

Localization of growth and secretion of proteins in *Aspergillus niger*

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Hyphal growth and secretion of proteins in *Aspergillus niger* were studied using a new method of culturing the fungus between perforated membranes which allows visualization of both parameters. At the colony level the sites of occurrence of growth and general protein secretion were correlated. In 4-d-old colonies both growth and secretion were localized at the periphery of the colony, whereas in a 5-d-old colony growth and secretion also occurred in a more central zone of the colony where conidiophore differentiation was observed. However, in both cases glucoamylase secretion was mainly detected at the periphery of the colonies. At the hyphal level immunogold labelling showed glucoamylase secretion at the tips of leading hyphae only. Microautoradiography after labelling with *N*-acetylglucosamine showed that these hyphae were probably all growing. Glucoamylase secretion could not be demonstrated immediately after a temperature shock which stopped growth. These results indicate that glucoamylase secretion is located at the tips of growing hyphae only.

Introduction

Filamentous fungi secrete large amounts of protein into the medium. For instance, some strains of *Aspergillus niger* secrete considerable quantities of glucoamylase (Ward, 1989). Such secreted proteins are larger than the estimated pore sizes of the walls of filamentous fungi as measured with isolated cell wall fractions (Trevithick & Metzberg, 1966) or determined by the solute-exclusion technique using living hyphae (Money, 1990). Measurements of wall porosity with isolated wall preparations relate to the bulk of wall material and not to the tiny fraction covering the growing hyphal apex. In fact, the apical wall fraction may be completely missing in such wall preparations (Wessels *et al.*, 1984). Yet, the cytological evidence (for a review see Grove, 1978) suggests that exocytosis in filamentous fungi mainly occurs at growing apices and proteins secreted into the medium thus probably pass the wall in this region. It has therefore been suggested that the growing apical wall is more porous than the rigid mature wall and allows for the rapid diffusion of proteins (Trevithick & Metzberg, 1966; Chang & Trevithick, 1974). The solute-exclusion technique used by Money (1990) would seem to include measurement of apical pores but nevertheless indicates a pore size not exceeding 2–3 nm, preventing diffusion of proteins larger than M_r 15000–20000. As an alternative it has been suggested that at the growing apex proteins

migrate through the wall together with visco-elastic wall polymers which are continuously driven to the outside of the wall by turgor and synthesis of new wall polymers at the inside (Wessels, 1989, 1990). This 'bulk flow' hypothesis does not necessarily require pores that traverse the wall; proteins could diffuse into the medium from the most stretched and probably most porous outer region of the wall. As a preliminary to obtaining experimental evidence for any of these hypotheses we examined the assumed relationship between secretion of proteins and apical growth. We studied protein secretion in *A. niger*, using a novel technique which permits visualization of growth and secretion at the same time. At the colony level the results do suggest a close correlation between wall growth and secretion of proteins into the medium. At the hyphal level glucoamylase secretion could only be demonstrated at the tips of growing leading hyphae.

Methods

Organism and culture medium. *A. niger* 402 is a morphological mutant with short conidiophores derived from the wild-type strain *A. niger* van Tieghem (CBS 120.49, ATCC 9029) (Bos *et al.*, 1988). Compared to industrial strains, this strain secretes only small amounts of glucoamylase (50 mg l^{-1}) in liquid medium (P. Punt, personal communication). Cultures were grown on minimal medium containing, per litre: 0.52 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 6.0 g NaNO_3 , 0.52 g KCl, 1.52 g KH_2PO_4 , 0.225 ml 10 M-KOH, (pH 6.8), 2.5 mg FeCl_3 , trace elements according to Whitaker (1951), and either 3% (w/v) xylose or 3% (w/v) soluble starch (Merck) as carbon sources.

Abbreviation: PVDF, polyvinylidenedifluoride.

