Characterization of chitin synthase from *Botrytis cinerea*

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Chitin synthase in a microsomal preparation from *Botrytis cinerea* had an apparent K_m for UDP-*N*-acetylglucosamine of 2.0 mM while nikkomycin Z and polyoxin D inhibited enzyme activity competitively with apparent K_i values of approximately 0.1 μ M and 6 μ M respectively. The organophosphorus fungicide edifenphos was a non-competitive inhibitor (K_{iapp} 54 μ M). Preincubation of microsomes for 2 h at 25 °C resulted in a maximum twofold stimulation of chitin synthase activity while preincubation with trypsin (25 μ g ml⁻¹) or cytosol (350 μ g cytosolic protein ml⁻¹) for 10 min at 25 °C resulted in approximately fourfold and 20-fold increases in chitin synthase activity, respectively. A range of protease inhibitors reduced the degree of activation of microsomal chitin synthase by cytosol. Most potent were phenylmethanesulphonyl fluoride and chymostatin; these compounds completely inhibited activation of enzyme activity. Two fragments (approx. 600 bp; *CHS1* and *CHS2*) were amplified from *B. cinerea* genomic DNA using degenerate PCR primers based on regions of complete amino acid homology between previously published chitin synthase gene sequences. When the DNA and predicted amino acid sequences of *CHS1* were used to probe computer databases for related sequences, *B. cinerea* *CHS1* was found to be most similar to *CHS1* from *Neurospora crassa*.

Keywords: *Botrytis cinerea*, chitin synthase, zymogen, polymerase chain reaction, phylogenetic analysis

INTRODUCTION

Botrytis cinerea is an important plant pathogen with a wide host range which includes vines, ornamental plants and salad vegetables (Groves *et al.*, 1988). A number of fungicides, including systemic compounds (fungicides which enter the plant and are transported in the xylem and phloem), have been used extensively to control diseases caused by *B. cinerea*. Unfortunately, serious problems of resistance have arisen during the use of systemic botryticides (Adams, 1994). For example, strains of *B. cinerea* resistant to benzimidazoles and dicarboximides have caused major set-backs in the field (Pommer & Lorenz, 1982; Leroux & Clerjeau, 1985; Locke & Fletcher, 1988). Consequently, there is an urgent requirement for new, broad-spectrum fungicides with novel modes of action which may permit more effective control of this plant disease.

Although some antifungal compounds disrupt fungal cell wall biosynthesis (Goody, 1990) it is unlikely that the potential of the cell wall as a target for fungicides has been fully realized. Currently, there is a paucity of information on the regulation of cell wall biosynthesis in agriculturally important fungal pathogens. Chitin, a (1 → 4) β polymer of *N*-acetylglucosamine (GlcNAc), is a major structural component of the cell walls of most fungi, including *B. cinerea* (Goody & Trinci, 1980; Backhouse & Willets, 1984). The enzyme chitin synthase (EC 2.4.1.16) catalyses the production of this macromolecule by polymerization of GlcNAc from the activated precursor UDP-GlcNAc. There is now compelling evidence that chitin synthase isozymes play a fundamental role during cell division and morphogenesis in fungi (Shaw *et al.*, 1991; Yarden & Yanofsky, 1991; Cabib *et al.*, 1992; Chen-Wu *et al.*, 1992). Chitin is not found in green plants or vertebrates (Goody, 1990). Therefore, chitin synthase and those cellular mechanisms which regulate the activity of this enzyme may present attractive targets for novel fungicides which lack host toxicity and toxicity to mammals and other animals.

The EMBL data library accession number for the nucleotide sequence reported in this paper is: X77937 *B. cinerea* (SCHBC1) *CHS1* gene.

Chitin synthase has been isolated from a wide range of fungal species and the enzymes characterized in detail (Cabib, 1987). At least three chitin synthase genes and gene products have been identified in *Saccharomyces cerevisiae* and genes for chitin synthase have been identified in several other fungi (Yarden & Yanofsky, 1991; Bower *et al.*, 1992; Chen-Wu *et al.*, 1992). In contrast, little information is available on the properties of chitin synthase from plant pathogens (Hori *et al.*, 1971, 1974a, b; Flemming *et al.*, 1982; Cohen *et al.*, 1986; Binks *et al.*, 1991). The aim of the current study was to undertake a detailed characterization of chitin synthase from *B. cinerea*. Ultimately, the enzyme will be used to determine the potential of mechanisms which regulate chitin synthase *in vivo* as targets for novel fungicides.

