Chitin synthesis in *Fusarium graminearum* and its inhibition by edifenphos (Hinosan)

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Membrane-bound chitin synthase from *Fusarium graminearum* A3/5 had a pH optimum of 6.5, an apparent temperature optimum of 30 °C and an apparent K_m for UDP-*N*-acetylglucosamine (UDP-GlcNAc) of 2.5 mM. Digitonin-solubilized chitin synthase had a lower (about 25% of the original activity) and more variable activity than the membrane-bound chitin synthase from which it was prepared. The activity of solubilized chitin synthase was stabilized by incorporating Allosamidin (a chitinase inhibitor) into the reaction mixture, and an approximately 3.6-fold increase in activity was observed when the reaction mixture contained dimyristoylphosphatidylcholine. Concentrations of the organophosphorus fungicide edifenphos (Hinosan) up to 25 µM had no effect on the *in vitro* activity of membrane-bound chitin synthase, but between 25 and 145 µM, chitin synthase activity decreased with increase in fungicide concentration. Edifenphos caused non-competitive inhibition of chitin synthase activity with an apparent K_i of 50 µM. Membrane-bound chitin synthase preparations from cultures of *F. graminearum* grown in 250 µM-edifenphos had a much lower *in vitro* activity than preparations from cultures grown in the absence of the fungicide. Pre-growth of *F. graminearum* in 250 µM (but not 80 µM) edifenphos also caused a reduction in incorporation of [³H]GlcNAc into chitin *in vivo* (washed biomass was incubated in [³H]GlcNAc-containing medium lacking fungicide).

Introduction

Edifenphos (Hinosan; *O*-ethyl-*S,S*-diphenylphosphorodithioate) was marketed in 1968 to control *Pyricularia oryzae* on rice (Scheinpfug & Jung, 1968), but there is still some uncertainty about its mode of action. Maeda *et al.* (1970) reported that iprobenfos (a related organophosphorus fungicide) inhibited incorporation of [¹⁴C]glucosamine into the cell wall of *P. oryzae* and they suggested that the fungicide inhibited chitin synthesis either directly, by inhibiting chitin synthase (EC 2.4.1.16), or indirectly, by altering membrane (cytoplasmic) permeability; chitin synthase in fungi is a membrane-associated enzyme (Gooday & de Rousset-Hall, 1975).

Although edifenphos and iprobenfos increased the permeability of membranes of *P. oryzae* (De Waard, 1974), iprobenfos had no effect on the membrane permeability of *Aspergillus nidulans* (Craig & Peberdy, 1983*a*). There are also contradictory reports of the effects of edifenphos and iprobenfos on phosphatidylcholine

synthesis; for example Akatsuka *et al.* (1977), Kodama *et al.* (1979) and Ross & Brady (1985) reported that organophosphorus fungicides inhibited phosphatidylcholine synthesis, but Craig & Peberdy (1983*b*) found that iprobenfos did not affect the phosphatidylcholine content of *A. nidulans*. Recently, Wiebe *et al.* (1990) showed that, at low concentrations (up to 246 µM), edifenphos acted as a paramorphogen (Tatum *et al.*, 1949), increasing branching of *Fusarium graminearum* A3/5, but not affecting its specific growth rate. They also showed that a concentration of edifenphos (123 µM) which did not affect specific growth rate caused a decrease in the phosphatidylcholine content of *F. graminearum*, and that this decrease was still observed when the fungus was grown in the presence of choline. This result, together with the observations of Kodama *et al.* (1980), suggests that edifenphos inhibits both the Kennedy (Kennedy & Weiss, 1956) and Greenberg (Bremner & Greenberg, 1961) pathways for phosphatidylcholine synthesis.

Edifenphos and iprobenfos have been reported to inhibit the incorporation of [^{14}C]glucosamine into mycelia of *P. oryzae* (De Waard, 1974), and both fungicides have been shown to inhibit the activity of chitin synthase preparations from various fungi (Kodama *et al.*, 1980; Craig & Peberdy, 1983a; Leighton *et al.*, 1981). However, Brillinger (1979) found that chitin synthase from *Coprinus cinereus* was not inhibited by iprobenfos, and De Waard (1974) reported that *Pythium ultimum*, an Oomycete with walls containing cellulose but not chitin, was inhibited by both edifenphos and iprobenfos.

