Unsaturated fatty acids are the active molecules of a glucan-synthase-inhibitory fraction isolated from entomophthoralean protoplasts

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A few entomophthoralean species are able to multiply in a protoplast form. The polysaccharide synthases which synthesize the cell wall are inactivated in this form. An inhibitor of one of the key enzymes of wall synthesis, glucan synthase, was isolated from entomophthoralean protoplasts, using silica column chromatography and HPLC. Thin-layer and gas chromatography revealed free fatty acids in the inhibitory fractions. These fatty acids, including long-chain unsaturated fatty acids, were shown to be responsible for the inhibition of glucan synthase. The fatty acids were generated during incubation of a protoplast homogenate for 36 h at 37 °C and were shown to be non-competitive and non-specific inhibitors of glucan synthase.

Keywords: glucan synthase, Entomophthorales, *Aspergillus fumigatus*, fatty acid, 1,3- β -glucan

INTRODUCTION

Entomophthorales are entomopathogenic fungi. Some species, such as *Entomophthora schizophorae*, *Entomophthora muscae* and *Entomophaga aulicae*, have the ability to develop and multiply *in vivo* as protoplasts, which lack a cell wall. These protoplasts, unlike hyphal bodies, are not recognized by the immune system of insects (Beauvais *et al.*, 1989). 1,3- β -Glucan and chitin are present in the hyphal wall but absent on the protoplast surface. These wall components are responsible for the recognition of the mycelium by the immune system, and their absence accounts for the ability of protoplasts to escape encapsulation (Beauvais *et al.*, 1989). Protoplasts and the corresponding walled form, the hyphal bodies, have been cultivated *in vitro* for *Entomophthora muscae* and *Entomophaga aulicae*. Only the protoplast stage has been cultivated *in vitro* for *Entomophthora schizophorae* (Beauvais & Latgé, 1988).

The major components of fungal cell walls, 1,3- β -linked glucan and chitin, are synthesized by the glycosyltransferases 1,3- β -glucan synthase (GS) and chitin

synthase, which catalyse the transfer of glucose or *N*-acetylglucosamine units from UDP-Glc (uridine diphosphate glucose) or UDP-*N*-acetylglucosamine into growing polysaccharide chains (Cabib *et al.*, 1988). The level of control which prevents the biosynthesis of cell wall in the protoplasts is not understood, although the membrane-bound polysaccharide synthases, which synthesize the cell wall, are somehow inactivated (Beauvais & Latgé, 1989). Regulatory mechanisms could operate at the transcriptional or at the translational level, or could involve a specific inhibitor only active in the protoplast stage. The presence of an mRNA specific for one of the chitin synthases found in this species suggested that the transcription of the polysaccharide synthases in the entomophthoralean protoplasts is not blocked (Thomsen & Beauvais, 1995). However, the transcription of the GS gene or the translation of the mRNAs of both polysaccharide synthases could not be studied, as no gene has been cloned for GS and these enzymes have not been purified. The presence of non-competitive inhibitors of polysaccharide synthases of natural origin has been detected in many fungi (Fèvre, 1979; Iwata *et al.*, 1982; Kang *et al.*, 1986; Sawistowska-Shröder *et al.*, 1984), including the protoplasts of two entomophthoralean species (Beauvais & Latgé, 1989).

Abbreviations: DG, diglyceride; FA, fatty acid; GS, 1,3- β -glucan synthase.

In this study, an inhibitor of GS was isolated from entomophthorean protoplasts and then identified in order to evaluate its specificity to the GS and its presence only in the protoplast form. Its activity was tested on the GS of *Aspergillus fumigatus*, a fungal pathogen of immuno-compromised patients. The low sensitivity or resistance of *A. fumigatus* to many antifungal compounds made it a good model for our study (Fromtling & Abruzzo, 1989; Beaulieu *et al.*, 1993).

