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Differential Effect of Purified Spruce Chitinases and β-1,3-Glucanases on the Activity of Elicitors from Ectomycorrhizal Fungi[1]

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Two chitinases (EC 3.2.1.14) and two β-1,3-glucanases (EC 3.2.1.39) were purified from the culture medium of spruce (*Picea abies* [L.] Karst.) cells to study their role in modifying elicitors, cell walls, growth, and hyphal morphology of ectomycorrhizal fungi. The 36-kD class I chitinase (isoelectric point [pI] 8.0) and the 28-kD chitinase (pI 8.7) decreased the activity of elicitor preparations from *Hebeloma crustuliniforme* (Bull. ex Fries.) Quél., *Amanita muscaria* (L.) Pers., and *Suillus variegatus* (Sw.: Fr.) O.K., as demonstrated by using the elicitor-induced extracellular alkalinization in spruce cells as a test system. In addition, chitinases released monomeric products from the walls of these ectomycorrhizal fungi. The β-1,3-glucanases (35 kD, pI 3.7 and 3.9), in contrast, had little influence on the activity of the fungal elicitors and released only from walls of *A. muscaria* some polymeric products. Furthermore, chitinases alone and in combination with β-1,3-glucanases had no effect on the growth and morphology of the hyphae. Thus, it is suggested that apoplastic chitinases in the root cortex destroy elicitors from the ectomycorrhizal fungi without damaging the fungus. By this mechanism the host plant could attenuate the elicitor signal and adjust its own defense reactions to a level allowing symbiotic interaction.

For most forest trees of the northern hemisphere formation of ectomycorrhizae is essential for survival. The fungal symbionts belong to the most advanced groups of Basidiomycetes (Boletales, Russulales, and Thelephorales) and Ascomycetes (Pezizales) (Kottke et al., 1997). Although many mycorrhizal associations show low specificity with regard to their symbiotic partners (Gianinazzi-Pearson and Gianinazzi, 1989; Hutchinson and Piché, 1995), compatibility of plant and fungus is a precondition for the formation of a symbiotic association. Whether mycorrhiza formation successfully occurs is determined by at least two modes of independent interactions. First, signaling events mediated by elicitors decide whether defense reactions are rapidly and strongly induced in the host plant by the foreign organism. Second, specific factors are responsible for triggering attachment and initiating developmental processes, such as the formation of the hyphal mantle and the Hartig net (Sirrenberg et al., 1995; for review, see Salzer et al., 1997).

An effective control of defense reactions in the plant is a precondition for mycorrhiza formation. This was impressively demonstrated on pea (*Pisum sativum*) myc$^-$ mutants. In these myc$^-$ mutants fungal strains of *Glomus mosseae*, which successfully formed arbuscular mycorrhizae on wild-type pea, failed to differentiate mycorrhizal structures because of the induction of physical barriers in the plant tissue located under the appressoria (Gollotte et al., 1993). It is known that elicitors can trigger processes such as lignification, callose apposition, and peroxidative cross-linking of extensin, which are the basis for an induced barrier formation in plant cell walls (Benhamou and Lafontaine, 1995; Brownleader et al., 1995). In this respect it was shown that elicitors released by the ectomycorrhizal fungus *Hebeloma crustuliniforme* (Bull. ex Fries.) Quél., a symbiotic partner of spruce (*Picea abies*), acted like those from pathogens.

Spruce cells responded to elicitors from the ectomycorrhizal fungus with a rapid efflux of K^+ and Cl^-, extracellular alkalinization, Ca^{2+} influx, protein phosphorylation, and synthesis of H_2O_2 (Salzer et al., 1996). All of these reactions are assumed to be part of the hypersensitive response (Atkinson et al., 1990, 1996; Viard et al., 1994). This raises the question of how mycorrhizae can be established in spite of these defense reactions in the host. One possibility could be a deactivation of the fungal elicitors by the enzymes that are released by the plant cells. Indeed, it was found that constitutively released proteins from spruce cells expressing high chitinase and β-1,3-glucanase activities destroy these signal molecules (Salzer et al., 1996). Originally, these signal molecules were components of the cell walls; chitin and β-glucans constitute the cell walls of Basidiomycetes and Ascomycetes (Wessels, 1993). Elicitors derived from chitin and β-glucan were demonstrated to induce lignification (Kurosaki et al., 1988; Barber et al., 1989; Okinaka et al., 1995; Cosio et al., 1996) and reactions that are assumed to be part of the hypersensitive

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Abbreviations: AA, acryl amide; DP, degree of polymerization; HIC, hydrophobic interaction chromatography; MS, mineral solution; QAE, quaternary aminoethyl.

response (Felix et al., 1993; Salzer et al., 1996). However, chitin-derived molecules (GlcNAc oligomers) can act as elicitors only if they fulfill some structural requirements, namely, they must have a DP > 3 (Felix et al., 1993; Baureithel et al., 1994). Likewise, β-glucan elicitors derived from *Phytophthora megasperma* (Sharp et al., 1984) are active as heptasaccharides. It is interesting that in the ectomycorrhiza-forming parts of spruce roots (the youngest part), the highest amount of chitinase was found (Sauter and Hager, 1989), and, as shown in this paper, in host cells a high number of chitinase isoforms are constitutively expressed.

We purified two chitinase and two β-1,3-glucanase isoforms from the medium of suspension-cultured spruce cells to study their effects on elicitors, cell walls, growth, and morphology on two ectomycorrhizal partners of spruce, *H. crustuliniforme* and *Amanita muscaria* (L.) Pers., and on a symbiotic partner of fir, *Suillus variegatus* (Sw.: Fr.) O.K. We demonstrate that only chitinases, not the β-1,3-glucanases, from spruce cells inactivate fungal elicitors, and that these chitinases and β-1,3-glucanases have no influence on fungal growth and morphology, although they act on fungal cell walls in vitro.

MATERIALS AND METHODS

Chemicals

Media for low-pressure chromatography and molecular weight standards for SDS-PAGE were from Pharmacia. The Aminex HPX 42A carbohydrate column, the de-ashing cartridges, AG 501 X8, and two-dimensional electrophoresis protein standards were obtained from Bio-Rad. Ultrafiltration membranes and Centricons were purchased from Amicon (Beverly, MA). Chemicals for protein sequencing were from Beckman, Riedl de Haën (Hannover, Germany), and Applied Biosystems; Protogel was from Hölzel (Manville, NJ), naphthalene acetic acid was from Serva Biochemicals (Paramus, NJ), 2,4-D was from Aldrich, and all other chemicals were from Sigma or Merck (Darmstadt, Germany). If available, chemicals of highest purity were used. All media were prepared with double-distilled water.