The present work was undertaken to determine the primary site of action of edifenphos in *F. graminearum*, and in particular to determine if chitin synthase is inhibited by the fungicide directly and/or indirectly through a primary effect on membrane phospholipid composition.

Methods

Organism and medium. *Fusarium graminearum* Schwabe strain A3/5 was obtained from Mr T. W. Naylor, Marlow Foods, Billingham, UK. The defined medium of Vogel (1956) was used with glucose (10 g l^{-1}) as the carbon source instead of sucrose. Vogel's mineral salts solution was prepared at $\times 50$ final concentration, sterilized by membrane filtration ($0.22\text{ }\mu\text{m}$ pore diameter, Whatman), and added to the glucose solution, which was sterilized by autoclaving at $121\text{ }^{\circ}\text{C}$ for 15 min. When necessary, the medium was solidified with 15 g l^{-1} agar (Taiyo technical grade; Davis Gelatine).

Chemicals. Edifenphos (Hinosan; *O*-ethyl-*S,S*-diphenylphosphorodithioate) was kindly provided by Imperial Chemical Industries PLC, Jealott's Hill Research Station, Bracknell, UK and contained 30% (w/v) of active ingredient (allowance for this is made when fungicide concentrations are given in Results). UDP-*N*-acetyl-D-[^{14}C]glucosamine (UDP-[^{14}C]GlcNAc) and *N*-acetyl-D-[^3H]glucosamine ([^3H]GlcNAc) were obtained from Amersham. Allosamidin was kindly provided by Professor Akita Isogai, University of Tokyo, Japan. Other chemicals were of Analaar grade and, unless stated otherwise, were obtained from BDH.

Cultural conditions. Batch cultures were grown in 50 ml volumes in 250 ml Nephlos flasks (Trinci, 1972). Each flask was inoculated with 1 ml of a suspension of macroconidia (to give a final concentration of about 5×10^4 macroconidia per ml medium) in distilled water prepared by harvesting macroconidia from 7–10-d-old cultures grown on agar medium. The flasks were incubated on a rotary shaker (with a throw of 2.5 cm) at 200 r.p.m. All cultures were grown at $25\text{ }^{\circ}\text{C}$. The fungus was grown in the presence and absence of edifenphos and exponential-phase (24 h) biomass was harvested by filtration through muslin.

Incorporation of [^3H]GlcNAc into chitin by biomass. Exponential-phase biomass grown in the presence and absence of edifenphos was washed in 2 vols Vogel's medium and incubated at $25\text{ }^{\circ}\text{C}$ in 25 ml Vogel's medium containing [^3H]GlcNAc ($0.08\text{ }\mu\text{Ci ml}^{-1}$; 2.96 kBq ml^{-1}) in the presence and absence of edifenphos. Control cultures were treated with 5% (v/v) trichloroacetic acid (TCA; final concentration) prior to being suspended in Vogel's medium containing [^3H]GlcNAc. All treatments

were incubated at $25\text{ }^{\circ}\text{C}$ for 30 min on a rotary shaker (200 r.p.m.). Growth was then stopped by the addition of TCA to give a final concentration in the culture of 5% (v/v). Four replicate 2 ml samples were taken from each flask, filtered through Whatman no.1 filter papers, and washed with 3 vols distilled water. The biomass and filters were then added to scintillation vials containing 4 ml volumes of scintillation fluid (Optiphase, 'Hisafell') and their radioactivity measured using a Packard Tricarb 1500 liquid scintillation analyser. The residual culture (17 ml culture + added TCA) in each flask was used for dry weight determinations.