Sandwiched cultures. *A. niger* 402 was grown in a thin layer as follows. A perforated polycarbonate membrane (diameter 76 mm, thickness 5–10 µm, 6×10^8 pores cm⁻² with a standard pore size of 0.08 µm; Poretics Corporation, Livermore, USA) was centrally placed on the surface of 15 ml solidified minimal medium (1.5%, w/v, agar) in a 9 cm Petri dish. Care was taken that the surface was perfectly level and that no air bubbles were trapped. The plate was heated to 70 °C and 2 ml of 1.25% (w/v) agarose (100 °C) was poured over the surface, giving an agarose layer of 0.3 mm thickness. The temperature of the plate was brought to room temperature and a small piece of mycelium was inoculated in the centre of the plate. Then, the surface was topped with a second perforated polycarbonate membrane. Sandwiched colonies were incubated in a water-saturated container at 25 °C. Polycarbonate membranes could be reused after washing and wet sterilization between filter paper.

Detection of secreted proteins at the level of the colony. Starch-degrading activity was detected by transferring a sandwiched colony to a fresh agar medium containing 3% soluble starch. At various times colonies were removed and the starch medium was sprayed with an iodine/potassium iodide solution (Lugol).

For detection of proteins secreted into the medium, the growing sandwiched colony was briefly lifted from the nutrient agar and a wetted polyvinylidenedifluoride (PVDF) membrane (Millipore) was positioned between the lower polycarbonate membrane and the agar medium. Glucoamylase was detected on the PVDF membrane using an antiserum raised against a purified glucoamylase from *A. niger*. Immunoreactions were done as described below for immunoblotting with goat-anti-rabbit-conjugated alkaline phosphatase as a second antibody (Harlow & Lane, 1988), with the modification that 1% (w/v) polyvinylpyrrolidone (PVP) was included in the blocking buffer to prevent non-specific signals due to secreted phenolic compounds. Incubations with normal rabbit serum were used as a control.

For detection of ³⁵S-labelled proteins the sandwiched colony was transferred to a PVDF membrane lying on an agar medium in which the concentration of sulphate was lowered from 0.52 to 0.026 g l⁻¹, MgCl₂ substituting for MgSO₄ to maintain the Mg²⁺ concentration. After 2 h a piece of rice paper with a diameter somewhat larger than the sandwiched colony and containing 185 kBq carrier-free ³⁵SO₄²⁻ (sp. act. 37×10^9 Bq mmol⁻¹, Amersham) was put on the top polycarbonate membrane and left there for 30 min to 6 h. After labelling, the sandwiched colony was fixed in freshly prepared 4% (w/v) paraformaldehyde for 30 min. Both the fixed colony and the PVDF membrane were washed three times for 60 min with 0.2% MgSO₄·7H₂O and subsequently dried overnight on filter paper.

Detection of secreted proteins at the level of the hypha. For localization of glucoamylase secretion, sandwiched colonies grown in 2% agarose were fixed in 4% (w/v) paraformaldehyde or 25% (w/v) trichloroacetic acid (TCA) for 2 h at 4 °C. After removal of the polycarbonate membranes the agarose slabs were washed six times for 5 min with phosphate-buffered saline, pH 7.0 (PBS) and non-specific binding sites were blocked by incubating in 5% (w/v) skimmed milk (Oxoid), 1% PVP, 0.02% sodium azide in PBS for 2 h. Incubations with the polyclonal antibodies against glucoamylase, or with normal rabbit serum as a control, were done overnight either at 4 °C or preferably at room temperature. The agarose slabs were then washed six times for 5 min with PBS and incubated for 2 h at room temperature with gold-conjugated goat-anti-rabbit antibodies (5 nm gold particles) to detect the primary antibody. Alternatively alkaline-phosphatase-conjugated goat-anti-rabbit antibodies were used for detection. After washing the agarose slabs six times for 5 min with water, silver enhancement was used according to the protocol supplied by the manufacturer of the reagents (Zymed Laboratories).

For localization of general protein synthesis at the level of individual hyphae, sandwiched colonies were labelled by placing a piece of rice

paper containing 1.85×10^6 Bq L-[4,5-³H]leucine (Amersham, 5.29×10^9 Bq mmol⁻¹) on the top membrane for various times. The colony was then fixed in 4% formaldehyde or 25% TCA for 2 h at 4 °C and washed six times for 5 min in 1 mmol unlabelled leucine. The colonies were then dried overnight on microscope slides coated with chrome-alum-gelatin and used for autoradiography (Rogers, 1969).