Culture of Seedlings and Suspension-Cultured Cells of Spruce

Seeds from *Picea abies* (L.) Karst., which were obtained from the Staatliches Forstamt Nagold (Staatsklenge Baden-Württemberg, Germany), were sterilized in 30% (v/v) H_2O_2 for 30 min and then washed in autoclaved, double-distilled water five times for 30 min. About 30 seeds were placed in Petri dishes (14 cm in diameter) containing 1% (w/v) agar with 1.5 g L^{-1} KH_2PO_4, 0.25 g L^{-1} $(NH_4)_2HPO_4$, 0.15 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.05 g L^{-1} $CaCl_2$, 0.05 g L^{-1} $FeCl_3$, and 0.025 g L^{-1} NaCl. For germination, the spruce seeds were incubated at 26°C in the dark for 4 to 5 d. After this time the radicula reached a length of about 2 cm. Then the seedlings were transferred to a greenhouse and were grown under conditions of 150 μmol quanta m^{-2} s^{-1} light for 16 h and 8 h of dark at 20 to 22°C and 40% RH. After 3 weeks, when the cotyledons had fully expanded and the radicles had reached a length of 4 to 6 cm, the seedlings were used for experiments.

Suspension cultures that were raised from calli from *P. abies* roots were grown in Gamborg's 4× medium, as described by Salzer et al. (1996). To isolate chitinases and β-1,3-glucanases from the culture medium, 40 mL of a 7-d-old suspension culture was transferred to 60 mL of Gamborg's 4× medium without auxins (9 μM 2,4-D, 2.7 μM naphthalene acetic acid, and 2.7 μM IAA were omitted).

Culture of Fungi

Hebeloma crustuliniforme (Bull. ex Fries.) Quél. (strain Tü 704) and *Amanita muscaria* (L.) Pers., which were isolated from fruit bodies growing under *P. abies*, and *Suillus variegatus* (Sw.: Fr.) O.K., which was isolated from a fruit body growing under *Pinus mugo* Turra, were grown on modified Melin-Norkrans agar in 8-cm Petri dishes, as described by Sirrenberg et al. (1995). To obtain suspension cultures, hyphae grown in two Petri dishes were inoculated in 200 mL of liquid modified Melin-Norkrans medium and were shaken at 100 rpm at 22 to 24°C in the dark.

Purification of Chitinases

Isolation and Concentration of Proteins from the Culture Medium

After growing in a liquid medium without auxins for 10 to 12 d, spruce cells were removed by filtration on a nylon net (10-μm mesh) and the culture filtrate was centrifuged at 17,700*g*, at 4°C for 60 min. All of the following purification procedures were conducted on ice or at 4°C in a cold room. Proteins contained in a 1.5-L culture filtrate were precipitated with ammonium sulfate at 70% saturation for 60 min, collected by centrifugation (17,700*g*, 60 min), and dissolved in 50 mL of a 20 mM NaPi-citrate buffer, pH 5.3. Insoluble precipitates were removed by centrifugation (38,000*g*, 120 min). Next, 100 mL of $NaHCO_3$ (20 mM) was added to the protein solution, which then was adjusted to pH 8.2 by the addition of NaOH.

Affinity Chromatography on Regenerated Chitin

All chromatographic procedures were performed on an Econo system (Bio-Rad). Regenerated chitin was freshly prepared from 3.5 g of chitosan according to the procedure of Molano et al. (1977), autoclaved, equilibrated with 20 mM $NaHCO_3$, and filled in a chromatography column (2.6 cm in diameter, LKB 2137). The resulting bed volume was about 46 mL.

Modifying the procedure of Molano et al. (1979), the proteins, which were adjusted to pH 8.2, were loaded on the column at a linear flow rate of 0.3 cm min^{-1}. The first washing was performed with 20 mM $NaHCO_3$ until the eluate was free of proteins. A second washing with 20 mM sodium acetate-acetic acid buffer, pH 5.6, followed for 90 min. Finally, the proteins were released from the chitin matrix with 20 mM acetic acid, pH 3.3, and immediately transferred to a 20 mM NaPi-citrate buffer, pH 7.3, by gel filtration on PD 10 columns (Pharmacia).

Cation-Exchange Chromatography of Chitin-Binding Proteins

After affinity chromatography, proteins were concentrated by ultrafiltration (Amicon cell, YM 10 Diaflo membranes) to a volume of about 5 mL, then loaded onto a column (1.6 cm in diameter, 28-mL bed volume, CM-Sepharose CL-6B), which was equilibrated with 20 mM NaPi-citrate buffer, pH 7.3. At a linear flow rate of 0.11 cm min^{-1} the NaCl concentration in the buffer was linearly increased from 0 to 0.15 M over a period of 180 min, followed by an increase from 0.15 to 0.4 M NaCl for the next 460 min. Fractions of 2 mL were collected and the chitinase activity determined as described by Sauter and Hager (1989). Those fractions with high chitinase activities were separately concentrated by ultrafiltration (Centricon, YM 10) to a final volume of about 60 μL. For experiments, fractions were used in which only a single protein band at 28 or 36 kD was detected by silver staining after SDS-PAGE.

Purification of β-1,3-Glucanases

QAE Chromatography at pH 5.3

The proteins that did not bind to the chitin matrix at pH 8.2 were concentrated by ultrafiltration (model 8200, Amicon cell, YM 10 Diaflo membranes) to a final volume of about 30 mL (Fig. 1). Then, they were dialyzed against 1 L of 20 mM NaPi-citrate buffer, pH 5.3, twice and loaded onto a QAE-Sephadex A-25 column (1.6 cm in diameter, 28-mL bed volume) at a linear flow rate of 0.18 cm min^{-1}. The sample application was followed by a 90-min washing step with 20 mM NaPi-citrate buffer, and the NaCl concentration in the buffer increased linearly from 0 to 0.5 M NaCl during the next 250 min. Fractions of 6 mL were collected and β-1,3-glucanase activity was measured as previously described (Salzer et al., 1996), with laminarin as a substrate.

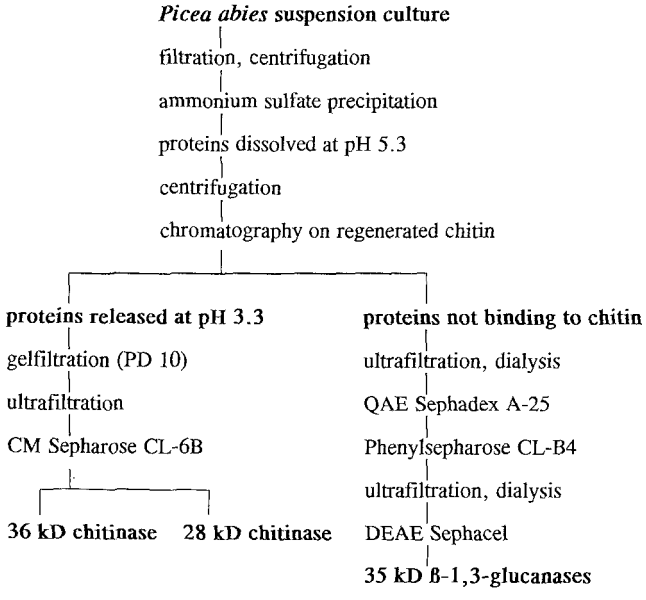

Figure 1. Scheme depicting purification of chitinases and β-1,3-glucanases from the culture medium of *P. abies* cells.