To confirm that [^3H]GlcNAc was incorporated into chitin, exponential-phase biomass was incubated with the precursor for 30 min as described above. Following incubation, the biomass was treated with TCA (see above) and 5 ml volumes of the suspension were homogenized in a Griffin homogenizer and then centrifuged at 1500 g for 10 min. The supernatant was removed and the residue was treated for 20 h in 6 M-HCl at $25\text{ }^{\circ}\text{C}$; or for 20 h in $2\text{ mg chitinase ml}^{-1}/50\text{ mM-potassium phosphate buffer at pH }6.0$ and $25\text{ }^{\circ}\text{C}$; or for 20 h in $1\text{ M-acetic acid at }55\text{ }^{\circ}\text{C}$; or for 20 h in $2\text{ mg boiled chitinase ml}^{-1}/50\text{ mM-potassium phosphate buffer at pH }6.0$ and $25\text{ }^{\circ}\text{C}$. After incubation, the reaction mixtures were centrifuged at 1500 g for 10 min. The residues were collected on $0.2\text{ }\mu\text{m}$ Millipore filters and washed with 5 ml distilled water. The filters were then transferred to scintillation vials and 4 ml of scintillation fluid was added to each. The radioactivity of the samples was then measured as described above. The remaining parts of the above cultures were used for dry weight determinations.

Separation of chitin, diacetylchitobiose and UDP-GlcNAc by TLC. The contents of tubes used for assays of membrane-bound chitin synthase were applied to silica gel plates (60 F_{254} , Merck, BDH) together with two $20\text{ }\mu\text{l}$ volumes of 50% (v/v) aqueous ethanol used to wash out the tubes. The plates were developed in propan-1-ol/water/ammonia (70:30:1, by vol.) and dried at room temperature. Areas on the plates which corresponded to chitin, diacetylchitobiose and UDP-GlcNAc (Gooday, 1973, as modified by Humphreys, 1984) were visualized by UV illumination, scraped into scintillation vials containing 4 ml scintillation fluid and their radioactivity measured.

Activity of membrane-bound and solubilized chitin synthase. Exponential-phase biomass grown in the presence and absence of edifenphos was washed with buffer (50 mM-MES , 10 mM-MgCl_2 , 1 mM-EDTA) at pH 6.5 and membrane-bound chitin synthase was extracted using the method of Vermeulen & Wessels (1983) and stored at $-70\text{ }^{\circ}\text{C}$. 'Solubilized' chitin synthase was prepared from membrane-bound chitin synthase by treating the preparations with $1\text{ mg digitonin ml}^{-1}$ as described by Adams & Gooday (1980). A modification of the method of Adams & Gooday (1980) was used to assay chitin synthase activity in both preparations. The standard reaction mixture consisted of $10\text{ }\mu\text{l}$ of the enzyme preparation, $35\text{ }\mu\text{l}$ of buffer (50 mM-MES , 10 mM-MgCl_2 , 1 mM-EDTA , final concentrations at pH 6.5) and $5\text{ }\mu\text{l}$ of substrate (250 mM-GlcNAc and $6\text{ mM-UDP-[}^{14}\text{C]GlcNAc}$ containing $13.75\text{ nCi [}^{14}\text{C]Bq}$). The mixture was incubated at $30\text{ }^{\circ}\text{C}$ for 30 min and the reaction was stopped by heating at $100\text{ }^{\circ}\text{C}$ for 2 min. The reaction mixture was then filtered through a Millipore filter (diameter, 25 mm; pore size, $0.45\text{ }\mu\text{m}$) that had been presoaked for 30 min in $20\text{ mM-Na}_4\text{P}_2\text{O}_7$ containing 5% (w/v) TCA and then washed in distilled water. The reaction tubes were washed twice with $50\text{ }\mu\text{l }1\%$ (w/v) Triton X-100 and twice with 2 ml distilled water. The filters were transferred to scintillation vials containing 4 ml volumes of scintillation fluid and counted. The effect of dimyristoylphosphatidylcholine on the activity of digitonin-solubilized chitin synthase was tested by adding $1\text{ }\mu\text{l}$ of 0, 0.1 or 0.5 mg ml^{-1} of dimyristoylphosphatidylcholine (Sigma) in chloroform/methanol (2:1, v/v) to the assay mixture (the solvents were included in the controls and had no effect on chitin synthase activity). In these assays, the reaction mixture contained $5\text{ }\mu\text{M}$ of the chitinase inhibitor, Allosamidin.

Fig. 1. Arrhenius plot of the effect of temperature on the activity of membrane-bound chitin synthase from *F. graminearum*. Membrane-bound chitin synthase was extracted from exponential-phase biomass harvested from shake flask cultures grown at 25 °C as described in Methods.

