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Chitin Biosynthesis in Protoplasts and Subcellular Fractions of *Aspergillus fumigatus*

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The biosynthesis of chitin has been obtained in broken mycelia and protoplasts of the fungus *Aspergillus fumigatus*. The specific activity of chitin synthase (EC 2.4.1.16) in a membrane preparation from protoplasts derived from the hyphal tips of *A. fumigatus* was 26.8-fold greater than that of the chitin synthase in broken mycelia, indicating that the active chitin synthase is located primarily in a membrane-bound site at the hyphal tip. Polyoxin D was a potent competitive inhibitor of the enzyme, having K_i 5.2±0.8μM with respect to the natural substrate UDP-*N*-acetyl-D-glucosamine, which has K_m 1.58mM.

Chitin synthase (EC 2.4.1.16) was first reported in *Neurospora crassa* (Glaser & Brown, 1957) and since then has been described in a number of yeasts and fungi. The biosynthesis of chitin *in vivo* requires an active chitin synthase enzyme which, in *Saccharomyces cerevisiae*, is produced by the action of a proteinase on chitin synthase zymogen (Cabib & Ulane, 1973). In mycelial fungi the use of protoplasts in biochemical investigations is in its infancy, and mycelial protoplasts have not previously been used in studies of chitin synthase. The present paper describes the production of protoplasts from the hyphal-tip region of *Aspergillus fumigatus* and their use in the study of chitin biosynthesis. *A. fumigatus* is an opportunistic pathogen for man and, as with other species of *Aspergillus*, chitin is a major component of the cell wall (Bartnicki-Garcia, 1968).

Polyoxin D is known to be a competitive inhibitor of chitin synthase in *Neurospora crassa* (Endo *et al.*, 1970), *Mucor rouxii* (Bartnicki-Garcia & Lippman, 1972), *Saccharomyces cerevisiae* (Bowers *et al.*, 1974) and *Aspergillus flavus* (Lopez-Romero & Ruiz-Herrera, 1976). The inhibition of chitin synthase by polyoxin D is due to the structural similarity between polyoxin D and UDP-*N*-acetyl-D-glucosamine (Isono *et al.*, 1969).

Experimental

Source and growth of Aspergillus fumigatus

Aspergillus fumigatus strain 2085 was a clinical isolate obtained from the Mycological Reference Laboratory, London School of Hygiene and Tropical Medicine, London W.C.1, U.K. Stocks were grown at 37°C on Czapek–Dox agar (Oxoid Ltd., London E.C.4, U.K.). Conidial suspensions were prepared from these agar plates by washing with 10ml of sterile 0.1% Tween 80. The conidia were then harvested by centrifugation for 10min at room temperature (20°C) and 1000*g* in a bench centrifuge, and washed twice with sterile water. *A. fumigatus* mycelia were obtained by suspending washed conidia at a final density of 10^6 spores/ml in Czapek–Dox medium supplemented with 0.1% casamino acids (Difco, Detroit, MI, U.S.A.) and incubating for 18h at 37°C in an orbital incubator. The mycelium was harvested by filtration through a sintered-glass filter and washed with sterile water.

Protoplast production

A 'lawn' of washed *A. fumigatus* conidia was spread on to a Cellophane disc covering the surface of glucose (1%, w/v)/yeast extract (Difco) (0.5%, w/v)/agar (1.5%, w/v; Difco).

After 18h growth at 37°C, the cellophan disc supporting the mycelium was removed from the surface of the agar and inverted over a solution of 0.7M-KCl containing a 50-fold dilution of β-glucuronidase [Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.]. This was incubated at 37°C for 1h with occasional shaking. Microscopic examination demonstrated that this procedure released protoplasts from the tips of the aerial hyphae. If left for longer than 1h, a greater yield of protoplasts was obtained, but these were from all parts of the aerial hyphae. The protoplasts were harvested from the 0.7M-KCl solution by passing this suspension through a sintered-glass filter to remove any contaminating mycelium and then centrifuging at

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1000g for 10min. The protoplasts were then washed twice by centrifugation in 0.7M-KCl.