HIC

To the fractions with the highest β-1,3-glucanase activity obtained after QAE-chromatography, NaCl was added to a final concentration of 3 M. Then the proteins were loaded onto a Phenyl-Sepharose CL-B4 column (2.6 cm in diameter, 77-mL bed volume), which was equilibrated with 3 M NaCl dissolved in 20 mM NaPi-citrate buffer, pH 5.3. Separation was achieved by linearly decreasing the NaCl concentration in the buffer from 3 to 1.2 M over a period of 480 min at a linear flow rate of 0.17 cm min^{-1}. Fractions of 6 mL were collected and tested for β-1,3-glucanase activity.

DEAE Anion-Exchange Chromatography at pH 7.3

After HIC the proteins were transferred to a 20 mM triethanolamine-HCl buffer, pH 7.3. To this end the fractions with the highest β-1,3-glucanase activities were pooled (about 48 mL) and concentrated by ultrafiltration to about 5 mL. Then, 50 mL of triethanolamine buffer was added to the concentrated proteins, which were ultrafiltrated a second time, and subsequently filled up with triethanolamine buffer to a final volume of 30 mL. The proteins (30 mL) were loaded onto a DEAE-Sephacel column (2.6 cm in diameter, 58-mL bed volume) equilibrated with 20 mM triethanolamine buffer, pH 7.3. At a linear flow rate of 0.18 cm min^{-1} the NaCl concentration in the buffer was linearly increased from 0 to 1 M NaCl during the following 480 min and fractions of 6 mL were collected. Fractions with the highest β-1,3-glucanase activities were pooled (about 30 mL) and concentrated by ultrafiltration to about 200 μL.

Electrophoretic Procedures

SDS-PAGE was performed on 5% (w/v) AA-bisAA stacking gels and 12.5% (w/v) AA-bisAA separation gels according to Westermeier (1990) using DTT and iodine acetamide for sample preparation. Silver staining was performed following the procedure of Ansorge (1985).

Native electrophoresis of chitinases and β-1,3-glucanases was conducted, as described by Trudel and Asselin (1989), in a Davis system with 5% (w/v) AA-bisAA stacking gels and 15% (w/v) AA-bisAA separation gels. Proteins were dissolved in 100 mM NaPi buffer, pH 7.0, containing 20% (w/v) Suc and 0.08% (w/v) bromphenol blue. For native electrophoresis of chitinases, 0.01% (w/v) glycol chitin (Molano et al., 1977) was included in the separation gel. Activity staining of chitinases was performed according to Trudel and Asselin (1989) using Calco Fluor White. The fluorescing gels (excitation 302 nm), which were lying on a blue glass plate, were photographed with a Polaroid camera in front of which a UV filter (Wratten, no. 2A, Kodak) was mounted. Activity staining of β-1,3-glucanase was performed according to Pan et al. (1991) with laminarin as a substrate.

Proteins concentrated by ultrafiltration were mixed with 5 volumes of O'Farrell (1975) buffer. Samples of 3 μL were

electrophoresed under the conditions described by Hoffmann and Hampp (1994). For chitinases, ampholyte gradients from pH 3.0 to 10.0 were used, and for glucanases, gradients from pH 2.0 to 11.0. After focusing, the gels were fixed in 10% (w/v) sulfosalicylic acid and stained with Coomassie brilliant blue according to Neuhoff et al. (1985). For a determination of the pI, two-dimensional protein standards were both focused in parallel to the chitinases and electrophoresed after being mixed with the enzymes. The pI was determined by calculating linear regression of the marker proteins versus the migration distances.

N-Terminal Sequencing of Spruce Chitinases

Before binding the chitinases (36 and 28 kD) to hydrophobic, solid-phase filter membranes (Glassybond membranes, Biometra, Göttingen, Germany), 10 of these membranes (2 mm in diameter), which were packed in polypropylene glycol-coated tips, were wetted with 20 μL of propanol and rinsed with 300 μL of water. The proteins (about 6 μL, 1.7 μg μL^{-1}) were washed with 100 μL of water and dried. The membranes with the adsorbed proteins were directly introduced into the reaction cartridge of the amino acid sequencer (3600 LF with on-line phenylthiohydantoin analyzer, Beckman). Sequencing was performed by Edman degradation, modified according to Hunkapiller et al. (1983). The resulting phenylthiocarbamyl adducts of each degradation cycle were analyzed on-line with a reverse-phase HPLC system (125 mm × 2 mm, Spherogel Micro PTH, Beckman) following the manufacturer's instructions. The amino acids were identified by a comparison of their retention times with those of a standard chromatogram obtained from a mixture of 20 amino acids.

Preparation of Fungal Cell Walls, Treatment of Elicitors by Chitinases and β-1,3-Glucanases, and Induction of Extracellular Alkalinization in Spruce Cells

Fungal cell walls were prepared as previously described (Salzer et al., 1996), except that centrifugation was at 500*g* for 3 min.

To release elicitors from the cell walls of *H. crustuliniforme*, *A. muscaria*, and *S. variegatus*, 6 mg (dry weight) of the fine, ground fungal walls were agitated for 20 min in 2 mL of MS containing 10 mM $NaNO_3$, 1 mM KCl, 1 mM Na_2SO_4, 1 mM $Mg(NO_3)_2$, and 1 mM $Ca(NO_3)_2$, which was buffered with 25 mM Mes-NaOH at pH 5.3. The soluble elicitors were separated from the insoluble wall particles by centrifugation (4000*g*, 5 min). To 400 μL of the supernatant, 2.5 μL of the 36-kD chitinase (2.3 pkat), 2.5 μL of the 35-kD β-1,3-glucanases (2.3 pkat), or 2.5 μL of both enzymatic activities (2.3 pkat of chitinase plus 2.3 pkat of β-1,3-glucanase activity) were added. After a 35-min incubation period at 37°C, the enzymatic reaction was stopped by boiling the samples for 2 min. In samples that were incubated without chitinases and β-1,3-glucanases, the enzymes were boiled separately and thereafter added to the samples. Twenty milligrams of AG 501 X8 ion-exchanger was added, which was previously washed with double-distilled water. After shaking the samples for 30 min the deionized solution was tested for the remaining elicitor activity with the aid of the elicitor-inducible extracellular alkalinization in spruce cells, which was measured as previously described (Salzer et al., 1996), except that 5- to 8-d-old suspension cultures of *P. abies* were used for experiments.

Analysis of the DP of the Digestive Products Released by Chitinases and β-1,3-Glucanases

Colloidal chitin (0.5 mg) was incubated with 1.5 pkat of chitinase activity, laminarin (0.5 mg) was incubated with 25 pkat of β-1,3-glucanase activity, and fungal cell walls (0.75 mg) were treated either with 1.5 pkat of chitinase activity or 25 pkat of β-1,3-glucanase activity. All reactions took place in a volume of 250 μL of MS, which was buffered with 25 mM Mes-NaOH (pH 5.3) in an Eppendorf thermoshaker at 37°C. The enzymatic reactions were stopped by boiling the samples for 2 min. Colloidal chitin and the fungal cell walls were removed by centrifugation (4000*g*, 3 min). Charged compounds were removed by the ion-exchanger AG 501 X8, of which 10 mg was added to 200 μL of the sample. After shaking for 30 min the deionized samples were removed from the ion-exchanger pearls and were injected into an injection loop (110 μL), which was coupled to an HPLC system (HPLC pump 420, data system 450, Kontron, Everett, MA) and an Aminex HPX 42A carbohydrate column in front of which two de-ashing refill cartridges were installed. Chromatography was at 80°C, at a flow of 0.4 mL min^{-1} with double-distilled, degassed water as an eluent. The cleavage products were detected with a 2142 LKB differential refractometer (range 1) using water as a reference. For determination of the DP, GlcNAc, *N,N′*-diacetylchitobiose, *N,N′,N″*-triacetylchitotriose, and *N,N′,N″,N‴*-tetraacetylchitotetraose were used as the standards.