Detection of wall synthesis. Chitin in sandwiched colonies was labelled for 10 min by contacting the upper polycarbonate membrane with a piece of rice paper containing 185 kBq *N*-acetyl-D-[1-¹⁴C]-glucosamine (sp. act. 2.17×10^9 Bq mmol⁻¹) or 185 kBq *N*-acetyl-D-[1,6-³H]glucosamine (sp. act. 1.22×10^{12} Bq mmol⁻¹) for macro- and microautoradiography, respectively. Colonies in the agarose slabs were then fixed for 30 min in freshly prepared 4% formaldehyde and washed three times for 60 min with 0.44 mM *N*-acetylglucosamine. They were then dried overnight on filter paper for macroautoradiography or on microscope slides coated with chrome-alum-gelatin for microautoradiography.

Autoradiography. Autoradiography of ¹⁴C- or ³⁵S-labelled colonies and PVDF membranes was done using X-OMAT AR films (Eastman Kodak). For microautoradiography ³H-labelled colonies dried on microscope slides were dipped mechanically (Rogers, 1969) in L4 emulsion (Ilford). Exposure was done in light-tight boxes containing silica gel for 3 to 15 d at 4 °C. X-OMAT AR film and L4 film were developed in Kodak D19 developer.

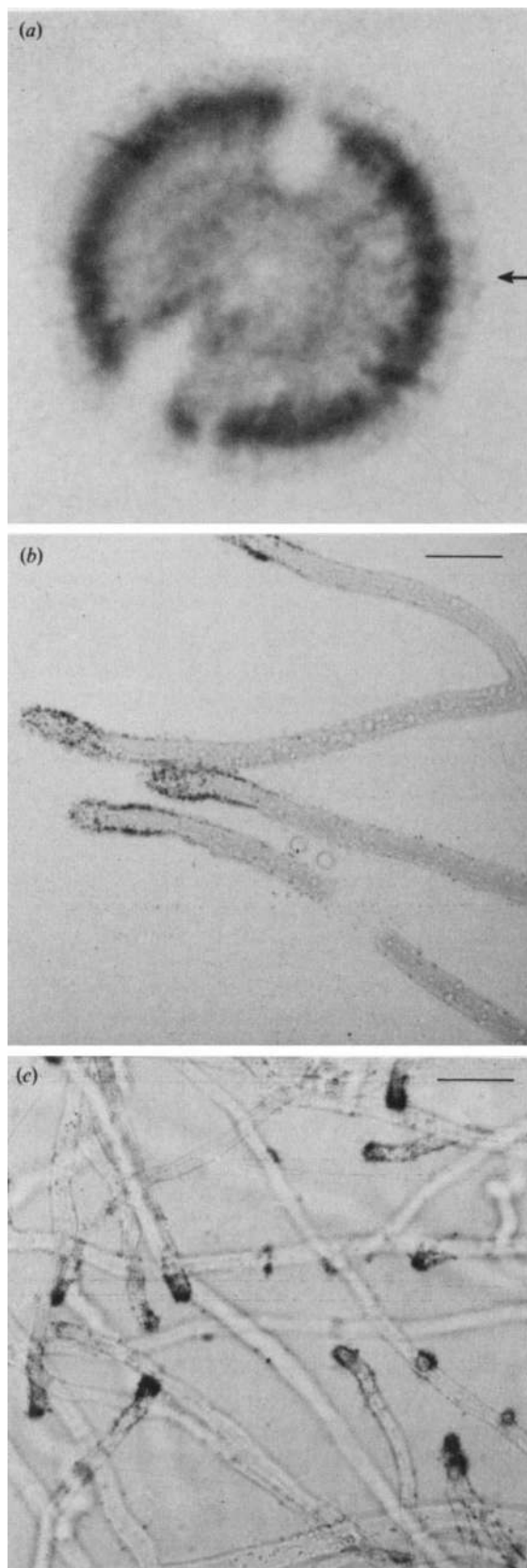
Results

Growth in sandwiched cultures

The method of growing a fungus in a thin agarose layer sandwiched between two perforated polycarbonate membranes allowed free diffusion of gases, water, nutrients and proteins, while hyphae could not grow through these membranes. Therefore the colonies grew nearly two-dimensionally, allowing microscopic examination of individual hyphae. Spore formation was prevented although conidiophore formation could be observed. Transfer of colonies to an inducing medium was possible without interference with growth. This was demonstrated by the uninterrupted apical incorporation of *N*-acetylglucosamine into chitin (results not shown). Most importantly, in this system, the agar medium on which the sandwiched cultures were growing acted as a sink for secreted proteins (see later). Therefore, only proteins recently secreted were detected in the sandwiched agarose layer or on a PVDF membrane positioned under the colony.

Localization of growth in a colony

Growth was localized in 4-d-old colonies, grown on starch or xylose, by determining incorporation of *N*-acetylglucosamine into chitin. Incorporation was highest in a zone just behind the edge of the colony (Fig. 1a). Microautoradiography (Fig. 1c) showed that this resulted from apical synthesis of chitin by numerous branches arising in this zone but the leading hyphae occurring in front of this zone were also heavily labelled