METHODS

Purification of the inhibitor from Entomophthorean species. Protoplasts were grown in 1 l GLEN medium (0.4% glucose, 0.5% yeast extract, 0.65% lactalbumin hydrolysate, 0.77% NaCl; Beauvais & Latgé, 1988), in a cell fermenter (Biolaftite) inoculated with 120 ml protoplasts of *Entomophthora schizophorae*, *Entomophthora muscae* (isolates Em2 and 44E respectively, provided by Dr J. Eilenberg, Royal Veterinary and Agricultural University, Denmark) or *Entomophaga aulicae* (isolate 2896, provided by Dr D. Boucias, University of Florida, USA). The protoplasts were grown at 25 °C for 3 d at 50 r.p.m. and an aeration rate of 0.2 l air min⁻¹ outlet located.

Protoplasts were recovered by centrifugation and washed in 10 mM phosphate buffer (pH 7.8) + 1 M sucrose. After breakage in a Potter piston homogenizer in TE buffer (50 mM Tris/HCl, 1 mM EDTA, pH 7.6), the protoplast suspension was incubated for 36 h at 37 °C and lyophilized to yield the crude inhibitor fraction. Since the inhibitory fraction was insensitive to pronase or trypsin (1–10 µg ml⁻¹ for 4–36 h at 37 °C), a lipidic structure was expected. A 100 mg sample of the crude inhibitor fraction was extracted (15 min vigorous shaking on a vortex) twice with 25 ml chloroform/methanol (2:1, v/v) at room temperature. The extract was then filtered through filter paper to eliminate particulates, and the sample concentrated under vacuum and finally dried under N₂. The product was resuspended in 500 µl chloroform (CHCl₃ fraction) and 10 µl was used for the GS assay test.

The CHCl₃ fraction was fractionated on a 3 ml Shandon Hypersep silica column for solid phase extraction, with 100 µl applied at a time. Columns were eluted sequentially with 1 column volume of hexane, 1 volume of chloroform, and 1 volume of 4:1 (v/v) chloroform/methanol. The 4:1 chloroform/methanol fraction, which was found to contain the inhibitor, was then concentrated and resuspended in 500 µl ethyl acetate. Ten microlitres of this 4:1 chloroform/methanol fraction was used to test the inhibitory activity.

The 4:1 chloroform/methanol fraction was used for HPLC on a Lichrosorb Si 5µ 250 × 4.6 column, using an isocratic system with hexane/chloroform/methanol (5:10:2, by vol.), at a flowrate of 1 ml min⁻¹. The run time was 30 min. Before injection, the solvent was totally evaporated and the sample resuspended in the running solvent. A peak of absorbance was observed between 3 and 6 min after injection. A major peak that eluted between 4 and 6 min (collected in tubes 8–12 and termed the 8–12 fraction) contained the inhibitory activity. Aliquots (1 ml) of this peak were pooled, dried down, and resuspended in 200 µl ethyl acetate. Ten microlitres of each fraction was used to test the inhibitory activity.

The purity of the inhibitory fractions was assayed by TLC. Samples were spotted on Kieselgel 60 F₂₅₄ DC-alufoil silica plates (Merck) and the plates were run in hexane/diethyl

ether/acetic acid (70:30:1). Fatty acid (FA) and diglyceride (DG) standards from Sigma were run for comparison. All plates were developed under I₂ vapour (Higgings, 1987).

Test of inhibitory activity on GS. To test the inhibitory activity of a fraction, solvent was evaporated under N₂ and the sample resuspended in TE buffer. The effects of various standards on GS activity were also assayed in TE buffer. Ten micrograms of DG standards (Sigma), containing equal amounts of dipalmitin, distearin and diolein, were added directly to the GS assay reaction mixture, giving a final concentration of 0.33 g l⁻¹. Free FAs were assayed, also with a concentration of 0.33 g l⁻¹. Both saturated (14:0, 16:0 and 18:0) and unsaturated (18:1, 18:2, 18:3 and 18:4) FAs were tested. The unsaturated long-chain FAs were also tested at lower concentrations, from 0.33 to 33 mg l⁻¹, using the following GS assay. A solubilized GS extract (P30) was isolated from *Aspergillus fumigatus* as described by Beauvais *et al.* (1993). For *Trichophyton rubrum* and *T. mentagrophytes*, the high-speed membrane extract (before solubilization) was used as a source of GS. The GS of *S. cerevisiae* was also used to test the inhibitor (E. Cabib, personal communication).