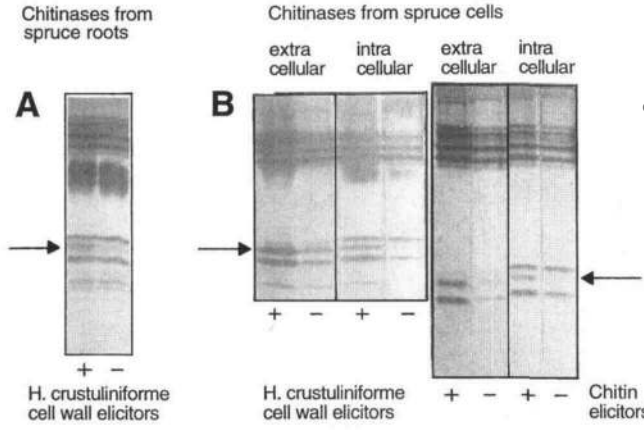

Figure 2. Constitutively expressed and elicitor-induced chitinase isoforms in roots (A) and suspension-cultured cells (B) of spruce. After 4 d of incubation with (+) or without (−) elicitors, chitinases were separated by native electrophoresis and activity-stained. Elicitors are autoclaved cell walls from *H. crustuliniforme* (A and B) and autoclaved colloidal chitin (B). Arrows indicate induced chitinase. Similar results were obtained in three independent experiments.

Table I. *Purification of the 36- and 28-kD chitinase isoforms and the 35-kD β-1,3-glucanase isoforms from the culture medium of spruce cells*

Purification started with a 1.5-L culture filtrate obtained from cells grown in a medium without auxins and fungal elicitors for 10 to 12 d.

Purification Step	Specific Activity	Total Protein	Purification	Yield
	pkat μg⁻¹	*μg*	*-fold*	*%*
Chitinase purification				
Culture filtrate	2.5	29,707	1	100
Ammonium sulfate precipitate	2.7	16,380	1.1	59.5
Chitin column	18.5	97	7.3	2.4
CM-Sepharose CL-6B[a]	20.0	60	7.8	1.6
Glucanase purification				
Culture filtrate	6.8	29,707	1	100
Ammonium sulfate precipitate	4.9	16,380	0.7	39.7
Chitin column (nonbinding proteins)	4.9	15,140	0.7	36.7
QAE Sephadex A-25	9.8	4,204	1.4	20.4
Phenyl Sepharose CL-B4	85.8	608	12.6	25.6
DEAE Sephacel	180	220	26.5	23.6

[a] In some fractions separation of the two isoforms was not complete due to their narrow pI; therefore, purification and recovery are given only for the sum of the 36- and 28-kD isoforms.

Induction of Chitinases in Spruce Roots

Fungal cell walls (1 mg dry weight) that were suspended in 10 mL of water were autoclaved for 20 min and dropped under sterile conditions to roots of 3- to 4- week-old spruce seedlings. Four days after an elicitor application the plants were removed from the agar and the roots were separated from the shoots with a razor blade. The roots (1 g) were homogenized in liquid nitrogen with a mortar and pestle and extracted with 3 mL of 150 mM NaPi buffer, pH 7.0, containing 10 μM benzamidine and 1 mM PMSF. Next, the extract was centrifuged (38,000*g*, 20 min) and the chitinase isoforms were analyzed by native electrophoresis.

Induction of Chitinases in Cultured Spruce Cells

Seven-day-old spruce cells were transferred to 60 mL of fresh culture medium, and 1 mL of autoclaved colloidal chitin (16 mg mL^{-1}) or 1 mL of autoclaved fungal cell walls (10 mg mL^{-1}) was added. To control cells 1 mL of autoclaved water was applied. To analyze the chitinases in the culture medium, 3-mL aliquots were removed with a pipette after 4 d of incubation and filtered with a nylon net (10-μm mesh). Next, 2.5 mL of the filtrate was loaded onto PD 10 columns, which were equilibrated with 150 mM NaPi buffer. Two milliliters of the eluate of the PD 10 column was concentrated by ultrafiltration to about 60 μL. A 6-μL aliquot of the concentrated proteins was mixed with 3 μL of 60% (w/v) Suc and 1 μL of bromphenol blue (0.8%, v/v) and analyzed by native electrophoresis.

To study intracellular chitinases, cells were washed in 150 mM NaPi buffer, pH 7.0, and dried by suction on a Büchner funnel. One gram of the cells was homogenized in liquid nitrogen with a mortar and pestle and extracted with 3 mL of a 150 mM NaPi buffer, pH 7.0, containing 10 μM benzamidine and 1 mM PMSF. The extract was centrifuged for 20 min at 38,000*g* and 2 mL of the supernatant was concentrated by ultrafiltration to about 60 μL, of which an aliquot of 6 μL was electrophoresed in the Davis system.

Inhibition of Fungal Growth and Influence on Hyphal Morphology

Chitinases and β-1,3-glucanases were transferred to 25 mM Mes-NaOH buffer, pH 5.3, by ultrafiltration (Centricon) and applied to cavities prepared in the agar in front of the growing hyphae. The hyphae of *S. variegatus* and *H. crustuliniforme* were grown for 17 d, and the hyphae of *A. muscaria* were grown for 4 weeks before the enzymes were added. Using sterile filtration (0.2-μm red-rim disposable filter holder, Schleicher & Schuell), about 10 pkat of chitinase activity (36- plus 28-kD isoforms), about 60 pkat of β-1,3-glucanase activity (35-kD isoforms), or a mixture of 5 pkat of chitinase and 30 pkat of β-1,3-glucanase activity was filled in the cavities. In control samples 25 mM Mes buffer without enzymes was filled in the holes. The hyphal growth was documented by photography for 7 d. Hyphal morphology was studied by light microscopy with a photomicroscope (Axioplan, Zeiss) at 100× magnification. For

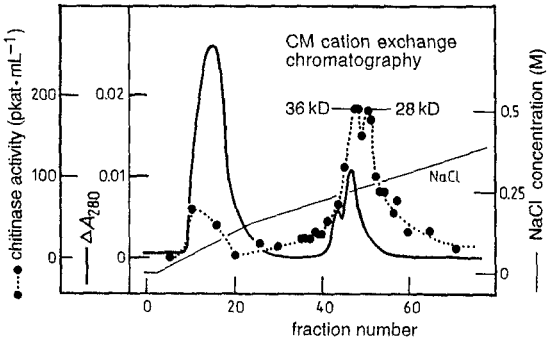

Figure 3. Separation of chitin-binding proteins by chromatography on CM-Sepharose CL-6B.