Microscopic examination

Samples of the aerial hyphae and protoplasts were viewed under phase contrast by using a Leitz Ortholux microscope (Ernst Leitz, Wetzlar, Germany) at a final magnification of ×1000. The presence of cell-wall material was detected by fluorescence microscopy in the presence of the optical brightener Tinopol BOPT [0.1%; Geigy (U.K.) Ltd., Manchester, U.K.] as described by Paton & Jones (1971).

Preparation of samples for use in the chitin synthase assay

The two preparations used predominantly in the chitin synthase assay were: (1) a crude broken-mycelial preparation and fractions prepared from it, and (2) a protoplast membrane preparation.

Preparation (1). The washed mycelial preparation from the 18h liquid culture was suspended in a minimum volume of 100mM-Tris/HCl buffer, pH 7.5, containing 20mM-$MgCl_2$ (hereafter called 'Tris/HCl/$MgCl_2$'), and broken in a Braun homogenizer (model MSK) with 0.45–0.50mm-diameter glass beads. The beads were allowed to sediment under gravity at 0°C for 10min and the broken mycelium was diluted with Tris/HCl/$MgCl_2$ to a final protein concentration of 5mg/ml; 10μl samples, containing 50μg of protein, were used for the chitin synthase assay.

In one experiment the mycelial homogenate was fractionated to give three particulate fractions and a supernatant. The first pellet (A) was obtained by sedimentation at 1000g for 10min and washing ten times by centrifugation with Tris/HCl/$MgCl_2$. After removal of pellet A, pellet B was obtained by sedimentation for 10min at 4°C and 15000g on an MSE 18 centrifuge and washing five times in Tris/HCl/$MgCl_2$. After the removal of pellets A and B, pellet C was obtained by sedimentation for 30min at 4°C and 300000g ($r_{av.}$ 8.5cm) in a Beckman model L5-65 ultracentrifuge by using a SW 50.1 rotor, and washing twice in Tris/HCl/$MgCl_2$. The supernatant from the first of the ultracentrifuge runs was the 'supernatant' sample. The three pelleted fractions were seen by phase-contrast microscopy to be predominantly cell wall, 'mitochondrial' and 'microsomal'.

Preparation (2). Protoplast membranes were prepared from the washed protoplast preparation by the addition of Tris/HCl/$MgCl_2$ to the pelleted protoplasts. This resulted in lysis of the protoplasts, and the membranes were then collected by sedimentation for 30min at 4°C and 300000g ($r_{av.}$ 8.5cm) in a Beckman model L5-65 ultracentrifuge by using a SW 50.1 rotor, and washing twice in Tris/HCl/$MgCl_2$. The membranes were then resuspended in Tris/HCl/$MgCl_2$ at a final protein concentration of 0.5mg/ml so that the 10μl sample for the chitin synthase assay contained 5μg of protein. Higher concentrations were not possible, owing to the low yield of protoplasts. The protoplast membranes contained a mixture of hyphal-tip plasma membranes and membrane vesicles trapped within the protoplasts when formed. Intra-hyphal vesicles not enclosed by protoplasts were harvested by sedimentation for 30min at 4°C and 300000g in a Beckman model L5-65 ultracentrifuge in a SW 50.1 rotor ($r_{av.}$ 8.5cm) after the 1000g sedimentation step to remove protoplasts, but no chitin synthase activity could be detected in this preparation.

In all cases, the protein composition was measured by using the Folin reagent (Lowry *et al.*, 1951), with bovine serum albumin as the standard.

Chitin synthase assay

The assay was performed in a final volume of 20μl and contained the following: 10μl of sample in Tris/HCl/$MgCl_2$; 5μl of a solution of UDP-*N*-acetyl-D-[U-^{14}C]glucosamine (The Radiochemical Centre, Amersham, Bucks., U.K.) in water (total 20nCi with broken mycelial samples and total 10nCi with protoplast membrane samples); 5μl of an aqueous solution containing 80mM-*N*-acetyl-D-glucosamine, 1mM-(unless varied for K_m determinations) UDP-*N*-acetyl-D-glucosamine, and polyoxin D when required. This mixture was incubated for the required time (up to 1h) at 30°C. The incorporation of radioactive material into acid-precipitable material was then determined by the addition of 50μl of 10% (w/v) trichloroacetic acid to the incubation mixture. The precipitate was allowed to form for 2h at 0°C and then collected by filtration through 0.45μm-diameter pore-size Nuflow membrane filters (Courtaulds, Spondon, Derby, U.K.) and washed with 10ml of water at 0°C. The filters were dried and the radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrometer, model 3375, by using 2,5-diphenyloxazole (4g/litre)/toluene scintillant.