GS activity was estimated by measuring the incorporation of UDP-[U-¹⁴C]glucose into insoluble glucan. To test the inhibitory activity of a fraction, the sample was resuspended in 10 µl TE. It was then incubated for 20 min with 15 µl P30 at room temperature before 5 µl of the reaction mixture, containing TE + 0.2 M NaF + 1 M sucrose + 2.5 mM UDP-glucose (with 2.4 µM UDP-[U-¹⁴C]glucose, specific activity 293 nCi nmol⁻¹, 108 Bq nmol⁻¹), was added. The enzyme reaction was allowed to take place for 45 min, after which the reaction product was precipitated with 50 µl ethanol and recovered by filtration on a GF/C glass-fibre filter. The filters were dried for 15 min at 80 °C, washed with 50 ml 60% (v/v) ethanol, and then incubated in 2 ml BCS scintillation fluid for 1 h. The radioactivity was measured in an LKB liquid scintillation counter. Percentage inhibition was measured as the ¹⁴C incorporated into insoluble glucan compared to the control without inhibitor. The specificity of the inhibitory activity (fraction 8–12) on GS was tested by incubating this fraction with a chitin synthase extract of *A. fumigatus* obtained as previously described (Beauvais & Latgé, 1989).

Kinetic analysis of *Entomophthora schizophorae* inhibitor on the GS. For K_m determination, different concentrations (0.34–2 mM) of the substrate UDP-glucose were used in the reaction mixture, and the reaction was only allowed to take place for 10 min. Since the insolubility of the inhibitor in the reaction mixture did not affect the linearity of the dose-response curve, K_i assays were done: the inhibitor concentrations were expressed in terms of c.p.m. of ¹⁴C added to the reaction since the molarity was not known. For this purpose, the protoplasts were grown in GLEN medium supplemented with 0.13 µM D-[U-¹⁴C]glucose for 3 d. The inhibitor was then purified as described above, and recovered in fraction 8–12. Concentrations corresponding to 225 and 300 c.p.m. were added for competition experiments. For all the combinations of inhibitor and substrate concentration 1/V was plotted against 1/S.

General lipase test on *Entomophthora schizophorae* inhibitor. The inhibitory fractions were treated with a lipase from *Candida cylindracea* (Sigma) dissolved in TE to a concentration of 0.2 mg ml⁻¹, i.e. 12000 enzyme units ml⁻¹. Fractions tested included CHCl₃ fraction, the 4:1 chloroform/methanol fraction, and fractions of the HPLC peak eluting between 4 and 6 min. Approximately 0.05 mg of each fraction

was combined with 0.5 ml of the enzyme/buffer mixture, then incubated for 1.5 h at 37 °C. Controls were also run with a DG standard (Sigma), consisting of equal amounts of dipalmitin, distearin and diolein, as well as with an enzyme preparation boiled for 10 min. Reactions were stopped by adding 1 ml chloroform to the reaction mixture and the reaction products were extracted by partitioning between the $\text{CHCl}_3/\text{H}_2\text{O}$ fractions. The chloroform fraction was then concentrated and resuspended in 500 μl ethyl acetate. The reaction products were assayed by using 42 μl for a GS assay and 10 μl for a TLC plate.

Effect of bovine serum albumin on inhibitor and GS activity.

Bovine serum albumin (BSA) is known to bind free FAs (Hamilton *et al.*, 1991). The protoplast inhibitor (10 μl CHCl_3 fraction) and 0.007 mg standard oleic acid (18:1) were combined with varying concentrations of BSA (1.6–8 g l^{-1}) and added to the GS assay. Residual effects of BSA on GS were tested by running controls with the same concentrations of BSA, without inhibitor. Percentage inhibition was then calculated by comparison with a control with neither inhibitor nor BSA, and plotted against concentration of BSA.