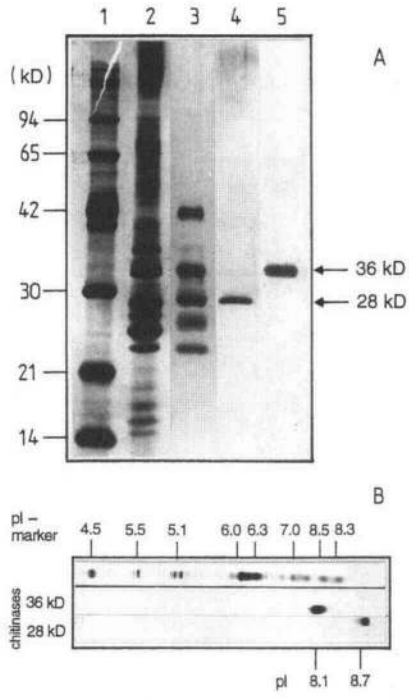

Figure 4. Purity control of chitinases by SDS-PAGE and IEF. A, SDS-PAGE. Lane 1, Molecular mass markers; lane 2, proteins (10 μL) before they were loaded to regenerated chitin; lane 3, proteins (10 μL) after the chitin column and after concentration to about 3 mL; lane 4, 28-kD chitinase from fraction 48 (5 μL); lane 5, 36-kD chitinase from fraction 43 (5 μL) after CM-chromatography and concentration to about 60 μL. The gels were silver-stained. B, IEF of the 36- and 28-kD chitinases. Aliquots of 0.5 μL from concentrated fractions 43 and 48 of the CM-Sepharose separation were focused and stained with Coomassie brilliant blue. Regression analysis of the migration distances plotted versus the pI values of the marker proteins was used to calculate the pI values of the chitinases. The values given result from two independent determinations with three samples electrophoresed in parallel.

this purpose the Petri dishes were put directly under the objective.

RESULTS

Constitutively Expressed and Elicitor-Induced Chitinase Isoforms in Roots and Suspension-Cultured Cells of *P. abies*

Roots of 3- to 4-week-old spruce seedlings expressed at least 11 chitinase isoforms. Native electrophoresis (Davis system) revealed similarities in the pattern of chitinases found in roots and in suspension-cultured cells. In both cases a group of four to five chitinase isoforms with low electrophoretic mobility was separated from a group of two to six isoforms with high mobility. In addition, one chitinase isoform was induced in the roots by an autoclaved cell wall preparation from the ectomycorrhizal fungus *H. crustuliniforme* (Fig. 2).

Colloidal chitin, as well as cell wall elicitors from the ectomycorrhizal fungi *H. crustuliniforme* (Fig. 2) and *A. muscaria* (not shown), induced the same pattern of new chitinase isoforms in spruce cells. These new isoforms were located intra- and extracellularly (Fig. 2).

Purification and Characterization of Chitinases from the Culture Medium of Spruce Cells

Both 36- and 28-kD chitinase isoforms were purified from the culture filtrate of suspension-cultured *P. abies* cells, which were grown without auxins and without fungal elicitors for 10 to 12 d (Fig. 1; Table I). Chitin-binding proteins were isolated by affinity chromatography on regenerated chitin. In some preparations up to five different chitin-binding proteins, always containing the 28- and 36-kD isoforms, could be released from the chitin matrix by 20 mM acetic acid. Subsequently, both isoforms were purified to electrophoretic homogeneity by chromatography on CM-Sepharose CL-6B (Figs. 3 and 4). SDS-PAGE and IEF of these chitinases resulted in single bands, demonstrating their purity. The pI of the 36-kD chitinase was determined to be 8.0, and that of the 28-kD chitinase to be 8.7 (Fig. 4B).

With colloidal chitin as a substrate both chitinases released primarily monomers and to a lesser extent dimers and trimers. Only the 36-kD isoform released a few tetramers (not shown). This indicates that both isoforms are exochitinases.

The N-terminal sequencing of the 36-kD isoform revealed a high homology of the mature protein to a hevein precursor protein of the para-rubber tree (Broekaert et al., 1990). Homology to class I chitinases, e.g. those of garden pea, *Theobroma cacao*, potato, and tobacco (Fig. 5), was also high, indicating that the spruce 36-kD chitinase was a class I chitinase. The N terminus of the 28-kD chitinase was blocked and therefore not accessible to N-terminal amino acid sequencing.

Purification and Characterization of β-1,3-Glucanases

Two 35-kD β-1,3-glucanase isoforms with pI values of 3.9 and 3.7 were purified from the culture medium of nonelic-

```
      1       5       10      15      20
Spruce E Q X G R Q A S G A L X P G G L X X S K W G W
Pea A2 * * X * T * * G * * * X * * * *
Pea B  * * X * * * * G * * T X * N N * X X * Q Y * Y

Cac    * * C * * * * G * * * C * * * * C C * Q F * *
Pot-28 * * C * S * * G * * * C A S * * C C * * F * *
Tob    * * C * S * * G * * R C A S * * C C * * F * *

Hevein * * C * * * * G * K * C * N N * C C * Q * * *
```

Figure 5. N-terminal amino acid sequence of the 36-kD chitinase from spruce cells. The sequence of the mature protein was determined by N-terminal protein sequencing. No residues were experimentally determined in positions 3, 12, 17, and 18, which are designated as X, but these positions correspond to Cys in other class I chitinases. For a comparison, the sequences from class I chitinases from *P. sativum* (Pea A2 and Pea B), which were determined by protein sequencing (Vad et al., 1991), from *T. cacao* (Cac) (Snyder-Leiby and Furtek, 1995), *Nicotiana tabacum* (Tob) (Shinshi et al., 1987), and *Solanum tuberosum* (Pot-28) (Meins et al., 1992), which were deduced from cDNA sequences, and the sequence of the hevein precursor protein from *Hevea brasiliensis* (Hevein) (Broekaert et al., 1990) are given. Identical positions to the spruce chitinase are marked by asterisks.

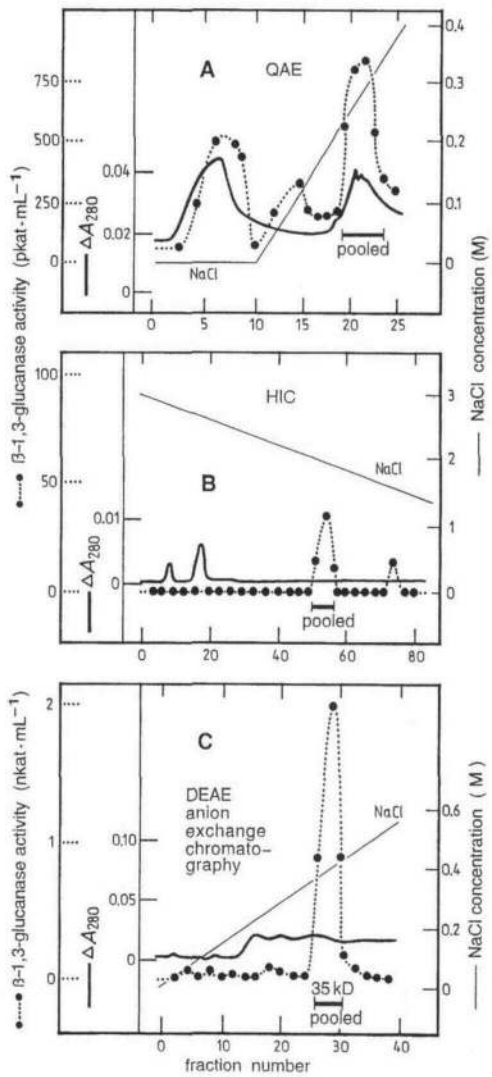

Figure 6. Chromatographic purification of β-1,3-glucanases. Subsequent chromatography on QAE-Sephadex (A), Phenyl-Sepharose CL-B4 (B), and DEAE-Sephacel (C).