at their apices (Fig. 1 *b*). The low general incorporation of *N*-acetylglucosamine in the central part of the colony was due to a more general incorporation of label in the hyphal walls. However, the centrally located ring of incorporation (Fig. 1 *a*) was due to apical growth of differentiating conidiophores (results not shown).

Localization of general protein synthesis and secretion

When 4- to 5-d-old colonies were exposed to [35 S]-sulphate, incorporation of label could be observed throughout the colony (Fig. 2 *a*). However, secretion of radioactive proteins in a 4-d-old colony was located at the periphery only (Fig. 2 *b*), which includes the zone of leading hyphae and high branching activity. In 5-d-old colonies secretion of radioactive proteins was also detected in a more centrally located ring (Fig. 2 *c*) where conidiophore differentiation could be observed (not shown).

These results, together with the data on *N*-acetylglucosamine incorporation, show a close correlation between areas of apical growth and general protein secretion.

Localization of glucoamylase secretion at the level of the colony

When 4-d-old colonies were transferred from xylose to starch and incubated overnight, starch-degrading activity was localized at the periphery of the colony, just behind the edge of the colony (Fig. 3 *a*). When growth on this plate was prolonged for another 8 h, all starch under the colony, but not beyond it, was degraded (Fig. 3 *b*). This shows that starch-degrading enzymes were primarily released from the growing zone but also more slowly from the more central part of the colony.

Using immunodetection of secreted glucoamylase on PVDF filters positioned under sandwiched colonies it was found that glucoamylase was mainly secreted at the periphery of the colony (Fig. 2 *d, e*). A signal could also be detected in more central parts, however, confirming the results of the activity tests.

Localization of glucoamylase secretion at the level of the hypha

After fixation of a sandwiched colony with 4% formaldehyde or preferably TCA, glucoamylase antibodies bound to a region of the agarose surrounding hyphal tips. This

Fig. 1. Growth pattern in a 4-d-old sandwiched colony as detected by incorporation of *N*-acetylglucosamine into the hyphal wall. (*a*) Macroautoradiography of a colony. The arrow indicates the edge of the colony. Note that the unlabelled areas are due to air bubbles preventing diffusion of label. (*b*) Microautoradiography of the leading hyphae. (*c*) Microautoradiography of the hyphae in the branching zone just behind the leading hyphae. Bars, 25 μ m.