METHODS

Materials. Unless otherwise indicated, all chemicals were of Analar grade and were obtained from BDH, and all biochemicals were purchased from Sigma. Media were obtained from Oxoid. Nikkomycin Z and polyoxin D were obtained from Calbiochem-Novabiochem. Edifenphos was provided by Schering Agrochemicals. All detergents with the exception of digitonin, sodium cholate and W-1 (Sigma) and Mega 9 (OXYL-GmbH), were purchased from Boehringer Mannheim. Radiochemicals were purchased from Amersham.

Organism and culture conditions. *B. cinerea* (field isolate designated SCHBC1) was maintained on plates of Potato Dextrose Agar (PDA; 39 g l⁻¹) at room temperature under daylight conditions and sub-cultured every 7–10 d. Spores were harvested by washing the surface of the culture with sterile Tween 20 (0.5%, v/v; 10 ml). Spore suspensions were filtered through glass wool (pre-washed with distilled water) and the filtrates pooled. The resultant spore suspension was used to inoculate Malt Extract (2%, w/v; 500 ml) to a final concentration of approximately 2×10^6 spores ml⁻¹ (measured microscopically using the Improved Neubauer Counting Chamber; Weber). Cells were shaken (100 r.p.m.) and incubated at 25 °C. After 16 h the culture was in the mid-exponential phase of growth and germlings were harvested by centrifugation in an MSE Mistral 6L centrifuge at 3000 r.p.m. for 15 min and washed once, by centrifugation, in cold (4 °C) homogenization buffer (50 mM Bis-Tris pH 6.5, 0.25 M sucrose, 1 mM EDTA).

Disruption of cells and centrifugation of lysate. All operations were performed at 4 °C. Germlings (approx. 20 g wet weight) were resuspended in homogenization buffer (15–20 ml) and mixed with glass beads (15 ml; 0.5–0.75 mm diameter) in 50 ml bottles. Cells were homogenized for 8 × 30 s at 4000 r.p.m., with intervals of 30 s for cooling, in a Braun MSK cell homogenizer. The homogenate was centrifuged at 9000 g (r_{av} , 6.98 cm) for 20 min in a Sorvall RC-5B superspeed centrifuge with SS-34 rotor. The supernatant was centrifuged at 144 800 g (r_{av} , 8.1 cm) for 1 h in a Sorvall OTD65B ultracentrifuge with TFT 50.38 rotor to pellet the microsomal fraction. The supernatant was stored at –20 °C as the cytosolic fraction and the pellet was washed once in 50 mM Bis-Tris (pH 6.5). The supernatant was discarded and the microsomal fraction resuspended in 50 mM Bis-Tris buffer (pH 6.5) to a final protein concentration of 0.5 mg ml⁻¹, and stored at –20 °C.

Enzyme assays. The chitin synthase assay was based on the method of Dickinson *et al.* (1991) with modifications. Unless otherwise stated the reaction mixture (50 µl) contained micro-

somal fraction (10 µl) and 40 mM Tris/HCl (pH 7.5), 6 mM MnCl₂, 32 mM GlcNAc and 1 mM UDP-GlcNAc containing 9 nCi (333 Bq) UDP-[¹⁴C]GlcNAc (272 mCi mmol⁻¹; 10.1 GBq mmol⁻¹). Incubations were at 25 °C for 60 min. The reaction was stopped by a further incubation at 100 °C for 2 min. The reaction mixture was filtered through glassfibre filter paper (Whatman GF/F glass microfibre filters; 2.5 cm diameter, 0.7 µm pore size) pre-soaked in distilled water for 60 min. The reaction tube was washed twice with 50 µl aqueous Triton X-100 (1%, v/v), and the washings filtered. Finally, the filter paper was washed with 10 ml distilled water. The filter papers were placed in 4 ml scintillant (Optiphase 'Safe', LKB Scintillation Products) and radioactivity measured in an LKB Wallac Rackbeta 1217 liquid scintillation counter.