Trypsin treatment of the enzyme preparations before the assay was carried out as follows. To the 10μl sample in Tris/HCl/$MgCl_2$ was added 0.1μg of trypsin (BDH Chemicals Ltd., Poole, Dorset, U.K.) and this mixture incubated for 5min at 30°C. Trypsin inhibitor (0.2μg; BDH) was added to give a final sample volume of 15μl. The final assay volume in this case was 25μl, and further additions were such that the final concentrations of all components (including the buffer) were the same as in the 20μl incubations.

Characterization of the chitin

Chitobiose units are known to occur in some glycoproteins in *A. fumigatus* (Rudick & Elbein, 1973, 1974, 1975), but the glycoprotein content is small compared with the amount of *N*-acetyl-D-glucosamine present in chitin in *A. fumigatus*. The procedures for

the characterization of chitin formed in similar assay systems in a number of organisms are now well documented, and the methods used in the present study were essentially the same as those described by McMurrough *et al.* (1971). Samples of the product formed in the chitin synthase assay were totally insoluble in 1 M-acetic acid (1 h at 100°C) and, although three 1 h extractions at 100°C in 3% (w/v) NaOH 'solubilized' 30.6% of the radioactivity, 52% of this was chromatographically immobile on a paper chromatogram developed in butan-1-ol/ethyl acetate/acetic acid/water (8:6:5:8, by vol.), showing that 84% of the product of the chitin synthase assay was unaffected by the NaOH treatment. 'Soluble' was defined as anything not sedimented by centrifugation at room temperature for 5 min at 1000**g**. One 4h extraction in 4M-HCl at 120°C solubilized 64% of the radioactivity, and this was all shown to be glucosamine after drying of the solubilized product over solid NaOH and analysis by paper chromatography, developed in the above solvent mixture. Treatment of the 'chitin' with chitinase (Koch–Light, Colnbrook, Bucks., U.K.) for 24 h at 30°C in 0.14 M-sodium acetate/0.06 M-acetic acid buffer, pH 5.0, solubilized 75.7% of the radioactivity, and this was all identified as *N*-acetyl-D-glucosamine by paper chromatography as above. That *N*-acetyl-D-glucosamine was formed rather than chitobiose is probably due to the presence of chitobiase (*β-N*-acetylglucosaminidase) in the chitinase.

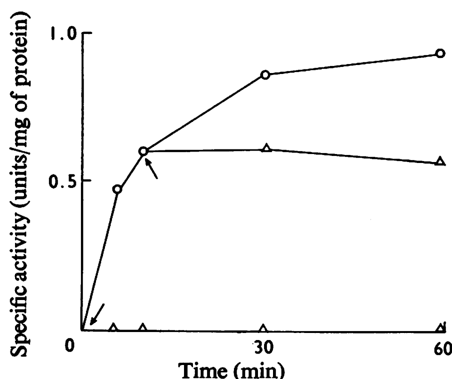

Fig. 1. *Synthesis of chitin in a broken-mycelial preparation of Aspergillus fumigatus*
A unit of chitin synthase activity is that amount catalysing the incorporation of 1 nmol of *N*-acetyl-D-glucosamine into chitin. The incorporation of *N*-acetyl-D-[U-^{14}C]glucosamine into chitin in a broken-mycelial preparation of *A. fumigatus* was determined over periods of up to 1 h at 30°C in the absence of polyoxin D (○), and in the presence of 100 μg of polyoxin D/ml (△), added at two different times, which are indicated by the arrows in the Figure.

These experiments confirmed that the product of the chitin synthase assay was indeed chitin.

Chemicals

All chemicals used were AnalaR grade. All solvents used were redistilled before use. Polyoxin D was a gift from Dr. S. Suzuki, The Institute of Physical and Chemical Research, Wako-Shi, Saitama, Japan.