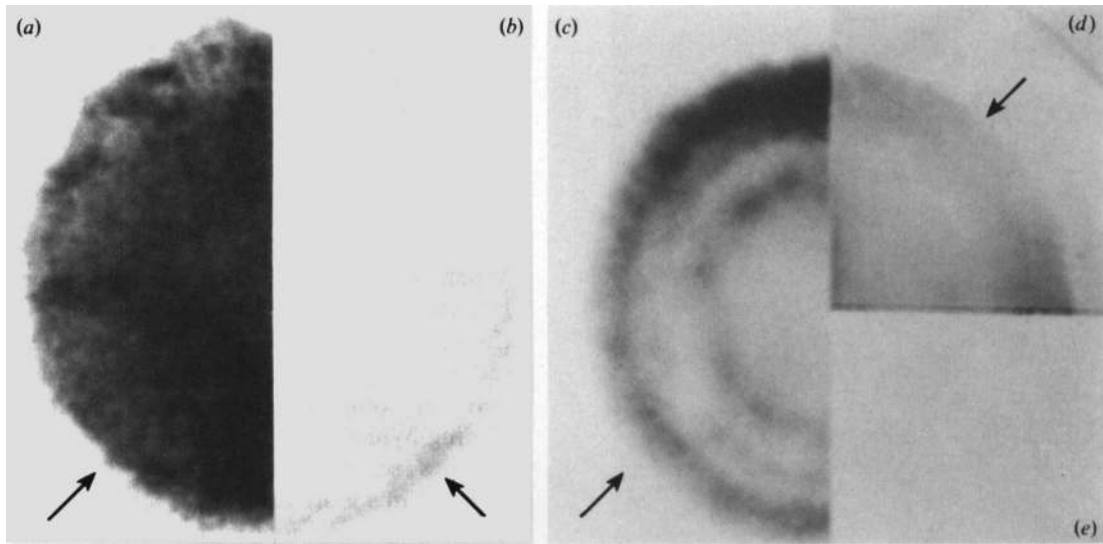


Fig. 2. Protein synthesis and secretion in sandwiched colonies growing on starch. (a) Autoradiograph of a 4-d-old colony after labelling with $[^{35}\text{S}]$ sulphate; (b) autoradiograph of a PVDF membrane underlying the colony shown in (a), representing the secreted proteins; (c) autoradiograph as in (b) but from a 5-d-old culture; (d) a PVDF membrane as in (c) but glucoamylase visualized with specific antibodies; (e) as (d) but reacted with normal rabbit serum. Arrows indicate edges of colonies.