ited spruce cells grown for 10 to 12 d without auxins (Fig. 1). The proteins, which did not bind to regenerated chitin at pH 8.2, were collected and chromatographed on QAE-Sephadex (Fig. 6A). Typically, three peaks of β-1,3-glucanase activity were obtained. At pH 5.3 positively charged β-1,3-glucanases did not bind to the matrix and were released without NaCl in the eluent. A minor peak of β-1,3-glucanase activity followed at NaCl concentrations of about 0.1 M. The main peak of β-1,3-glucanase activity, which appeared at NaCl concentrations of about 0.3 M, was used for further purification.

HIC resulted in the separation of a major β-1,3-glucanase peak containing the 35-kD isoforms appearing at NaCl concentrations of about 2 M and a minor peak at 1.6 M NaCl (Fig. 6B). β-1,3-Glucanases of both peaks differed in their mode of cleaving laminarin. Only the enzymes of the minor peak produced monomers from laminarin, but not the 35-kD form. DEAE anion-exchange chromatography at pH 7.3 resulted in one peak of β-1,3-glucanase activity (Fig. 6C), which appeared as a single protein band after SDS-PAGE and silver staining (Fig. 7A). However, using native electrophoresis and IEF, this band (35 kD) was shown to contain two β-1,3-glucanase isoforms (Fig. 7, B and C).

HPLC analysis of the products that were released from laminarin by the 35-kD β-1,3-glucanases revealed an endo-action of the enzymes because no monomers and dimers were generated. The DP of most of the glucan oligomers ranged between 4 and 10 (not shown).

Effect of Chitinases and β-1,3-Glucanases on the Activity of Elicitors Released from Ectomycorrhizal Fungi

Both exochitinases of spruce cells, the 36-kD class I chitinase (Fig. 8) and the 28-kD chitinase (not shown), reduced the effectiveness of the elicitors released from the cell walls of *S. variegatus*, *A. muscaria*, and *H. crustuliniforme*. Chitinase treatment of the elicitors strongly decreased their ability to induce extracellular alkalinization in spruce cells. In contrast, treatment of elicitors with the 35-kD β-1,3-glucanases had no or only slight effects on their capability to induce this reaction. Maintaining full activity after incubating the elicitors in MS containing BSA confirmed that

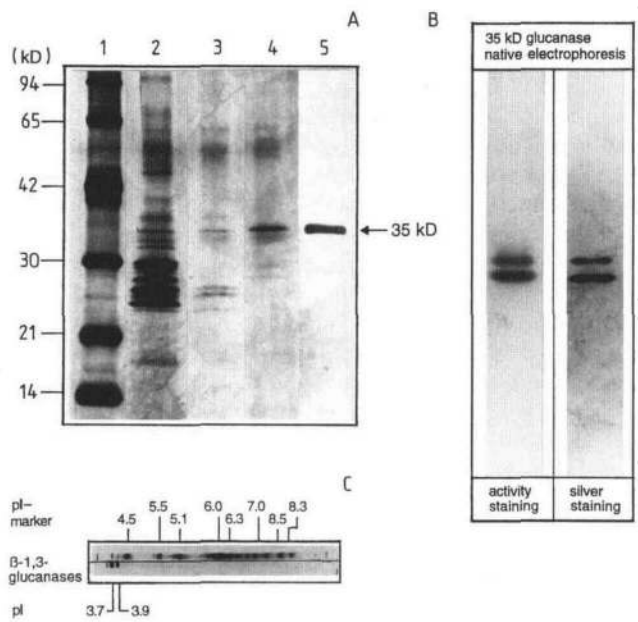

Figure 7. Purification steps and detection of β-1,3-glucanases by SDS-PAGE, native electrophoresis, and IEF. A, SDS-PAGE. Lane 1, Molecular mass markers; lane 2, proteins not binding to the chitin column (10-μL aliquot); lane 3, β-1,3-glucanase after QAE chromatography (10-μL aliquot); lane 4, β-1,3-glucanase (10 μL) after HIC and concentration to about 5 mL; lane 5, β-1,3-glucanase after DEAE (10 μL) after concentration to about 200 μL. B, Native electrophoresis of the purified and concentrated 35-kD β-1,3-glucanase (10-μL aliquots were loaded). One part of the gel was silver-stained, the other part was activity-stained. C, IEF of the 35-kD β-1,3-glucanases. An aliquot of 0.5 μL of the concentrated proteins was focused and stained with Coomassie blue. The values given were calculated from two independent determinations, as described for chitinases.

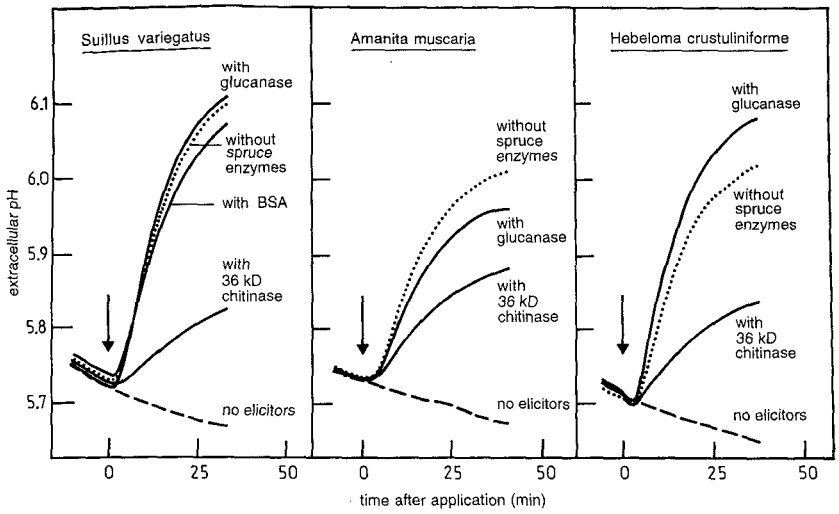

Figure 8. Effect of 36-kD chitinase and 35-kD β-1,3-glucanases on the activity of elicitors from *S. variegatus*, *A. muscaria*, and *H. crustuliniforme* in inducing extracellular alkalinization in spruce cells. Soluble elicitors were released from the fungal cell walls and were incubated with chitinase, β-1,3-glucanase, or both enzymes (2.3 pkat) for 35 min at 37°C before they were added to spruce cells. Identical results concerning the chitinase were obtained in three independent experiments. Results with β-1,3-glucanase were nonuniform, but never showed strong inhibition or increase of elicitor activity.

inactivation by chitinases was not due to nonspecific elicitor-protein interactions.