Results

Properties of chitin synthase

Membrane-bound chitin synthase from *F. graminearum* had an apparent temperature optimum of about 30 °C and maintained 90% of its initial activity after 6 weeks storage at -70 °C (results not shown). Arrhenius plots of the effect of temperature on the activity of membrane-bound chitin synthase from *F. graminearum* showed a deflection at about 20 °C (Fig. 1), confirming that the enzyme was membrane-associated (Vermeulen & Wesels, 1983).

Membrane-bound chitin synthase exhibited optimal activity at pH 6.5, and for incubation periods up to 60 min, preparations at pH 6.5 and 30 °C incorporated UDP-[14 C]GlcNAc into chitin at a linear rate (results not shown). Judged by the absence of diacetylchitobiose in the reaction mixture (as indicated by TLC analyses), and by the lack of an effect of 5 μ M-Allosamidin (an inhibitor of chitinase activity; Sakuda *et al.*, 1987) on chitin synthesis, no appreciable degradation of newly formed chitin (by membrane-bound chitinase) occurred at pH 6.5 (a pH which is above the optimum for chitinase activity) during the 30 min assay period (results not shown). A Lineweaver-Burk plot of reaction velocity against substrate (UDP-[14 C]GlcNAc) concentration gave a K_m value for membrane-bound chitin synthase of about 2.5 mM and a saturation velocity, V , of 3×10^{-3} nmol UDP-GlcNAc min^{-1} (mg protein) $^{-1}$ (Fig. 2).

Chitin synthase activity in digitonin-solubilized preparations was variable and was only stabilized when 5 μ M-

Fig. 2. Lineweaver-Burk plots of the effect of substrate (UDP-[14 C]GlcNAc) concentration on the activity at pH 6.5 and 30 °C of (1) membrane-bound chitin synthase isolated from exponential-phase biomass of *F. graminearum* grown at 25 °C in the absence of edifenphos (□), and (2) membrane-bound chitin synthase isolated from exponential-phase biomass of *F. graminearum* grown at 25 °C in the presence of 30 (Δ), 60 (○), or 250 (▲) μ M-edifenphos. Each result is the mean of four replicates.

Allosamidin was included in the reaction mixture. Addition of 1 μ l of a 0.5 mg ml^{-1} dimyristoylphosphatidylcholine solution to the assay mixture resulted in a 3.6-fold [from 2.3×10^{-4} to 8.2×10^{-4} nmol UDP-GlcNAc min^{-1} (mg protein) $^{-1}$] increase in the activity of digitonin-solubilized chitin synthase.

Inhibition by edifenphos of the activity of membrane-bound chitin synthase. Fig. 3 shows the effect of edifenphos on the *in vitro* activity of membrane-bound chitin synthase from *F. graminearum*. Edifenphos concentrations up to 25 μ M had no effect on chitin synthase activity, but between 25 and 145 μ M, chitin synthase activity decreased linearly with fungicide concentration, giving an IC_{50} (concentration of edifenphos that reduced chitin synthase activity by 50%) of 92 μ M. Fig. 4 shows that when edifenphos was added to the reaction mixture, it caused non-competitive inhibition of the activity of membrane-bound chitin synthase, with an apparent K_i of about 50 μ M.

Activity of membrane-bound chitin synthase preparations isolated from *F. graminearum* cultured in the presence and absence of edifenphos

Membrane-bound chitin synthase preparations were isolated from *F. graminearum* cultures which had been grown in the presence and absence of edifenphos. Fig. 2

Fig. 3. Effect of edifenphos on the *in vitro* activity at pH 6.5 and 30 °C of membrane-bound chitin synthase from exponential-phase biomass of *F. graminearum* grown in the absence of fungicide. In the absence of edifenphos, the preparation had an activity of 5.7×10^{-3} nmol GlcNAc incorporated min^{-1} (mg protein) $^{-1}$. Each result is the mean ($\pm$ SE) of four replicates.

shows Lineweaver-Burk plots of the reaction velocities of these preparations at various substrate (UDP-[^{14}C]GlcNAc) concentrations. Preparations of membrane-bound chitin synthase from cultures of *F. graminearum* grown in the presence of 30 or 60 μM -edifenphos gave Lineweaver-Burk plots which were indistinguishable from those obtained with preparations from cultures grown in the absence of fungicide (Fig. 2). However, membrane-bound chitin synthase preparations from cultures of *F. graminearum* grown in medium containing 250 μM -edifenphos had an activity (per mg protein) which was only about 40% of that of preparations isolated from cultures grown in the absence of fungicide.