Results

Protoplast formation

Microscopic examination showed the emergence of protoplasts from the hyphal tip of *Aspergillus fumigatus* and that although the Tinopol BOPT bound readily to the hyphal wall, it did not bind to the protoplasts. The final, washed preparation of protoplasts showed the protoplasts to be completely spherical and unaffected by Tinopal BOPT. These observations are consistent with the lack of cell-wall material on the protoplasts. As would be expected for true protoplasts, they were osmotically highly sensitive and readily lysed by the addition of water or Tris/HCl/$MgCl_2$.

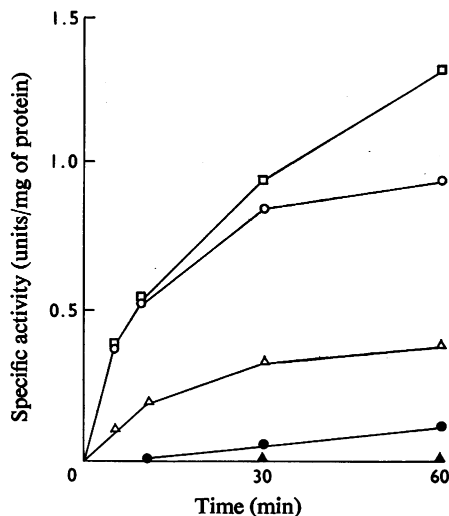

Fig. 2. *Formation of chitin in different fractions of a broken-mycelial preparation of Aspergillus fumigatus*
The unit of chitin synthase activity is defined in Fig. 1. The incorporation of *N*-acetyl-D-[U-^{14}C]-glucosamine into chitin in the following subcellular fractions of *A. fumigatus* was determined after periods of up to 1 h at 30°C; microsomal membrane (□), mitochondrial membrane (△), cell wall (●), soluble fraction (▲), and an unfractionated broken-mycelial preparation (○). The preparation of these various cell fractions is described in detail in the Experimental section.

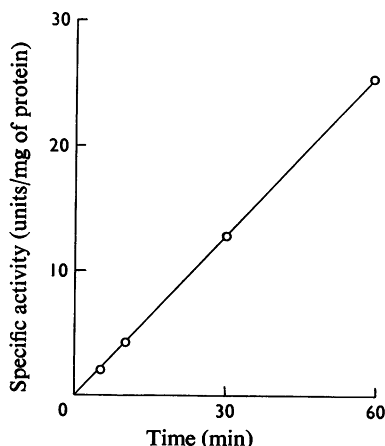

Fig. 3. *Formation of chitin in a protoplast membrane preparation from Aspergillus fumigatus*
The unit of chitin synthase activity is defined in Fig. 1. The sample protein was membrane-derived from protoplasts of the hyphal tips of *A. fumigatus*, and the incorporation of *N*-acetyl-D-[U-^{14}C]glucosamine into chitin was determined over periods of up to 1 h at 30°C.

Chitin synthase activity in broken mycelium

The formation of chitin by the broken-mycelial preparation over a period of 1 h is illustrated in Fig. 1. The incorporation is non-linear with time and may be arrested at any time by the addition of polyoxin D.

Partial fractionation of this system into three pelleted fractions and one soluble fraction, and their use in the chitin synthase assay, yielded the results in Fig. 2. The incorporation profile with the broken mycelial preparation is included for comparison. No chitin synthase activity was detected in the soluble fraction, and the specific activity of the enzyme was very low in the cell-wall fraction. The highest specific activities of the chitin synthase were found in the two membranous fractions. However, the specific activities of the chitin synthase in these fractions were still low because protein from the whole mycelial mass was included in the assay, whereas most of the active chitin synthase activity was probably located at the hyphal tips. A membrane preparation from the protoplasts should then give a significant improvement in the specific activity of the chitin synthase.