Cleavage Products Released from Cell Walls of Ectomycorrhizal Fungi

Spruce chitinases released mainly monomeric cleavage products from the walls of *A. muscaria* (Fig. 9A), *H. crustuliniforme*, and *S. variegatus* (not shown). Only differences in the number of dimers and trimers generated by the spruce chitinase were found. Whereas the 36-kD class I chitinase also released dimers and trimers from the cell walls of *H. crustuliniforme*, only monomers were released from the walls of *S. variegatus* (not shown). Among the digestive products released from cell walls of *A. muscaria*, monomers and trimers were identified (Fig. 9A).

In contrast, the decomposing action of β-1,3-glucanase on the cell walls of *H. crustuliniforme* and *S. variegatus* was small (not shown). Only minor amounts of monomeric cleavage products were found. However, glucanases released some carbohydrates of a DP > 10 from the walls of *A. muscaria* (Fig. 9B).

The action of chitinases and β-1,3-glucanases generated no elicitors from the cell walls of the ectomycorrhizal fungi, as was demonstrated by using the extracellular alkalinization response of spruce cells as a bioassay (data not shown).

Spruce Chitinases and β-1,3-Glucanases and Fungal Growth and Hyphal Morphology

Chitinases (36- and 28-kD isoforms) and the 35-kD β-1,3-glucanase isoforms had no inhibitory effect on growth of the mycorrhizal fungi *A. muscaria*, *H. crustuliniforme*, and *S. variegatus* (data not shown). After a 7-d incubation period with 10 pkat of chitinase activity, 60 pkat of β-1,3-glucanase activity, or 5 pkat of chitinase plus 30 pkat of β-1,3-glucanase activity, there was no difference in growth in relation to control hyphae in which an equivalent amount of buffer (25 mM Mes-NaOH, pH 5.3) was applied. The same results were obtained in experiments in which apoplastic spruce proteins concentrated by ammonium sulfate precipitation (Fig. 1) were used. Comparative light-microscopy studies of hyphae in contact with enzymes and control hyphae gave no hints that the enzymes cause swelling of hyphal tips. Also, induction of mycorrhiza-like structures as found in the dual cultures of *A. muscaria* and *H. crustuliniforme* and spruce callus cells (Sirrenberg et al., 1995) did not occur (data not shown).

DISCUSSION

To get an insight into the role of chitinases and β-1,3-glucanases in the early interactions of ectomycorrhizal partners, in a first step we purified two constitutively expressed chitinases and β-1,3-glucanases from the culture medium of *P. abies* cells. Suspension cultures were preferred for isolating apoplastic enzymes for two reasons: (a) culture medium can be obtained easily and in high quantity from suspension cultures, and (b) only minor amounts of intracellular proteins occur in the apoplastic space of the cells. For instance, one intracellular chitinase isoform was not found in the culture medium of the spruce cells (Fig. 2). To minimize the disadvantage of the artificial system and to come closer to conditions in spruce roots at the beginning of mycorrhiza formation, the *P. abies* cells, which were raised from root callus, were cultured at low auxin concentrations and without fungal elicitors in the medium.

From the medium of suspension-cultured spruce cells a 36-kD chitinase isoform (pI 8.0) and a 28-kD isoform (pI 8.7) were purified. The 36-kD chitinase was found to have an N-terminal sequence that showed high homology to sequences of class I chitinases, e.g. 85% of the experimentally determined amino acid positions of the 36-kD spruce chitinase were identical to those from pea and *T. cacao* class I chitinases (Vad et al., 1991; Snyder-Leiby and Furtek, 1995). Further examples of homologies are given in Figure 5. The affinity of the 28-kD chitinase to regenerated chitin indicated that this isoform also possesses a hevein domain (Raikhel et al., 1993), which is typical for class I chitinases. Contrary to class I chitinases, which are located in the plant vacuole (Iseli et al., 1993), the spruce chitinases were found

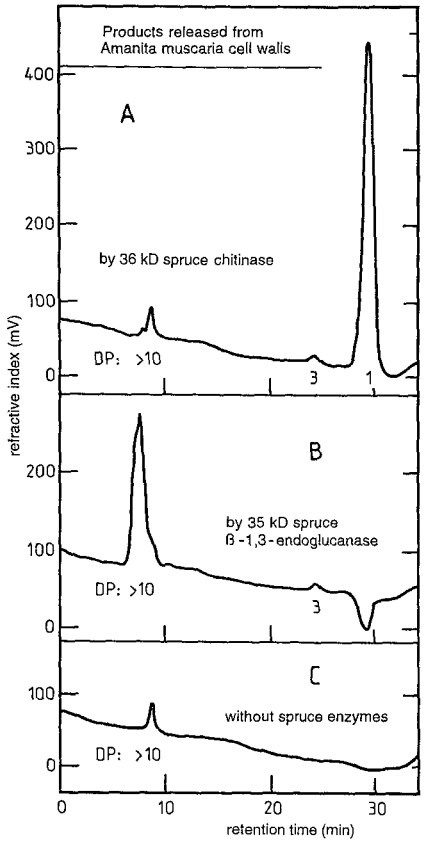

Figure 9. Action of spruce chitinase and β-1,3-glucanases on cell walls of the ectomycorrhizal fungus *A. muscaria*. Cell walls (0.75 mg) were incubated with either 36-kD chitinase (1.5 pkat) (A), 35-kD β-1,3-glucanases (1.5 pkat) (B), or without enzymes (C) for 120 min at 37°C. Analysis of the cleavage products was by HPLC with an Aminex HPX 42A column and refractometric detection. The DP was determined by a comparison with the retention times of GlcNAc, *N,N'*-diacetylchitobiose, *N,N',N''*-triacetylchitotriose, and *N,N',N'',N'''*-tetraacetylchitotetraose. Identical results were obtained in three independent experiments.

in the apoplastic space, as was also reported for the class I chitinase from *Cicer arietinum* (Vogelsang and Barz, 1993). The property of the spruce chitinases to release predominantly monomers from colloidal chitin was an indication that they act as exochitinases like those from parsley and carrot (Kurosaki et al., 1989; Kirsch et al., 1993). However, the majority of plant chitinases release GlcNAc oligomers from chitin with a DP > 3 (Kurosaki et al., 1989; Jacobsen et al., 1990; Kragh et al., 1990).

The localization of acidic β-1,3-glucanases (35 kD, pI 3.7 and 3.9) in the apoplastic space of suspension-cultured spruce cells resembles that of acidic hydrolases found in other plant systems (Meins et al., 1992).