Chitinase, β-N-acetylglucosaminidase and lysozyme were assayed as described by Dickinson *et al.* (1989), Rast *et al.* (1991) and Lundblad *et al.* (1974), respectively.

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard.

Characterization of the chitin synthase reaction product.

Chitin synthase reaction mixtures (50 µl) containing microsomes (10 µl) were incubated at 25 °C for 60 min. One of the following solutions (50 µl) was added: 50 mM potassium phosphate, pH 6.0 (buffer A); 0.05 units of chitinase from *Streptomyces griseus* (Sigma, product no. C1525) in buffer A; 0.05 units of chitinase boiled for 3 min in buffer A; 11.6 M HCl; 2 M KOH. Reaction vessels were incubated for 1 h at 100 °C (KOH) or shaken (160 r.p.m.) for 20 h at 20 °C. Incubations were filtered and radioactivity measured as described above.

Effect of trypsin on chitin synthase activity. Equal volumes (50 µl) of the microsomal fraction (0.5 mg protein ml⁻¹) and trypsin in 50 mM Bis-Tris, pH 6.5, were mixed and incubated for 10 min at 25 °C. Soybean trypsin inhibitor (25 µl) was added at twice the trypsin concentration. Ten microlitres of the final mixture was used as enzyme preparation in the chitin synthase assay.

Effect of cytosol on microsomal chitin synthase. Cytosol (16 ml) was concentrated by ultrafiltration using a Centricon 10 microconcentrator (Amicon) and resuspended to a concentration of 0.7 mg protein ml⁻¹ in 50 mM Tris, pH 7.5. Equal volumes (25 µl) of a microsomal suspension (0.5 mg protein ml⁻¹) and cytosol, or cytosol diluted in 50 mM Tris, pH 7.5, were mixed and incubated at 25 °C for 10 min. Ten microlitres of the mixture was used in a standard assay for chitin synthase activity. In control incubations, cytosol was replaced with 50 mM Tris, pH 7.5.

Effect of protease inhibitors on the activation of chitin synthase by cytosol.

One of the following protease inhibitors was included in incubations of microsomes with concentrated cytosol (final inhibitor concentration indicated in parentheses): phenylmethanesulphonylfluoride (PMSF; 0.5 mM), benzamidin (1 mM), aprotinin (0.3 µg ml⁻¹), chymostatin (0.1 mM), leupeptin (0.1 mM), antithrombin III (0.013 µg ml⁻¹), lima bean trypsin inhibitor (0.3 µg ml⁻¹), soybean trypsin inhibitor (0.3 µg ml⁻¹), EDTA (1 mM), 1,10-phenanthroline (5 mM), cystatin (0.3 µg ml⁻¹), or pepstatin A (0.01 mM). Protease inhibitors were used at concentrations described previously as effective for the inhibition of specific classes of proteases (Beynon & Salvesen, 1989). The inhibitors were also included in control incubations.

Isolation of genomic DNA from *B. cinerea*. The method was based on that of Murray & Thompson (1980) with the following modifications: freeze-dried germlings (500 mg) were re-

suspended in extraction buffer (15 ml) which included RNase ($40 \mu\text{g ml}^{-1}$) and proteinase K (1 mg ml^{-1}); 50 mM Tris/HCl, pH 8.0, cetyl trimethylammonium bromide (1%, w/v), 10 mM EDTA (30 ml) was added to the aqueous phase (15 ml) following the second chloroform/octanol extraction, the DNA was precipitated with ammonium acetate/ethanol, washed with ethanol (70%, v/v), dried briefly under vacuum and re-suspended in TE buffer (5 ml).