Effect of edifenphos on the incorporation of [^3H]GlcNAc into chitin of cultures grown and incubated in the presence and absence of fungicide

When *F. graminearum* was washed and then incubated at 25 °C in Vogel's medium containing [^3H]GlcNAc, incorporation of radioactivity into chitin occurred at a linear rate for 45 min (results not shown). The labelled product produced *in vivo* when *F. graminearum* was incubated with [^3H]GlcNAc had the following properties in common with chemically purified chitin: (a) incubating it with 2 mg ml^{-1} chitinase (but not boiled chitinase) for 20 h at 25 °C gave 76% degradation to GlcNAc, (b) the product was hydrolysed to glucosamine

Fig. 4. Lineweaver-Burk plots of the effect of substrate (UDP-[^{14}C]GlcNAc) concentration on the activity at pH 6.5 and 30 °C of membrane-bound chitin synthase isolated from exponential-phase biomass of *F. graminearum* grown at 25 °C and incubated in the presence of 0 ($\square$), 60 ($\circ$), 100 ($\blacksquare$), and 120 ($\bullet$) μM -edifenphos. Each result is the mean of four replicates.

when treated with 6 M-HCl, and (c) the product was insoluble in 1 M-acetic acid.

Pre-growth of *F. graminearum* in medium containing 80 μM -edifenphos had no significant ($P > 0.05$) effect on the subsequent incorporation of [^3H]GlcNAc into chitin (Table 1); 80 μM -edifenphos had no effect on maximum specific growth rate μ_{max} (Wiebe *et al.*, 1990), but did affect chitin synthase activity *in vitro* (Fig. 3). However, addition of 80 μM -edifenphos to the incubation medium caused a significant ($P < 0.05$) decrease in chitin synthesis (Table 1). This decreased rate of chitin synthesis was not significantly ($P > 0.05$) affected when the biomass was both pre-grown and incubated in the presence of the fungicide (Table 1).

In contrast to the result obtained with 80 μM -edifenphos, biomass pre-grown in medium containing 250 μM -edifenphos, washed and suspended in the incubation medium lacking fungicide, had a significantly ($P < 0.05$) lower rate of incorporation of [^3H]GlcNAc into chitin than control cultures grown and incubated in medium lacking fungicide (Table 1); 250 μM -edifenphos affects membrane composition but does not affect μ_{max} (Wiebe *et al.*, 1990). Chitin synthesis was further decreased when 250 μM -edifenphos was present in the incubation medium as well as the pre-growth medium.

Table 1. Effects of edifenphos on the incorporation of [^3H]GlcNAc into chitin by *F. graminearum* biomass pre-grown in the presence and absence of fungicide and incubated *in vivo* in the presence and absence of fungicide

Exponential-phase biomass was harvested from shake flask cultures grown at 25 °C in the presence or absence of edifenphos, washed and incubated in medium containing [^3H]GlcNAc for 30 min. Rate of [^3H]GlcNAc incorporation is expressed as a percentage of the rate [3.6×10^{-3} nmol GlcNAc min $^{-1}$ (g dry wt $^{-1}$)] of control cultures pre-grown and incubated in the absence of fungicide. Each result is the mean ($\pm$ SE) of four replicates. Figures with the same superscript letter are not significantly different ($P > 0.05$).