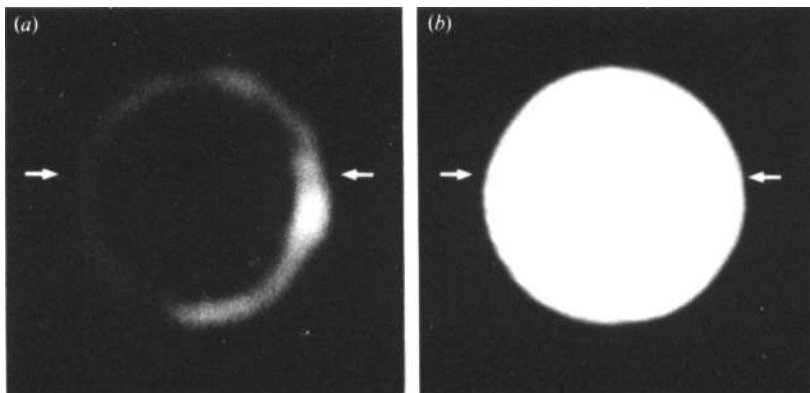


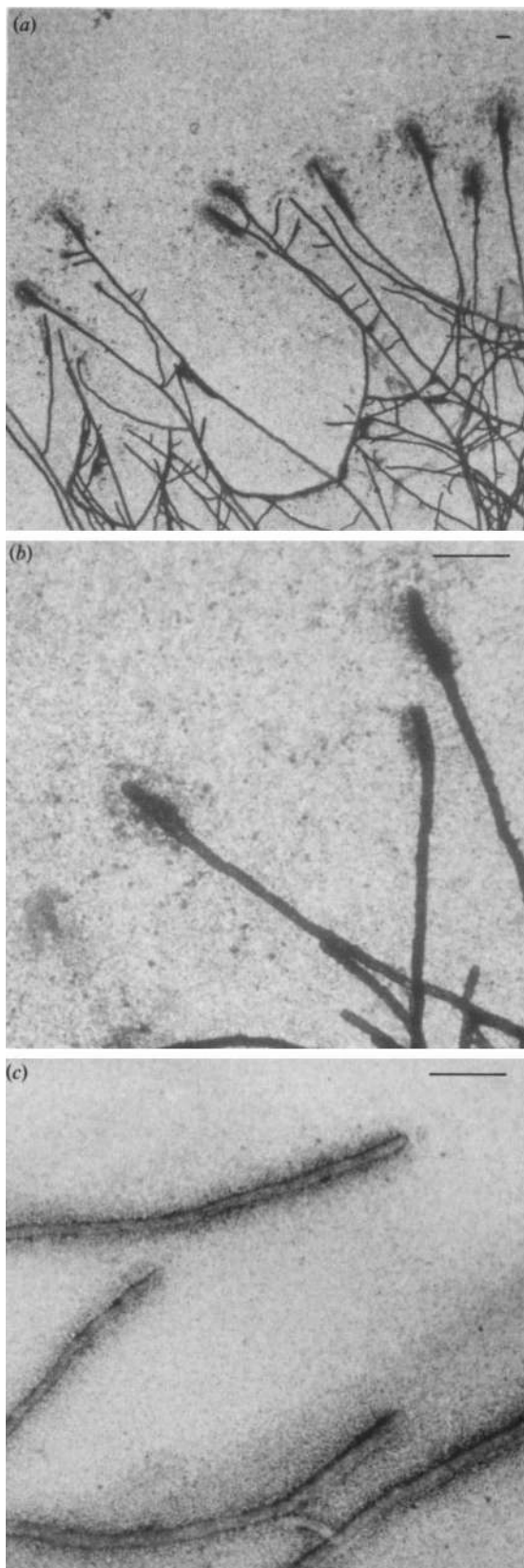
Fig. 3. Starch-degrading activity of a 4-d-old colony grown on xylose. Degradation 16 h (a) and 24 h (b) after transfer to a starch-containing medium. Arrows indicate the edge of the colony.

could be demonstrated by detection of the primary antibody with goat-anti-rabbit antibodies coupled to alkaline phosphatase (results not shown) or gold particles (Fig. 4a, b). Apart from heavy labelling of the walls, glucoamylase was observed around almost all tips of leading hyphae, but was hardly seen in the surroundings of subapical parts of these hyphae. Localization of glucoamylase in the branching zone was more difficult due to the high concentration of hyphae.

The importance of the existence of a steep gradient in glucoamylase, due to the sink provided by the agar medium, was shown by placing a sheet of cellophane membrane between the agar medium and the lower polycarbonate membrane. As a consequence, a stronger

immunological signal was observed in the agarose and high glucoamylase concentrations were detected surrounding subapical parts of the hyphae (Fig. 4c). When cultures were labelled with $[^3\text{H}]$ leucine, labelling of external proteins was difficult to discern because of the heavy labelling of internal proteins in the hyphae. However, these experiments proved that the presence of large amounts of glucoamylase around tips could not have been caused by bursting of tips, e.g. during fixation.

Microautoradiography after feeding labelled *N*-acetylglucosamine indicated that virtually all leading hyphae were growing. When a sandwiched colony was transferred to a cold (4 °C) agar medium and after 10 min re-transferred to the original plate at 25 °C, labelling



with *N*-acetylglucosamine showed that all hyphae had stopped growing. At the same time immunosignals of glucoamylase could no longer be demonstrated around hyphae. No interference with growth and secretion occurred when colonies were transferred to a fresh agar medium at 25 °C and after 10 min re-transferred to the original plate. Apparently, glucoamylase was only released by growing tips.

Discussion

Because filamentous fungi typically secrete large amounts of enzymes into their surroundings they are of great importance in degrading polymers in nature and in the industrial production of enzymes such as glucoamylase. Because of this property they have also been proposed as organisms of choice for the expression and recovery of heterologous proteins (Van Brunt, 1986). Attempts to achieve this, using promoters and signal sequences derived from, for example, glucoamylase of *A. niger* (Ward, 1989) or cellobiohydrolase in *Trichoderma reesei* (Harkki *et al.*, 1989), have met with limited success, for unknown reasons. Obviously too little is known about the secretion mechanisms in these organisms.