PCR amplification of genomic DNA. Incubations were as described by Bowen *et al.* (1992) with modifications. Briefly, each reaction (100 μl) consisted of 2.5 units *Taq* DNA polymerase, 20 nmol of each dNTP, 10 mM Tris/HCl, pH 8.3, 50 mM KCl, gelatin (0.1 mg ml^{-1}), 2 mM MgCl_2 , genomic DNA (100 ng) and 25 pmol of each primer (supplied by British Biotechnology). The primers used were as follows:

(A) 5'-CTG AAG CTT ACA ATG TAT AAT GAG GAA-3'

G C C A T
C C
G

(B) 5'-GTT CTC GAG CTT GTA CTC GAA GTT CTG-3'

T A T A A T

Forty cycles were run consisting of a 94°C , 1 min step, a 50°C , 1 min step and a 72°C , 3 min step using an Omnigene Temperature Cycler (Hyaid). The conserved sequences encoded by the primers are not included in the analysed sequences.

Cloning using the pCRII vector and preparation of plasmid DNA from transformed *E. coli*. The TA cloning system (Invitrogen Corporation) and Qiagen Hi Purity 'Plasmid' kit were used in accordance with the manufacturers' instructions.

DNA sequencing. PCR products were sequenced by the method of Sanger *et al.* (1977) using the Sequenase kit (version 2.0; United States Biochemical) and [^{35}S]dATP αS . Several sequence-specific primers were constructed by Dr G. D. Elliott, Department of Microbiology, University of Leeds.

Analysis of DNA sequences. The entire DNA sequence of the *B. cinerea* *CHS1* fragment was used to probe the GenBank (release 77.0) and EMBL (release 35) databases using the FASTA program. The amino acid sequence of the 600 bp fragment was derived using the DNASIS program (Hitachi Software Engineering Co.) and used to probe the Owl database (version 20.1) for related sequences, using a Sweep program generated with Sooty. The amino acid sequences were aligned and a phylogenetic tree generated using CLUSTALV (Higgins *et al.*, 1991). FASTA, Sooty, Sweep and CLUSTALV were run on the BioVax system at the Department of Biochemistry and Molecular Biology, University of Leeds.

RESULTS

Isolation and analysis of nucleic acids

The PCR primers used during the present study were based on regions conserved in *S. cerevisiae* *CHS1* and *CHS2*, and *Candida albicans* *CHS1*. Therefore, the two fragments (approximately 600 bp) amplified from *B. cinerea* genomic DNA using these primers were designated *CHS1* and *CHS2* [the latter was described previously as '*CHS3*' (Causier, 1993)]. The *CHS1* fragment was

completely sequenced (Fig. 1). Comparison with the partial sequence of the *CHS2* fragment (133 bp, data not shown) suggested that the *CHS1* and *CHS2* fragments were from similar but distinct genes. The DNA sequence of *CHS1* was used to probe the GenBank and EMBL databases for related sequences. Of 34 chitin synthase sequences identified, *CHS1* from *Neurospora crassa* was most closely related to *B. cinerea* *CHS1* (74.7% identity for an overlap of 475 nucleotides). Similarly, when the predicted amino acid sequence of *B. cinerea* *CHS1* was used to probe the Owl database, the deduced gene product from *B. cinerea* was most closely related to the predicted amino acid sequence for *N. crassa* *CHS1* (85.7% identity for an overlap of 161 amino acids). When the predicted amino acid sequences retrieved from the database were aligned and used to generate a phylogenetic tree, the sequences were divided into three groups (data not