Concn of edifenphos in pre-growth medium (μM)	Concn of edifenphos in incubation medium (μM)	Rate of incorporation of [^3H]GlcNAc into chitin in incubation medium (% of control rate)
80	0	98 $\pm$ 32 ^a
0	80	51 $\pm$ 21 ^b
80	80	49 $\pm$ 13 ^b
250	0	47 $\pm$ 23 ^b
0	250	8 $\pm$ 2 ^c
250	250	31 $\pm$ 8 ^d

Discussion

The properties (pH optimum, apparent temperature optimum and K_m) of membrane-bound chitin synthase from *F. graminearum* are similar to those for chitin synthase from other fungi (Gooday & Trinci, 1980). The deflection in the Arrhenius plot (Fig. 1) of the effect of temperature on the activity of membrane-bound chitin synthase is characteristic for membrane-bound enzymes (Vermeulen & Wessels, 1983), and the temperature at which this deflection occurred (20 °C) was similar to one of the deflections observed for chitin synthase isolated from *Schizophyllum commune* (12 and 20 °C; Vermeulen & Wessels, 1983) and *Coprinus cinereus* (16 and 25 °C; Montgomery & Gooday, 1985). Digitonin-solubilized chitin synthase from *F. graminearum* had a much lower activity than the membrane-bound chitin synthase from which it was prepared. This reduced activity was probably at least partly due to the activity of chitinase in the preparation, because addition of a chitinase inhibitor to the reaction mixture increased chitin synthase activity. The phospholipid requirement of chitin synthase has been demonstrated for preparations from *Schizophyllum commune*, *Coprinus cinereus* and *Saccharomyces cerevisiae* (Vermeulen & Wessels, 1983; Montgomery & Gooday, 1985; Humphreys & Gooday, 1984; Duran & Cabib, 1978). In these studies, fatty acid chain length and the nature of the polar head group were shown to be important for maximal stimulation of chitin

synthase activity. In the present study the activity of digitonin-solubilized chitin synthase from *F. graminearum* was increased 3.6-fold by addition of 1 μl of a 0.5 mg ml $^{-1}$ (11.64 μM) dimyristoylphosphatidylcholine solution to the reaction mixture. This supports previous results demonstrating the requirement for particular lipids for the activity of membrane-associated enzymes. However, even in the presence of Allosamidin and dimyristoylphosphatidylcholine, digitonin-solubilized chitin synthase still only had about 80% of the activity of the membrane-bound chitin synthase from which it was prepared.

Edifenphos may affect chitin synthase activity (a) directly, (b) indirectly by altering the phospholipid composition of the membrane which in turn modulates chitin synthase activity, or (c) a combination of (a) and (b). At edifenphos concentrations between 60 and 120 μM (Fig. 4), non-competitive inhibition of membrane-bound chitin synthase activity *in vitro* was observed. This result suggests that the inhibitory effect of edifenphos on *F. graminearum* at 60 μM and above may be caused by a direct inhibitory effect on chitin synthase activity. Wiebe *et al.* (1990) showed that concentrations of edifenphos between 41 and 246 μM caused a reduction in the mean hyphal extension rate and hyphal growth unit length of *F. graminearum*, but had no effect on specific growth rate. Thus, at these concentrations, edifenphos acted as a paramorphogen (Tatum *et al.*, 1949) rather than as a fungicide. The results obtained here and those of Wiebe *et al.* (1990) suggest that the paramorphogenic effect of low concentrations of edifenphos on *F. graminearum* could be explained in terms of the fungicide causing direct inhibition of chitin synthase activity. Inhibition of chitin synthase activity would in turn cause a reduction in hyphal extension rate, but, if this was accompanied by increased hyphal branching, it would not necessarily be associated with an inhibition of specific growth rate (Robson *et al.*, 1990). However, caution must be exercised in translating *in vitro* inhibition data directly to effects *in vivo*.

As Akatsuka *et al.* (1977), Kodama *et al.* (1979) and Ross & Brady (1985) observed with other fungi, Wiebe *et al.* (1990) found that 123 μM -edifenphos caused a decrease in the phosphatidylcholine content of *F. graminearum*. Growing *F. graminearum* in 250 μM -edifenphos (but not in 30 or 60 μM -edifenphos) caused a reduction in the activity of membrane-bound chitin synthase isolated from the biomass (Fig. 3). Also, pre-growth of *F. graminearum* in medium containing 250 μM (but not 80 μM) edifenphos caused a subsequent reduction in [^3H]GlcNAc incorporation into chitin when the biomass was suspended in medium lacking the fungicide (Table 1). We are currently investigating the hypothesis that pre-growth of *F. graminearum* in high

concentrations of edifenphos causes changes in membrane composition which in turn affect chitin synthase activity.

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