Chitinases and β-1,3-glucanases have been intensely studied in relation to plant defense reactions (for review, see Meins et al., 1992). Most work has focused on elicitor-inducible chitinases and β-1,3-glucanases and their roles as pathogenesis-related proteins (Linthorst, 1991). In many cases direct antifungal activity of induced chitinases and β-1,3-glucanases was demonstrated in vitro (Schlumbaum et al., 1986; Mauch et al., 1988; Arlorio et al., 1991, 1992). In planta the protecting effect of chitinases against pathogenic fungi was demonstrated by overexpressing these enzymes in tobacco roots (Broglie et al., 1991; Vierheilig et al., 1993). Furthermore, chitinases from wheat and carrot are assumed to act as signal enhancers by generating elicitors from the cell walls of pathogenic fungi (Kurosaki et al., 1988; Ride and Barber, 1990). In roots and suspension-cultured cells from *P. abies* chitinases were also induced by elicitors from the ectomycorrhizal fungi *H. crustuliniforme* and *A. muscaria* (Sauter and Hager, 1989; Salzer et al., 1997), and as shown here, elicitors from these fungi, as well as colloidal chitin, induce synthesis of new chitinase isoforms and stimulate synthesis of constitutively expressed isoforms. Also, during ectomycorrhiza formation chitinases were induced (Albrecht et al., 1994a). However, the function of these enzymes in a symbiotic organ is quite different from that in plant-pathogen interactions. For instance, mycorrhiza formation was best when the highest chitinase activity was induced (Albrecht et al., 1994b). Sauter and Hager (1989) proposed that the plant chitinases probably facilitate the growth of the hyphae into the plant cell walls. In a previous paper we suggested that chitinases and β-1,3-glucanases could be involved in suppression of plant defense reactions during ectomycorrhiza formation (Salzer et al., 1996). We demonstrated that elicitors from the ectomycorrhizal fungus *H. crustuliniforme* induced the same rapid reactions in spruce cells as elicitors from pathogens induce in cells of nonhost plants (Conrath et al., 1991; Viard et al., 1994). On the other hand, spruce cells released proteins to the culture medium, which were able to decrease the activity of the fungal elicitors to induce such rapid reactions in spruce cells. Differing from plant-pathogen interactions, there was no indication that the proteins acted as suppressor molecules; rather, they showed high chitinase and β-1,3-glucanase activities (Salzer et al., 1996).

In this paper we present evidence that inactivation of these elicitors is due to the action of constitutively released chitinases from spruce cells. Two apoplastic chitinases, a 28- and a 36-kD isoform, which were purified from the culture medium of spruce cells, were able to inactivate fungal elicitors from the ectomycorrhizal fungi *H. crustuliniforme*, *A. muscaria*, and *S. variegatus*. In contrast, treatment of the fungal elicitors by purified β-1,3-glucanases from spruce cells had no significant influence on the activity of the elicitors from these ectomycorrhizal fungi. The inactivation of elicitors by chitinases is most probably caused by cleavage of elicitor-active GlcNAc oligomers to inactive fragments. Generally, GlcNAc oligomers are no more active as elicitors if their DP is less than 4 (Ride and Barber, 1990; Felix et al., 1993). The finding that the 36- and the 28-kD chitinases from spruce cells released primarily monomeric cleavage products from the fungal cell walls supports the view that chitin-derived elicitors were cleaved to monomers. This indicates that constitutively expressed chitinases, which are localized in the apoplastic space of the host root, could degrade part of the chitinous elicitors on their way across the plant cell wall before they reach their receptors in the plant plasma membrane. High-

affinity binding proteins for GlcNAc elicitors were demonstrated in the plasma membranes of tomato cells (Baureithel et al., 1994). However, the spruce chitinases exhibited no differences in inactivating elicitors from the mycorrhizal partners *H. crustuliniforme* and *A. muscaria*, and *S. variegatus*, a symbiotic partner of fir. The elicitors from the compatible spruce pathogen *Heterobasidion annosum* were inactivated by a partially purified preparation of chitinases from spruce cells (P. Salzer, unpublished data). Many ectomycorrhizal associations are nonspecific regarding the combination of symbiotic partners. Thus, inactivation of GlcNAc elicitors by plant chitinases is presumably a more general precondition for a compatible interaction between plants and fungi with chitin in their cell walls. The fact that chitin is a constituent of all mycorrhiza-forming and many pathogenic fungi rules out that GlcNAc oligomers are species-specific signals. Therefore, plants might distinguish between slow-growing ectomycorrhizal fungi and fast-growing pathogenic fungi on the basis of the amount of GlcNAc elicitors released.

Another example of the turning off of microbial signals by plant enzymes should be mentioned, namely, the inactivation of lipooligosaccharides by plant chitinases (Staehelin et al., 1994).

We found no evidence that spruce chitinases exert antifungal activity against the ectomycorrhizal fungi *H. crustuliniforme*, *A. muscaria*, and *S. variegatus*. It is interesting that chitinases from herbaceous plants, although active against fast-growing fungi, did not impair the growth of mycorrhizal fungi and the formation of arbuscular mycorrhizae (Arlorio et al., 1991; Vierheilig et al., 1993). The finding that the spruce chitinases identified cannot generate elicitors from cell walls of *H. crustuliniforme*, *A. muscaria*, and *S. variegatus* further supports the conclusion that chitinases in ectomycorrhizal interactions have different functions compared with those in plant-pathogen interactions.

Our studies on β-1,3-glucanase from spruce cells showed that the enzyme does not influence the effectiveness of fungal elicitors and does not generate elicitors, as was reported in the case of walls from *Phytophthora megasperma* (Yoshikawa and Sugimoto, 1993; Okinaka et al., 1995). Likewise, β-1,3-glucanase alone and together with chitinases did not inhibit the growth of the ectomycorrhizal fungi *A. muscaria*, *H. crustuliniforme*, and *S. variegatus*.

Growth of the spruce pathogen *H. annosum* was not influenced by partially purified chitinases and β-1,3-glucanases from the culture medium of spruce cells (P. Salzer, unpublished result).

Eleven isoforms of constitutively expressed chitinases were identified in roots of spruce seedlings by native electrophoresis. Such a high number of isoforms in healthy organs is common in many plant species. In seeds and cotyledons of cucumber, for instance, 13 chitinase isoforms were found (Majeau et al., 1990; Zhang and Punja 1994). An indication of apoplastic localization of chitinases in spruce roots was the finding of chitinase activity in root exudations (not shown). In addition, apoplastic chitinases from suspension-cultured spruce cells had an electrophoretic mobility similar to that determined for root chitinases. However, the 28- and the 36-kD chitinase could not be ascribed to one of the bands shown in Figure 2. Proof of constitutively expressed chitinases in the apoplastic space of cell walls was shown for *Lupinus albus* organs (Regalado and Ricardo, 1996). For ectomycorrhizal interactions, therefore, it is reasonable to assume that chitinases are present in the apoplastic space of the root cortex from the beginning of mycorrhiza formation.

In spruce cells and in spruce roots fungal elicitors induced de novo synthesis of chitinase. The best formation of ectomycorrhizae was reported on eucalyptus roots, where the highest chitinase activity was induced (Albrecht et al., 1994b). Moreover, in pea it was shown that induced chitinases play a decisive role in the formation of arbuscular mycorrhizae (Dumas-Gaudot et al., 1994). The finding that colloidal chitin could also induce chitinase in spruce cells allows us to believe that stimulated synthesis of chitinase might enhance the effectiveness of the chitin elicitor-inactivating system. Such chitin-induced synthesis of chitinase was also demonstrated in *Parthenocissus quinquefolia* cells, rice, and yam callus (Inui et al., 1991; Koga et al., 1992; Flach et al., 1993). Thus, inactivation of fungal chitin-derived elicitors might be one of many instrumental preconditions required to create a compatible interaction of plant and fungus in the mycorrhiza.

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