1	D V L F A R T M H G V F K N I E F M C T	20
1	GATGTATTGTTTGGTAGAACAAATGCACGGAGTATTCAAGAACATTGAATTTATGTGCACT	60
21	R K D S K T W G K D A W K K I V V C V V	40
61	CGTAAAGACAGTAAACATGGGGCAAGGATGCTTGGAAAGATCGTAGTGTGTGGTTC	120
41	S D G R A K I N P R T R A V L A G L G V	60
121	AGTGATGGACGTGCCAAATCAATCCAGAACAAAGGGCTGTTTGGCTGGTTTGGGGGT	180
61	Y Q D G I A K Q Q V N G K D V T A H I Y	80
181	TATCAGGACGGAATTCGGAAGCAACAAGTCAATGGCAAGGACGTGACAGCACATCTAT	240
81	E Y T T Q V G I S L K K D I V T L T P K	100
241	GAATATACGACTCAAGTCGGAATTTCTTTGAAAAGGATATTGTACACTCACACCAAAA	300
101	Q Q P V Q L L F C L K E K N Q K K I N S	120
301	CAGCAACCCGTTCAATTGCTTTCTGTTTGAAGGAAAGATCAGAAAAGATCAACTCT	360
121	H R W F F Q A F G R V L D P N I C V L L	140
361	CACAGATGGTTCTTCCAAGCCTTTGGTAGAGTACTGTATGCCAACATTTGTGTACTCCTC	420
141	D A G T K P G K D S I Y H L W K A F D L	160
421	GATGCTGGTACAAAACAGGAAAGGACTCAATTTATCATCTATGGAAGCTTTTCGATTG	480
161	E P H C A G A C G E I K A M L G P G G K	180
481	GAACACATTTGTGCAGGAGCTTGTGGTGAGATCAAGCTATGTTGGGCCAGGAGGAAAG	540
181	N L V N P L V A T	
541	AATTGGTCAATCCATTGGTTGTACTAC	

Fig. 1. The nucleotide and predicted amino acid sequence of the *CHS1* fragment from *B. cinerea*.

Fig. 2. Effect of GlcNAc on chitin synthase activity. Values shown are the means of three experiments $\pm$ SEM.

Fig. 3. Effect of cations (chloride salts, 6 mM) on chitin synthase activity. Values shown are the means of three experiments $\pm$ SEM.

shown). Bootstrap analysis of the phylogenetic data demonstrated, in 100% of trials, that *CHS1* from *B. cinerea* was grouped with *CHS1* from *N. crassa*.

Properties of the microsomal chitin synthase preparation

Plots of product formed as a function of time (0–3 h) and rate of reaction against enzyme concentration (0–2 mg protein ml⁻¹) were straight lines (data not shown). Lineweaver–Burk analysis yielded an apparent K_m of 2.0 mM for UDP-GlcNAc and the apparent temperature optimum was 35 °C (data not shown). Free GlcNAc stimulated enzyme activity (Fig. 2), which was dependent upon the presence of a divalent cation. Mn²⁺ was most effective (Fig. 3).

The reaction product was identified as chitin on the basis of the following properties: (a) incubating it with chitinase (0.025 units), but not boiled chitinase, for 20 h at 20 °C gave 98% degradation to soluble sugars; (b) 70% of the product was hydrolysed when treated with concentrated hydrochloric acid (5.8 M); and (c) the product was insoluble in KOH (1 M).

Microsomal and cytosolic preparations contained chitinase [0.02 and 0.2 pmol GlcNAc released min⁻¹ (mg protein)⁻¹, respectively] and β -N-acetylglucosaminidase [0.3 and 5 μ g 4-nitrophenol released min⁻¹ (mg protein)⁻¹, respectively] activities. Lysozyme activity was not detected in microsomal or cytosolic preparations.

Effect of nikkomycin Z, polyoxin D, UDP and edifenphos on microsomal chitin synthase

The nucleoside-dipeptide antibiotics nikkomycin Z and polyoxin D were potent, competitive inhibitors of enzyme activity with apparent K_i values of approximately 0.1 μ M

Fig. 4. Effect of nikkomycin Z and polyoxin D on chitin synthase activity in the presence of 0.5 mM UDP-GlcNAc (▲), giving regression lines of $y = 0.01x + 0.004$ ($r = 0.98$; nikkomycin), $y = 0.001x + 0.007$ ($r = 0.98$; polyoxin), and 1 mM UDP-GlcNAc (△), giving regression lines of $y = 0.002x + 0.002$ ($r = 0.98$; nikkomycin), $y = 0.0005x + 0.004$ ($r = 0.99$; polyoxin).

and 6 μ M, respectively (Fig. 4). Dixon plot analysis indicated that UDP inhibited competitively (apparent K_i of 190 μ M) and the organophosphorus fungicide edifenphos was a non-competitive inhibitor (apparent K_i of 54 μ M) (data not shown).