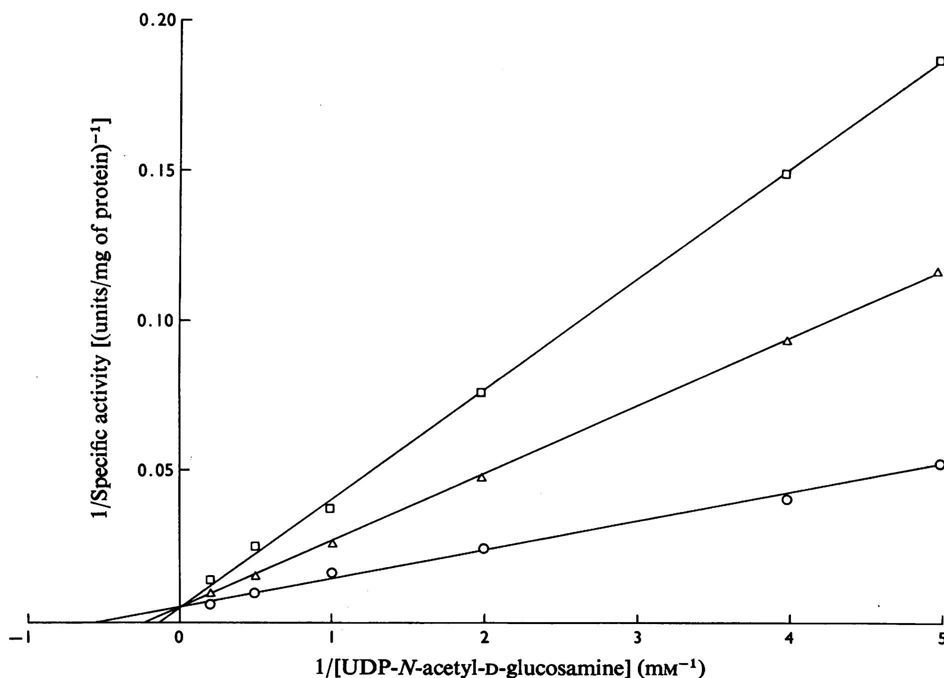

Fig. 4. *Effect of polyoxin D on chitin synthase activity in a protoplast membrane preparation from Aspergillus fumigatus*
A unit of chitin synthase activity is that amount catalysing the incorporation of 1 nmol of *N*-acetyl-D-glucosamine (at 30°C) into chitin/h. Chitin synthesis was measured over a range of substrate concentrations in the absence of polyoxin D (○) and in the presence of 10 μg of polyoxin D/ml (△) and 20 μg of polyoxin D/ml (□). The results are presented as a double-reciprocal plot. The sample protein was membrane-derived from protoplasts of the hyphal tips of *A. fumigatus*, and the incorporation of *N*-acetyl-D-[U-^{14}C]glucosamine into chitin was determined at 30°C for 1 h.

Table 1. *Comparative aspects of the chitin synthesis in different chitin synthase preparations from Aspergillus fumigatus*
These are the results of one typical experiment. The variation from one experiment to another was never more than 10%. Chitin synthesis was measured by the incorporation of *N*-acetyl-D-[U-^{14}C]glucosamine into chitin after 1h at 30°C. The amount of chitin synthesis was measured in a broken-mycelial preparation of *A. fumigatus*, with and without prior trypsin treatment, and in membranes from protoplasts derived from the hyphal tips.

System ...	Broken mycelium	Broken mycelium (trypsin-treated)	Protoplast membranes
Percentage utilization of substrate/μg of protein	0.04	0.14	1.08
Percentage utilization of substrate/assay	1.7	7.0	5.4
Radioactivity (d.p.m.) incorporated/μg of protein	14.7	63.1	240
Radioactivity (d.p.m.) incorporated/assay	733	3152	1199

Chitin synthase activity in the protoplast membrane preparation

Fig. 3 demonstrates that the specific activity of the chitin synthase from protoplast membranes was very high in comparison with that in the broken-mycelial system. After 1h, the specific activity of the chitin synthase in protoplast membranes was 26.8-fold greater than that in the broken-mycelial preparation. Also, the incorporation of radioactivity into chitin proceeded linearly with time over the period of 1h, although it did not continue so beyond 90min.

