1981) Biochemical mechanisms of disease resistance. Annu Rev Plant Physiol **32:** 21–81
4. **Boller T** (1985) Induction of hydrolases as a defense reaction against pathogens. *In* JL Key, T Kosuge, eds, Cellular and Molecular Biology of Plant Stress. Alan R Liss, New York, pp 247–262
5. **Boller T, Gehri F, Mauch F, Vögeli U** (1983) Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. Planta **157:** 22–31
6. **Boller T, Mauch F** (1988) Colorimetric assay for chitinase. Methods Enzymol **161:** 430–435
7. **Broekhaert WF, Van Parijs J, Allen AK, Peumans WJ** (1988)

Comparison of some molecular, enzymatic and antifungal properties of chitinases from thorn-apple, tobacco and wheat. Physiol Mol Plant Pathol **33:** 319–331

8. **Broglie KE, Gaynor JJ, Broglie RM** (1986) Ethylene-regulated gene expression: molecular cloning of the genes encoding an endochitinase from *Phaseolus vulgaris*. Proc Natl Acad Sci USA **83:** 6820–6824
9. **Cabib E, Bowers B** (1971) Chitin and yeast budding: localization of chitin in yeast bud scars. J Biol Chem **246:** 152–159
10. **Gaynor JJ** (1988) Primary structure of an endochitinase mRNA from *Solanum tuberosum*. Nucleic Acids Res **16:** 5210
11. **Hirano S, Ohe Y, Ono H** (1976) Selective *N*-acetylation of chitosan. Carbohydr Res **47:** 315–320
12. **Kombrink E, Schroder M, Hahlbrock K** (1988) Several "pathogenesis-related" proteins in potato are 1,3-β-glucanases and chitinases. Proc Natl Acad Sci USA **85:** 782–786
13. **Kurosaki F, Tashiro N, Nishi A** (1987) Induction, purification and possible function of chitinase in cultured carrot cells. Physiol Mol Plant Pathol **31:** 201–210
14. **Legrand M, Kaufmann S, Geoffroy P, Fritig B** (1987) Biological function of pathogenesis-related proteins: four tobacco pathogenesis-related proteins are chitinases. Proc Natl Acad Sci USA **84:** 6750–6754
15. **Mauch F, Hadwiger LA, Boller T** (1988) Antifungal hydrolases in pea tissue. I. Purification and characterization of two chitinases and two β-1,3-glucanases differentially regulated during development and in response to fungal infection. Plant Physiol **87:** 325–333
16. **Mauch F, Mauch-Mani B, Boller T** (1988) Antifungal hydrolases in pea tissue II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiol **88:** 936–942
17. **Mauch F, Staehelin LA** (1989) Functional implications of the subcellular localization of ethylene-induced chitinase and β-1,3-glucanase in bean leaves. Plant Cell **1:** 447–457
18. **Meyerowitz EM, Pruitt RE** (1985) *Arabidopsis thaliana* and plant molecular genetics. Science **229:** 1214–1218
19. **Molano J, Duran A, Cabib E** (1977) A rapid and sensitive assay for chitinase using tritiated chitin. Anal Biochem **83:** 648–656
20. **Pearce RB, Ride JP** (1982) Chitin and related compounds as elicitors of the lignification response in wounded wheat leaves. Physiol Plant Pathol **20:** 119–123
21. **Reissig JL, Strominger JL, Leloir LF** (1955) A modified colorimetric method for the determination of *N*-acetylamino sugars. J Biol Chem **217:** 959–966
22. **Roberts WK, Selitrennikoff CP** (1986) Isolation and partial characterization of two antifungal proteins from barley. Biochim Biophys Acta **880:** 161–170
23. **Roberts WK, Selitrennikoff CP** (1986) Isolation and partial characterization of two antifungal proteins from barley. J Cell Biochem Suppl **10C:** 26
24. **Rokem JS, Klein D, Toder H, Zomer E** (1986) Degradation of fungal cell walls taking into consideration the polysaccharide composition. Enzyme Microbiol Technol **8:** 588–592
25. **Samac DA, Hironaka CM, Yallaly PE, Shah DM** (1990) Isolation and characterization of the genes encoding basic and acidic endochitinase in *Arabidopsis thaliana*. Plant Physiol **93:** 907–914
26. **Shinshi H, Mohnen D, Meins F Jr** (1987) Regulation of a plant pathogenesis-related enzyme: Inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. Proc Natl Acad Sci USA **84:** 89–93
27. **Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC** (1985) Measurement of protein using bicinchoninic acid. Anal Biochem **150:** 76–85