Effect of pre-incubating microsomes alone or with trypsin or high-speed cytosol on chitin synthase activity

A slight (maximum twofold) increase in chitin synthase activity was observed following incubation of microsomes (0.3 mg protein ml⁻¹) for 2 h at 25 °C. Microsomal chitin synthase was activated by trypsin; maximal activation (3.75-fold) occurred at a concentration of 25 μ g trypsin ml⁻¹ (Fig. 5a). Pre-incubation with cytosol resulted in a

Fig. 5. Effect of trypsin (a) and concentrated cytosol (b) on microsomal chitin synthase. Microsomes were incubated with trypsin or cytosol as described in Methods, and assayed for chitin synthase activity. Values are based on a control incubation which did not contain trypsin or cytosol [activity in the control incubation was $2.8 \text{ nmol GlcNAc incorporated min}^{-1} (\text{mg protein})^{-1}$]. For trypsin incubations, values shown are the means of four experiments $\pm$ SEM. For cytosol incubations, the experiment was repeated three times with essentially the same result.

20-fold increase in chitin synthase activity at a concentration of $350 \mu\text{g cytosolic protein ml}^{-1}$ (Fig. 5b). No chitin synthase activity was detected in cytosol prior to incubation with microsomes.

Effect of protease inhibitors on activation of chitin synthase by cytosol

All of the protease inhibitors reduced the degree of activation of microsomal chitin synthase by cytosol (Fig. 6). Most potent were PMSF and chymostatin; these compounds completely inhibited activation of enzyme activity.

Fig. 6. Effect of protease inhibitors on the activation of chitin synthase by concentrated cytosol. Protease inhibitors (for concentrations, see Methods; trypsin inhibitors 1 and 2 were from lima bean and soyabean, respectively) were included in incubations of microsomes with cytosol (final concentration $200 \mu\text{g cytosolic protein ml}^{-1}$) as described in Methods, and assayed for chitin synthase activity. Values are based on a control incubation which contained boiled cytosol. Activity in the control incubation was $2.8 \text{ nmol GlcNAc incorporated min}^{-1} (\text{mg protein})^{-1}$. All assays were performed in triplicate and varied by less than 10%. The experiment was repeated twice with essentially the same result.

DISCUSSION

Bowen *et al.* (1992) designed degenerate PCR primers based on two small regions of complete amino acid homology between the chitin synthase genes of *Saccharomyces cerevisiae* (*CHS1* and *CHS2*) and *Candida albicans* (*CHS1*). The primers were used to amplify fragments (approximately 600 bp) homologous to chitin synthase from the genomic DNA of 14 fungal species. During the present study, similar primers were used to amplify fragments (approximately 600 bp; *CHS1* and *CHS2*) from the genomic DNA of *B. cinerea*. When the DNA and predicted amino acid sequences of *CHS1* were used to probe computer databases for related sequences, *B. cinerea CHS1* was found to be most similar to *CHS1* from *N. crassa*. Phylogenetic analysis of aligned amino acid sequences divided the sequences into three classes (data not shown). Sequences from like species grouped together within these classes in broad agreement with the results of Bowen *et al.* (1992).

The properties of microsomal chitin synthase from *B. cinerea* were similar to those of particulate chitin synthases from other fungi (for references, see Cabib, 1987; Machida & Saito, 1993). It is important to note that chitinase and *N*-acetylglucosaminidase activities were also detected in the *B. cinerea* microsomes. The specific activities of these