All secreted proteins must cross the cell wall, whether destined for the outer layers of the wall or the culture medium. 'Slime' strains of *Neurospora crassa*, which are defective in wall synthesis (Emerson, 1963) over-secrete enzymes including invertase (Bigger *et al.*, 1972; Casanova *et al.*, 1987; Pietro *et al.*, 1989), alkaline phosphatase (Burton & Metzberg, 1974), and alkaline protease and aryl- β -glucosidase (Pietro *et al.*, 1989). This suggests that the wall acts as a barrier in protein secretion. The passage of this barrier is the more intriguing because studies using different techniques (Trevithick & Metzberg, 1966; Money, 1990) all indicate that the general porosity of the wall limits passage of proteins larger than M_r 15000–20000. The cytological evidence suggests that the major exocytotic machinery of these filamentous fungi is located at the growing apex (reviewed by Grove, 1978), the older evidence being augmented by more recent studies using freeze substitution for fixation (Hoch, 1986), minimizing the chance that artifacts were observed. However, this evidence has not been generally appreciated probably because many enzymes are secreted in large amounts during the idiophase in which growth (i.e. increase in biomass) of the fungus is limited.

Fig. 4. Localization of glucoamylase by immunogold labelling in the agarose layer of a sandwiched colony after TCA fixation. (a, b) After standard procedure of growth in a sandwiched culture (see Methods); (c) after placing a cellophane membrane between the bottom polycarbonate membrane and the agar medium. Bars, 50 μ m.

Some reports have shown concentration of secreted enzymes in hyphal tips of filamentous fungi (Chung & Trevithick, 1970; Pugh & Cawson, 1977; Sprey, 1988); however, no studies have actually demonstrated extrusion of enzymes at hyphal tips. Clearly such a demonstration is impossible with hyphae growing in liquid medium but even in solid medium it is difficult because of diffusion of the enzymes and the continuous growth of hyphae while secreting. To circumvent these difficulties we devised the sandwiched culture technique as described in this paper, which allows for a number of experimental manipulations while providing a sink for secreted enzymes. Unfortunately, with the specific enzyme studied another useful property of the system, induction of formation of the enzyme by transfer to a starch medium, was not very successful. In accordance with Nunberg *et al.* (1984) we found that in liquid cultures *A. niger* forms no glucoamylase on xylose but is induced when grown on starch. However, in sandwiched cultures with xylose some glucoamylase was observed, both on Western blots and on PVDF filters positioned under the colonies, which was not due to impurities in the agar (results not shown).

The results obtained with these sandwiched cultures showed that protein secretion in general, as measured by labelling proteins with [³⁵S]sulphate, is limited to growing areas of the culture, that is where apical extension occurs. Similar results were obtained for glucoamylase, as detected by its activity and by using specific antibodies. With the latter technique high concentrations of glucoamylase could be demonstrated to occur specifically around growing hyphal tips of leading hyphae. However, the walls also seem to contain glucoamylase and some glucoamylase seemed to leak from older, presumably non-growing, parts of the culture. Because these parts failed to secrete radioactive proteins when pulsed with [³⁵S]sulphate, we presume that the glucoamylase release in this area originated from enzyme earlier secreted into the wall during apical exocytosis. Glucoamylase may also have a particular affinity for the wall as suggested by Hayashida *et al.* (1982).

It is interesting that proteins are also secreted in the area of conidiophore differentiation. This process, which can be considered as idiophasic, is also associated with hyphal growth. Preliminary evidence obtained in our laboratory with the idiophasic enzyme lignin peroxidase produced by *Phanerochaete chrysosporium* in sandwiched cultures also indicates that this enzyme is primarily secreted into wall and medium by growing branches arising in older parts of the culture (S. M. Moukha and others, unpublished).

These studies which indicate the growing apical wall as the major point of exit of secreted proteins justify a

further investigation of the mechanism by which this apical wall is traversed by proteins. Because of the mode of formation of this apical wall (Wessels, 1986, 1990) two hypotheses, which are not mutually exclusive, seem attractive. The proteins may traverse the apical wall through large pores present in this area (Chang & Trevithick, 1974) or they may be pushed through the wall together with nascent wall polymers (Wessels, 1989, 1990), which would also explain retention of some of these proteins in the lateral walls.

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