Electrospray ionization of *Entomophthora schizophorae* inhibitor.

After HPLC purification, samples were analysed by electrospray ionization MS on a triple-quadrupole Sciex API III instrument (PE/SCIEX; Kerwin *et al.*, 1994). Samples were infused into the electrospray source via a 50 μm i.d. fused silica transfer line using a Harvard Apparatus pump at 3 $\mu\text{l min}^{-1}$. Positive-ion MS or MS/MS was run with an orifice voltage of 70 V, and the corresponding negative-ion spectra were run at 70 V. The interface temperature was maintained at 52 °C. For tandem mass spectrometry (MS/MS), precursor ions were selected with the first of three quadrupoles (Q1) for collision-induced dissociation with argon in the second quadrupole (Q2). The third quadrupole (Q3) was scanned with a mass step of 0.20 Da and 1 ms per step. Parent ion transmission was maximized by reducing the resolution of Q1 to transmit a 2–3 m/z window about the selected parent ion, and Q3 resolution was adjusted to approximately 50% valley between peaks 3 Da apart. Spectra were collected and analysed using software from Sciex Corporation.

GC and GC-MS of fatty acid methyl esters (FAME). Free FAs in the HPLC fractions were methylated using ethereal diazomethane (Kates, 1986). FAME were analysed using a Hewlett Packard 5890 series II gas chromatograph. Using helium as a carrier gas at 2 ml min^{-1} , samples were injected (splitless injection) onto a 30 m DB-23 column (J&W Scientific). The initial temperature of 120 °C was held for 2 min and raised at 4 °C min^{-1} up to 240 °C and held for 10 min. FAs were identified and quantified using retention times and GC-MS on a Kratos mass spectrometer.

RESULTS

Purification of the *Entomophthora schizophorae* inhibitor

The results are summarized in Fig. 1. The active fraction was first found in the CHCl_3 fraction, then in the 4:1 chloroform/methanol fraction, and finally in tubes 8–12 eluted after 3–6 min during the HPLC run. TLC on silica plates revealed at least three different products in the fraction from tubes 8–9. The spots corresponded roughly to those of standards of DGs and free FAs. The non-active 6–7 fraction consisted mainly of DGs, while the active 10–12 fraction consisted mainly of FAs. Without the 36 h incubation period at 37 °C of the protoplasts,

Fig 1. Purification of the GS inhibitor from protoplasts of *Entomophthora schizophorae*. The percentage inhibition of GS caused by the various fractions is shown in parentheses (bold type); see Methods for details.

there was no inhibitory activity and there was no TLC spot for FAs (data not shown).

The inhibitory fraction was found to correspond to a TLC spot with an R_F value of 0.36–0.40, similar to the area to which unsaturated FAs migrated. Kinetic studies showed that the apparent K_m for UDP-glucose in the standard enzyme preparation, determined by using Michaelis-Menten plots, was 1 mM. The inhibitor is a non-competitive inhibitor of GS activity: the slope of double-reciprocal plots increased with increasing concentrations of the inhibitor without change in K_m .

The crude lyophilized fraction was also active on the GS of fungi other than *A. fumigatus*, including *S. cerevisiae* (E. Cabib, personal communication), *Trichophyton rubrum* and *T. mentagrophytes* (data not shown). Fraction 8–12 inhibited chitin synthase activity by 90% at a concentration similar to that which gave 100% inhibition of *A. fumigatus* GS. A GS-inhibitory (8–12) fraction was also isolated from protoplasts of two other species of Entomophthorales: *Entomophthora muscae* and *Entomophthora anilicae*.