enzymes were approximately one-tenth of the activities detected in cytosolic preparations. Nonetheless, the presence of chitinase and *N*-acetylglucosaminidase in chitin synthase preparations should be borne in mind as these enzymes may have influenced the results obtained during studies of enzyme kinetics. Apparent membrane-bound chitinases have been detected in a number of fungi (Adams *et al.*, 1993), and Rast *et al.* (1991) proposed a model for the controlled lysis of chitin in the cell wall of *Mucor rouxii*, through the concerted action of chitin synthase with microsomal and cytosolic chitinase and *N*-acetylglucosaminidase activities. The apparent K_m for UDP-GlcNAc was approximately 2 mM and enzyme activity [typically 3–4 nmol GlcNAc incorporated into chitin min^{-1} (mg protein^{-1})] was stimulated by free GlcNAc. High concentrations of GlcNAc were required for stimulation (Fig. 2); the physiological significance of this effect is unclear (Cabib, 1987). Polyoxin D and nikkomycin Z were potent, competitive inhibitors of chitin synthase in *B. cinerea* microsomes; the kinetics of inhibition closely resembled those reported previously for other fungi (reviewed by Gooday, 1990). Polyoxins have been used with some success in the treatment of fungal diseases of rice plants and black spot disease of pears (caused by *Alternaria kickuchiana*) in Japan (Hori *et al.*, 1974c; Fiedler *et al.*, 1982). Nikkomycins were active against *B. cinerea* growing on bean plants (Fiedler *et al.*, 1982) and when *B. cinerea* was incubated with nikkomycin *in vitro*, the antibiotic inhibited spore germination and caused swelling of germ tubes (Zhu & Gooday, 1992). During the current study, germ tube morphology was affected in a similar manner by polyoxin D (50 μM ; data not shown).

The modulation of membrane-bound enzyme activities by alterations in membrane fluidity (which result from changes in the lipid composition of the membrane) is well-documented (Houslay & Stanley, 1982). Chitin synthase is an integral membrane protein and, predictably, studies *in vitro* and *in vivo* suggest that the lipid environment may play a role in regulating enzyme activity (Adams *et al.*, 1993). The organophosphorus fungicide edifenphos, which is thought to inhibit phosphatidylcholine synthesis *in vivo* (Kodama *et al.*, 1980), reduced the incorporation of [^3H]GlcNAc into chitin in *Fusarium graminearum* (Binks *et al.*, 1991). Furthermore, membrane-bound chitin synthase preparations from cultures of *F. graminearum* grown in the presence of edifenphos had much lower activities *in vitro* than preparations grown in the absence of the fungicide. Microsomal chitin synthase from *F. graminearum* was inhibited non-competitively by edifenphos with an apparent K_i of 50 μM . A similar result was obtained during the present study: microsomal chitin synthase from *B. cinerea* was inhibited non-competitively by edifenphos with a $K_{i\text{app}}$ of 54 μM . As Binks *et al.* (1991) suggested, edifenphos may inhibit chitin synthase activity indirectly *in vivo* following an alteration in the phospholipid composition of the cell membrane. In addition, our results, and those of Binks *et al.* (1991) suggest that the fungicide may also affect chitin synthase activity directly.

Chitin synthase preparations from most species of fungi contain enzyme which may be activated by partial proteolysis. During the present study, limited proteolysis with trypsin increased chitin synthase activity approximately fourfold. One interpretation of this phenomenon is that, *in vivo*, regulation of localized chitin synthesis is achieved through synthesis of chitin synthase as a zymogen, with subsequent activation of the enzyme at the required time and place in the cell (Cabib & Farkas, 1971). The results of Machida & Saito (1993) appear to lend weight to this hypothesis. These authors purified a chitin synthase zymogen (molecular mass 30 kDa) from *Absidia glauca*. Incubation with trypsin converted the zymogen to an active enzyme (28.5 kDa). Incubations of *B. cinerea* microsomes with cytosol from this organism caused a marked stimulation of chitin synthase activity (Fig. 5b). This effect was completely inhibited by the serine/cysteine protease inhibitors PMSF and chymostatin (Fig. 6). However, a wide range of compounds representing all of the major classes of protease inhibitor caused partial inhibition of chitin synthase activation by cytosol. These results suggest that many proteases endogenous to *B. cinerea* may, potentially, activate chitin synthase *in vivo*. Indeed, using ion-exchange chromatography, we identified a number of proteins in *B. cinerea* cytosol which activated chitin synthase zymogen *in vitro* (Adams *et al.*, 1993). Furthermore, *B. cinerea* microsomes also appear to contain a factor which activates chitin synthase. To date, the nature of the endogenous proteolytic activator(s) of chitin synthase zymogen has not been determined for any fungus. If proteolytic activation is an important mechanism for the regulation of chitin synthase *in vivo*, then it is essential that the nature and sub-cellular location of the activator should be established, as such a protease would present an attractive target for novel fungicides.

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