Effect of polyoxin D on chitin synthesis in the protoplast membrane system

Chitin synthesis was measured over a 1h period at 30°C with a variety of substrate concentrations in the absence of polyoxin D and in the presence of 10 and 20μg of polyoxin D/ml (19 and 38μM respectively). The results are plotted as a double-reciprocal plot in Fig. 4. All intercept values for K_m, K_i and $V_{max.}$ determinations were obtained from a Sumlock Compucorp 342 Statistician calculator. Fig. 4 demonstrates that the inhibition of the chitin synthase by polyoxin D (K_i 5.2$\pm$0.8μM) is competitive with respect to UDP-*N*-acetyl-D-glucosamine (K_m 1.58mM; $V_{max.}$ 194$\pm$31μmol/min). This gives a K_m:K_i ratio of 304. These values are consistent with other published values describing the effect of polyoxin D on the chitin synthase in *Neurospora crassa* (Endo *et al.*, 1970), *Mucor rouxii* (Bartnicki-Garcia & Lippman, 1972), *Saccharomyces cerevisiae* (Bowers *et al.*, 1974) and *Aspergillus flavus* (Lopez-Romero & Ruiz-Herrera, 1976).

Comparative aspects of chitin synthesis from different systems

Some comparative aspects of the synthesis of chitin in the different systems described in the present paper are given in Table 1. The action of trypsin in the protoplast membrane system is not included because no activation of the chitin occurred under conditions optimal in the broken-mycelial preparation. Indeed, the chitin synthase activity in the protoplast membranes was progressively decreased by the action of trypsin. The results in Table 1 are the results from one typical experiment, although the variation from experiment to experiment was never more than 10%.

Discussion

The formation of protoplasts from a number of mycelial fungi is now well established, and it has been observed that the first-formed protoplasts usually appear from the hyphal tips (Peberdy, 1976), although protoplast formation from *A. fumigatus* has not previously been reported. It has been reported on the basis of radioautographic studies in *Mucor rouxii* that chitin biosynthesis occurs predominantly in the apical region of the hyphae (McMurrough *et al.*, 1971), and that the same is true in *A. nidulans*, although in the presence of cycloheximide, chitin biosynthesis occurs along the length of the *A. nidulans* hyphae (Katz & Rosenberger, 1971). From these reports it seems that the active chitin synthase is located at the hyphal tips of fungi, and the results given in the present paper provide biochemical support for this.

The 26.8-fold increase in the specific activity of the active chitin synthase when hyphal-tip protoplasts are used in the assay system instead of broken mycelia suggests that the hyphal-tip region of the *A. fumigatus* mycelium contains the active chitin synthase. Trypsin treatment resulted in a 4.1-fold stimulation in chitin synthase activity with the broken-mycelial preparation, but had no activating effect on the protoplast membrane preparation, suggesting that although the active chitin synthase is located at the hyphal tips, an inactive form of the enzyme exists elsewhere, probably along the length of the hyphae, which is activated by trypsin treatment. The absence of cell wall from the protoplast membrane preparation indicates that the active chitin synthase is membrane-bound, either to the plasma membrane at the hyphal tip or in the tip vesicles which are enclosed by the plasma membrane during protoplast formation. It is noteworthy that the membrane-bound vesicles that are released from the hyphae after protoplast formation (i.e. from a region further from the hyphal tip) have no detectable chitin synthase activity.

That the active chitin synthase is membrane-bound

is consistent with observations made with other fungi, including *Allomyces macrogynus* (Porter & Jaworski, 1966) and *Mortierella vinacea* (Peberdy & Moore, 1975), although activity has also been reported in cell-wall preparations from *Mortierella vinacea* (Peberdy & Moore, 1975) and *Mucor rouxii* (McMurrough *et al.*, 1971). In *Saccharomyces carlsbergensis* (Keller & Cabib, 1971) and *Saccharomyces cerevisiae* (Duran *et al.*, 1975) there seems no doubt that the chitin synthase activity is located on the plasma membrane. Indeed, it seems that the activating factor for the chitin synthase zymogen in *S. cerevisiae* is located in membrane-bound vesicles (Cabib & Ulane, 1973; Ulane & Cabib, 1976). It may be that the vesicles present inside the protoplasts from *Aspergillus fumigatus* contain a chitin synthase-activating factor and that the chitin synthase itself is located on the plasma membrane; further work is required to show whether this is the case.

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