GC analysis of the inhibitory fraction 8–12 from *Entomophthora schizophorae* revealed that the sample contained approximately 32 μg of FAs. The four major FAs and their relative composition were: palmitic acid, 16:0 (32%); stearic acid, 18:0 (15%); oleic acid, 18:1 ω_9 (43%); and linoleic acid, 18:2 ω_6 (10%). Other long-chain polyunsaturated FAs were also present in trace amounts. Electrospray ionization confirmed this result and revealed the presence of other compounds, including

Fig. 2. (a) Negative ion electrospray MS spectrum of *Entomophthora schizophorae* DGs, with representative peaks marked. Note that many of these peaks consist of two to eight different DG molecular species. (b) Negative ion electrospray MS/MS spectrum of the peak at m/z 607 (*) in (a). This peak is due to the synthesis of at least eight different ether-linked molecular species: 16:1/20:3 alkenyl/acyl, 16:1/20:4 alkyl/acyl, 16:0/20:5 alkyl/acyl, 18:4/18:0 alkenyl/acyl, 18:3/18:1 alkenyl/acyl, 18:2/18:2 alkenyl/acyl, 20:2/16:0 alkenyl/acyl and 20:2/mono-OH-15:0 alkyl/acyl moieties. Species are reported as [*sn*-1/*sn*-2 bond type], with the ether-linked moieties assumed to be in the *sn*-1 positions. The ether-linked (alkyl and alkenyl) FAs are not seen in the spectrum because they are lost as neutral species (Kerwin *et al.*, 1994). Their identity is obtained by subtracting the mass of the FA in the *sn*-2 position from the parent ion.

several modified DGs (Fig. 2). An unexpected observation was the presence, in addition to the expected diacyl-linked DGs, of a very large amount of alkyl/acyl and alkenyl/acyl DG moieties (Fig. 2b and unpublished observations). Fig. 2 also demonstrates the versatility of electrospray MS and MS/MS, in which a complex mixture of incompletely purified compounds can be rapidly and definitively identified using picogram or nanogram quantities of material.

Inhibitory activity of FA

Dipalmitin, distearin and diolein did not have significant inhibitory activity, nor did the saturated FAs palmitic acid (16:0) and stearic acid (18:0). The shorter chain myristic acid (14:0) was more active, with 67% inhibition at 0.33 g l⁻¹. The unsaturated C₁₈ FAs, however, including oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), and 6,9,12,15-octadecatetraenoic acid (18:4), at a concentration of 0.33 g l⁻¹, caused 90–100% inhibition. These results suggest that either a short chain, or a long unsaturated chain, is necessary for inhibition. Inhibition

was much less, however, when the unsaturated FAs were tested at lower concentrations. None of them gave any inhibition at 3.33 or 0.33 mg l⁻¹. Oleic acid gave 16% inhibition at 33 mg l⁻¹, while 18:2, 18:3 and 18:4 failed to give any significant inhibition at any of the lower concentrations. The concentration of 18:1 at 0.33 g l⁻¹, i.e. 10 µg per assay, is approximately equal to the amount of 18:1 found in the inhibitory HPLC fraction by GC.

Effect of BSA on inhibitory activity

At 1.5 g l⁻¹ BSA was found to have a protective effect against inhibition (Fig. 3). Greater amounts of BSA added to either the reaction mixtures with the protoplast inhibitor (CHCl₃ fraction) or the 18:1 FA standard resulted in lower percentages of inhibition. Increasing concentrations of BSA added to the assay with no added inhibitor resulted in an activation of GS. This suggests that the BSA was interacting with the protoplast inhibitor and the 18:1 FA standard, as well as with FAs present in the solubilized GS extract.

Fig. 3. Effect of BSA on inhibition of GS activity by CHCl_3 fraction (■) and by the 18:1 FA standard (▲). Percentage inhibition decreased with increasing concentration of BSA. Activity increased (negative inhibition) when BSA was added to the reaction mixture without inhibitor (◆).

Effect of treatment with commercial lipase on inhibitory activity

Inhibitory activity did not significantly increase with lipase treatment of the least purified CHCl_3 fraction. Treatment of the 4:1 chloroform/methanol fraction resulted in an increase of inhibitory activity from 64% to 93%.

Lipase treatment had different effects on the three pooled HPLC fractions eluted between 3 and 6 min (fractions 6–7, 8–9, 10–12). Fraction 8–9, consisting of both DGs and free FAs, had inhibitory activity that was unaffected by lipase treatment. The inhibitory activity of the 10–12 fraction, which consisted only of FAs, was also unaffected by lipase treatment. The 6–7 fraction, however, consisting mainly of DGs, inhibited only 3.5% without lipase treatment, but 34.5% after lipase treatment. This was accompanied by a change on the TLC plate: a diminution of the DG spots and the appearance of a FA spot. The effect of commercial DGs when treated with a lipase was similar. DGs by themselves gave no inhibition of GS, but after lipase treatment they gave 98% inhibition. The TLC also showed the disappearance of DGs and the appearance of a spot with an R_F value corresponding to that of FAs. This high level of inhibition was due to free FAs. The presence of DGs in the 6–7 fraction from protoplasts after lipase treatment compared to commercial DGs, similarly treated, indicated that protoplast DGs were modified and less sensitive to lipase. This observation was confirmed by electrospray MS and MS/MS, and may be due to the high percentage of ether-linked DGs synthesized by the fungus. Treatment with boiled enzyme gave the same results as no lipase treatment. The lipase alone, with no substrate, caused no inhibition.

Extensive mass spectrometric examination of the inhibitory fractions, while confirming the presence of DGs and FAs, did not reveal any lipopeptides or glycopeptides similar to the previously documented GS inhibitors aculeacin, papulacandin and echinocandin (Iwata *et al.*, 1982; Kang *et al.*, 1986; Sawistowska-Shröder *et al.*, 1984).

DISCUSSION

Unsaturated long-chain FAs present in the extract from *Entomophthora schizophorae* protoplasts were responsible for the inhibition of GS. Among all FAs present in this extract, oleic acid was the most potent inhibitor. Amounts of standard unsaturated FAs comparable to those found in the purified fractions gave very high inhibition, making it unlikely that inhibition was caused by other compounds present in the purified fraction. Protoplast extracts which were not incubated at 37 °C were found to contain DGs but neither FAs nor inhibitory activity. This result suggests that the FAs are generated by the action of one or more lipases in the protoplast extract when incubated for 36 h at 37 °C. FAs were also found to be active against the chitin synthase of *A. fumigatus*. Since chitin synthase of *A. fumigatus* or GS of *T. rubrum* and *T. mentagrophytes* were not solubilized by CHAPS as was GS of *A. fumigatus*, the action of FAs on GS was not due to a disruption of micelles formed by CHAPS. Instead, FAs presumably, by disrupting the membrane equilibrium, affected the activity of the polysaccharide synthases, which are multi-enzyme complexes. The lack of inhibition at low concentrations of FAs supports the idea that the mechanism of inhibition may involve disruption of the membrane, for which relatively large concentrations of FAs are necessary.

Although an inhibitor of GS can be purified from entomophthoralean protoplasts, it is not a novel or unique compound. Many of the antifungal drugs targeted against GS, including cilofungin and papulacandin B, contain FA side-chains. These compounds have been shown to be ineffective without side-chains (Taft & Seletrennikoff, 1990; Varona *et al.*, 1983). It has also been shown that FAs and monoglycerides cause a concentration-dependent decrease in the activity of yeast GS due to minor perturbations of the plasma membrane (Ko *et al.*, 1994). Likewise, DGs, which do not inhibit GS, are unable to alter membrane fluidity (Ko *et al.*, 1994). Maresca *et al.* (1994) also showed that desaturation of membrane lipids allowed a change in the morphogenesis of *Histoplasma*. Our results, that long-chain unsaturated free FAs, at relatively high concentrations, are responsible for inhibition, are consistent with these previous observations.

The unsaturated FAs cannot be the sole cause of the complete inhibition of the GS activity in protoplasts, since similar inhibitory activity was found in a (8–12) fraction isolated from hyphal body extract of *Entomophaga aulicae* (data not shown). This result suggests that the inhibitory activity due to FA is not specific. While free FAs by themselves offer little hope for the development of a pharmaceutical antifungal agent, it is possible that any existing drug compounds that rely upon FA side-chains for activity could be made more effective with a long-chain unsaturated FA side-chain.

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